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Decreased Cell Kill of Vincristine and Methotrexate against 9L Rat Brain Tumor Cells *in Vitro* Caused by α-Difluoromethylornithine-induced Polyamine Depletion[1]

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ABSTRACT

The effect of polyamine depletion on the cell kill caused by the cell cycle-specific agents vincristine (VCR) and methotrexate (MTX) was studied in 9L rat brain tumor cells *in vitro* using a colony-forming efficiency assay as the experimental end point. The cell kill produced by a 24-hr treatment with VCR or MTX was decreased in 9L cells pretreated with 1 mM α-difluoromethylornithine, an irreversible inhibitor of ornithine decarboxylase. Reversal of the α-difluoromethylornithine-induced polyamine depletion with 1 mM exogenous putrescine prevented the decrease in VCR and MTX cytotoxicity. After a 48-hr treatment with 1 mM α-difluoromethylornithine, the number of mitotic cells in asynchronously growing 9L cell cultures was reduced markedly. The decreased cell kill of VCR and MTX appeared to be the result of polyamine depletion-induced inhibition of 9L cell cycle traverse, which reduced the number of cells in drug-sensitive phases of the cell cycle and thereby reduced the cell kill caused by the drugs.

INTRODUCTION

Since the publication of the first reports that DFMO[4] depletes intracellular polyamine levels and that the cell-killing effects of some anticancer drugs are potentiated in polyamine-depleted cells, the effects of treatment with combinations of polyamine biosynthesis inhibitors and clinically useful anticancer drugs with different mechanisms of action have been studied. The cell kill caused by 1,3-bis(2-chloroethyl)-1-nitrosourea and other chloroethylnitrosourea is increased in 9L cells pretreated with DFMO (6, 8). We postulated that polyamine depletion changes the conformation of cellular DNA in such a way that the formation of DNA interstrand cross-links, which, for this agent, is thought to be the molecular event that kills cells, is increased. An increase in cross-link formation caused by this drug combination has been confirmed experimentally (21).

By simple analogy, the cell killing of other cross-linking agents should be increased in polyamine-depleted cells. However, we found that the cell kill caused by the cross-linking agents *cis*-diamminedichloroplatinum(II) (7) and aziridinylbenzoquinone (1) is decreased by DFMO-induced polyamine depletion. The change in DNA structure caused by DFMO-induced polyamine depletion should be a constant factor; however, *cis*-diamminedichloroplatinum(II), aziridinylbenzoquinone and chloroethylnitrosoureas form cross-links through different mechanisms, and polyamine depletion-induced modification of cell kill obviously is agent dependent.

In addition to influencing the cross-linking of DNA, polyamine depletion influences drug action by modifying transmembrane drug transport. The cytotoxic agent MGBG, a spermidine analogue that is an inhibitor of *S*-adenosylmethionine decarboxylase, is taken up faster by polyamine-depleted cells than by untreated cells (2). Pretreatment of Ehrlich ascites cells with DFMO causes MGBG to be accumulated rapidly to intracellular levels that are toxic (16). The concept of using sequential administration of DFMO and MGBG is being investigated as a possible treatment for acute childhood leukemias (17).

Sunkara *et al.* (19) have shown that HeLa cells treated with DFMO accumulate in S phase and are killed more effectively by the S-phase-specific agent ara-C than are HeLa cells treated with ara-C alone. Rupniak and Paul (14) and Sunkara *et al.* (18) have shown that a variety of "normal" cell lines accumulate in G_1 phase but that a variety of transformed cells accumulate in S phase after treatment with polyamine biosynthesis inhibitors. This result suggests that, *in vivo*, pretreatment with a polyamine biosynthesis inhibitor might increase the relative cell kill of tumor cells treated with S phase-specific agents while simultaneously protecting normal tissues. In mice bearing L1210 leukemia, the percentage of normal bone marrow cells in S phase is decreased by DFMO treatment, whereas leukemic cells accumulate in S phase; treatment with DFMO and ara-C increases the survival of mice bearing L1210 leukemia (13). However, we have found that pretreatment of 9L cells with DFMO dramatically inhibits the cell killing effect of ara-C (9). Although 9L is a transformed line, flow cytometric analysis has shown that cells do not accumulate in S phase after being treated with DFMO (15). Thus, treatment with the same combination of agents produces different results when used against different cell lines, which emphasizes the need to investigate the response of several cell lines.

In the study reported here, we investigated the effect of DFMO pretreatment on the cell kill of 9L monolayer cells caused by the cell cycle-specific drugs VCR and MTX. VCR inhibits polymerization of microtubules and is toxic to cells in mitosis (22). Bartholeyns and Koch-Weser (3) have shown that the combination of vindesine and DFMO is effective *in vivo*. MTX, a folate analogue that inhibits dihydrofolate reductase and blocks synthesis of deoxythymidylate, is specific for cells in S phase (4). We found that, compared to 9L cells not pretreated with DFMO, the cell kill caused by VCR or MTX was decreased by DFMO-induced polyamine depletion.

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[4] The abbreviations used are: DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); ara-C, 1-β-D-arabinofuranosylcytosine; MTX, methotrexate; VCR, vincristine; PU, putrescine.

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MATERIALS AND METHODS

Drugs. DFMO was generously provided by the Merrell-Dow Research Institute (Cincinnati, OH). VCR (Lilly) and MTX (Bristol Laboratories) were clinical preparations for i.v. injection obtained from the hospital pharmacy. PU was purchased from Calbiochem-Behring Corp. (La Jolla, CA).

Stock solutions of DFMO and PU (500 mM) were prepared in Hanks' balanced salt solution, neutralized, sterile filtered, and stored at −20°. Sterile solutions of VCR and MTX were diluted in Hanks' balanced salt solution and stored at 8°.

Cell Culture. 9L rat brain tumor cells were grown in monolayer culture in Eagle's minimal essential medium supplemented with 10% newborn calf serum, nonessential amino acids, and gentamicin (50 μg/ml). Cells were plated in 75-sq cm tissue culture flasks in 15 ml of medium approximately 24 hr before drug treatment.

In order to minimize differences in cell densities at the beginning of treatment with either VCR or MTX, control flasks were seeded with 1×10^5 cells, flasks to be pretreated with 1 mM DFMO were seeded with 4×10^5 cells, and flasks to be treated with PU after 48 hr of DFMO pretreatment (reversal studies) were seeded with 2×10^5 cells. In the polyamine replenishment experiments, cells were pretreated with 1 mM DFMO for 48 hr, and then 1 mM PU was added for 24 hr. At 72 hr, cells were treated with various concentrations (given in the charts) of VCR or MTX for an additional 24 hr. After treatment, cells were trypsinized and plated for the colony-forming efficiency assay (5). Controls cells were treated with Hanks' balanced salt solution. DFMO alone was not toxic to 9L cells.

Determination of Mitotic Indices. Control flasks were seeded with 5×10^5 cells, and flasks to be treated with 1 mM DFMO for 48 hr were seeded with 1×10^6 cells. After 48 hr of DFMO treatment, cells were trypsinized and treated with Carnoy's fixative, and chromosomes were stained with Giemsa using a reported procedure (11). Approximately 2000 cells were scored in each instance, and the fraction of mitotic cells was calculated.

RESULTS

Survival Studies. Survival plots for 9L cells treated for 24 hr with VCR under various conditions are shown in Chart 1. The maximum cell kill caused by treatment with VCR alone for 24 hr was approximately 1 log. Pretreatment of cells with 1 mM DFMO for 72 hr before treatment with VCR decreased the cell kill by approximately 50%. When polyamine depletion was reversed by addition of 1 mM PU for 24 hr before treatment, the decrease in VCR cell kill was prevented.

Survival plots for cells treated with MTX for 24 hr under various conditions are shown in Chart 2. The maximum cell kill caused by MTX alone was approximately 2 logs. Depletion of polyamines by a 72-hr treatment with 1 mM DFMO reduced the cell kill of MTX by approximately 1 log. Addition of 1 mM PU to DFMO-treated cultures 24 hr before treatment prevented the decrease in MTX cell kill.

Determination of Mitotic Indices. The mitotic index of untreated 9L cells was 1.80, and the mitotic index for 9L cells treated with 1 mM DFMO for 48 hr was 1.06.

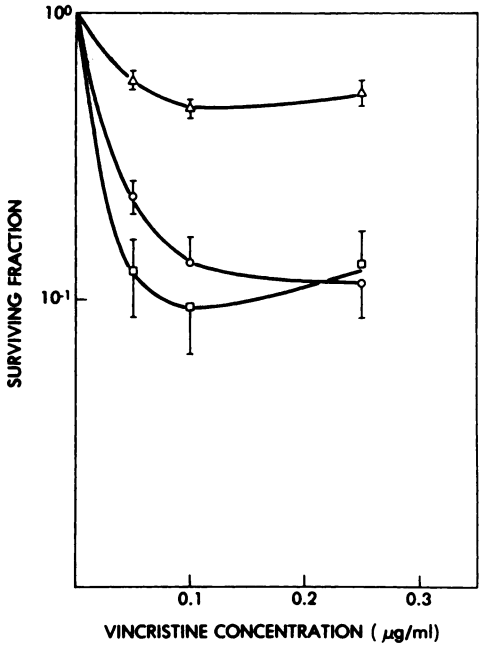

Chart 1. Effect of polyamine depletion by DFMO on the cytotoxicity of VCR in 9L cells. O, untreated for 72 hr, followed by VCR for 24 hr; Δ, 1 mM DFMO for 96 hr and VCR for the last 24 hr; □, 1 mM DFMO for 96 hr, 1 mM PU for the last 48 hr, and VCR for the last 24 hr. *Points*, means; *bars*, S.D. of 4 to 8 dishes.

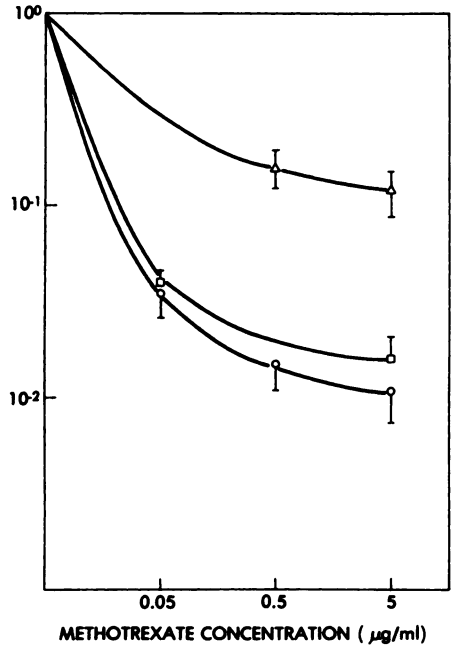

Chart 2. Effect of polyamine depletion by DFMO on the cytotoxicity of MTX in 9L cells. O, untreated for 72 hr, followed by MTX for 24 hr; Δ, 1 mM DFMO for 96 hr and MTX for the last 24 hr; □, 1 mM DFMO for 96 hr, 1 mM PU for the last 48 hr, and MTX for the last 24 hr. *Points*, means; *bars*, S.D. of 4 to 8 dishes.

DISCUSSION

It has been reported that polyamines induce polymerization of actin *in vitro* (10), that MGBG decreases the amount of actin filaments in human fibroblasts (20), and that polyamine starvation causes the disappearance of microfilaments in a CHO variant cell line (12). These findings suggest that DFMO-induced polyamine depletion might alter these cellular constituents in 9L cells and perhaps increase the cell-killing effects of VCR. However, we have found that the presence and distribution of actin filaments, intermediate filaments, and microtubules, as measured by indirect immunofluorescence, are unchanged from controls in 9L, V79, and NRK fibroblast cells treated with either DFMO or MGBG and that treatment of 9L cells with DFMO and MGBG does not affect repolymerization of microtubules that have been

depolymerized with nocodazole.[5] Thus, because polyamine depletion did not affect these structures in 9L cells, *a priori*, the cell kill of VCR should not be increased by polyamine depletion secondary to that mechanism.

However, DFMO pretreatment causes a decrease in the cell-killing effect of VCR. This finding is consistent with the fact that DFMO treatment delays progression of 9L cells through the cell cycle (15) and with the fact that the mitotic index of 9L cells treated with DFMO is reduced compared with untreated controls. It is possible that, if synergistic effects were operative, the effect would be masked by the overpowering effect of DFMO-induced perturbation of cell cycle progression.

We have shown that the cell killing effect of ara-C, an S phase-specific agent, is markedly decreased in 9L cells pretreated with DFMO (9). Based on the results of flow cytometric studies, we have shown that this decrease is caused by the effects of polyamine depletion on the 9L cell cycle. It seems reasonable to suggest that the decrease in VCR and MTX cell kill caused by DFMO-induced polyamine depletion is the result of polyamine-mediated perturbation of cell cycle progression. Cells were treated for 24 hr with both VCR and MTX, a period of sufficient length that all cycling cells would have entered phases affected by either drug if cells grew at the normal rate (doubling time of approximately 20 hr). However, because continuous treatment with DFMO markedly slows the traverse of 9L cells through the cycle and eventually causes cytostasis (15), DFMO pretreatment undoubtedly decreased the number of target cells available for VCR and MTX at the time of treatment. Because it is not possible to distinguish between G_2 and M phase cells using flow cytometry, we determined the percentage of mitotic cells with a Giemsa staining technique. Our results suggest that DFMO treatment decreased the fraction of 9L cells passing through M phase.

Whether or not polyamine depletion affects a specific cellular constituent that is important to the cell kill caused by a chemotherapeutic agent, the effects of polyamine depletion on cell cycle traverse may affect the outcome of subsequent treatment with any chemotherapeutic agent, particularly with cell cycle-specific agents. Study of more *in vitro* and *in vivo* models that are affected differently by polyamine depletion may provide data with which appropriate therapies based on combinations of polyamine inhibitors and a variety of chemotherapeutic agents can be developed.

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