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Inhibition of Ornithine Decarboxylase Activity and Cell Growth by Diamines: A Comparison Between the Effects of Two Homologs, 1,3-Diaminopropane and 1,4-Diaminobutane (Putrescine)

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The ornithine decarboxylase (ODC) activity of Ehrlich ascites tumor cells was almost completely inhibited by treatment with either putrescine (10 mM) or 1,3-diaminopropane (5 mM). 1,3-Diaminopropane treatment eradicated the cellular content of putrescine and reduced that of spermidine and spermine. Putrescine treatment caused a dramatic increase in cellular putrescine content and a temporary decrease in spermidine and spermine content. Despite the fact that 1,3-diaminopropane and putrescine inhibited the ODC activity more effectively than did α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, they were considerably less antiproliferative in action. However, as compared to DFMO the diamines were less effective in reducing the total polyamine (putrescine + spermidine + spermine) content of the cells.

Key words: 1,3-diaminopropane, putrescine, α-difluoromethylornithine, polyamines, ornithine decarboxylase, cell size, cell proliferation, cell cycle, flow cytometry, DNA histogram

Ornithine decarboxylase (ODC; EC 4.1.1.17) is a key enzyme in polyamine synthesis and cell growth [1,2]. It has a remarkably short half-life [3–5] and is subject to many control mechanisms [2,6]. Upon enzyme-activated irreversible inhibition of the ODC activity by treatment with α-difluoromethylornithine (DFMO), cells are depleted of their putrescine and spermidine content and, as a consequence, cease to proliferate [2]. The ODC activity is also inhibited by treatment with di- and polyamines (for references, see [2]). Using a competitive radioimmunoassay procedure, Seely and Pegg [4,5] have recently demonstrated that the ODC activity and the amount of enzyme protein decrease in parallel as a result of diamine treatment. Certain diamines, particularly 1,4-diaminobutane (putrescine) and its lower homolog

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1,3-diaminopropane, have been shown to induce the synthesis and/or release of a 26,000 molecular weight ODC inhibitory protein, termed antizyme [6–10]. This protein may be involved in the rapid degradation of ODC.

These findings raise the question whether 1,3-diaminopropane, by inhibiting the ODC activity, may be used in a similar manner as DFMO to deplete the cellular polyamine content and thereby exert an antiproliferative effect. Although it has been reported that administration of 1,3-diaminopropane inhibits the incorporation of [^{3}H]thymidine into DNA [11–14], the relationship between polyamine depletion and the antiproliferative effect has not been analyzed in detail.

The present study was designed to (1) substantiate the effects of treatment with 1,3-diaminopropane on polyamine metabolism and cell growth and (2) compare the effects of 1,3-diaminopropane with those of the higher homolog putrescine, which is a product of the ODC-catalyzed reaction and therefore should counteract polyamine depletion and growth inhibition. In these experiments the concentrations of putrescine and 1,3-diaminopropane were carefully chosen such that they almost completely eradicated the ODC activity and yet did not produce any apparent cytotoxicity.

MATERIALS AND METHODS

Cell Culture

A hyperdiploid subline of the Ehrlich ascites tumor was grown in suspension culture in a medium supplemented with 10% fetal calf serum [15]. At time 0, 1.0×10^7 plateau phase cells were suspended in 100 ml of growth medium and seeded in a 150-cm^2 Costar tissue culture flask. In addition, the same number of cells was seeded in 100 ml of growth medium containing putrescine (0.01, 1, or 10 mM) (Fluka, Buchs, Switzerland), 1,3-diaminopropane (5 mM) (Aldrich-Chemie, Steinheim, West Germany), or DFMO (5 mM). Stock solutions of the diamines and DFMO were made up at 100× concentrations in complete culture medium. Before addition to the cell cultures, the solutions were adjusted to pH 7.4 and sterilized by passage through 0.20-μm pore size filters (Flow, Irvine, Scotland). A Coulter counter (Industrial Model D) was used to determine the cell number. Every 12 hr, samples were removed for cell counting, ODC assay, polyamine analysis, cell size distribution analysis, and cell cycle distribution analysis. Cells intended for biochemical analyses were harvested by centrifugation at 1,000g for 10 min at 4°C. The cell pellets were stored at −70°C.

Cells that had been subjected to putrescine treatment were washed once in a large volume (10 ml) of phosphate-buffered saline (pH 7.2) to remove excess putrescine from the cell pellet. This wash reduced the amount of putrescine associated with the cell pellet without affecting the spermidine and spermine content of the cells. The putrescine content of these cells has to be considered an approximation, however, because we cannot exclude the possibility that some intracellular putrescine may have been lost during the wash. Inasmuch as there were negligible amounts of polyamines in the medium, 1,3-diaminopropane-treated cells and untreated control cells were not washed.

ODC Assay

Cells were disrupted by sonication in ice-cold 10 mM Tris-HCl buffer (pH 7.2) containing 5 mM dithiothreitol, 0.05 mM pyridoxal 5′-phosphate, and 0.5 mM Na_2

EDTA. The ODC activity was assayed in the presence of a saturating (1 mM) L-ornithine concentration essentially as described by Jänne and Williams-Ashman [16].

Polyamine Analysis

Cells were disrupted by sonication in ice-cold 0.2 M perchloric acid. After 1 hr on ice, the homogenate was centrifuged at 1,000g for 10 min at 4°C. A 200-μl aliquot of the supernatant was dansylated and analyzed by thin-layer chromatography [17,18].

Cell Size Distribution Analysis

Approximately 1×10^6 cells, grown in the absence or presence of 10 mM putrescine or 5 mM 1,3-diaminopropane for 96 hr, were suspended in 100 ml of saline containing 1% formaldehyde. The cell size distribution was analyzed using a Coulter counter (Industrial Model D).

Flow Cytometric Analysis

Cells were harvested by centrifugation at 500g for 5 min, fixed in absolute ethanol ($1–2 \times 10^6$ cells/ml) at −20°C, and analyzed for their cell cycle distribution by flow cytometry. Before analysis the cells were treated with pepsin, RNase, and Nonidet P40, and they were stained with propidium iodide as previously described [19]. The stained cell nuclei were analyzed using a Cytofluorograph 50-H (Ortho Instruments).

RESULTS

Figure 1 shows the effects of treatment with various putrescine concentrations on Ehrlich ascites tumor cell growth in culture. Inhibition of cell growth exerted by putrescine was dose-dependent. The reduction in growth rate, caused by putrescine concentrations up to 10 mM, did not exceed 21% (Figs. 1,3A).

The effects of treatment with the same putrescine concentrations on the cellular ODC activity are shown in Figure 2. In control cells three peaks of ODC activity were observed during the 96-hr growth period: at 3, 10, and 24 hr after seeding. Putrescine treatment reduced all three increases in ODC activity in a dose-dependent manner. A 10 mM putrescine concentration was required to completely prevent the increases in ODC activity.

The effects of treatment with 5 mM 1,3-diaminopropane and 10 mM putrescine on cell growth and polyamine metabolism are compared in Figure 3. These concentrations were required to almost completely inhibit (by 96–100%) the ODC activity (Fig. 3B,G). Both diamines exerted a similar, approximately 20%, inhibitory effect on tumor cell growth rate (Fig. 3A,F). Administration of putrescine caused a rapid intracellular accumulation of this diamine (Fig. 3C). Conversely, 1,3-diaminopropane treatment eradicated the cellular putrescine content within 12 hr (Fig. 3H). In cells treated with putrescine, the spermidine content decreased during the first 24 hr and returned to the control level by 36 hr (Fig. 3D). Treatment with 1,3-diaminopropane resulted in a continuous decrease in the cellular spermidine content (Fig. 3I). Treatment with either diamine first resulted in a decrease in the cellular spermine content, but 2 days after seeding the control level was reached (Fig. 3E,J). Although the basal polyamine levels varied among experiments (Fig. 3, Table I), the changes observed

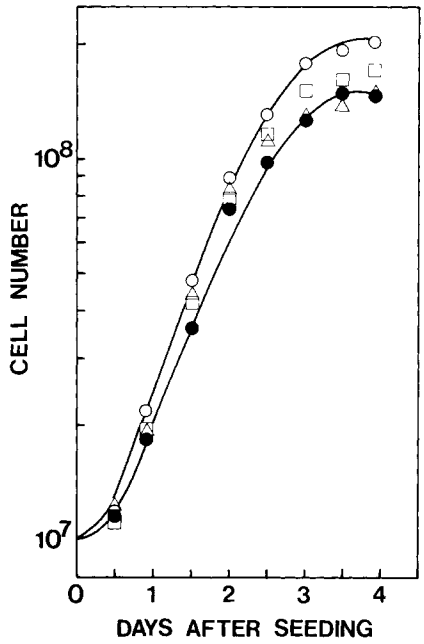

Fig. 1. Effect of putrescine on Ehrlich ascites tumor cell growth. Putrescine was added at time 0 to a final concentration of 0.01 mM (□), 1.0 mM (△), or 10 mM (●). Untreated control culture (○).

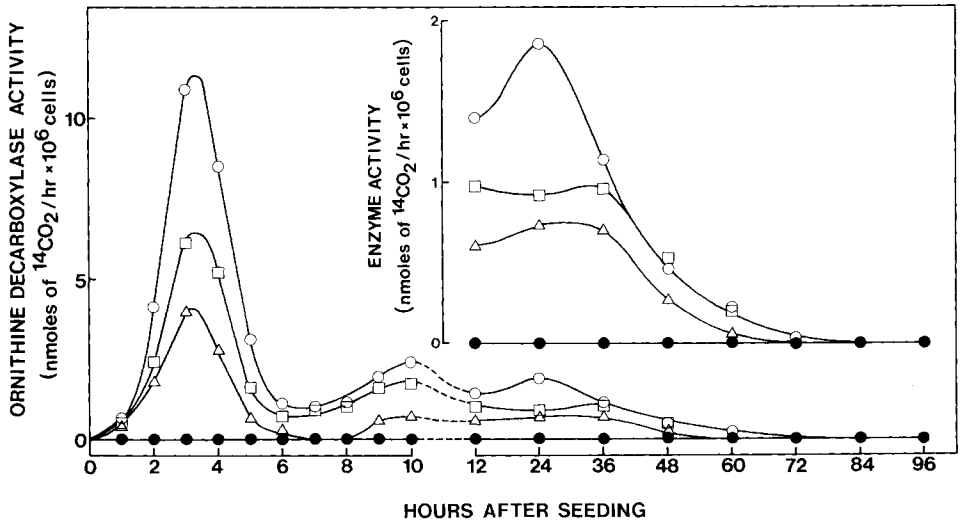

Fig. 2. Effect of putrescine on the activity of ornithine decarboxylase in Ehrlich ascites tumor cells. Putrescine was added at time 0 to a final concentration of 0.01 mM (□), 1.0 mM (△), or 10 mM (●). Untreated control culture (○). To elucidate the effects occurring after 12 hr of growth, the ordinate was expanded fivefold (inset).

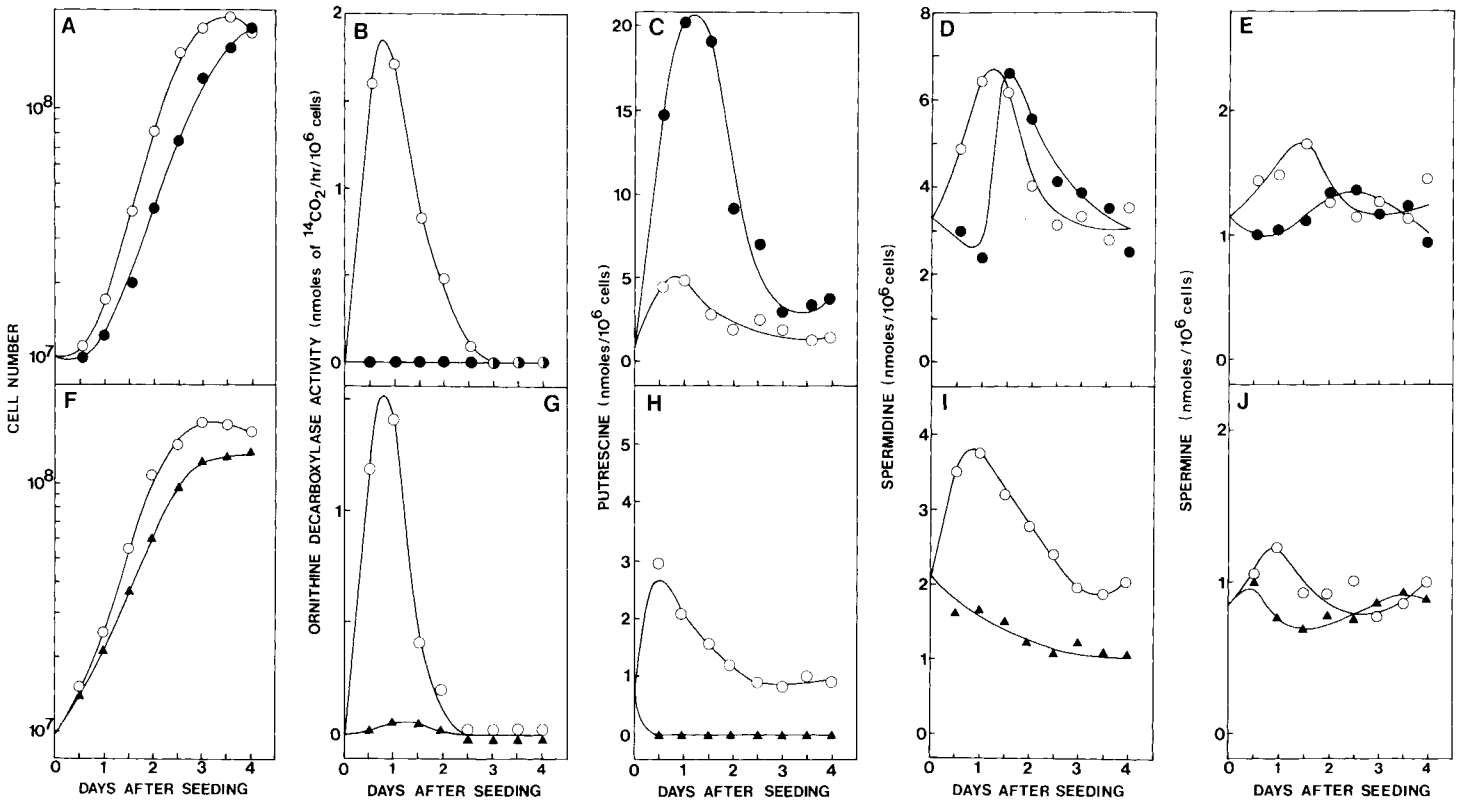

Fig. 3. Effects of 10 mM putrescine (A–E) and 5 mM 1,3-diaminopropane (F–J) on Ehrlich ascites tumor cell growth and polyamine synthesis and content. Putrescine (●) and 1,3-diaminopropane (▲) were added at time 0. Untreated control cultures (○).

TABLE I. Effects of Treatment With Diamines and DFMO on Total Polyamine Content and Proliferation of Ehrlich Ascites Tumor Cells

Treatment[a]	Cell number ($\times 10^8$)	Total polyamine content (nmoles/10^6 cells)
Control	2.12 (100)[b]	6.51 (100)[b]
Putrescine (10 mM)	1.33 (63)	8.01 (123)
Control	1.78 (100)	2.74 (100)
1,3-Diaminopropane (5 mM)	1.23 (69)	1.96 (72)
Control	2.42 (100)	5.14 (100)
DFMO (5 mM)	0.86 (36)	1.00 (19)

[a]Putrescine, 1,3-diaminopropane, and DFMO were added to the cell cultures at the time of seeding, and the cells were harvested after 72 hr of treatment.
[b]Figures in parentheses are the percent of control.

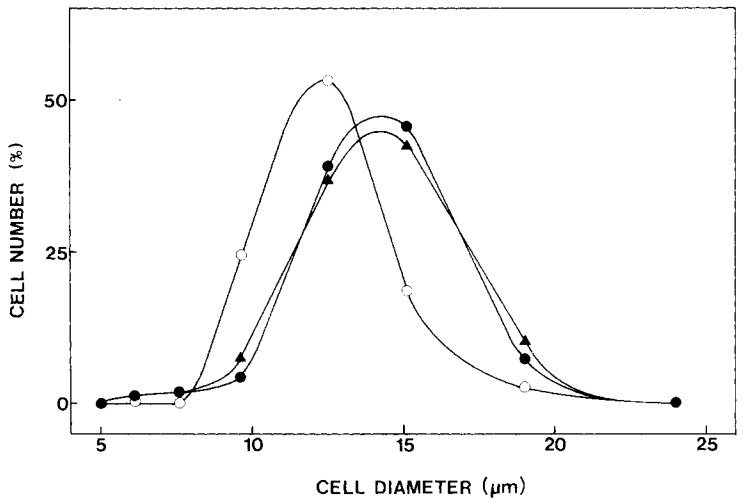

Fig. 4. Effect of 10 mM putrescine and 5 mM 1,3-diaminopropane on cell size distribution of Ehrlich ascites tumor cells. Putrescine (●) and 1,3-diaminopropane (▲) were added at time 0 and the cells were harvested 96 hr later. Untreated 96-hr control culture (○). Individual data points represent the percentage of cells exhibiting a certain diameter (in each of the three cell populations).

after diamine treatment were temporally consistent and quantitatively proportional. This was established by using a control in each experiment.

Putrescine and 1,3-diaminopropane treatment caused a significant increase in average cell size (Fig. 4). Nevertheless, flow cytometric analyses of cells treated with putrescine or 1,3-diaminopropane revealed only a slight change or no change at all in cell cycle distribution (Fig. 5). A slight increase in the G2 phase fraction and a corresponding decrease in G1 and S was consistently observed in putrescine-treated cells (Fig. 5B). Because of the increase in size of diamine-treated cells (Fig. 4), the actual concentrations of the polyamines in these cells are even further decreased than indicated by their cellular content (Fig. 3, Table I).

Table I shows the effects of treatment (72 hr) with putrescine and 1,3-diaminopropane, compared to that of DFMO, on total polyamine (putrescine + spermidine + spermine) content and cell proliferation. Only 1,3-diaminopropane and DFMO

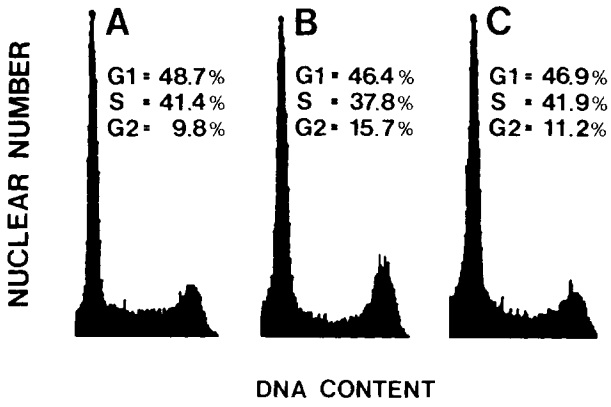

Fig. 5. Effect of 10 mM putrescine and 5 mM 1,3-diaminopropane on the cell cycle distribution of Ehrlich ascites tumor cells. Putrescine (B) and 1,3-diaminopropane (C) were added at time 0 and the cells were harvested 96 hr later. Untreated 96-hr control culture (A). The DNA histograms were obtained by flow cytometry and the percentage of cells in G1, S, and G2 was calculated essentially as described by Dean [28].

treatment reduced the total polyamine content; DFMO was more effective than 1,3-diaminopropane at the concentration used (5 mM). Putrescine and 1,3-diaminopropane inhibited cell growth to approximately the same extent. DFMO exerted a significantly greater antiproliferative effect (Table I).

DISCUSSION

In agreement with many previous reports (for references, see [2]), the present study shows that treatment with the diamines putrescine and 1,3-diaminopropane inhibits cellular ODC activity. Thus, a 96–100% inhibition of the ODC activity in Ehrlich ascites tumor cells was achieved by treatment with 10 mM putrescine or 5 mM 1,3-diaminopropane. Studies on Chinese hamster ovary (CHO) cells, Ehrlich ascites tumor cells, and diet-stimulated and regenerating rat liver show that diamine-induced ODC inhibition causes a reduction in the incorporation of [^{3}H]thymidine into DNA [11–14,20] and a lower mitotic index [14,20].

In this report we assess the antiproliferative effects exerted by putrescine and 1,3-diaminopropane and analyze whether they are due to interference with any particular phase of the cell cycle. The diamine concentrations were chosen such that they produced a total eradication of the ODC activity, yet did not show any apparent cytotoxic effect. Even though the cells were incubated with rather high diamine concentrations and for long time periods in the presence of fetal calf serum, there appeared to be no toxicity owing to serum oxidases inasmuch as treatment with aminoguanidine, a potent inhibitor of diamine oxidase, failed to reduce the antiproliferative effect of putrescine [21]. That fetal calf serum does not oxidize putrescine is also suggested by the fact that all radioactive putrescine was recovered intact after a 4-hr incubation with 100% fetal calf serum [22].

Putrescine and 1,3-diaminopropane exerted a similar antiproliferative action. 1,3-Diaminopropane treatment did not change the cell cycle distribution, while pu-

trescine treatment caused a slight accumulation of cells in the G2 phase. Nevertheless, treatment with either diamine resulted in a cell size increase. Since there was no major change in cell cycle distribution in diamine-treated cells, it appears that all cell cycle phases are prolonged and that cell enlargement is due to unbalanced growth. This is at variance with the results of a previous study in which we demonstrated that putrescine treatment of the Ehrlich ascites tumor *in vivo* caused an accumulation of cells in the S phase, mainly owing to a decrease in the rate of DNA synthesis [20]. Furthermore, the results of Sunkara et al [11] suggest that 1,3-diaminopropane treatment causes an accumulation of CHO cells in the S phase. The more pronounced cell cycle effects in these previous studies may be due to the fact that the diamines were given at higher, possibly toxic concentrations.

Despite the increase in cellular putrescine content during treatment with exogenous putrescine, the spermidine and spermine content decreased initially, as compared to the control. The decrease in spermidine content may be due to the fact that putrescine, like 1,3-diaminopropane, inhibits S-adenosylmethionine decarboxylase (SAMDC) at high concentrations [23]. The decrease in spermine content is probably due to the fact that putrescine acts as a competitive inhibitor of spermine synthase. Thus, putrescine competes with spermidine for the active site of the enzyme [24].

1,3-Diaminopropane treatment totally inhibited putrescine synthesis by eradicating the ODC activity. The substantial decrease in spermidine content resulting from this treatment may be due partly to a lack of putrescine (by 12 hr) and partly to a direct inhibitory effect of 1,3-diaminopropane on the SAMDC activity [23]. The decrease in spermine content after 1,3-diaminopropane administration is unexpected when considering the effects of DFMO treatment. Thus, treatment with DFMO very effectively depletes the putrescine and spermidine content and increases the spermine content of Ehrlich ascites tumor cells [15]. Since putrescine is a competitive inhibitor of spermine synthase, putrescine depletion relieves this inhibition and the spermine content increases. That 1,3-diaminopropane instead causes a decrease in cellular spermine content, despite the depletion of putrescine, therefore suggests that 1,3-diaminopropane inhibits spermine synthase in a similar manner as does putrescine [24]. An inhibitory effect of 1,3-diaminopropane on the activity of spermine synthase has been observed *in vitro* [25].

Even if 10 mM putrescine inhibits the cellular ODC activity completely, there should be no reduction in putrescine, spermidine, and spermine content since putrescine is a product of the ODC-catalyzed reaction. Nevertheless, putrescine treatment reduced the spermidine and spermine content, possibly owing to inhibition of the SAMDC and spermine synthase activities at this rather high concentration (10 mM). The antiproliferative effect of putrescine treatment therefore seems to be a result of the decrease in spermidine and spermine content. At a lower (1 mM) concentration, putrescine completely reverses the antiproliferative effect of DFMO-induced putrescine and spermidine deficiency [21,26]. There is a corresponding decrease in the cellular content of spermidine and spermine during the initial phase of 1,3-diaminopropane treatment, ie, when the antiproliferative effect is established. The fact that 1,3-diaminopropane treatment, in addition, causes putrescine depletion but is not more inhibitory to cell proliferation (than is putrescine treatment) suggests that 1,3-diaminopropane can partly replace putrescine in its cellular function. In experiments in which Ehrlich ascites tumor cells were treated with a combination of DFMO and 1,3-diaminopropane, there was a further decrease in spermine content (compared to

that observed in cells treated with DFMO alone) but no major potentiation of the antiproliferative effect [21,27]. A possible interpretation of this, which is consistent with the present data, is that 1,3-diaminopropane partly replaces putrescine in its function at the same time as it blocks spermine synthesis. In fact, 1,3-diaminopropane can partly replace the role played by putrescine in rat hepatoma cell growth [26]. That DFMO exerts a more potent antiproliferative effect than does putrescine and 1,3-diaminopropane may be a consequence of the depletion of putrescine (in addition to that of spermidine) with no diamine substitute present. Furthermore, DFMO-treated cells have a lower content of total polyamines (putrescine + spermidine + spermine) (Table I).

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