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# ANTI-PROLIFERATIVE PROPERTIES OF DL-α-DIFLUOROMETHYL ORNITHINE IN CULTURED CELLS. A CONSEQUENCE OF THE IRREVERSIBLE INHIBITION OF ORNITHINE DECARBOXYLASE

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SUMMARY

Both DL-α-Methyl ornithine (α-MeOrn), a competitive inhibitor of ornithine decarboxylase (ODC) (L-ornithine carboxy-lyase EC 4.1.1.17) and DL-α-difluoromethyl ornithine (α-DF MeOrn), a catalytic irreversible inhibitor of this enzyme, decrease the concentrations of putrescine and spermidine but not of spermine in rat hepatoma (HTC) cells and in mouse leukemia cells cultured in vitro. The depletion of the two amines is followed by a striking decrease in the rate of cell replication in both cell lines. Growth of human prostate adenoma cells is inhibited by α-DF MeOrn but not by α-MeOrn, illustrating the greater effectiveness of the irreversible inhibitor. These findings again support an essential function for putrescine and spermidine in cell division processes.

## INTRODUCTION

We recently reported (1) that DL-α-Methyl ornithine (α-MeOrn), a competitive inhibitor of ornithine decarboxylase (ODC) (2,3) (L-ornithine carboxy)lyase EC 4.1.1.17), blocked the increases of putrescine and spermidine in proliferating rat hepatoma tissue culture (HTC) cells. The depletion of intracellular putrescine and spermidine was accompanied by a decrease in the growth rate of these cells, supporting the concept that theses amines play an essential function in cell division processes and particularly

Abbreviations used are : α-MeOrn, DL-α-Methyl ornithine ; α-DF MeOrn, DL-α-difluoromethyl ornithine ; DTT, dithiothreitol ; HTC, hepatoma tissue culture ; ODC, ornithine decarboxylase.

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in DNA replication. Experiments with other inhibitors of the polyamine biosynthesis have led to the same conclusion (4,5,6,7,8,9).

To further ascertain the relationship between polyamine deficiency and decrease of cell growth rate, we studied the effects of DL-α-difluoromethyl ornithine (α-DF MeOrn) (10), a catalytic irreversible inhibitor of ODC, on rat hepatoma and other tumor cells.

## MATERIALS AND METHODS

Rat hepatoma tissue culture (HTC) cells were grown as previously described (1). Ascitic mouse leukemia L1210 cells (donated by Dr. BOLLAG - Roche Institute, Basel - Switzerland), were adapted to in vitro spinner cultures by serial cultures during 4 weeks. They were routinely grown under air atmosphere in Swim's 77 medium supplemented as described for HTC cells (14) and with 10 % horse serum. Human prostatic adenoma MA 160 cells (Microbiological Associates) were grown under 5 % $CO_2$ - 95 % air atmosphere in Eagle minimum essential medium (EMEM) (Gibco) with Earle's salts containing 2 mM glutamine, 0.1 mM Eagle's non-essential amino-acids (Gibco), 0.1 mM pyruvate and 10 % foetal calf serum. Cell growth and cell viability was determined as described previously (1).

For ODC assay, 1.5 - $3x10^6$ cells were centrifuged, washed with cold phosphate-buffered saline and sonicated in 0.7 ml 30 mM sodium phosphate buffer pH 7.2, 5 mM DTT, 0.1 mM pyridoxal phosphate and 0.1 mM EDTA. ODC activity was determined essentially as described (12) on the crude cell sonicate in the same buffer supplemented with 0.2 mM L-ornithine and 2.5 μCi (1-$^{14}$C) DL-ornithine (58 mCi/mol, Amersham). Protein was estimated by the fluorescamine method (15).

DL-α-difluoromethyl-ornithine (α-DF MeOrn) (10) was dissolved before use in phosphate-buffered saline and adjusted to pH 7.5 with NaOH.

For the determination of polyamines, aliquots ($3x10^6$ cells) were centrifuged and the pellet washed twice with cold phosphate-buffered saline. The cells were disrupted by sonication in 0.9 ml HCl 0.1 N. Proteins were precipitated by adding 0.1 ml of 2 N perchloric acid. After centrifugation, polyamines were assayed in 10-80 μl aliquots of the supernatant using a Durrum D-500 amino-acid analyzer with a slight modification of the previously described method (11).

## RESULTS AND DISCUSSION

HTC cell proliferation was initiated by dilution of a high density cell culture with fresh medium supplemented with 10 %

calf serum. As shown in Fig. 1-A, a biphasic increase of ODC occurred after induction of cell proliferation. Addition of α-DF MeOrn to the cultures immediately after dilution blocked this increase, the maximum effect occurring at concentration $\geq$ 0.1 mM (Fig. 1-A). Dialysis of the enzymatic HTC cell extracts did not result in recovery of any enzyme activity, suggesting that the inhibition was irreversible.

Intracellular polyamine content was determined under the same experimental conditions. 0.01 mM α-DF MeOrn almost completely prevented the accumulation of putrescine and spermidine (Fig. 1-B). Higher concentrations of α-DF MeOrn decreased basal levels of both putrescine and spermidine. This depletion correlated with inhibition of ODC activity (Fig. 1-A). Spermine concentrations did not decline in the presence of the drug, as they had not during exposure to α-MeOrn (1).

From previous findings with α-MeOrn we postulated that stationary HTC cells had sufficient basal contents of putrescine and spermidine to allow one cycle of DNA synthesis (1). Subsequent inhibition of DNA synthesis was related to the decrease in basal levels of these amines. Accordingly, we did not expect cell growth inhibition at 0.01 mM α-DF MeOrn, since initial levels of putrescine and spermidine were maintained at this inhibitor concentration. As expected, higher concentrations of the drug slowed down cell multiplication after a 24 h lag period (Fig. 2).

Cell growth inhibition by α-DF MeOrn was partially antagonized by 1 mM L-ornithine (Fig. 3-A), presumably because this substrate competes with the inhibitor for the active site of ODC (10). Inhibition was also prevented by 10 μM putrescine or spermidine or 1 μM spermine (Fig. 3-B). A likely explanation of the surprising effect of spermine is its conversion back into spermidine and as discussed previously

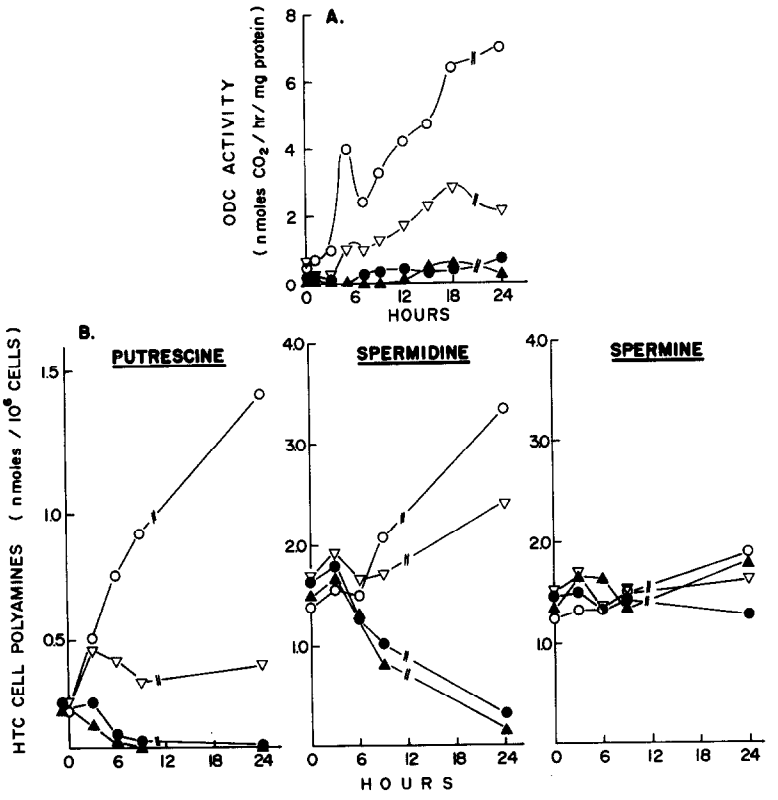
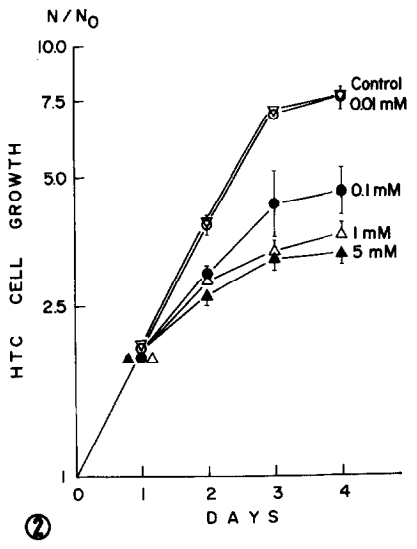



Figure 1. Effect of α-DF MeOrn on HTC cell ODC (A) and intracellular polyamine concentration (B).
HTC cells were incubated from time 0 in the absence (o) or in the presence of 0.01 mM (▽), 0.1 mM (●), 5 mM (▲) α-DF MeOrn.

(1). Growth was restored by 10 μM putrescine when added after 8 days of incubation with α-DF MeOrn (Fig. 4-A). This addition of putrescine replenished the normal spermidine level (Fig. 4-B). Therefore, it is likely that the growth inhibitory effect of α-DF MeOrn results from its ODC-inhibitory activity (10).

Inhibition of cell prolifetation resulting from polyamine deficiency could be limited to the rat hepatoma cell line or be a much more general phenomenon of cultured eukaryotic cells. Newton and Abdel-Monem (13) failed to demonstrate any modification of the DNA content of cultured L1210 leukemia cells incubated for two generations with α-MeOrn. In order



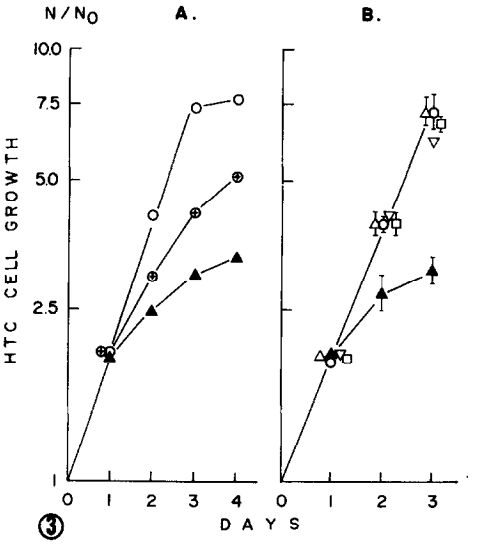




Figure 2. Effect of α-DF MeOrn on HTC cell division.
HTC cell spinner culture ($1x10^5$ cells per ml) were incubated from time 0 in the absence (o) or in the presence of 0.01 mM (▽), 0.1 mM (●), 1 mM (△), 5 mM (▲) α-DF MeOrn. Results are expressed as the ratio $N/N_0$, where N = number of cells per ml at day 1, 2, 3, 4 and $N_0$ = number of cells per ml at day 0, and are the means ± SEM.

Figure 3. Reversal of inhibition of HTC cell division by L-ornithine (A) and polyamines (B).
HTC cell spinner cultures ($1x10^5$ cells per ml) were incubated from time 0 in the absence (o) or in the presence of 5 mM α-DF MeOrn (▲). At day 1, A- 1 mM L-ornithine ( ⊕ ) B - 10 μM putrescine (△), 10 μM spermidine (▽) or 1 μM spermine ( □ ) were added to α-DF MeOrn-treated cultures.

to follow the effect of both α-MeOrn and α-DF MeOrn over a much more extended period, ascitic mice L1210 leukemia cells were first adapted to grow in spinner culture in Swim's 77 medium supplemented with 10 % horse serum. In these particular culture conditions the doubling time of the population (15 h) was found to be constant if the medium was changed every 2 days (Fig. 5-A). Then, intracellular polyamine levels and growth rates were determined for 8 days in the presence of 5 mM α-MeOrn and 5 mM α-DF MeOrn.

As illustrated in figure 5-B, putrescine accumulation was observed in the control cultures to each medium change. Intracellular spermidine and spermine levels showed less marked fluctuations. Contrary

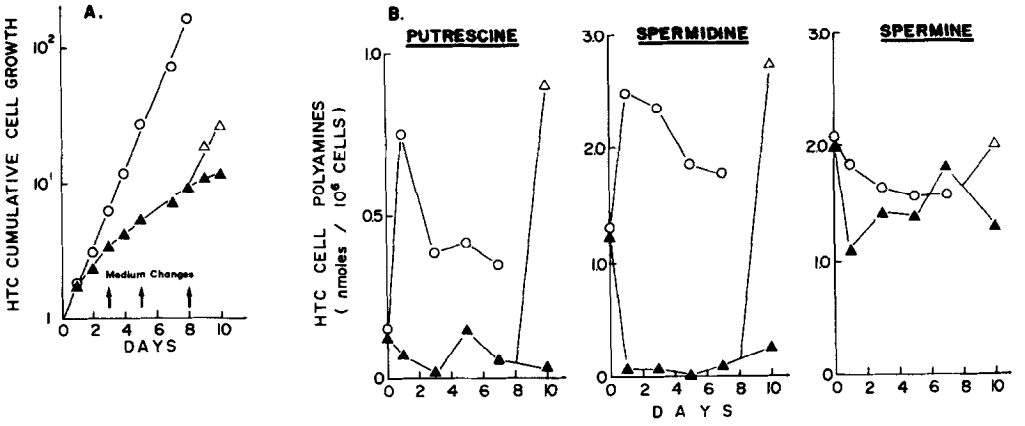



Figure 4. Long-term effect of α-DF MeOrn on HTC cell growth (A) and polyamine concentration (B). Reversal effect of putrescine. HTC cell spinner cultures were incubated from time 0 in the absence (o) or in the presence of 5 mM α-DF MeOrn (▲). At day 3, 5 and 8, cells were gently centrifuged (200Xg) and resuspended in fresh medium in the presence or absence of the drug at a cell density of 1.5 - $3x10^5$ cells per ml. 10 μM putrescine (Δ) was added to α-DF MeOrn treated culture at day 8. Cumulative growth curves were established taking account of the dilution factor (A) and polyamine concentrations were determined at the indicated time (B).

to the report of Newton and Abdel-Monem (13), 5 mM α-MeOrn completely depleted spermidine in one and a half generations even though cellular putrescine was only decreased by 60-65 %. Spermine concentration remained unaffected. Similar results were obtained using 5 mM α-DF MeOrn (Figure 5-B). In parallel with depletion of spermidine, L1210 cell growth rate was strinkingly decreased by both ODC inhibitors after a lag period of 24 h (Fig. 5-A).

In contrast to the results with HTC and L1210 cell lines, 5 mM α-MeOrn did not significantly affect the rate of proliferation of prostate adenoma cells (Fig. 6), while the irreversible inhibitor of ODC was effective. At present, the resistance of these cells to α-MeOrn is not readily explained but underlines the greater effectiveness of the catalytic irreversible over competitive inhibitors.

The maintenance of cellular spermine concentrations after long-term incubation with ODC inhibitors (Figs. 4-B, 5-B) suggests

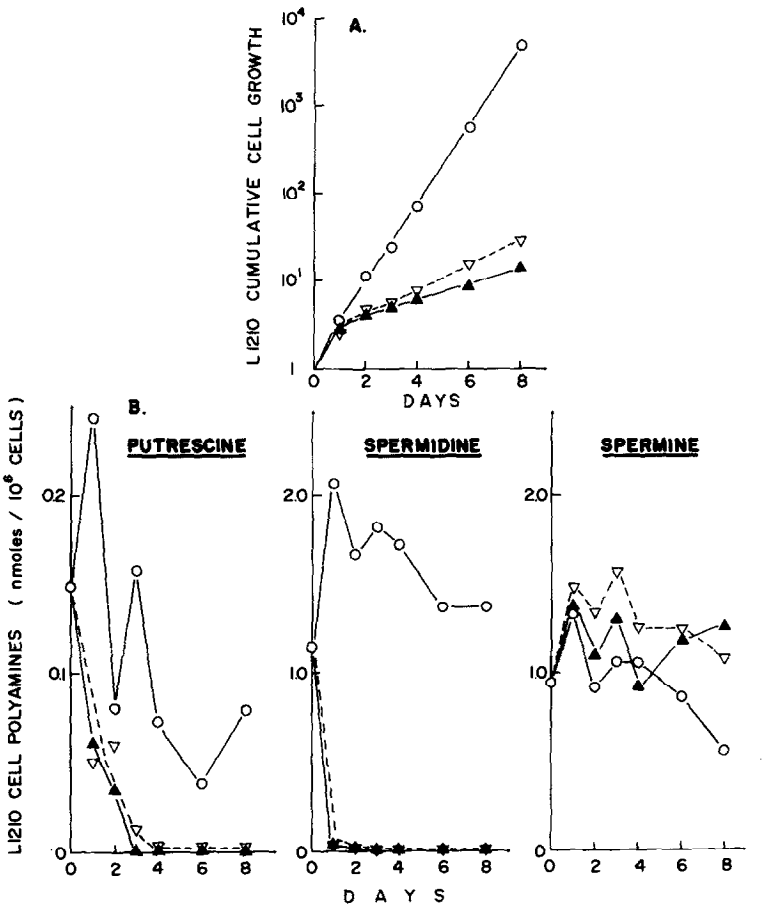



Figure 5. Effect of α-MeOrn and α-DF MeOrn on L1210 cell multiplication (A) and polyamine concentration (B).
L1210 cell spinner cultures ($1x10^5$ cells per ml) were incubated from time 0 in the absence (o) or in the presence of 5 mM α-MeOrn (▽) or 5 mM α-DF MeOrn (▲). Every two days media were changed as described in Fig. 4.

that the small amount of putrescine synthetized under this condition allows the accumulation of spermine especially as α-MeOrn and α-DF MeOrn increase S-adenosyl-L-methionine decarboxylase activity (16). Preliminary experiments using short pulse labelling of HTC cells with trace amounts of ornithine appear to indicate that, though reduced by 96 %, radioactivity was found in the polyamine fraction of HTC cells, incubated 3 days with α-DF MeOrn, and was essentially associated with the spermine fraction.

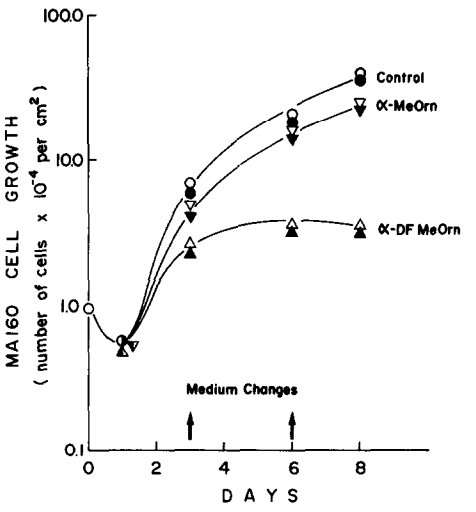



Figure 6. Effect of 5 mM α-MeOrn and 5 mM α-DF MeOrn on MA 160 cell multiplication. MA 160 prostate adenoma cells growing in monolayer were trypsinized, counted and $5x10^5$ cells were seeded in replicate 80 mm dishes. 30 min. later, the compounds to be tested, were added. Cell counts and viability were estimated at the indicated times after trypsinization of duplicate dishes. Medium were changed every three days. Control (o,●), α-MeOrn (▽,▼), α-DF MeOrn (△,▲).

Our results clearly show that inhibition of putrescine synthesis by ODC inhibitors which causes a deficiency of putrescine and spermidine, leads to a decreased rate of cell proliferation. It is worth noting, however, that prolonged treatment (8 days) with these ODC inhibitors of HTC cells (Fig. 4-A) and L1210 cells (Fig. 5-A) did not completely arrest cell growth. This observation suggests that depletion of putrescine and spermidine does not totally block progression of the cells through their cycle. It remains to be determined whether intracellular spermine can partially fulfill the functions of putrescine and spermidine in the maintenance of growth or whether the residual growth of tumor cells is polyamine-independent.

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