Comparisons between Sensitive and Resistant Human Tumor Cell Lines Regarding Effects of Polyamine Depletion on Chloroethylnitrosourea Efficacy

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ABSTRACT

We have reported that 2-difluoromethylornithine (DFMO)-induced polyamine (PA) depletion sensitized five chloroethylnitrosourea (CENU)-resistant, O6-alkylguanine repair-proficient (Mer*) human tumor cell lines to 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU), but failed to alter BCNU efficacy in a single CENU-sensitive, repair-deficient (Mer-) line. Further, alkaline elution assays of DNA interstrand cross-links (ISC) found no BCNU-induced ISC in either PA-depleted or control Mer+ cells, suggesting that targets other than ISC may be involved in the DFMO/BCNU drug interaction. To verify that DFMO-induced enhancement of BCNU action segregates with Mer phenotype, we tested three additional Mer- lines for effects of DFMO pretreatment on BCNU efficacy. We found no potentiation of BCNU by PA depletion in any of our human Mer- lines. We also used streptozotocin (STZ) to deplete the repair capacity of Mer+ cell lines, thus converting their BCNU sensitivity to near that of Mer- cells. Combined pretreatment with DFMO then STZ did enhance BCNU cell kill relative to STZ pretreatment alone. Exogenous putrescine restored BCNU sensitivity of (DFMO plus STZ)-pretreated cells to that of cells pretreated with STZ alone. Measurements of O6-alkylguanine DNA alkyltransferase activity verified that in at least one of the Mer+ lines (HT-29), STZ did deplete repair capacity to below detectable limits. These results suggest that in HT-29 cells, STZ and DFMO probably act via differing mechanisms to potentiate BCNU. Our observations also imply that targets for CENU may differ between Mer+ and Mer- cells, with importance of ISC possibly limited to Mer- cells. Our data further suggest that PA depletion may potentiate CENUs only at targets critical in Mer+ cells. We also noted that 48-h treatments with DFMO markedly reduced clonogenicity of Mer- cells. Exogenous putrescine restored Mer- cell survival after DFMO to near that of controls. In contrast, Mer+ cells showed little, if any, effect of DFMO treatment on plating efficiency. These results suggest that PA depletion may be cytotoxic to some Mer+ cells.

INTRODUCTION

Reports from several laboratories have demonstrated increased cytotoxic efficacy for some CENU antitumor drugs in rodent (1-4) and human (5, 6) tumor cell lines pretreated with inhibitors of PA biosynthesis (see Refs. 7-9 for reviews). For BCNU, enhanced effectiveness was also demonstrated in DFMO-treated animals utilizing the 9L rat gliosarcoma system in vivo (10). DFMO is an enzyme-activated irreversible inhibitor of ODC (11), the initial enzyme in de novo biosynthesis of PAs (12).

For 9L cells in vitro, sensitization to BCNU by DFMO is accompanied by increased BCNU-induced ISC as measured by alkaline elution analysis (13). Similarly, BCNU produces higher amounts of alkaline-labile DNA strand breaks in DFMO-pre-treated L1210 cells than in untreated controls (4). These observations have been interpreted to suggest that PA depletion enhances BCNU action by altering in situ DNA structure or conformation so as to increase levels of DNA damage from fixed concentrations of the CENU drug (4, 7, 9, 13, 14). In support of this hypothesis, Hung et al. (15) used viscoelasticity to demonstrate that DFMO-induced PA depletion significantly altered DNA conformation in cultured 9L cells. However, a direct causal relation between altered DNA structure secondary to PA depletion, and DFMO-mediated potentiation of BCNU has not yet been firmly established.

We have been investigating the mechanism by which DFMO sensitizes cultured human adenocarcinoma cells to BCNU (5, 6). We first demonstrated that potentiation occurs in a series of five cell lines pretreated with DFMO, and that addition of exogenous PUT, the product of ODC, reverses this effect (5). However, alkaline elution assays of ISC in three of these cell lines failed to detect any BCNU-induced ISC in either DFMO-pretreated or control cultures (6). In addition, we found no differences between PA-deficient cells and controls with regard to DNA single-strand breaks or DNA-protein cross-links resulting from BCNU treatment. Since the three cell lines tested were all BCNU-resistant Mer+ cell lines with high levels of OAGTase, we also tested for sensitization to BCNU by DFMO in a BCNU-sensitive Mer- cell line (6). We found no effect of PA depletion on the cytoidal effects of BCNU in the Mer- BE cell line. As this was the sole human Mer- line tested thus far, we have compared BCNU survival curves for DFMO-treated and control cultures of three additional repair-deficient cell lines. Herein we report our findings that DFMO-induced chemosensitization to BCNU consistently segregates with Mer phenotype in a panel of nine human cell lines.

To investigate further the hypothesis that BCNU-induced ISC may be the target at which DFMO potentiates BCNU in Mer+ human adenocarcinoma cells, we depleted the OAGTase activity of the repair-proficient cells with STZ. Zlotogorski and Erickson (16, 17) and Erickson et al. (18) have demonstrated that nontoxic doses of DNA-methylating agents can be used to sensitize Mer+ cells to BCNU, presumably by saturating the repair capacity of the cells. After such pretreatment, cell lines which show no measurable ISC after BCNU in the absence of STZ yield significant levels of BCNU-induced ISC. We reasoned that if DFMO and STZ were potentiating BCNU at the same intracellular targets (i.e., by enhancing formation of ISC from fixed numbers of single-strand lesions) we should find no sensitization to BCNU by DFMO in cells fully depleted of OAGTase activity by STZ. Even if PA depletion does alter DNA structure so as to increase the rate of ISC formation, in the absence of any monoalkylation repair capacity the same number of ISCs per cell should result. The data presented below demonstrate that, in at least one Mer+ cell line, combined pretreatment with DFMO then with STZ further enhances BCNU cytotoxicity relative to sensitization by saturating levels of STZ alone.

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3 The abbreviations used are: CENU, chloroethylnitrosourea; PA, polyamine; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; DFMO, 2-difluoromethylornithine; ODC, ornithine decarboxylase (EC 4.1.1.17); ISC, DNA interstrand cross-link; PUT, putrescine; OAGTase, O6-alkylguanine-DNA alkyltransferase; Mer+, Mer-, methylation repair-proficient or -deficient, respectively; STZ, streptozotocin; SPD, spermidine; SPM, spermine.
MATERIALS AND METHODS

Chemicals and Drugs. PUT dihydrochloride and 1,6-diaminohexane were obtained from Sigma Chemical Co. (St. Louis, MO), while SPD trihydrochloride and SPM tetrahydrochloride were purchased from Calbiochem-Behring (La Jolla, CA). DFMO was a generous gift of the Merrell Dow Research Institute (Cincinnati, OH). Stock solutions of DFMO (150 mM) were prepared in serum-free culture medium, adjusted to pH 7.2-7.4, stored at -20°C, and sterilized by membrane filtration (0.22-µm pores) immediately before use. BCNU was kindly provided by the Pharmaceutical Research and Development Division, Bristol-Meyers Co. (Wallington, CT) and was dissolved in absolute ethanol immediately before use. STZ was obtained from the Pharmaceutical Resources Branch, Division of Cancer Treatment, National Cancer Institute (Bethesda, MD), and was dissolved in dimethyl sulfoxide immediately before use. All other chemicals used were of analytical grade and were obtained from standard commercial sources.

Cell Culture. Routine culture of HT-29 (colon adenocarcinoma) and HuTu-80 (duodenal adenocarcinoma) human tumor cells was performed as described (5, 6, 19). The A1235 (human glioma), VA-13 (SV40 transformed human fibroblast) and A427/LE (human lung adenocarcinoma) cells were obtained from the laboratory of Dr. L. C. Erickson (Loyola University Medical School, Maywood, IL) and were cultured under conditions identical to those used for HT-29 and HuTu-80 cells (5, 6, 19). BE human colon carcinoma cell cultures were cultured as described by Gibson and Erickson (20). The Mer phenotype of each cell line has been reported (21), based on its ability to reactivate chemically methylated adenovirus. Growth rates of these cell lines are roughly comparable, with doubling times varying from 18 to 22 h. Plating efficiencies for untreated cultures ranged between 40 and 80% for Mer+ lines, and was lower (20 to 40%) for the Mer~ lines.

Survival Assays. To determine the effects of DFMO pretreatment on the cytotoxic efficacy of BCNU, 75-cm² plastic flasks were seeded with 5 x 10⁵ to 1 x 10⁶ cells and had DFMO stock solution added at 24 h to a final concentration of 0.5 to 5 mM. Control flasks were treated with an equal volume of vehicle at the same time. Cultures were then incubated for 48 h at 37°C to maximally deplete PAs by cell growth in the presence of DFMO. To test for reversibility of DFMO effects, exogenous PUT was added at a final concentration of 100 µM after DFMO treatment. In those experiments utilizing pretreatment with both DFMO and STZ, a 60-min incubation with STZ took place immediately before BCNU addition. BCNU in ethanol was added to achieve the indicated final concentrations (see figures) and was then removed after a 60-min incubation at 37°C. Controls received an equal volume of ethanol (150 µl added to 15 ml media) for the identical time period, which had no effect on plating efficiency. The monolayers were washed, cells were harvested by gentle trypsinization, dispersed into single-cell suspensions, and counted in a Coulter Electronics (Hialeah, FL) Model ZBI particle counter. Survival of the treated and control cells was determined by plating efficiency assay, as described (5, 6, 19). For cultures pretreated with DFMO, STZ, or both drugs, calculation of surviving fractions after BCNU treatment was done relative to plating efficiencies of cultures subjected to the identical pretreatments but not incubated with BCNU. In those experiments testing the effect of DFMO alone on clonogenicity, cells were harvested at 48 h after DFMO addition with no additional drug or vehicle treatments. The survival curves shown are representative of two to five repeat experiments yielding similar results.

Polyamine Content. Aliquots of trypsinized and counted cells from DFMO-pretreated and control cultures were pelleted, washed, and extracted as described (5, 19) by sonication in 0.2 N perchloric acid containing 20 µM diaminoethane as internal standard for estimation of recovery. Concentrations of PAs in supernatants of the cell extracts were determined by paired-ion reverse-phase high-pressure liquid chromatography (22).

OAGTase Measurements. Quantitation of OAGTase activity in extracts of control and STZ-treated Mer~ cells was kindly performed in the laboratory of Dr. A. E. Pegg, Pennsylvania State University College of Medicine, Hershey, PA. The assay measures cell extract-catalyzed removal of the H-methyl moiety from the O-position of guanine in calf thymus DNA methylated by reaction with N-[methyl-3H]-nitrosourea. Procedures for preparation of H-methylated DNA, for incubation of radiolabeled DNA with cell extracts, and for high-pressure liquid chromatography analysis of reaction products have been published (23, 24).

RESULTS

BCNU Sensitivity in Mer~ Cell Lines. We have previously reported that in the Mer~ cell line BE, DFMO does not potentiate BCNU efficacy (6). The survival curves shown in Fig. 1 document that DFMO fails to sensitize three additional Mer~ human cell lines to the cytotoxic effects of BCNU. In A1235 cell cultures pretreated with 1.0 mM DFMO, PA depletion afforded a slight degree of protection from BCNU lethality. In contrast, with either VA-13 cells (treated with 5.0 mM DFMO) or A427/LE cells (treated with 0.5 mM DFMO), BCNU survival curves for PA-deficient cultures were similar to those of untreated controls. We found close agreement between our survival curves for each of the three lines after BCNU alone and previously published data from other laboratories (25). The concentration of DFMO used for pretreating each cell line was chosen so as to minimize loss of clonogenicity after 48 h of DFMO treatment (see below).

Chemosensitization by DFMO in Mer~ Lines with or without STZ. In contrast to the data of Fig. 1 obtained with Mer~ cell lines, both HT-29 and HuTu-80 cells are sensitized to BCNU by a 48-h pretreatment with 5 mM DFMO (Fig. 2). These observations replicate our earlier results with the two Mer~ cell lines (5). One possible cause for this difference between the Mer~ and Mer+ cell lines in the effect of DFMO on BCNU sensitivity might have been a lesser degree of PA depletion by DFMO in the Mer~ cells. Given the need to use lower concentrations of DFMO in two of the three Mer~ lines (see below), this point was of particular concern. Fig. 3 shows that in all five lines studied, DFMO (at the concentrations used with each cell line for the survival curves of Figs. 1 and 2) depletes PUT and SPD to undetectable levels. Fig. 3C also shows that SPM depletion in HT-29 cells, in which the greatest degree of potentiation for BCNU was observed, was not substantially greater than in VA-13 or A427/LE. To investigate further the mecha-
nism by which PA depletion enhances BCNU action in the two Mer+ cell lines, we used STZ to deplete these cells of OAGTase activity. The concentrations of STZ used for each cell line were chosen as the highest dose tolerated in a 60-min incubation without reducing plating efficiency below that of untreated controls. For HT-29, this was reported by others to be 2.5 mM (18), while preliminary experiments in this laboratory (data not shown) found that 1.0 mM STZ was the highest nontoxic concentration tolerated by HuTu-80 cells. Fig. 2 includes survival curves after BCNU treatment for cultures of both Mer+ cell lines that were pretreated either for 1 h with STZ alone or with DFMO for 48 h, followed by STZ for 1 h. Our results clearly show that DFMO plus STZ sensitizes Mer+ cells to BCNU by more than does STZ alone. It is noteworthy that in HuTu-80 cells enhancement by DFMO in STZ-pretreated cultures is even greater than in the cultures not treated with STZ.

Measurements of OAGTase activity in control and STZ-pretreated cultures of the two Mer+ cell lines are shown in Fig. 4. For HT-29 cells, the 1-h incubation with 2.5 mM STZ reduced OAGTase activity below the limits of detection (30 femol of the 3H methyl moiety transferred/mg of protein). In HT-29 cells, therefore, OAGTase activity was reduced to <10% of control levels. In contrast, HuTu-80 cells treated for 1 h with 1.0 mM STZ (the maximally tolerated noncytocidal concentration) only shows loss of approximately 58% of OAGTase activity.

Reversal of DFMO Effect in STZ-treated Mer+ Cells by PUT. In an earlier report we demonstrated the causal relationship between DFMO-induced ODC inhibition and sensitization to BCNU in both HT-29 and HuTu-80 cells in the absence of STZ (5). In those studies we utilized exogenous PUT added 24 h before BCNU treatment to bypass the inhibition of ODC and restore concentrations of all three PAs to near control levels. Fig. 5 shows results of similar experiments with cultures of both cell lines pretreated with both DFMO and STZ. In each case, exogenous PUT restored the sensitivity of (DFMO plus STZ)-pretreated cultures to levels not distinguishable from those of cultures pretreated with STZ alone. Measurements of PA concentrations (data not shown) in extracts prepared from aliquots of the cells utilized for the survival assays of Fig. 5 verified: (a) that DFMO depleted PUT, SPD, and SPM in STZ-pretreated cultures to the same degrees as shown in Fig. 3 for cells treated only with DFMO and BCNU; (b) that exogenous PUT restored PA contents to near control levels; and (c) that STZ had no effect on PA concentrations of control cells not treated with DFMO.

Effects of PA Depletion on Plating Efficiencies of Mer+ and Mer Cells. Fig. 6 compares survival of cells after 48-h incubations with 5.0 mM DFMO for four Mer+ cell lines (HT-29, HuTu-80, ME-180, and MCF-7) and four Mer- lines (A1235, VA-13, A427/LE, and BE). The data for the Mer+ lines, taken from our earlier report (19), show at least 50% survival after 48 h with 5.0 mM DFMO for each cell line. In contrast, two of the Mer- lines had plating efficiencies between 20 and 35% of untreated controls, while the remaining two showed 1.5 to 5% survival, after identical treatments. We also examined the

Fig. 2. DFMO-induced polyamine depletion potentiates BCNU efficacy in two human Mer+ cell lines with or without streptozotocin. Cultures were treated with 5 μM DFMO at 24 h after seeding 5 × 10^5 cells/75-cm² flask. After a further 48 h, the indicated cultures were treated for 60 min with STZ (2.5 mM for HT-29 and 1.0 mM for HuTu-80) to deplete O'-alkylguanine-DNA alkyltransferase activity. In separate paired experiments, cell survival after BCNU treatment was compared between DFMO-pretreated cultures and controls, and between (DFMO + STZ)-pretreated cultures and cultures pretreated only with STZ. Cell survival was measured by colony formation assays, and was normalized to identically pretreated cultures receiving no BCNU for each of the four pretreatment groups. Each pair of survival curves is from a single representative experiment. Points, means of 4-12 replicate dishes/BCNU dose for each pretreatment group; bars, SD.

Fig. 3. Polyamine contents of DFMO-pretreated Mer+ and Mer- cells. Aliquots of the cell suspensions used for colony formation assays in Figs. 1 and 2 were pelleted by centrifugation and washed with phosphate-buffered saline. Polyamine contents in 0.2 N HClO4 extracts of the cell pellets were measured by reverse-phase high-pressure liquid chromatography on C-18 columns, using ion-pairing buffers and post-column derivatization with o-phthaladhyde. Columns, means of results from 2-5 experiments, with 3 or 4 replicates/experiment; bars, SD. The numbers given above the data for spermine contents of DFMO-treated cultures indicate percentages of control spermine levels.

Fig. 4. OAGTase activity in control and STZ-pretreated cultures of the two Mer+ cell lines. For HT-29 cells, the 1-h incubation with 2.5 mM STZ reduced OAGTase activity below the limits of detection (30 femol of the 3H methyl moiety transferred/mg of protein). In contrast, HuTu-80 cells treated for 1 h with 1.0 mM STZ (the maximally tolerated noncytocidal concentration) only shows loss of approximately 58% of OAGTase activity.
**Fig. 4.** Effects of streptozotocin on O'-alkylguanine-DNA alkyltransferase activities in two human Mer* cell lines. Cultures were incubated for 72 h after seeding 5 x 10^4 cells/75-cm^2_ flask, then either treated or not treated for 60 min with STZ (2.5 mM for HT-29 and 1.0 mM for HuTu-80). Alkyltransferase activity in buffer extracts of cell pellets was measured by transfer of the ^3_H methyl moiety from the O'-position of guanine in DNA, using published procedures (23, 24). Columns, means for three replicate assays on extracts of single pooled samples for each treatment condition; bars, SD.

**Fig. 5.** Putrescine restores BCNU sensitivity of (DFMO + STZ)-pretreated Mer* cells to that of cultures pretreated with STZ alone. Cultures given one of three pretreatments were subsequently treated with the indicated doses of BCNU and survival was measured by clonogenicity assays. The three pretreatments were: STZ only (at 2.5 mM for HT-29 and 1.0 mM for HuTu-80) for 1 h immediately before BCNU treatment at 73 h; 5 mM DFMO added at 24 h, then STZ (at the above doses) added at 72 h, followed by BCNU at 73 h; and 5 mM DFMO added at 24 h, followed by 100 µM PUT added at 60 h and STZ (at the above doses) at 72 h, and finally BCNU treatment at 73 h. Each set of survival curves is from a single representative experiment. Points, means of 4–12 replicate dishes/BCNU dose for each pretreatment group; bars, SD.

**Fig. 6.** Cell survival after 48 h with 5 mM DFMO segregates with Mer phenotype in a series of eight human tumor lines. Colony formation efficiencies were measured for control and for DFMO-treated cultures of four Mer* cell lines (HT-29, HuTu-80, ME-180, and MCF-7) and four Mer* lines (A1235, VA-13, A427/LE, and BE). Surviving fractions after DFMO were calculated as the ratios of treated to control efficiencies for each cell line. Columns, means of results from 2-5 experiments with 4-12 replicates/treatment condition in each experiment. Dotted line, 50% survival level.

**Fig. 7.** Dose dependence and putrescine reversibility for reduced survival after 48-h treatments with DFMO in the two most sensitive Mer* cell lines. Cultures were treated with the indicated concentrations of DFMO at 24 h (O, A) and one-half were then treated with 100 µM PUT at 60 h (•, △). At 72 h, cultures were harvested and cell survival was measured by colony formation assay. Points, means of 4–12 replicate determinations/treatment condition from a single representative experiment; bars, SD.

**DISCUSSION**

The experiments presented here, together with our previous reports (5, 6) show that in a total of nine human tumor and transformed cell lines, the ability of DFMO-induced PA depletion to sensitize cells to the cytotoxic effects of BCNU is consistently dependent on their alkylation-repair phenotype. Thus, five Mer* lines all show chemosensitization to BCNU by DFMO pretreatment, while four Mer* lines show no more than equal sensitivity to BCNU in DFMO-pretreated and control cultures. Measurements of PA contents in cell extracts demonstrate that this difference could not be attributed to inadequate PA depletion by DFMO in the Mer* cell lines (Fig. 3). It also seems unlikely that the ability to remove alkyl groups from...
the O6 position of guanine in DNA bears a direct causal relationship to the ability of PA depletion to alter CENU efficacy in a given cell line. Nonetheless, a direct test of the latter hypothesis is possible, using Mer* cells maintained for several doublings in the presence of low concentrations of DNA-methyllating agents so as to continuously inactivate their OAGTase activity. If PA depletion of these cells continues to sensitize them to BCNU, one could conclude that Mer phenotype and DFMO-induced potentiation of CENU were two independent cellular characteristics which happen to segregate together. Experiments to test this possibility are currently in progress.

Several lines of evidence strongly suggest (but do not conclusively prove) that, at least in human Mer* cells, ISC are the lesions most responsible for BCNU-induced cell lethality (16-18, 21, 26). Although it remains possible that ISCs are not the most critical targets in either phenotype, the need for 5- to 10-fold higher concentrations of BCNU to kill comparable fractions of human Mer* cells has generally been interpreted to result from the increased ability of these cells to remove O6-chloroethyl groups from guanine in DNA. Thus, it has been assumed that concentrations of BCNU which more than saturate Mer* cell OAGTase activity are ultimately lethal by ISC formation. Our inability to detect ISCs in either control or PA-depleted human Mer* cells by alkaline elution analysis (6), even at BCNU concentrations which reduce survival by several logs, seems to challenge the above assumption. Instead, our data suggest that the relevant target(s) for BCNU in human Mer* cells might be different from that in human Mer* cells (i.e., ISC). Such putative targets for CENUs could require substantially higher drug concentrations for inactivation than those used to produce lethal levels of ISCs in Mer* cells. If that were the case, our finding that DFMO pretreatment of human Mer* cells does not increase their sensitivity to BCNU might be explained by the low concentrations of BCNU used with the more sensitive Mer* cells. Since these BCNU doses seem to be inadequate to inactivate hypothetical targets at which PA depletion enhances BCNU efficacy in human cells, DFMO may not sensitize human Mer* cells to BCNU. It is important to note here that our results with human Mer* cells differ markedly from those reported in two rodent tumor cell lines. DFMO-mediated PA depletion does sensitize 9L and LI210 cells to BCNU (1, 4), despite the fact that the BCNU sensitivities and OAGTase levels of these cells resemble those of human Mer* lines. We are as yet unable to reconcile these differing effects of DFMO on responses to BCNU between human and rodent cells.

Our results with combined DFMO and STZ pretreatment provide further support for the hypothesis that ISCs may not be the relevant targets for enhanced efficacy of BCNU in PA-deficient cells. If the two pretreatments act at the same target, one would expect that STZ doses which completely saturate cellular OAGTase activity would result in maximal enhancement of ISC formation, and thus the greatest possible BCNU-induced cell kill. The data of Fig. 4 show that 2.5 mM STZ reduces HT-29 OAGTase activity below the limit of detection. Nonetheless, as shown by the data of Figs. 2 and 5, DFMO followed by STZ potentiates BCNU in HT-29 cells by more than does STZ alone. The latter observation is also true for HuTu-80 cells. However, since the 1.0 mM STZ treatment did not abolish HuTu-80 OAGTase activity, it remains unclear if DFMO and STZ act at the same or at different targets in the HuTu-80 cells. We are currently investigating STZ treatments at reduced concentrations for periods longer than the 60 min used here so that OAGTase activity can be fully eliminated from HuTu-80 cells under conditions which do not reduce clonogenicity. If DFMO plus STZ continues to sensitize HuTu-80 to BCNU by more than does STZ alone, then the two pretreatments must also be acting via different mechanisms in this cell line. Even in the HT-29 cells, though, our observations do not yet prove that DFMO mediates increased BCNU efficacy at targets other than ISCs. One can envision other mechanisms, besides complete depletion of OAGTase-dependent repair capacity, by which ISC production by CENUs may be enhanced as a result of PA deficiency. Examples include altered chromatin structure resulting in either increased reactivity at guanine O6 or more efficient ISC formation from a fixed number of monoalkylations. To investigate this issue further, we are currently using alkaline elution assays to compare BCNU-induced ISCs in HT-29 cells pretreated with both DFMO and STZ to ISC levels in cells pretreated with STZ alone.

An additional point needing further study is the BCNU concentration dependence for inactivating putative Mer* cell-specific target(s). Our results with Mer* cells (Fig. 1) predict that complete elimination of OAGTase activity in HT-29 cells by STZ should reduce the minimal cytocidal doses of BCNU below the levels needed to inactivate these targets. We thus expected that DFMO pretreatment should not alter BCNU sensitivity after STZ is added to Mer* cells. Comparison of Figs. 2 and 5 to Fig. 1 shows that STZ pretreatment brings the cytocidal dose range for HT-29 cells down to concentrations near those used in the Mer* cells (5 to 20 mM). Nonetheless, PA deficiency further enhances BCNU action under these conditions. Unless the STZ pretreatment sensitizes HT-29 cells at the non-ISC targets in addition to eliminating AGT activity, it remains unclear why DFMO enhances BCNU efficacy at the lower dose range used in STZ-treated Mer* cells, yet fails to sensitize Mer* cells at comparable concentrations. One hypothesis worth further investigation is that sensitivity of non-ISC targets to BCNU may be a second independent phenotypic difference between Mer* and Mer* cells, unrelated to expression of OAGTase activity.

A final noteworthy difference between these two human tumor cell types appears to be their ability to survive 48-h incubations with DFMO and STZ without substantial declines in clonogenicity (Figs. 6 and 7). In nearly all cell lines tested thus far, DFMO is cytostatic rather than cytocidal (reviewed in Refs. 27 and 28). The sole exceptions noted thus far are a series of human small cell lung carcinoma lines showing declines in cell numbers after longer incubations with DFMO, interpreted as indicating cell death (29, 30). The Mer* cell lines used here all show substantial cytostatic responses to 48-h treatments with DFMO, as measured by clonogenicity assays. It would thus be of interest to determine the Mer phenotypes of the small cell lines used in the earlier reports. It is also intriguing that a large cell lung tumor line responds to DFMO with cytostasis, yet is killed by concentrations of bis(ethyl) PAs which cause no greater PA depletion in the treated cells than does DFMO (31). These observations imply that factors besides PA contents after treatment must determine whether cells will show cytotastic or cytoidal responses to biosynthetic inhibitors or analogues of the PAs. Further work to elucidate the factors causing cytodial responses to drugs which interfere with PA biosynthesis is of obvious importance to the ultimate use of such compounds in antineoplastic chemotherapy.

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