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Possible Factors in the Potentiation of 1-(2-Chloroethyl)-3-*trans*-4-methylcyclohexyl-1-nitrosourea Cytotoxicity by α-Difluoromethylornithine in 9L Rat Brain Tumor Cells*

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Abstract—*Depletion of intracellular levels of polyamines in 9L rat brain tumor cells by α-difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ornithine decarboxylase, significantly enhanced the cytotoxicity of* 1-(2-*chloroethyl*)-3-trans-4-*methylcyclohexyl*-1-*nitrosourea (MeCCNU)* in vitro *as measured by a colony-forming efficiency assay. Administered as a single agent, DFMO was not cytotoxic to 9L cells. Treatment for* 48 hr *with* 10, 1, 0.5 *or* 0.1 mM *DFMO produced similar levels of polyamine depletion and similar potentiation of MeCCNU cytotoxicity. Restoration of intracellular polyamine levels by the addition of exogenous putrescine* (1 mM) *to treated cells prevented the potentiation of MeCCNU, which indicates that this phenomenon might be the result of polyamine depletion. DNA adduct formation in polyamine-depleted and control cells was studied with* [^{14}C]-*MeCCNU; no difference in monoadduct formation was found between polyamine-depleted and control cells. Experiments to determine whether polyamine depletion has an effect on enzymes involved in the repair of alkylated bases showed that the activity of* O^6-*methylguanine-DNA demethylase,* 7-*methylguanine-DNA glycosylase and* 3-*methyladenine-DNA glycosylase were unaffected by* 48 hr *of treatment with* 10 mM *DFMO. DFMO treatment causes a substantial increase in the intracellular content of decarboxylated* S-*adenosyl*-L-*methionine, which was reversed by addition of putrescine. The possibility that the elevation of decarboxylated* S-*adenosyl*-L-*methionine rather than the depletion of polyamines is responsible for the effects of DFMO is discussed.*

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Abbreviations: Pu, putrescine; Sd, spermidine; Sp, spermine; DFMO, α-difluoromethylornithine; MGBG, methylglyoxal bis(guanylhydrazone); BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; *cis*-platinum, *cis*-dichlorodiammineplatinum II; MeCCNU, 1-(2-chloroethyl)-3-*trans*-4-methylcyclohexyl-1-nitrosourea; AdoMet, S-adenosyl-L-methionine; m^6G demethylase, O^6-methylguanine-DNA demethylase; m^7G glycosylase, 7-methylguanine-DNA glycosylase; m^3A glycosylase, 3-methyladenine-DNA glycosylase; HBSS, Hanks' balanced salt solution; DER, dose enhancement ratio; CFE, colony-forming efficiency.

INTRODUCTION

DMFO is an enzyme-activated, irreversible inhibitor of ornithine decarboxylase, the first enzyme in the biosynthesis of the polyamines [1]. Pu and Sd levels in 9L rat brain tumor cells treated *in vitro* with a non-toxic (10 mM) concentration of DFMO for 48 hr are less than 5% of control levels; Sp levels are essentially unchanged [2]. The cytotoxicity of the chloroethylnitrosourea BCNU, which is thought to be the result of alkylation and subsequent cross-linking of DNA, is significantly increased in polyamine-depleted cells [3]. If Pu was added to the culture medium of DFMO-pretreated cells before treatment with BCNU, potentiation was prevented. In contrast, DFMO pretreatment decreased the cytotoxicity of *cis*-platinum against 9L cells [4]; this decreased cytotoxicity was also prevented by the addition of Pu.

The stabilizing effect of polyamines on DNA is well documented [5–8]. We have postulated that reduction of intracellular polyamine levels leads to the destabilization of DNA, after which DNA bases are more susceptible to alkylation and subsequent inter- and intrastrand cross-linking reactions of chloroethylnitrosoureas [3, 9]. In contrast, platinum complexes react with nucleophiles on DNA bases; monoadducts can subsequently form base–Pt–base inter- and intrastrand cross-links [10–13]. The two chlorine atoms, the leaving groups in *cis*-platinum, are separated by 3.3 Å [14, 15], which is similar to the interplanar base distance (3.4 Å) of DNA [16]; a number of nucleophilic groups in DNA are separated by 3.4 Å [10, 17–19]. Thus it is possible that the alteration in DNA structure caused by the depression of polyamine levels might change the distance between these nucleophilic groups on DNA bases and thereby prevent cross-linking [4]. To study an aspect of this mechanism for chloroethylnitrosoureas, polyamine-depleted and control 9L cells were treated with [^{14}C]-MeCCNU and the number of monoadducts formed was measured.

Polyamine depletion might affect a number of cellular processes that are connected with or have an influence on the alkylation event. A number of enzymes are involved in the repair of alkylation damage of DNA [20–23]. It is possible that DFMO-mediated enhancement of BCNU toxicity might be the result of an effect that polyamine depletion has on the activity of these enzymes; we studied the effect on the activities of m^{6}G demethylase, m^{7}G glycosylase and m^{3}A glycosylase.

A substantial increase in the content of decarboxylated AdoMet has been reported in DFMO-treated fibroblasts [24] and hepatoma cells [25]. It has been suggested that the high levels of decarboxylated AdoMet could contribute to the synergism between DFMO and BCNU [24]. Levels of decarboxylated AdoMet in DFMO-treated 9L cells were investigated.

Polyamine biosynthesis inhibitors are currently undergoing clinical investigation as cancer chemotherapeutic agents. Because the high but non-toxic DFMO concentrations frequently used *in vitro* might not be achievable *in vivo*, it is important to investigate the effective doses of DFMO that cause potentiation. We report here the effect on MeCCNU cytotoxicity of pretreating 9L cells *in vitro* with 10, 1, 0.5 and 0.1 mM DFMO.

MATERIALS AND METHODS

Drugs

DFMO was generously provided by the Merrell-Dow Research Center (Cincinnati, OH). MeCCNU and [^{14}C]-MeCCNU (both carbons in the chlorethyl group labeled; sp. act. 18.2 mCi/mmol) were generously provided by Dr Robert R. Engle of the National Cancer Institute. Pu was purchased from Calbiochem (LaJolla, CA).

Stock solutions of DFMO (300, 30, 15 and 3 mM) and Pu (31 mM) were made in HBSS, adjusted to pH 7.2, sterile filtered and stored at −20°C. Both cold and [^{14}C]-MeCCNU were dissolved and serially diluted in absolute ethanol immediately before use. To achieve the final concentration of MeCCNU, 150 μl of an appropriate dilution were added to medium in the treatment flasks. The final concentration of ethanol in the medium did not affect the plating efficiency of the cells.

Cell culture

9L rat brain tumor cells were grown in monolayer culture in Eagle's minimum essential medium supplemented with non-essential amino acids, 10% newborn calf serum and gentamicin (50 μg/ml).

For toxicity experiments, cells were plated into 75-cm^2 Falcon tissue culture flasks (Oxnard, CA) in 14.5 ml of medium. To allow cells to reach early exponential growth, they were incubated for 24 hr before drug treatment. To minimize differences in cell densities at the beginning of MeCCNU treatment, flasks to be preincubated with DFMO were plated with 5×10^5 cells and those to be pretreated with HBSS were plated with 2.5×10^5 cells. For the study of adduct formation, activity of repair enzymes and content of decarboxylated AdoMet, 9L cells were seeded into 175-cm^2 Falcon tissue culture flasks in 43.5 ml of medium and incubated for 24 hr before drug treatment. Flasks to be treated with DFMO were

plated with 1.2×10^6 cells and those to be treated with HBSS were plated with 0.6×10^6 cells.

Cytotoxicity studies

Cells were pretreated with either 0.5 ml of 300, 30, 15 or 3 mM DFMO (final concentration, 10, 1, 0.5 and 0.1 mM respectively) or 0.5 ml of HBSS (control). After 48 hr of incubation both DFMO-treated and control cells were treated with MeCCNU for 1 hr at 37°C, after which drug-containing medium was decanted and cells were trypsinized, counted and plated for CFE [26].

In the polyamine replenishment experiment 0.5 ml of 31 mM Pu (final concentration, 1 mM) was added to cells that had been incubated for 48 hr with 10 mM DFMO. Control cells incubated with 0.5 ml of HBSS for 48 hr also received 1 mM Pu. After an additional 24 hr of incubation cells were treated with MeCCNU and processed for the CFE assay as described above.

Cells for polyamine analysis were pelleted at 2000 rev/min for 10 min at 4°C and stored at −20°C before assaying [27].

Adduct formation

Cells that had been treated for 48 hr with either 10 mM DFMO or HBSS were treated with [^{14}C]-MeCCNU (6 μg/ml) for either 1 or 24 hr at 37°C. After drug treatment cells were trypsinized and counted; and an aliquot of cells was used for the CFE assay to determine whether the cytotoxic effect of the [^{14}C]-MeCCNU was equivalent to that of the cold MeCCNU. The remaining cells were pelleted at 2000 rev/min for 10 min at 4°C and kept at −70°C before being assayed. The analysis of adduct formation was carried out as described [28] with the exception that the suspended cell pellet was homogenized in 1 ml of 0.1 M Tris–HCl, 0.1 M NaCl, 5 mM EDTA, pH 8.0, and incubated at 37°C for 30 min with 0.1 mg of protease K before extraction of the DNA. The protease treatment improved the yield of DNA. Although all of the monofunctionally alkylated bases were not identified, all radioactivity was accounted for in the HPLC analysis, allowing us to determine any differences between the products of DFMO-treated and non-treated cells.

Activity of repair enzymes

DFMO-treated cells (10 mM, 72 hr), DFMO/Pu-treated cells (DFMO, 10 mM, 48 hr; then Pu, 24 hr) and control cells were trypsinized, counted and pelleted at 2000 rev/min for 10 min at 4°C. Cells were kept at −70°C before being assayed. The activity of m^6G demethylase, m^7G glycosylase and m^3A glycosylase were measured by a described method [29].

Decarboxylated AdoMet content

Cells were treated as described above for measurement of repair enzyme activity. Levels of decarboxylated AdoMet were estimated with a reported method [24].

RESULTS

Polyamine levels in cells treated for 48 hr with 10, 1 or 0.5 mM DFMO were essentially the same; Pu was not detectable, Sd was less than 5% of control and Sp was slightly increased compared to control cells (Table 1). Pu, Sd and Sp levels in DFMO-pretreated cells incubated with 1 mM Pu for 24 hr were 79, 91 and 108%, respectively, of polyamine levels in control cells receiving only HBSS.

Pretreatment of 9L cells with 10 mM DFMO for 48 hr significantly increased the cytotoxicity of MeCCNU (Fig. 1). Untreated 9L cells had plating efficiencies of approximately 70%, and concentrations of 10–0.5 mM DFMO alone had virtually no

Table 1. Polyamine levels in 9L cells treated with different doses of DFMO

Concentration of DFMO (mM)	Putrescine	Spermidine (nmol/10^6 cells)	Spermine
0	3.11	3.70	1.88
10	n.d.*	0.19	2.45
1	n.d.	0.16	2.10
0.5	n.d.	0.15	2.12

n.d., not detectable.

Table 2. Potentiation of MeCCNU cytotoxicity against 9L cells by pretreatment with DFMO

Concentration of MeCCNU (μg/ml)	Surviving fraction: Concentration of DFMO (mM) 0	0.1	0.5	1.0
5	$1.57 \pm 0.24^* \times 10^{-1}$	$8.74 \pm 0.98 \times 10^{-2}$	$5.71 \pm 1.08 \times 10^{-2}$	$4.62 \pm 0.68 \times 10^{-2}$
10	$6.10 \times 0.84 \times 10^{-3}$	$7.00 \pm 1.24 \times 10^{-4}$	$2.34 \pm 0.49 \times 10^{-4}$	$2.48 \pm 0.67 \times 10^{-4}$

*Standard deviation calculated from 4–8 Petri dishes per experiment.

cytotoxic effect on 9L cells as measured by the CFE assay. The increase in MeCCNU cytotoxicity caused by DFMO pretreatment was prevented by the addition of Pu to polyamine-depleted cultures 24 hr before MeCCNU treatment (Fig. 2). Pretreatment with 10, 1 and 0.5 mM DFMO potentiated the cytotoxicity of MeCCNU; the DERs at 10, 1 and 0.1% survival levels were approximatly 1.3 (Fig. 3).

In an independent experiment, treatment with 1, 0.5 and 0.1 mM DFMO for 48 hr similarly potentiated MeCCNU cytotoxicity against 9L cells (Table 2). Polyamine depletion was virtually identical for these three doses of DFMO.

No difference in monofunctional adduct formation was found between polyamine-depleted and control cells incubated with 6 μg/ml of [^{14}C]-MeCCNU for 1 hr. Figure 4 shows the histogram of alkylated bases from cells isolated immediately after the 1-hr drug treatment. A dramatic increase in radioactivity, mainly in guanine, adenine and pyrimidine oligonucleotides, was found when cells were incubated for 24 hr after [^{14}C]-MeCCNU treatment (data not shown); however, there was still no difference in monofunctional adduct formation between polyamine-depleted and control cells. The increase in radioactivity was probably the result of non-specific incorporation into nucleosides of ^{14}C from degradation of the [^{14}C]-MeCCNU. The potentiation of the cytotoxicity of [^{14}C]-MeCCNU at 6 μg/ml caused by 10 mM DFMO was slightly less than that obtained for the same dose of cold MeCCNU (data not shown).

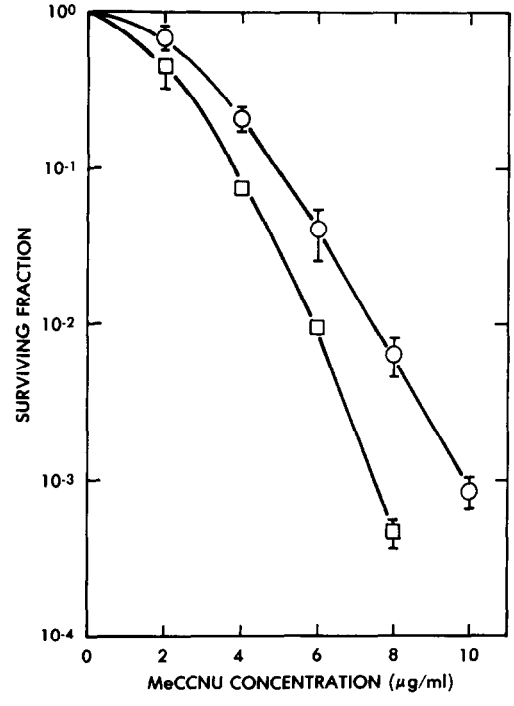

Fig. 1. Effect of polyamine depletion by DFMO on the cytotoxicity of MeCCNU on 9L cells in vitro. *○ denotes cells pretreated with HBSS for* 48 hr *followed by MeCCNU for* 1 hr; *□ denotes cells treated with* 10 mM *DFMO for* 48 hr *followed by MeCCNU for* 1 hr. *Points, means of* 4–8 *Petri dishes; bars, S.D.; the absence of bars indicates that the S.D. was within the symbol.*

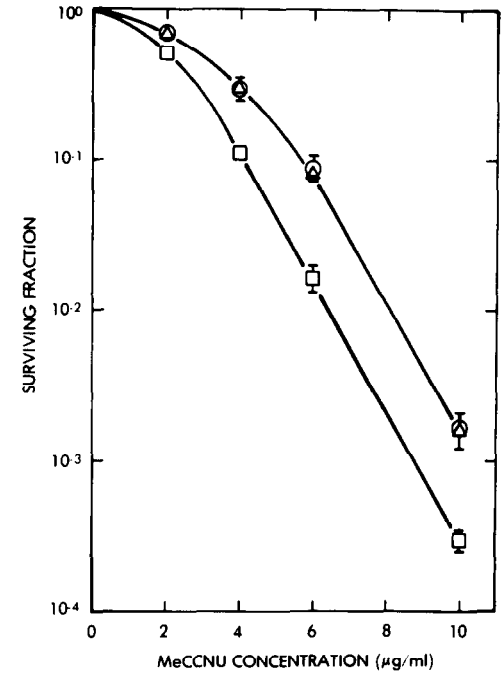

Fig. 2. Effect of DFMO/Pu treatment on the cytotoxicity of MeCCNU on 9L cells in vitro. *○ denotes cells receiving HBSS for* 48 hr, 1 mM *Pu for* 24 hr and then MeCCNU for 1 hr; *□ denotes cells receiving* 10 mM DFMO for 48 hr, *HBSS for* 24 hr *and then MeCCNU for* 1 hr; *△ denotes cells receiving* 10 mM *DFMO for* 48 hr, 1 mM *Pu for* 24 hr *and then MeCCNU for* 1 hr. *Points, means of* 4–8 *Petri dishes; bars, S.D.; the absence of bars indicates that the S.D. was within the symbol.*

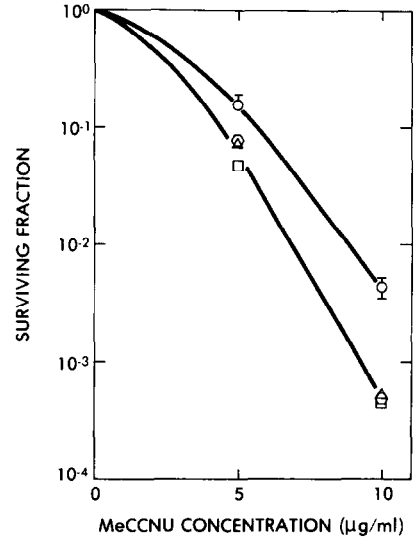

Fig. 3. Effect of polyamine depletion by different doses of DFMO on the cytotoxicity of MeCCNU on 9L cells in vitro. *The cells were pretreated with either DFMO (□,* 10 mM; *○ ,* 1 mM; *△,* 0.5 mM) *or HBSS (○) for* 48 hr *followed by MeCCNU for* 1 hr. *Points, means of* 4–8 *Petri dishes; bars, S.D.; the absence of bars indicates that the S.D. was within the symbol.*

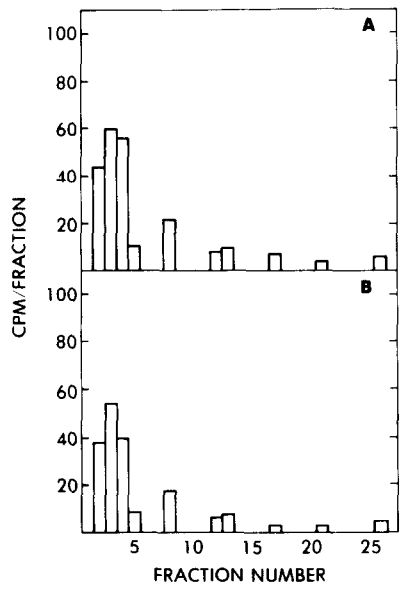

Fig. 4. Radioactivity of bases alkylated with 6 μg/ml [^{14}C]-MeCCNU for 1 hr. (A) Control; (B) DFMO-treated (10 mM, 48 hr). Bases were separated by high-performance liquid chromatography and each fraction was counted. Marker bases were identified chromatographically and correspond to the following fraction numbers: guanine, 4; 7-methylguanine, 7; adenine, 8; 7-methyladenine, 11; 3-methyladenine, 22.

Table 3. Activity of DNA repair enzymes for methylated bases in DNA of 9L cells

Treatment	m^6G demethylase	m^7G glycosylase (fmol/mg/hr)	m^3A glycosylase
Control	22	5	82
10 mM DFMO	24	7	108
DFMO/Pu	20	7	76

Duplicate samples agreed ± 15%.

Table 4. Decarboxylated AdoMet levels in 9L cells

Treatment	Decarboxylated AdoMet (pmol/10^6 cells) Experiment 1	Experiment 2
Control	2	1
10 mM DFMO	89	61
DFMO/Pu	2	2

The values for control and DFMO/Pu cells are at the limit of detection of the assay system and are approximate estimations.

Experiments were carried out to determine whether polyamine depletion had any effect on enzymes involved in the repair of alkylated bases. The activity of the enzymes m^6G demethylase, m^7G glycosylase and m^3A glycosylase were not affected by the 48-hr pretreatment with 10 mM DFMO (Table 3).

The concentration of decarboxylated AdoMet in control 9L cells is very low (Table 4). A striking increase in decarboxylated AdoMet content was found in cells treated with DFMO. When Pu was added to the medium of DFMO-treated cells 24 hr before cells were harvested for the assay, decarboxylated AdoMet levels were similar to control levels. In HPLC chromatograms of extracts from DFMO-treated cells there was a peak for an unidentified compound near the peak for decarboxylated AdoMet. This peak was not detected in the Pu-treated cells and could represent a metabolite of decarboxylated AdoMet. Using a different chromatographic technique, Wagner *et al.* [30] have found a similar peak.

DISCUSSION

As found for BCNU [3], pretreatment of 9L cells for 48 hr with 10 mM DFMO potentiated the cytotoxicity of MeCCNU. The DERs of 1.3 were similar to those found for BCNU. The effect was prevented by the addition of Pu to the medium of DFMO-treated cells 24 hr before treatment with MeCCNU, which suggests that the potentiation might be the result of polyamine depletion. Treating cells for the same period with DFMO concentrations of 10–0.1 mM produced very similar levels of potentiation. This is of importance because if DFMO is to be used to potentiate the effects of cytotoxic agents in human patients, potentiation must be obtained at clinically achievable doses of DFMO.

A striking accumulation of decarboxylated AdoMet has been found in mouse fibroblasts [24] and hepatoma cells [25] when Pu and Sd production are inhibited by DFMO. We also found a significant increase in the content of decarboxylated AdoMet in 9L cells treated with 10 mM DFMO. As expected, the elevated level of decarboxylated AdoMet was reduced by addition of Pu. The absence of Pu and Sd, which, with decarboxylated AdoMet as an aminopropyl group donor, serve as substrates for aminopropyl transferases, and the enhanced activity of AdoMet decarboxylase that is caused by the depletion of Sd cause the increase seen in decarboxylated AdoMet with DFMO treatment [24]. Although there is indirect evidence obtained in HTC cells grown *in vitro* to show that decarboxylated AdoMet does not mediate the antiproliferative effects of DFMO [25], the effects of decarboxylated AdoMet in other cell lines, or on other cellular processes, are unknown. Thus it has been suggested that high intracellular concentrations of decarboxylated AdoMet, which is thought to be a weak methylating agent, might contribute to DFMO potentiation of chloroethylnitrosourea cytotoxicity [24]. Clearly, if methylation by

decarboxylated AdoMet occurs it would influence cytotoxicity only synergistically, because DFMO alone is not toxic to 9L cells even though levels of decarboxylated AdoMet are increased.

MGBG is an inhibitor of AdoMet decarboxylase [31]; it therefore reduces polyamine levels but does not increase the levels of decarboxylated AdoMet [24]. Nevertheless, we found a potentiating effect on BCNU cytotoxicity, similar to that obtained with DFMO pretreatment, by pretreating 9L cells with 40 μM MGBG for 48 hr [32].

The stabilizing effect of polyamines on the structure of DNA is a well-documented phenomenon [5–8]. We have postulated that decreased intracellular levels of polyamines caused by DFMO destabilizes DNA and renders it more susceptible to alkylation and subsequent interstrand cross-linking [3, 9]. There was no significant difference in the number of DNA monoadducts formed in polyamine-depleted and control cells treated with [^{14}C]-MeCCNU. This is consistent with our recent finding that the cytotoxicities of two monofunctional alkylating agents, *N*-methyl-*N*-nitrosourea and *N*-ethyl-*N*-nitrosourea, are not potentiated by polyamine-depletion of 9L cells [33]. However, these two compounds can only monoalkylate DNA and therefore cannot form cross-links. Thus, although monoalkylation reactions are not enhanced by polyamine depletion, our results with MeCCNU and BCNU, both of which can alkylate and subsequently cross-link DNA, might be explained as a result of increased formation of DNA interstrand cross-links. This explanation is consistent with the fact that while the cytotoxicities of the monofunctional alkylating agents are not potentiated by polyamine depletion, the cytotoxicity of the alkylating and cross-linking agent 2[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose (chlorozotocin) is potentiated with a DER identical to that obtained for MeCCNU and BCNU [33]. Experiments using alkaline elution techniques indicate that after DFMO-pretreatment, BCNU-induced cross-linking is increased and that addition of Pu to DFMO-pretreated cells before treatment with BCNU prevents the increase in cross-linking [34].

The sister chromatid exchange assay is a sensitive and relatively simple method for the measurement of damage to chromosomes [35]. DFMO-induced polyamine depletion increases the number of SCEs induced by BCNU; the increase is prevented by the addition of Pu [9]. It is not known if this phenomenon is the result of an increase in bifunctional alkylation and DNA cross-link formation, although it appears to be consistent with it.

A number of enzymes are involved in the repair of akylation damage [20–23]. A correlation has been found between the ability to remove alkylation damage from DNA and resistance to alkylating agents [36–42]. Erickson *et al.* [37] reported that cell lines that have the capability to remove O^6-substituents are resistant to cross-link formation. Tong *et al.* [43] recently showed that a cytosine–guanine cross-link is formed by a slow, stepwise reaction, the first reaction of which is alkylation at the O^6 of guanine; if the O^6-guanine adduct is repaired, cross-linking cannot occur. Obviously, an inhibition of the activity of repair enzymes could increase the cytotoxicity of alkylating agents. In the studies reported here we found that polyamine depletion did not affect the intracellular activity of m^{6}G demethylase, m^{7}G glycosylase or m^{3}A glycosylase; therefore we do not believe that they are involved in the potentiation of MeCCNU cytotoxicity caused by DFMO.

This and other studies we have reported show that potentiation of the cytotoxicity of chloroethylnitrosoureas by DFMO-induced polyamine depletion is a general phenomenon for this class of drugs. While the mechanism has not been defined, the results reported here suggest that even though polyamine depletion does not increase the number of monoadducts formed, an increase in cross-link formation seems probable.

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