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Time Dependence of the Potentiation of 1,3-Bis(2-chloroethyl)-1-nitrosourea Cytotoxicity Caused by α-Difluoromethylornithine-induced Polyamine Depletion in 9L Rat Brain Tumor Cells[1]

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ABSTRACT

Treatment of 9L rat brain tumor cells with 1.0 mM α-difluoromethylornithine (DFMO) produced a time-dependent depletion of cellular putrescine and spermidine. An increase in the potentiation of 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) cytotoxicity, measured with a colony-forming efficiency assay, followed the time course of polyamine depletion, reaching its maximum at 48 hr, the time at which maximum polyamine depletion was achieved.

Treatment with DFMO at concentrations as low as 0.05 mM for 48 hr effectively depleted putrescine and spermidine and potentiated BCNU cytotoxicity. Treatment for 96 hr with 0.01 mM DFMO produced a partial decrease in putrescine and spermidine levels and a moderate potentiation of BCNU cytotoxicity. The amount of polyamine depletion in 9L cells treated with 0.05, 0.1, and 0.5 mM DFMO was identical at both 48 and 96 hr, but potentiation of BCNU cytotoxicity was greater at 96 hr than at 48 hr.

When 9L cells were treated for 48 hr with 1 mM DFMO and DFMO was then removed from the cultures, polyamine levels did not reach control levels by 96 hr after change of medium. The potentiation of BCNU cytotoxicity during this 96-hr period correlated with the extent of polyamine depletion. When 100 μM putrescine was added to the culture medium after DFMO pretreatment (1 mM), polyamine levels approached those of control cells by 24 hr, and the amount of potentiation of DFMO cytotoxicity decreased. These results show that potentiation of BCNU cytotoxicity correlates closely with the amount of DFMO-induced polyamine depletion in 9L cells.

INTRODUCTION

We have shown that, in 9L rat brain tumor cells, the cytotoxicities of the chloroethylnitrosoureas (4, 6, 9, 10), *cis*-platinum[4] (3, 8), and aziridinylbenzoquinone (1), anticancer drugs that are thought to affect cellular DNA, can be modified by pretreating cells with DFMO or MGBG, inhibitors of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase, respectively (7, 14). We suggested that DFMO-induced polyamine depletion destabilizes DNA and, depending on the structure of and/or the mechanism of action of the drug, either increases or decreases the cytotoxicity of the drug. This hypothesis is supported by data obtained with viscoelastometry that showed that conformational changes in 9L cell DNA are caused by DFMO-induced polyamine depletion (5). DFMO pretreatment potentiated BCNU-induced sister chromatid exchanges in 9L cells but decreased the number of exchanges induced by *cis*-platinum (13). Recently, we have shown that the number of DNA cross-links caused by BCNU in 9L cells is increased but that the number of DNA cross-links caused by *cis*-platinum is decreased by DFMO pretreatment (12). If polyamine levels in 9L cells were restored by adding exogenous PU to culture medium after cells were treated with DFMO, all of these effects were prevented.

In the study reported here, we examined the time course of polyamine depletion caused by DFMO and correlated potentiation of BCNU cytotoxicity with the amount of polyamine depletion at the time of BCNU treatment. No potentiation was observed until intracellular SD levels had decreased by approximately 40%, and PU by approximately 70% of control levels, with essentially no change in SP levels. The increase in polyamine levels and the decrease of the potentiation of BCNU cytotoxicity were temporally related after PU was added to restore polyamine levels in polyamine-depleted cells.

MATERIALS AND METHODS

Drugs. DFMO was generously provided by the Merrell-Dow Research Institute (Cincinnati, OH). BCNU was provided by the National Cancer Institute. PU was purchased from Calbiochem-Behring Corp. (La Jolla, CA). Stock solutions of DFMO (500 mM) and PU (500 mM) were prepared in Hanks' balanced salt solution, sterile filtered, and stored at −20°. Immediately before use, BCNU was dissolved in ethanol to give a 5 mM stock solution. The final ethanol concentration in culture medium did not affect the plating efficiency of 9L cells.

Cell Culture. 9L rat brain tumor cells were grown in monolayer culture in Eagle's minimal essential medium supplemented with nonessential amino acids, 10% newborn calf serum, and gentamicin (50 μg/ml). To obtain approximately equal cell numbers in control and DFMO-pretreated cultures at the time of BCNU treatment, different numbers of cells were seeded into culture flasks at the start of an experiment. Three sets of experiments were performed. In the first, 2.5×10^5 cells (control) and 5×10^5 cells (to be pretreated with 1 mM DFMO) were seeded in 15 ml of medium. DFMO was added to cultures after early exponential growth had been established. Polyamine levels and potentiation of BCNU cytotoxicity were measured at 0, 8, 24, 48, 72, and 96 hr after DFMO addition as described below. In the second set of experiments, 1 to 2.5×10^5 cells (control) and 4 to 5×10^5 cells (to be pretreated with 0.01, 0.05, 0.1, and 0.5 mM DFMO) were seeded in 15 ml of medium. Polyamine levels and potentiation of BCNU cytotoxicity were measured at 48 and 96 hr after DFMO addition. In the third set of experiments, 1×10^4 to 2.5×10^5 cells (control) and 2.5 to 5×10^5 cells (to be pretreated with 1

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[4] The abbreviations used are: *cis*-platinum, *cis*-diamminedichloroplatinum(II); DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; PU, putrescine; SD, spermidine; SP, spermine.

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mM DFMO for 48 hr) were seeded in 15 ml of medium. After pretreatment, the medium was changed in all flasks, and a number of the DFMO-pretreated flasks received 100 μM PU. Polyamine levels and potentiation of BCNU cytotoxicity were measured at 0, 24, 48, 72, and 96 hr after the medium change.

To determine the potentiation of BCNU cytotoxicity at each time point in each set of experiments, cells were treated with a final concentration of 30 μM of BCNU for 1 hr. Cells were then trypsinized and replated for the colony-forming efficiency assay (2). In all experiments, DFMO alone was not cytotoxic to 9L cells.

For polyamine analysis, trypsinized cells were pelleted at 1000 rpm for 5 min at 4°, and pellets were stored at −20° until analyzed. The assay has been described (11).

RESULTS

Treatment of 9L cells with 1 mM DFMO produced a rapid decrease in intracellular PU levels, a somewhat slower depletion of SD levels, and no significant change in SP levels (Chart 1). At each time point, the potentiation of BCNU cytotoxicity was measured after incubating the cells for 1 hr with 30 μM BCNU, a dose that caused more than 1.5 log cell kill when used alone.

As a relative measure of the potentiation of BCNU cytotoxicity, a potentiation factor, the ratio of the surviving fraction of BCNU-treated cells to the surviving fraction of BCNU-treated cells that were pretreated with DFMO, was calculated. The potentiation factor is quite sensitive to the actual surviving fractions of untreated and DFMO-pretreated cells and may differ because of minor variations in BCNU concentrations in culture medium. Thus, although potentiation factors may not be directly comparable in magnitude from one experiment to another, they nevertheless provide an estimate of potentiation at a given time point. The results shown are representative of 3 to 4 experiments. Data for the relative potentiation factor are plotted in Chart 1 as a function of the time of DFMO pretreatment. PU and SD depletion was accompanied by potentiation of BCNU cytotoxicity, but, from data for the 8-hr time point, it appears that PU depletion alone is insufficient to induce potentiation; significant potentiation of BCNU cytotoxicity occurs only after SD levels have been depleted (by 24 hr after treatment). However, the potentiation factor falls off at 96 hr despite the fact that polyamine content remained depleted at a constant level.

We found that confluent 9L cells are significantly more sensitive to BCNU than are exponentially growing cells. By comparing the actual surviving fractions listed in Table 1, it is apparent that the same dose of BCNU results in an almost 1-log-lower surviving fraction when given to confluent cells at 96 hr than to exponentially growing cells at 72 hr. When the potentiation factor is assessed at 96 hr, an artificially low value may be obtained, because of the increased sensitivity to BCNU of control confluent cells compared to nonconfluent DFMO-treated cells. This increased sensitivity may be related to the lower polyamine levels (50, 75, and 87% for PU, SD, and SP, respectively) in confluent cells compared to exponentially growing 9L cells.

Table 1

Potentiation of BCNU cytotoxicity by pretreatment with 1 mM DFMO in 9L cells

Length of pretreatment (hr)	DFMO present	Surviving fraction	Potentiation factor
8	No	$7.97 \pm 0.56 \times 10^{-2}$ [a]	
	Yes	$7.56 \pm 0.93 \times 10^{-2}$	1.1
24	No	$4.71 \pm 0.77 \times 10^{-2}$	
	Yes	$1.80 \pm 0.18 \times 10^{-2}$	2.6
48	No	$2.77 \pm 0.36 \times 10^{-2}$	
	Yes	$6.60 \pm 1.0 \times 10^{-3}$	4.2
72	No	$2.18 \pm 0.21 \times 10^{-2}$	
	Yes	$5.78 \pm 0.87 \times 10^{-3}$	3.8
96	No	$2.90 \pm 0.90 \times 10^{-3}$	
	Yes	$1.12 \pm 0.06 \times 10^{-3}$	2.6

[a] Mean ± S.D. of 4 to 8 dishes for each point.

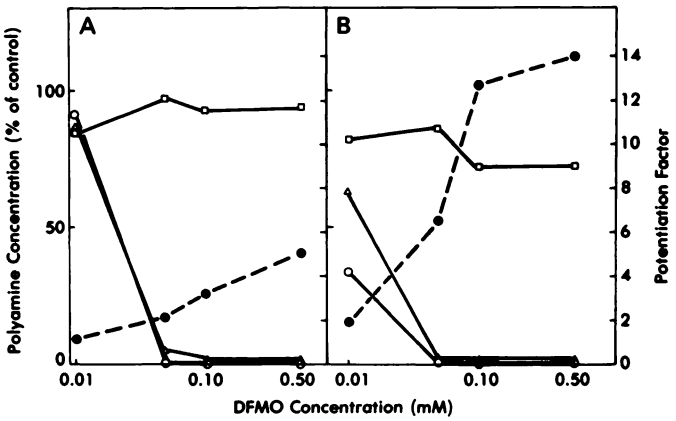

Chart 2. Effect of pretreatment of 9L cells with low DFMO concentrations on the potentiation of BCNU cytotoxicity. The cells were pretreated with 0.01, 0.05, 0.1, or 0.5 mM DFMO for 48 hr (*A*) or 96 hr (*B*). Polyamine concentrations in cells pretreated with DFMO are expressed as the percentage of respective control levels. ○, PU; △, SD; □, SP; ●, potentiation of BCNU cytotoxicity by DFMO.

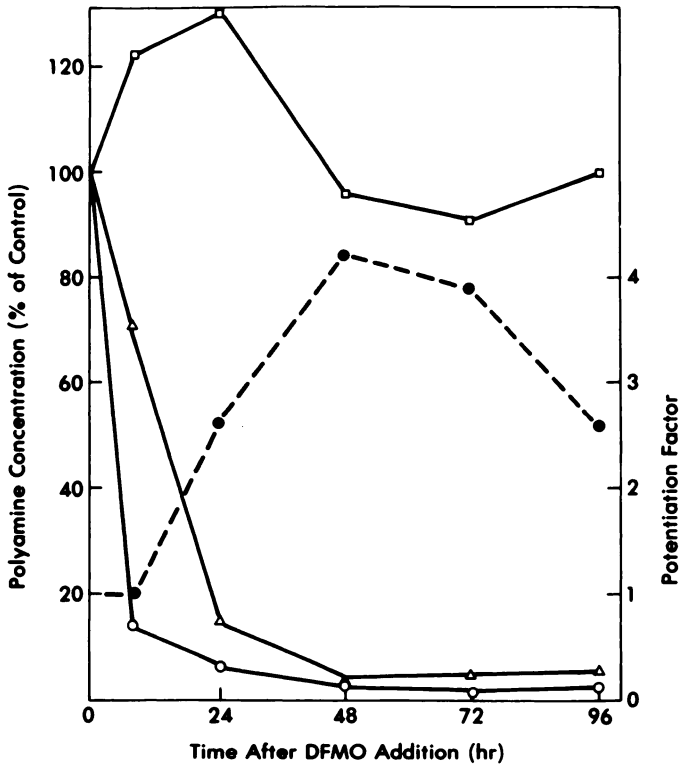

Chart 1. Relation of polyamine depletion by DFMO to the potentiation of BCNU cytotoxicity against 9L cells. Polyamine concentrations in cells pretreated with 1 mM DFMO are expressed as the percentage of control levels at each time point. ○, PU; △, SD; □, SP; ●, potentiation of BCNU cytotoxicity by DFMO.

Table 2

Effect of restoration of depleted polyamine pools on the potentiation of BCNU cytotoxicity caused by DFMO

9L cells were pretreated with 1 mM DFMO for 48 hr, after which cells were grown in fresh medium either with or without 100 μM PU. Polyamine levels and potentiation are expressed relative to control cells that were not pretreated with DFMO.

Time after DFMO removal (hr)	PU present	Polyamines (% of control)			Potentiation factor
		PU	SD	SP	
0	No	3.5	4.3	119	3.4
24	No	3.3	2.7	80	10.1
	Yes	25	103	116	1.4
48	No	2.7	3.5	81	10.4
	Yes	39	85	106	0.7
72	No	3.4	4.9	83	3.7
	Yes	57	89	97	0.3
96	No	4.0	5.7	96	5.7
	Yes	72	104	102	0.5

To determine the minimum DFMO concentration that could cause potentiation of BCNU cytotoxicity, we pretreated 9L cells with 0.01, 0.05, 0.1, and 0.5 mM DFMO for 48 and 96 hr. Data plotted in Chart 2 show that, after a 48-hr pretreatment (*A*), PU and SD levels were depleted fully for treatment with concentrations of 0.05 mM or greater, whereas treatment with 0.01 mM did not significantly affect polyamine levels. BCNU cytotoxicity was potentiated in cells treated with 0.05, 0.1, and 0.5 mM DFMO; a 96-hr treatment with 0.01 mM DFMO only partially depleted polyamine levels and therefore produced only a moderate potentiation of BCNU cytotoxicity (Chart 2*B*). Pretreatment with higher DFMO concentrations for 96 hr produced greater polyamine depletion and a more pronounced potentiation of BCNU cytotoxicity than was achieved by equimolar DFMO treatment for 48 hr (Chart 2*B*). Based on these data and on the fact that depletion of PU alone is insufficient to cause potentiation (Chart 1), it appears that a concomitant reduction of SD levels by approximately 40% must be achieved, with essentially no change in SP levels, before significant potentiation of BCNU cytotoxicity occurs in 9L cells.

Polyamine levels in cells that had been pretreated with 1 mM DFMO for 48 hr were determined for various times after the inhibitor had been removed. Immediately after pretreatment, BCNU cytotoxicity was potentiated by a factor of 3.4, and PU and SD levels were depleted to less than 5% of the control levels (Table 2). After depletion, polyamine levels in cells were unchanged by 96 hr after DFMO was removed, and potentiation of BCNU cytotoxicity remained evident. It should be noted that, in this experiment, control cells were not confluent at 96 hr. If 100 μM exogenous PU was added to DFMO-pretreated cells at the time medium was changed, because PU that had been taken up by cells was converted rapidly to SD, SD levels were restored by 24 hr, and the potentiation factor decreased from 10.1 to 1.4 (Table 2). Restoration of SD levels to 63% of control levels could be achieved by adding PU to achieve a concentration in medium of 10 μM for 24 hr, which resulted in complete loss of potentiation (data not shown). These data lend additional support to the observation that an approximate 40% depletion (*versus* control) of SD with depleted PU and with essentially no change in SP levels is needed for any potentiation of BCNU cytotoxicity to occur. The fact that, after addition of PU, potentiation factors fell below unity at 48 to 96 hr seems to indicate that cells were actually protected against BCNU by exogenous PU, although intracellular polyamine levels did not exceed control levels (Table 2).

DISCUSSION

We have reported the DFMO-induced potentiation of the cytotoxicity caused by BCNU (4) or by other bifunctional chloroethylnitrosoureas (9, 10). In previous studies, pretreatment for 48 or 72 hr with 10 mM DFMO was used to produce polyamine depletion. Results reported here show that considerable potentiation of BCNU cytotoxicity can be seen, even after only a 24-hr pretreatment with 1 mM DFMO, at which time PU and SD levels were depleted significantly (Chart 1). It appears that depleting levels of PU alone are not sufficient to cause potentiation. However, with a concomitant 40% depletion of SD and with levels of SP essentially unchanged, potentiation is achieved. The level of potentiation remains high as long as polyamine depletion is maintained, if DFMO-treated cells are compared with exponentially growing cells that are synthesizing polyamines. Polyamine depletion and potentiation of BCNU cytotoxicity are produced after prolonged treatment with concentrations of DFMO as low as 0.01 mM. These findings are relevant to the clinical use of combinations of BCNU and DFMO because even higher levels of DFMO can be achieved in humans (3).

We have regarded the prevention of potentiation after reversal of polyamine depletion by exogenous PU as strong evidence for the polyamine dependence of BCNU potentiation. Unfortunately, we were not able to show that potentiation decreased when polyamine levels in DFMO-pretreated cells were restored by reinitiation of intracellular polyamine synthesis, because such reinitiation of synthesis did not take place in the experiments described (Table 2). Although the half-life of ornithine decarboxylase in 9L cells is brief, as has been found for other cell lines, removal of DFMO from the culture medium was not adequate to allow for the initiation of polyamine synthesis during the experimental period. Perhaps DFMO is sequestered in 9L cells at sufficient levels that enzyme activity continues to be inhibited, or perhaps the covalent bonding of DFMO to the enzyme alters the synthesis and/or metabolism of the enzyme. Because our control cells reach confluency by the end of the experimental period, it was not possible to extend the length of the experiments.

We have shown that MGBG pretreatment also potentiates BCNU cytotoxicity against 9L cells (6). SD and SP, but not PU, are depleted by MGBG. The effects of lowered levels of SD and SP are probably critical in any induced change in DNA conformation, and an adequate depletion of one of these compounds, or a lesser depletion of both, may induce the same changes. In confluent 9L cells, SD is not depleted to 40% of levels in exponentially growing cells; however, SP is significantly depleted in contrast to cells treated with DFMO. This combined depletion may be adequate to cause BCNU potentiation. Of interest is the fact that *cis*-platinum is less cytotoxic to confluent than to exponentially growing 9L cells,[5] which is in agreement with our finding that DFMO-induced polyamine depletion decreases the cytotoxicity of *cis*-platinum against 9L cells (8).

Potentiation of BCNU cytotoxicity does not appear to correlate strictly with total polyamine levels. First, a prolonged treatment

[5] P. J. Tofilon, D. F. Deen, and L. J. Marton, unpublished results.

(96 hr) with 0.05 to 0.5 mM DFMO appeared to increase potentiation, even though levels of polyamines appear to be unchanged from those observed after 48 hr of treatment (Chart 2). Second, an almost complete reversal of DFMO-induced polyamine depletion by PU not only prevented potentiation but appeared to protect cells against BCNU, although intracellular polyamine levels did not exceed control levels (Table 2). These results may reflect the occurrence of other polyamine-dependent cellular effects, such as direct effects on alkylation, cross-linking, or repair mechanisms, in addition to changes in DNA conformation. Intracellular compartmentation of polyamines is also a matter that must be considered. We have studied only the acid-soluble free polyamines. The subcellular distribution of polyamines may be an important factor that should be considered when appropriate methods for the analysis of intracellular distribution of these compounds become available.

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