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INHIBITION OF POLYAMINE BIOSYNTHESIS BY α-DIFLUOROMETHYL ORNITHINE POTENTIATES THE CYTOTOXIC EFFECTS OF ARABINOSYL CYTOSINE IN HELA CELLS

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SUMMARY: The object of this study was to examine the effect of inhibition of polyamine biosynthesis on the cell cycle traverse of HeLa cells using α-difluoromethyl ornithine (DFMO), a catalytic irreversible inhibitor of ornithine decarboxylase. The results of this study indicate that DFMO inhibits HeLa cell growth by causing a decrease in the intracellular levels of putrescine and spermidine without any significant effect on concentration of spermine. The inhibition is readily reversible by exogenous supply of putrescine to the medium. The DFMO treatment also results in an accumulation of cells in S phase. Further, the use of an S phase-specific drug like Ara-C following DFMO treatment results in a synergistic killing of the tumor cells as revealed by the inhibition of cell growth. These observations suggest that exploitation of regulation of the cell cycle by the depletion of polyamines with the use of inhibitors like DFMO might help in designing better therapeutic regimes in combination with other cytotoxic drugs.

INTRODUCTION

The availability of specific inhibitors of polyamine biosynthesis has enabled us to demonstrate that putrescine and spermidine play important roles in the regulation of DNA synthesis (1, 2, 3, 4) and cytokinesis (5) of mammalian cells. Inhibition of polyamine biosynthesis either with methyl-glyoxal bis-(guanyl-hydrazone) (MGBG) or α-methyl ornithine (α-MO) has been reported to block human, mouse, and rat embryo fibroblasts in G1 phase (6, 7, 8), whereas the depletion of polyamine levels

Abbreviations: DFMO, α-difluoromethyl ornithine; Ara-C, Arabinosyl cytosine, α-MO, α-methyl ornithine; MGBG, methyl-glyoxal bis-(guanyl-hydrazone), G1, pre-DNA synthetic period; S, DNA synthetic period. G2, post-DNA synthetic period; PCC, prematurely condensed chromosomes.

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in a majority of the transformed cells resulted in the arrest of these cells in the S phase of the cell cycle (8, 9). Recently, Mamont et al (10) have shown that α-difluoromethyl ornithine (DFMO), a catalytic irreversible inhibitor of ornithine decarboxylase (11), decreased the concentrations of putrescine and spermidine and caused a rapid inhibition of cell growth in rat hepatoma and mouse leukemia cells in culture. In addition, DFMO has been shown to inhibit the growth of experimental tumors in mice (12, 13). In the present study, we have investigated the effect of DFMO on the growth and cell cycle traverse of HeLa cells. The results of our study indicate that DFMO causes a rapid inhibition of HeLa cell growth as a results of depletion of intracellular levels of putrescine and spermidine, and that it arrests a majority of the cells in S phase. Further, we found that a sequential administration of DFMO and Ara-c, an S phase-specific drug, results in a synergistic antiproliferative effect on HeLa cells.

MATERIALS AND METHODS

Cells and cell synchrony. HeLa cells were routinely grown as monolayer cultures as described earlier (14). These cells have a cell cycle time of 22 hr, consisting of 10.5 hr of pre-DNA synthetic (G2) period, and 1.0 hr of mitosis (15). To obtain mitotic cells for cell fusion experiments, an exponentially growing culture was partially synchronized by a single thymidine block that was followed by N_2O block for 9 hr, and the separation of mitotic cells by selective detachment (16). Cells thus harvested had a mitotic index of about 98%.

Cell kinetics. HeLa cells were plated in 150-mm culture plates at a cell density of 2.5×10^5 cells per plate. The cells were grown either in the presence or absence of DFMO (2.5 mM) at 37°C. At appropriate incubation periods, cells were collected by trypsinization and counted, and a portion of the cells (2×10^6 cells) was used to determine cell cycle kinetics of both the populations.

To find where the DFMO treated cells were blocked, we applied the technique of premature chromosome condensation (17). This method, which involves the Sendai virus-mediated fusion between mitotic and interphase cells, makes it possible to determine the position of an interphase cell in the cell cycle on the basis of the morphology of its prematurely condnesed chromosomes (PCC). Recently, this method has been used for cell cycle

analysis (8, 18, 19). HeLa cells were treated with DFMO for 4 days. At the specified periods, the treated cells were fused with a synchronized population of mitotic HeLa cells to induce PCC, chromosome preparations were made and scored for the frequency of the various types of PCC, i.e., G1, S, and G2. The percentage of cells in S phase was further confirmed by pulse labeling the interphase cells for 20 min just before fusion and then determining the labeling indices after autoradiography.

Measurement of polyamine levels. The cell pellet was resuspended in 0.2 ml of 4% sulfosalisylic acid and sonicated using a Branson Sonifier. The homogenate was then centrifuged at 10,000 x g in a Sorvall RC2B centrifuge at 4°C. The supernatant so obtained was used to estimate the polyamine levels in a Durrum amino acid analyzer according to Marton and Lee (20). The results presented represent the average of at least two estimates.

Effect of sequential administration of DFMO and Ara-C on HeLa cell growth. For this study, we have grown exponential cultures of HeLa cells with or without a 2.5-mM concentration of DFMO for days. At the end of the incubation period one half of both, the control and the DFMO-treated cells were subjected to a 16-hr Ara-C (50 µg/ml) treatment followed by incubation in regular medium. While the other half served as control i.e., grown in regular medium. The increase in cell number was determined over a period of 4 days by trypsinizing the cells and counting them by the use of a Coulter particle counter. The data presented are an average from two experiments.

RESULTS AND DISCUSSION

Effect of DFMO on polyamine levels and growth of HeLa cells.

Our initial studies indicated that DFMO at a concentration of 2.5 mM caused the maximum inhibition of growth of HeLa cells without any obvious cell damage. Hence, this concentration was used throughout the study. The inhibition of HeLa cell growth by DFMO was apparent from the second day of the treatment (Fig. 1), at which time both the intracellular levels of putrescine and spermidine were found to be very low (Fig. 2) without any significant change in the levels of spermine. This observation confirms that both putrescine and spermidine are required for optimal cell proliferation (1, 2, 3, 10, 21). In spite of the total inhibition of the biosynthesis of putrescine and spermidine, some increase in cell number was observed in DFMO-treated cultures, probably because of the compensation for the polyamine requirement by spermine. Replacement of DFMO-containing medium

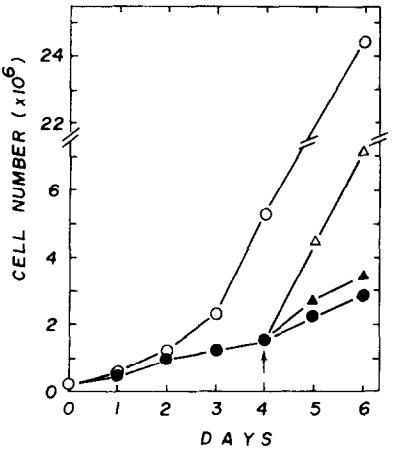

Fig. 1. Effect of DFMO on HeLa cell growth. O , control; ●, DFMO (2.5 mM) treatment. ▲ , replaced with regular medium following DFMO treatment; Δ , replaced with putrescine (50 μM) containing medium following DFMO treatment.

with fresh regular medium on the fourth day caused a slow recovery of HeLa cell growth (Fig. 1). However, addition of putrescine (50 μM) to the DFMO-treated cultures on the fourth day caused a rapid increase in growth, which was comparable to that of the untreated cultures. The addition of putrescine caused an increase in the levels of both putrescine and spermi-

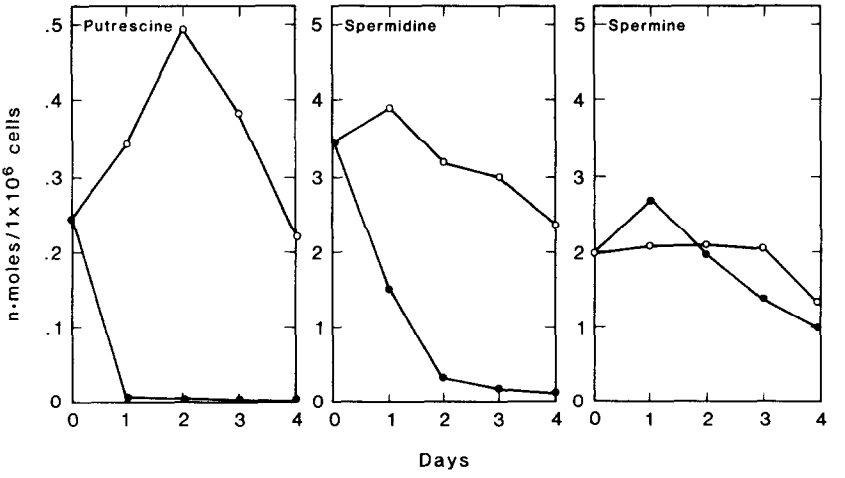

Fig. 2. Effect of DFMO on polyamine levels in HeLa cells. O, control; ● , DFMO-treated.

dine without significant effect on the spermine levels (not presented). Therefore, the growth inhibition caused by DFMO is probably due to the depletion of putrescine and spermidine levels as a results of its inhibitory effect on the ornithine decarboxylase, the rate-limiting enzyme in the polyamine biosynthetic pathway (11).

Effect of DFMO on the cell cycle traverse of HeLa cells.

We have employed the PCC method to determine the cell cycle kinetics of DFMO-treated cutlrues. The results indicate that by 24 hrs there was a significant accumulation of cells in G1 phase (Table-1). During the following days, a gradual increase in the S-phase cells was observed with a simultaneous decrease in the G1 population. By the fourth day, there was a significant increase (74%) in the S-phase population, with about 24% of the cells in G1 phase of the cell cycle. Further, all these G1 cells showed a late G1 morphology. These observations indicate that the intracellular depletion of putrescine and spermidine causes a slow progression of cells through S phase. This is further confirmed by a rapid increase (about threefold) in the cell growth observed between fourth and fifth day after the

Table 1.

EFFECT OF DFMO (2.5 mM) TREATMENT ON THE CELL CYCLE TRAVERSE OF HELA CELLS.

TREATMENT	Frequency (%) PCC in G1	S	G2
Control	46.0	44.0	10.0
Days of Treatment			
Day 1	80.0	18.0	2.0
Day 2	73.0	25.0	2.0
Day 3	64.0	36.0	0
Day 4	24.0	74.0	2.0

addition of putrescine-containing medium. Since the generation time of HeLa cells is about 22-hr to 24 hr, this increase would only be possible if the HeLa cells are synchronized nearer to mitosis before the reinitiation of growth by putrescine. These results are in agreement with our earlier study wherein we found an increase in the S-phase population in HeLa cells using other inhibitors of polyamine synthesis, namely, MGBG and α-MO (8).

Synergistic effect of sequential administration of DFMO and Ara-C on HeLa cell growth.

Since a significant increase in the S-phase population was observed after a four-day treatment with DFMO, we decided to examine whether exposure to an S phase-specific durg, i.e., Ara-C, would increase the toxicity to HeLa cells. The results presented in Fig. 3 indicate that 16-hr treatment with Ara-C had a significant effect on the DFMO-treated populations when compared to exponentially growing control cells. The DFMO-treated cells reversed wtih fresh medium, slowly recovered, and started growing by the second day. However, cells treated both

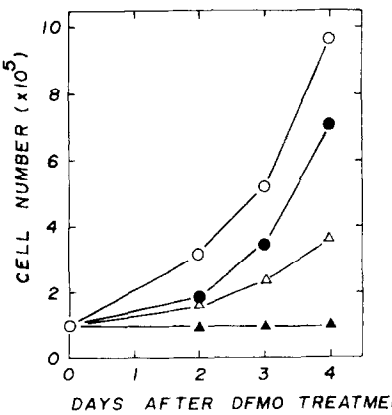

Fig. 3. Synergistic cytotoxicity of Ara-C following DFMO treatment. ○ , control; ● , control cultures treated with Ara-C (50 μg/ml) for 16 hr and replaced with regular medium; △ , DFMO-treated cultures replaced with regular medium; ▲ , DFMO-treated cultures treated with Ara-C (50 μg/ml) for 16 hr and replaced with regular medium.

with DFMO and Ara-C did not recover, and no change in the cell counts were seen even 4 days following the drug treatments. This indicates a synergistic killing of the tumor cells by the sequential treatment of DFMO and Ara-C. Currently, we are testing whether this observation holds true in experimental animal tumors.

The results of this study indicate that α-difluoromethyl ornithine inhibits HeLa cell growth by causing a decrease in the intracellular levels of putrescine and spermidine. This inhibition is readily reversible by an exogenous supply of putrescine to the medium. The DFMO treatment also causes an accumulation of cells in S phase and the use of an S phase-specific drug like Ara-C following DFMO treatment results in a synergistic killing of the tumor cells as revealed by the complete inhibition of cell growth. Thus, it may be possible to design effective therapeutic protocols combining the inhibitors of polyamine biosynthesis with other cytotoxic drugs.

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