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α-DIFLUOROMETHYLORNITHINE EFFECTS ON NITROSOUREA-INDUCED CYTOTOXICITY AND CROSSLINKING IN A METHYLATION EXCISION REPAIR POSITIVE (MER$^+$) HUMAN CELL LINE*

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Abstract—We investigated the cytotoxic effects of nitrosoureas with and without a 42-hr preincubation with the ornithine decarboxylase (EC 4.1.1.17) inhibitor α-difluoromethylornithine (DFMO, 1 mM) in a MER$^+$ (methylation excision repair positive) human cell line. DFMO combined with a chloroethyl nitrosourea [1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU) or 1-(2-chloroethyl)-1-nitrosourea (CNU)] yielded increased toxicity with D_{37} ratios of 1.9 and 3.3 respectively. There was no enhanced toxicity with the monofunctional nitrosourea 1-ethyl-1-nitrosourea (ENU). BCNU or CNU did not induce DNA–DNA interstrand crosslinks in cells with or without a DFMO pretreatment. DNA single-strand breakage was not increased by addition of DFMO. BCNU-induced DNA-protein crosslinking was decreased in cells pretreated with DFMO. These findings are similar to those in MER$^-$ cells in that the chloroethyl carbonium alkylating species is required for the enhanced cytotoxicity seen with DFMO. The ability to form DNA interstrand crosslinks, however, does not appear to be necessary for this toxicity enhancement.

In studying the mechanism of action of bifunctional alkylating agents, we are measuring drug-induced DNA damage after changing cytotoxicity with drug modifiers. The ornithine decarboxylase (EC 4.1.1.17) inhibitor α-difluoromethylornithine (DFMO‡) has been shown to interact with chloroethyl nitrosoureas to increase both DNA damage and cytotoxicity in rodent cells [2–9]. We examined this relationship in a human cell line that is relatively resistant to nitrosourea cytotoxicity and exhibits the MER$^+$ (methylation excision repair positive) phenotype [10–12]. MER positivity has been correlated with the levels of O^6-methylguanine methyltransferase in cell lines that have been studied [13–16].

MATERIALS AND METHODS

Cell culture. The human Burkitt's lymphoma cell line, designated BHM, was provided by Dr. Ian Magrath of the Pediatric Oncology Branch of the National Cancer Institute. This B-cell neoplasm is EBV nuclear antigen negative. BHM cells were grown in log phase in suspension culture in RPMI medium 1640 with 15% heat inactivated fetal calf serum (HIFCS) (Dutchland Laboratories, Denver, PA), penicillin, streptomycin, and 1 mM L-glutamine. Cultures were maintained in a forced draft incubator at 90% humidity and 7% carbon dioxide.

Drug treatment. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU) and 1-(2-chloroethyl)-1-nitrosourea (CNU) were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. 1-Ethyl-1-nitrosourea (ENU) was obtained from the Sigma Chemical Co., St. Louis, MO. Drugs were made up fresh for each experiment at a concentration of 0.01 M in ethanol. α-Difluoromethylornithine (DFMO) (MDL 71,782) was a gift from the Merrell-Dow Research Institute (Merrell-Dow Pharmaceuticals, Inc., Cincinnati, OH). DFMO was dissolved in distilled water at a concentration of 300 mM, sterile filtered, and kept refrigerated.

For drug treatments, cells were removed from the growth medium and resuspended in RPMI 1640 with 1% HIFCS plus glutamine, penicillin and streptomycin with or without 1 mM DFMO. A nitrosourea was added at the desired dose, and cells were reincubated for 1 hr. The drug was removed by centrifugation, and the cells were washed with RPMI 1640 with 15% HIFCS. The cells were then counted, put into flasks, and reincubated at a concentration of 5×10^5 cells/ml. DFMO-treated cells were preincubated in medium containing 1 mM DFMO for 42 hr prior to drug treatment. Cells were counted and resuspended in fresh medium with or without DFMO 12 hr prior to nitrosourea treatment.

Cytotoxicity studies. Following drug treatment, cells were diluted in medium and cloned in tubes with soft agar (0.1%) (Agar Noble, Difco, Detroit, MI) according to the method of Chu and Fischer [17].

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‡ Abbreviations: DFMO, α-difluoromethylornithine; MER, methylation excision repair; BCNU, 1,3-bis-(2-chloroethyl)-1-nitrosourea; CNU, 1-(2-chloroethyl)-1-nitrosourea; ENU, 1-ethyl-1-nitrosourea; and RPMI, Roswell Park Memorial Institute.

Cloning tubes were incubated for approximately 14 days until clones were visible with the naked eye, at which time colonies were counted with the aid of a magnifying glass.

Radionuclide labeling. Cells were grown for 30 hr in log phase in medium containing [^{14}C]thymidine at 0.02 μCi/ml (58 mCi/mmol, New England Nuclear, Boston, MA) in the presence or absence of 1 mM DFMO. The label containing medium was removed 12 hr prior to drug treatment, and the cells were counted and resuspended in fresh RPMI 1640 with 15% HIFCS with or without DFMO as appropriate. Cells used as internal standards (L1210 leukemia cells) were grown for 24 hr in medium (RPMI 1630 with 10% HIFCS plus penicillin, streptomycin and glutamine) containing [^{3}H]thymidine at 0.05 μCi/ml (20 Ci/mmol, New England Nuclear).

Alkaline elution. The alkaline elution of DNA from membrane filters was performed as previously described [18, 19]. At intervals following drug treatment, cells were removed from culture and placed in plastic tubes on ice. For single-strand break and DNA interstrand crosslinking assays, BHM cells were mixed with internal standard cells and the mixtures were applied by gravity to 3 μm pore-sized polycarbonate filters (Nucleopore Corp., Pleasanton, CA) and lysed *in situ* with 2% sodium dodecyl sulfate (SDS, 99% purity, BDH Biochemicals, Poole, England), 0.02 M EDTA and 0.1 M glycine, pH 10. Following the lysis solution, 2 ml of a deproteinizing solution (proteinase K, Fisher Scientific, 0.5 mg/ml in the lysis solution) was layered onto the filters, and this was followed by the elution solution of tetrapropyl ammonium hydroxide (RSA Corp., Ardsley, NY) 0.02 M EDTA (acid form), and 0.1% SDS at pH 12.1. For DNA interstrand crosslinking assays, [^{14}C]-labeled cells received 3 Gy of [^{137}Cs] radiation (Gammacell 40, Atomic Energy of Canada, Ltd.). The elution pump rates were 0.035 to 0.045 ml/min, and fractions were collected at 3-hr intervals for 15 hr. Samples were processed as previously described [19] and were counted in Budgetsolve (RPI Corp., California) in a liquid scintillation counter. For DNA–protein crosslinking assays, the modifications of Kohn and Ewig [20] were utilized. Cells received 10 Gy of radiation, were mixed with internal standard cells, and were applied by gravity to 2 μm pore-sized polyvinyl chloride filters (Millipore Corp., Massachusetts). They were lysed with SDS lysis (see above), and this was followed by 5 ml of a 0.02 M EDTA wash, pH 10. The deproteinizing solution was omitted, and the elution solution consisted of tetrapropyl ammonium hydroxide, pH 12.1, without added SDS.

Calculations. DNA single-strand breakage is calculated according to the equation:

$$P_{BD} = P_{BR}\left[\frac{\log (r_1/r_0)}{\log (R_{300}/r_0)}\right]$$

where P_{BD} equals the strand breakage in rad equivalents, P_{BR} is the X-ray dose in rads, r_1 is the retention of DNA on the filter at the end of the elution in the drug-treated cells, R_{300} is the retention of DNA at the end of the elution for cells receiving 300 rads of X-ray, and r_0 is the retention of DNA at the end of the elution for control cells [19]. DNA interstrand crosslinking was calculated according to the formula

$$P_C = \left[\left(\frac{1 - R_0}{1 - R_1}\right)^{1/2} - 1\right](P_{BR} + P_{BD})$$

where P_C is the crosslink frequency in rad equivalents, R_1 is the retention of DNA on the filter at the end of the elution for irradiated drug-treated cells, and R_0, P_{BR}, and P_{BD} are defined as above [19]. Relative retentions at the end of the elution were corrected as described by Ducore *et al.* [21]. Where appropriate, the value of R_0 in the crosslinking equation was corrected for drug-induced single-strand breakage according to the formula:

$$\log R_0' = \left[1 + \left(\frac{P_{BD}}{P_{BR}}\right)\right]\log R_0$$

where R_0' is the corrected value of R_0 and the other terms are as described above [19].

Protein crosslinking was calculated by the equation

$$PXL = [(1 - r)^{-1/2} - (1 - r_0)^{-1/2}]P_{br}$$

where PXL is the protein crosslinking in crosslinks per nucleotide, r is the retention of DNA from the slow eluting portion of the curve from drug-treated cells, r_0 is the retention of the slow eluting portion of the curve from control cells, and P_{br} is the frequency of single-strand breaks induced by the radiation in breaks per nucleotide [20]. r and r_0 were calculated at the 0 time point of the least squares linear regression lines for the last three eluted fractions (9, 12 and 15 hr of elution).

RESULTS

DFMO pretreatment prolonged the doubling time of BHM cells 20.6 ± 8.7 hr versus 35.0 ± 20.6 hr (mean ± 1 SD) for cells without and with 1 mM DFMO respectively ($P < 0.2$, paired t-test, $N = 5$) in the 12 hr just prior to nitrosourea treatment. Because cells were resuspended in fresh medium 12 hr prior to drug treatment, there was little difference in cell density at the time of nitrosourea addition [1.3×10^6 cells/ml (±0.2) and 1.0×10^6 cells/ml (±0.1) for cells without and with 1 mM DFMO respectively].

DFMO pretreatment alone decreased the colony formation of BHM cells from 49.7% (±28.4) to 20.1% (±14.6) ($P < 0.005$, paired t-test, $N = 10$). Figure 1 shows a representative experiment measuring the survival of BHM cells following a 1-hr treatment with BCNU with or without a DFMO pretreatment. The BHM cell survival from six experiments is shown in Table 1. The slopes of the least squares linear regression lines for BCNU dose versus log survival show the variation in results from experiments performed on different days. On any given day, however, the slope of the line from cells pretreated with DFMO was always steeper than that of non-pretreated cells, denoting greater cytotoxicity in pretreated cells. The slopes (mean ± SD) were −0.0586 (±0.0582) and −0.0800 (±0.0682) for cells treated without and with DFMO pretreatment, respectively, with $P < 0.01$ (paired t-test, $N = 6$).

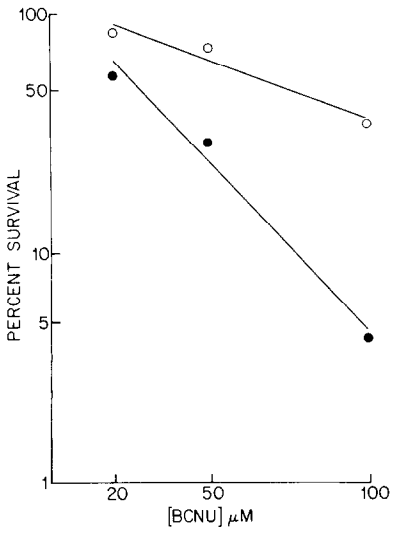

Fig. 1. Least squares linear regression lines for BHM cell survival after a 1-hr BCNU treatment. One hundred percent survival is for cells treated with or without DFMO as appropriate. Key: (○) cells treated with BCNU alone; and (●) cells pretreated with 1 mM DFMO for 42 hr prior to BCNU treatment.

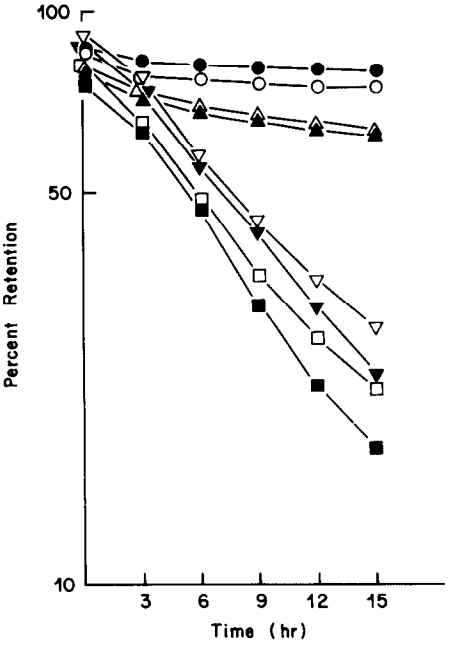

Fig. 2. Alkaline elution profiles of BHM cell DNA measuring single-strand breaks or interstrand crosslinks 6 hr after a 1-hr BCNU treatment with or without a 42-hr DFMO pretreatment. Key: (open symbols) no DFMO; (solid symbols) 1 mM DFMO; (○, ●) strand break controls (no BCNU), no radiation; (△, ▲) 100 μM BCNU, no radiation; (▽, ▼) crosslink controls (no BCNU), 3 Gy gamma radiation; and (□, ■) 100 μM BCNU, 3 Gy gamma radiation.

The ratio of D_{37} values (BCNU dose yielding 37% survival) for cells without and with pretreatment yielded a mean of 1.9 (±0.6).

Figure 2 shows the results from a representative experiment measuring DNA single-strand breakage following BCNU treatment in cells with or without DFMO pretreatment. DFMO alone did not induce strand breakage in control cells. While BCNU induced single-strand breaks (as has been seen previously [22]), the break frequency was not increased by DFMO pretreatment. A 100 μM concentration of BCNU induced 148 ± 106 rad equivalents of single-strand breaks in BHM cells versus 92 ± 88 rad equivalents for cells pretreated with DFMO (mean ± SD, P = 0.16, paired *t*-test, N = 5).

Figure 2 also shows a representative alkaline elution profile for DNA interstrand crosslinks induced by BCNU in BHM cells with or without a DFMO pretreatment. The fall off in the elution profile for cells treated with drug plus X-ray below the DNA of cells treated with X-ray alone suggests that interstrand crosslinking is low to non-existent in BHM cells.

Table 1. Survival of BHM cells after BCNU treatment* with and without DFMO preincubation†

Expt. No.	Slope of survival curve‡ −DMFO	Slope of survival curve‡ +DMFO	Ratio of D_{37} values§
1	−0.0589	−0.0761	1.4
2	−0.0035	−0.0071	2.2
3	−0.1538	−0.1899	1.4
4	−0.0099	−0.0313	3.0
5	−0.0976	−0.1287	1.4
6	−0.0281	−0.0466	1.9
Mean (±SD)	−0.0586‖ (0.0582)	−0.0800 (0.0682)	1.9 (0.6)

* One-hour treatment.
† One millimolar DFMO for 42 hr.
‡ From least squares linear regression line of BCNU dose vs log survival.
§ D_{37} = BCNU dose yielding 37% survival; ratio is dose without DFMO/dose with DFMO.
‖ $P < 0.01$, compared to +DMFO, paired *t*-test, N = 6.

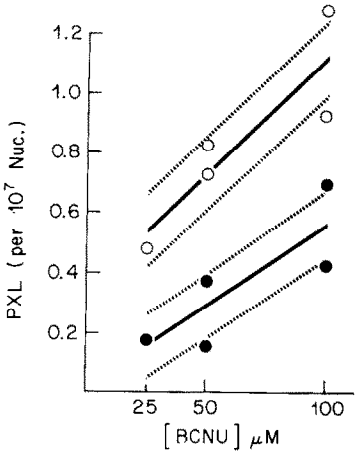

Fig. 3. DNA–protein crosslinking immediately following a 1-hr BCNU treatment as a function of nitrosourea dose and DFMO pretreatment in BHM cells. Points are from two separate experiments. Key: (○) no DFMO, (●) 1 mM DFMO; (——) least square linear regression lines from the data points; and (– – –) ± 1 standard deviation.

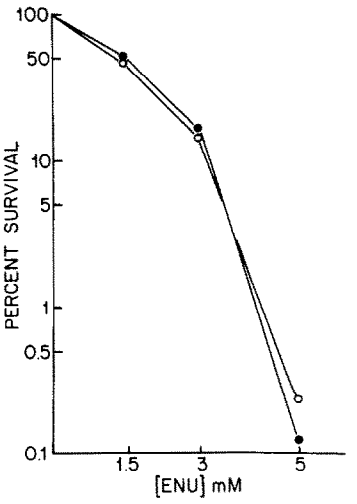

Fig. 4. BHM cell survival after a 1-hr ENU treatment. One hundred percent survival is for cells treated with or without DFMO as appropriate. Key: (○) cells treated with ENU alone; and (●) cells pretreated with 1 mM DFMO for 42 hr prior to ENU treatment. Points are means of experiments (N = 3, no DFMO; N = 2, 1 mM DFMO).

We have reported [12] that BHM cells most likely possess the O^6-methylguanine methyltransferase enzyme system because they fail to demonstrate DNA interstrand crosslinking following nitrosourea treatment, the MER$^+$ phenotype [10, 11, 13–16]. [The level of the O^6-methylguanine methyltransferase* was measured by Dr. Thomas Brent at the St. Jude Children's Research Hospital in Memphis, TN, using methodology he has described previously [23, 24]; in this preliminary experiment the level of activity of the enzyme in BHM cells appeared to be comparable to that in an enzyme-positive cell line used as a control and to levels from enzyme positive cells previously described from his laboratory [24] (data not shown).] Even when the strand breakage effect of BCNU is taken into account [19], only extremely low levels of crosslinking were calculated at 100 μM BCNU (mean ± SD, 20 ± 20 rad equivalents versus 8 ± 19 rad equivalents for cells without or with DFMO pretreatment respectively). Similar low levels of DNA interstrand crosslinking were seen with CNU at doses up to 300 μM. CNU does not cause as much strand breakage as BCNU [20]. Such low levels are consistent with measurements obtained in other MER$^+$ cell lines [11], and well within the estimated limits of sensitivity of the alkaline elution procedure of approximately 50–100 rad equivalents of damage [19]. In addition, even at such low levels, there was no trend toward increased DNA interstrand crosslinking in the DFMO pretreated cells (data not shown).

We next measured DNA–protein crosslinking immediately after BCNU removal. Such measurements have been used as an estimate of the amount of active drug reaching the DNA [25, 26]. As Fig. 3 shows, there were fewer DNA–protein crosslinks in cells pretreated with DFMO. Because of inter-experimental variability, the data are treated as data pairs within each experiment and there was a significant decrease in protein crosslinking, $P < 0.02$ (paired t-test) for cells pretreated with DFMO.

Since the DNA–protein crosslinking experiments suggested that the increased cytotoxicity was not due to more drug attaching to the DNA, we hypothesized that DFMO increased the cytotoxicity of a monofunctional nitrosourea alkylation. We tested this with the monofunctional nitrosourea ENU. The survival of BHM cells treated with ENU with or without a DFMO pretreatment is shown in Fig. 4. There was

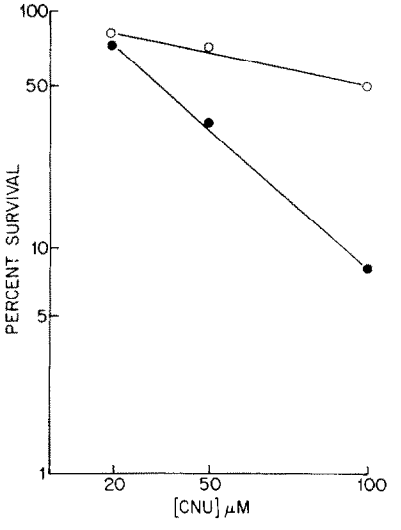

Fig. 5. BHM cell survival after a 1-hr CNU treatment. One hundred percent survival is for cells treated with or without DFMO as appropriate. Key: (○) cells treated with CNU alone; and (●) cells pretreated with 1 mM DFMO for 42 hr prior to CNU treatment. Points are means from experiments (N = 2). Lines are least squares linear regression lines from individual data points.

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no difference in survival with or without the pretreatment when we controlled for the toxicity of DFMO. Finally, we tested the question of whether it was the toxicity of the alkylating species of BCNU that was enhanced by DFMO. BCNU spontaneously hydrolyzes into two moieties, a chloroethyl carbonium ion which alkylates and an isocyanate which carbamoylates [22]. It has been shown previously that the carbamoylating portion has toxic and DNA repair inhibitory properties of its own [27–30]. The bifunctional nitrosourea CNU also hydrolyzes, but its isocyanate portion self-inactivates and for this reason may be used as a "pure" alkylator [22, 30]. The cytotoxicity induced by this drug with or without DFMO is shown in Fig. 5. There was a significant enhancement of toxicity by DFMO pretreatment with a D_{37} ratio of 3.3 and a D_0 ratio of 4.5 ($P < 0.001$, *t*-test for comparison of regression slopes).

DISCUSSION

We report here the increased cytotoxicity of the combination of DFMO and chloroethyl nitrosoureas in a human cell line which possesses the O^6-methylguanine methyltransferase DNA repair system and exhibits the MER$^+$ phenotype [10, 11]. Oredsson *et al.* [4] alluded to DFMO pretreatment sensitizing a human cell line to the effects of BCNU (no data are shown), and Seidenfeld and Komar [31] demonstrated sensitization to BCNU by DFMO in a number of human carcinoma cells; the MER status of these lines was not detailed. While Table 1 shows that there was a wide variation in the clonogenic measurement of BHM cell sensitivity to BCNU on any given day, DFMO pretreatment always sensitized the cells to the nitrosourea cytotoxicity. The reason for this variability is not clear and may reside in the fact that BCNU must kill BHM cells by mechanisms other than those associated with interstrand crosslinking and perhaps is partially a function of carbamoylation. This is also suggested by the fact that there was less day-to-day variation when the non-carbamoylating nitrosourea CNU was used instead of BCNU.

The enhanced toxicity of the drug combinations resides in the chloroethyl carbonium part of the nitrosourea molecule since CNU, an agent which alkylates but does not carbamoylate [22, 30], also demonstrated enhanced toxicity. The monofunctional alkylator ENU in combination with DFMO did not yield enhanced toxicity, signifying that there was no increased sensitivity to monofunctional alkylations in general. DFMO does not presumably inhibit the methyltransferase repair enzyme (although we have not measured this directly) since DNA interstrand crosslinking was not seen in BHM cells after chloroethyl nitrosourea treatment either with or without a DFMO pretreatment. Finally, increased sensitivity to DNA–protein crosslinks is unlikely since such crosslinks were decreased with a DFMO pretreatment.

These results are similar to previous reports using rodent cells [5, 6], which lack high levels of O^6-methylguanine methyltransferase (are said to be MER$^-$ [10, 11, 13–16]), and show DNA interstrand crosslinking after chloroethyl nitrosourea treatment. In those cells, DFMO pretreatment also yielded enhanced cytotoxicity with alkylating chloroethyl nitrosoureas independent of carbamoylation, and there was no enhanced cytotoxicity with monofunctional nitrosoureas [5]. DFMO pretreatment yielded increased DNA interstrand crosslinking and this was proposed as the mechanism of the enhanced cytotoxicity [5, 6]. The increased crosslinking was also not a function of inactivation of the O^6-methylguanine methyltransferase enzyme where it could be measured [6]. Our results show that the enhanced killing does not require DNA interstrand crosslinks. Additionally, while cytotoxicity and DNA interstrand crosslinking increase in L-phenylalanine mustard treated BHM cells pretreated with DFMO, the cytotoxic enhancement is greater than the increased crosslinking *per se* [32].

cis-Diamminedichloroplatinum(II) causes both DNA interstrand and intrastrand crosslinks, the latter of which are cytotoxic [33–35], but not measured by alkaline elution. It is possible that the DNA conformational changes resulting from DFMO-induced decreases in polyamines result in the formation or increased formation of nitrosourea intrastrand crosslinks. (A preliminary experiment provided by Dr. L. Marton, University of California, San Francisco, suggests that both putrescine and spermidine are undetectable in BHM cells after 48 hr in DMFO.*) Changes in chromatin structure (including alterations induced by polyamine depletion) have been shown to alter 1-methyl-1-nitrosourea (MNU)- and ENU-induced alkylations [36, 37]. Intrastrand crosslinking after nitrosourea treatment is possible although not widely recognized [38], and such lesions would be "invisible" in an assay such as alkaline elution.

In conclusion, treatment of cells with DFMO and a chloroethyl nitrosourea resulted in enhanced cytotoxicity. This is most likely not related to inactivation of DNA repair systems [6] or to the increased toxicity of monofunctional alkylations [5, 6]. It is unlikely to be related to changes in DNA–protein crosslinking. Although the enhanced toxicity involved the chloroethyl carbonium alkylating species, it was not dependent on the existence of DNA interstrand crosslinks, as was suggested using MER$^-$ rodent cells. Whether the increased toxicity resides in a monofunctional alkylation of low frequency or whether there is a bifunctional lesion not measured by alkaline elution (e.g. DNA intrastrand crosslinking) remains to be determined.

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