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Nitrogen mustard-DNA interaction in melphalan-resistant mammary carcinoma cells with elevated intracellular glutathione and glutathione-S-transferase activity*

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Summary. We examined the relationship between intracellular levels of glutathione (GSH), glutathione-S-transferase (GST) activity, and the kinetics of DNA cross-links induced by the bifunctional alkylating drugs melphalan (MLN), chlorambucil (CLB), and mechlorethamine (HN2) in a rat mammary carcinoma cell line (WT) and in a subline selected in vitro for primary resistance to MLN (MLNr, 16-fold resistance). MLNr cells exhibit a 2-fold increase in intracellular GSH concentration and an approximately 5-fold increase in GST activity as compared with the parent cells. They are cross-resistant to a variety of drugs, including CLB (6-fold) and HN2 (14-fold). Treatment of WT cells with 30 μM MLN or CLB induced a significant accumulation of DNA-DNA cross-links for up to 8 h, which decreased over a 24-h period. In MLNr cells, no significant cross-link formation was induced by either MLN of CLB at any time between 0 and 24 h. Doses of up to 100 μM MLN failed to induce cross-links in MLNr cells. Formation of cross-links was observed immediately after treatment with HN2 in both cell lines and was followed by a subsequent decrease during a 24-h incubation in drug-free medium. At an equimolar concentration (30 μM), the mumbers of HN2-induced cross-links were significantly lower in MLNr cells than in WT cells. However, treatment of MLNr cells with 60 μM HN2 resulted in cross-link levels similar to those obtained using 30 μM HN2 in WT cells. The 35% decrease in MLN accumulation observed in MLNr cells could not entirely explain the absence of cross-links, since thin-layer chromatographic analysis demonstrated that both cell lines accumulate a significant amount of MLN and metabolize it to the same extent. Significant amounts of MLN were also detected in nuclei isolated from WT and MLNr cells that had been treated with 30 μM [^{14}C]-MLN. Intracellular depletion of GSH by a nontoxic concentration of L-buthionine-(S, R)-sulfoximine (BSO, 100 μM; about 70% GSH depletion) significantly sentisized MLNr cells to MLN and increased cross-link formation. A nontoxic concentration (50 μM) of ethacrynic acid (EA, an inhibitor of GST showing some specificity for Yc/Yp subunits) also sensitized MLNr cells to MLN and increased cross-link formation. Our data demonstrate that both EA and BSO are effective modulators of nitrogen mustard cytotoxicity in tumor cells resistant to alkylating drugs. The limited number of cross-links formed in MLNr cells after treatment with MLN or even CLB suggests that efficient repair of drug-DNA monoadducts is operative in these cells and that the increases obtained in the presence of BSO and EA may be related to the involvement of both GSH and GST in drug-DNA interactions such as monoadduct repair.

Introduction

The development of tumor resistance to bifunctional alkylating drugs is a common problem in the clinical setting. One of the most important alterations associated with this type of resistance is the elevation of intracellular levels of glutathione (GSH) as well as several enzymes involved in its metabolism and in catalysis of the formation of GSH conjugates (i.e., glutathione-S-transferases, GST) [7, 21, 28, 35, 38].

GSH plays a major role in the conjugation and subsequent detoxification of many electrophiles. GSTs are phase II enzymes that catalyze the conjugation of GSH to drug or to drug-DNA adducts. GSTs are a family of distinct isoenzymes that are encoded by different genes, have

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Abbreviations: MLN, melphalan; CLB, chlorambucil; HN2, mechlorethamine; EA, ethacrynic acid; BSO, L-buthionine-(S,R)-sulfoximine; GSH, glutathione; GST, glutathione-S-transferase; BCNU, Carmustine; CDDP, *cis*-diamminedichloroplatinum(II)

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Table 1. Cross-resistance profile of MLNr cells

Drug	Resistance[a]
Drugs:	
MLN	16-fold
HN2	13-fold
CLB	6-fold
BCNU	17-fold
CDDP	12-fold
Adriamycin	2.3-fold
Vincristine	2-fold
EA	2-fold
X-rays[b]:	
WTcells:	
Do	174
n	3.2
MLNr cells:	
Do	246
n	3.2

Data represent mean values for 3 experiments in which the SE was <10% of the value reported

[a] Ratio of the IC_{50} value obtained for resistant cells to that found for sensitive cells following continuous exposure on agar as described elsewhere [32]

[b] Do is the exponential slope of the survival curve and *n* is the extrapolation number that represents the size of the shoulder [25]

different substrate specificities [26, 35], and are inducible by specific electrophiles to different degrees [16]. GSH may act nonenzymatically [6, 34] or through GST-catalyzed reactions [6, 35] to compete with DNA for drug interaction, the critical event leading to cell killing by alkylating drugs, through the inactivation of reactive metabolites and the reduction of drug-induced DNA damage [7, 21, 28, 35, 38]. GSH has been found to quench DNA monoadducts in alkylated DNA and thus, to inhibit DNA cross-link formation [4]. It has also been suggested that both GSH and GST modulate other processes such as DNA repair [17, 23, 39]. Further characterization of the role of GSH and GST in resistance to alkylating drugs is required for the establishment of strategies to overcome tumor resistance.

In the present study, we examined a subline of a rat mammary carcinoma cell line (MatB) that was selected for MLN resistance in vitro (MLNr) and found to be cross-resistant to other alkylating agents as well as to other unrelated drugs and to radiation (Table 1). Increased intracellular GSH and GST activity associated with a specific overexpression of Yc and Yp GST mRNA and increased organic peroxidase activity have previously been reported in MLNr cells [33]. Since DNA is a target for alkylating drugs, we performed a detailed study of the kinetics of DNA cross-links induced by melphalan (MLN), chlorambucil (CLB), and mechlorethamine (HN2) in both sensitive and resistant cell sublines. The relationship between DNA damage, MLN accumulation, intracellular levels of GSH, and GST activity was investigated.

Materials and methods

Drugs. MLN and L-buthionine-(S,R)-sulfoximine (BSO) were obtained from Sigma Chemical Company. CLB was supplied by ICN Biomedicals Canada Ltd. HN2 was purchased from Merck Sharp & Dohme Canada, and EA was obtained from Merck Frosst Canada. Proteinase K was supplied by BDH Chemicals Co. Ltd. [^{14}C]-Thymidine (sp. act., 51.7 mCi/mmol) and [^{3}H]-thymidine (sp. act. 20 Ci/mmol) were purchased from New England Nuclear; [chloroethyl-^{14}C]-MLN was obtained from the Drug Development Branch, Division of Cancer Treatment, National Cancer Institute (Silver Spring, Md.; NSC number 8806); and [carboxy-^{14}C]-inulin (sp. act., 17.1 mCi/mmol) and tritiated water (sp. act., 0.5 mCi/ml) were supplied by Amersham Corporation (Oakville, Ontario, Canada).

Cell lines and culture. The parental drug-sensitive MatB 13762 cell line (WT) was originally obtained from a rat mammary adenocarcinoma. A MLN-resistant subline (MLNr) was obtained by continuous exposure of cells to escalating MLN concentrations ranging from 10^{-8} to 10^{-5} M [33]. Cultured cells were maintained in monolayers in alpha minimal essential medium (α-MEM, Gibco) supplemented with 1.3% sodium pyruvate, 2.6% glutamine, 1.3% nonessential amino acids, 10% fetal bovine serum, and 100 units gentamicin/ml. Cells were maintained in a humidified atmosphere containing 5% CO_2 and were passaged twice weekly at approximately 1×10^5 cells/ml.

Cytotoxicity assays. The cytotoxicity of the drugs was determined using a 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Exponentially growing cells were seeded in 96-well microtiter culture plates (Falcon) at a density of 1,000 cells/well in 200 µl α-MEM supplemented with 2.6% nonessential amino acids, 10% fetal bovine serum, and antibiotics. After 18 h cultivation, cells were treated with drug. After 72 h, cell growth was determined spectrophotometrically as described elsewhere [8].

DNA-DNA cross-link studies. DNA interstrand cross-links were quantified by the alkaline elution assay [24]. Exponentially growing cells were labeled for 48 h with either 0.075 µCi/ml [^{14}C]-thymidine or 0.5 µCi/ml [^{3}H]-thymidine at a final concentration of 10^{-6} M. The labeled cells were washed twice with cold phosphate-buffered saline (PBS), and the radioactivity was chased by an additional 16-h incubation in medium containing 10^{-5} M cold thymidine. The ^{14}C-labeled cells were treated in serum-free medium for 1 h and treatment was then stopped by immediate chilling of cells on ice. Aliquots of cells were used immediately (time zero) or were further incubated in drug-free medium for the accumulation and removal of DNA cross-links. Control or drug-treated ^{14}C-labeled cells (0.5×10^6) were then mixed with tritiated cells (0.5×10^6) and irradiated with 6 Gy ^{60}Co γ-rays using a dosing rate of approximately 1 Gy/min at 4° C. The combined cell suspension was immediately prepared for alkaline elution as previously described [3]. Briefly, 1×10^6 combined tritium- and ^{14}C-labeled cells were layered onto a polyvinyl chloride filter, washed with cold PBS and lyzed with 5 ml lysis solution [2% sodium dodecyl sulfate (SDS), 0.025 ethylenediaminetetracetic acid (EDTA), 0.5 mg porteinase K/ml; pH 9.7] for 45 min. Filters were then washed with 2×2 ml 0.02 M EDTA (pH 10.3), and the DNA was eluted with 30 ml tetrapropylammonium hydroxide-EDTA buffer (pH 12.1) containing 0.5% SDS solution using a flow rate of approximately 0.035 ml/min in a dark environment at room temperature. The ^{14}C and tritium activities on filters and fractions were analyzed by liquid scintillation counting, and the DNA cross-link frequency expressed as the DNA-DNA cross-link index was estimated as:

$$(1-Ro)^{1/2}/(1-R)^{1/2}-1,$$

where *Ro* and *R* represent the final fractions of [^{14}C]- and [^{3}H]-DNA retained on the filter, respectively.

GSH and GST determination. Total intracellular content and GST activity were assayed spectrophotometrically using 5,5′-dithio-bis(2-nitrobenzoic) acid (DTNB) and 1-chloro-2,4-dinitrobenzene (CDNB) as the substrate, respectively, as described elsewhere [14, 20]. Protein concentrations were determined according to the method of Lowry et al. [27].

Melphalan transport and metabolism. Exponentially growing cells (2×10^6 cells/ml) were incubated wiht [chloroethyl-^{14}C]-MLN for different periods at 37° C. At the end of each incubation period, 400 µl of the

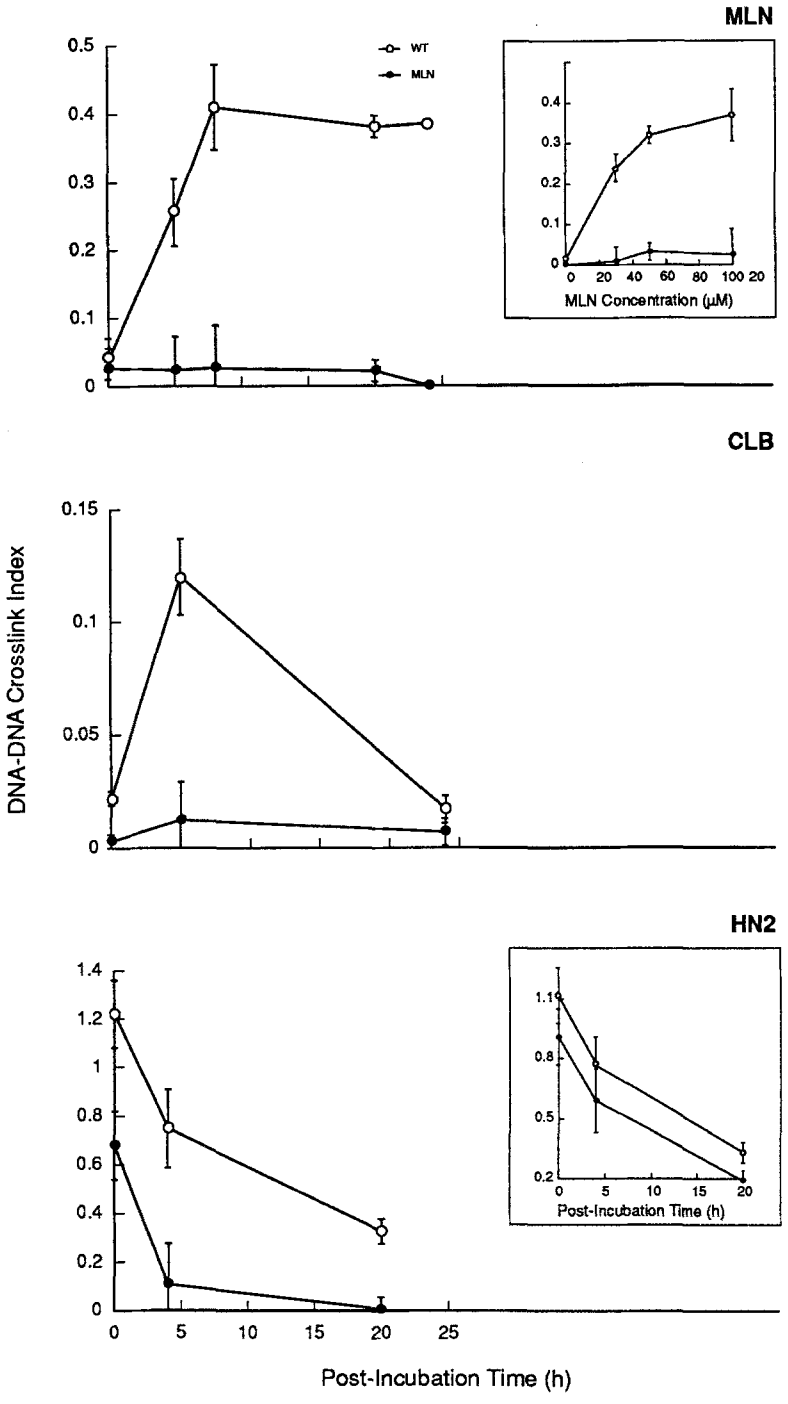

Fig. 1. DNA cross-links induced by MLN, CLB, and HN2 in WT (○) and MLNR (●) cells. Exponentially growing cells (1×10^6) were labeled with [^{14}C]-thymidine and treated with 30 μM MLN, CLB, or HN2 for 1 h in serum-free medium. Cells were then either used immediately for alkaline elution or further incubated in drug-free medium for the accumulation and removal of cross-links over the periods indicated. Each point represents the mean value ± SE calculated from 3 independent experiments. *Inserts:* Dose effect on MLN-induced cross-links after 1 h exposure followed by 5 h post-incubation in drug-free medium (*top*) and kinetics of cross-links formed in MLNr cells treated with 60 μM HN2 for 1 h (●) as compared with WT cells treated with 30 μM (○) (botton). Each point represents the findings obtained independently of the other data

incubation mixture was layered onto 1 ml vestibule F-50 silicone oil in microcentrifuge tubes and centrifuged at 12,000 *g* for 1 min at room temperature. The radioactivity in the medium and in the cell pellet was determined as previously described [30]. Nonspecific absorption of labeled drug was determined by layering 200 μl untreated cells onto 200 μl medium containing labeled MLN at 4° C followed by immediate centrifugation as described above. Extracellular concentrations of MLN trapped in the cell pellet by the oil layer were estimated using [carboxy-^{14}C]-inulin as a marker for extracellular fluid. Intracellular water space was estimated using tritiated water as described elsewhere [10, 30]. The correction factor was 1.08 ± 0.66 for WT cells and 1.13 ± 0.85 for MLNr cells and was used to evaluate the net uptake. The cell/medium ratio for MLN was calculated as the ratio of radioactivity per cell valume to that in an equivalent volume of extracellular medium. For investigation of the accumulation of MLN in nuclei, WT and MLNr cells were treated with radiolabeled MLN and the nuclei were isolated as previously described [12]. Nuclei were lyzed with 1 *N* NaOH and then neutralized, and the radioactivity was determined by scintillation counting (in this case, no correction was made for intracellular water space). Duplicate samples were used to determine percentages of recovery of MLN and its metabolites by thin-layer chromatography. The amount of radioactivity recovered was calculated as a percentage of the total radioactivity recovered under each condition.

Cell-cycle analysis. Cell-cycle-phase distribution was determined by flow cytometry using a FACS I cell sorter (Becton-Dickinson, Mountain View, Calif.) as described elsewhere [25].

Table 2. Cell-cycle analysis

Cells	Fraction of cell phase of cycle (%)[a]:			Doubling time (h)[b]
	G1	S	G2/M	
WT	82	12	6	28
MLNr	71	19	10	22

[a] Exponentially growing cells were stained with propidium iodide and analyzed by flow cytometry as described in Materials and methods
[b] As determined by Coulter counter

Results

DNA-DNA cross-links induced by MLN, CLB, and HN2

The DNA cross-linking patterns obtained using MLN, CLB, and HN2 in WT and MLNr cells are summarized in Fig. 1. Exponentially growing cells were treated for 1 h with 30 μM of each drug and were cultured for various intervals for the accumulation and removal of DNA cross-links. In WT cells, MLN and CLB induced the formation of cross-links, which increased over time to a maximum at 8 h and then decreased significantly for up to 24 h. In MLNr cells, MLN produced no significant cross-link formation at any time between 0 and 24 h, even at concentrations of up to 100 μM. A similar kinetic profile was obtained using 30 μM CLB (Fig. 1). In contrast, the formation of DNA cross-links was seen in both cell sublines immediately following HN2 treatment, and the number of cross-links decreased over time on incubation of the sublines in drug-free medium (Fig. 1). However, at an equimolar concentration (30 μM), HN2 induced significantly fewer cross-links in MLNr cells than in WT cells ($P < 0.05$). Treatment of MLNr cells with 60 μM HN2 induced approximately the same number of initial cross-links that were obtained using 30 μM in WT cells ($P < 0.5$). Since differences in cell-cycle distribution between WT and MLNr cells might theoretically account for some of the observed kinetics of DNA cross-link formation and removal, we studied cell-cycle obtained by flow cytometry. No significant difference between the two cell lines was found through the G1, S, G2, and M phases (Table 2).

MLN transport and metabolism

MLN accumulation was investigated in WT and MLNr cells that had been treated with 30 μM [^{14}C]-MLN under conditions similar to those described for the alkaline elution assay. Figure 2 shows the cell/medium ratio for [^{14}C]-MLN as corrected for [^{14}C]-MLN surface binding and for intracellular water concentration. In both cell lines, the MLN-uptake pattern was linear for up to 3 min but thereafter rapidly became nonlinear and remained so from 10 to 60 min. This indicates a rapid transport saturation, in agreement with previous reports [10, 37]. The maximal accumulation was observed at 10 min for WT (cell/medium ratio, 3.9 ± 1.4) and MLNr cells (cell/medium ratio, 2.8 ± 0.38). The cell/medium ratio at the steady-state MLN concentration was approximately 35% lower in MLNr cells relative to WT cells (Fig. 2). The accumulation of MLN in nuclei isolated from pretreated cells (30 μM [^{14}C]-MLN for 1 h) revealed that MLN is significantly distributed in both WT and MLNr cell nuclei

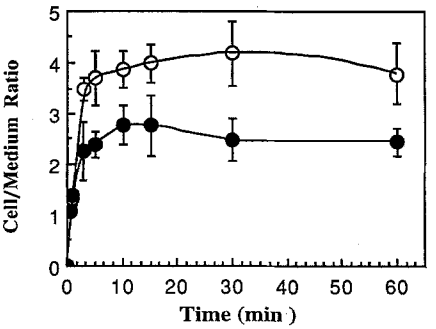

Fig. 2. MLN accumulation in WT (○) and MLNr (●) cells. Exponentially growing cells (2×10^6) were incubated with [^{14}C]-MLN for different intervals at 37° C. Accumulation experiments were carried out as described in Materials and methods, and the cell/medium ratio was determined after corrections for MLN membrane binding and intracellular water. The volume of intracellular water was 0.74 ± 0.21 ml for WT cells and 0.74 ± 0.30 ml for MLNr cells. Each point corresponds to the mean value ± SE generated from at least 2 experiments

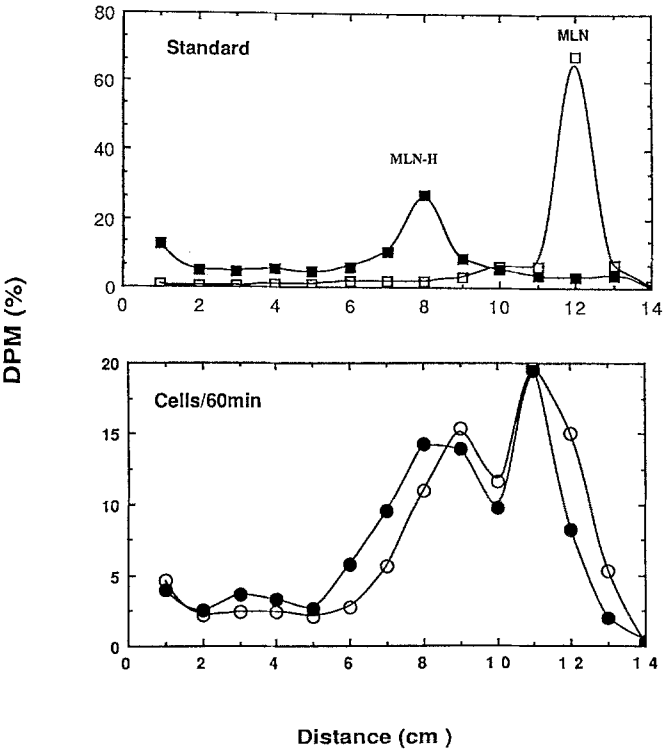

Fig. 3. MLN metabolism in WT and MLNr cells. Cell pellets obtained from 60-min transport experiments were used to estimate MLN metabolism by thin-layer chromatography as described in Materials and methods. Cell pellets were isolated from WT (○) and MLNr (●) cells. Each point represents the mean value for 2 determinations in which the SD was <10%. The reference chromatogram (*Standard*) shows the data obtained using MLN (□) and its inactive metabolite hydroxy-MLN (■). The R_f value was 0.9 ± 0.007 for MLN and 0.6 ± 0.007 for hydroxy-MLN (*MLN-H*)

Table 3. Effect of BSO on GSH levels in WT and MLNr cells

	GSH (nmol/mg protein)	
	WT[a]	MLNr[a]
Control	37.75 ± 2.23	90.76 ± 1.73
BSO (μM)[b]:		
20	22.69 ± 2.68 (40%)	60.30 ± 1.58 (34%)
50	18.38 ± 1.63 (51%)	45.93 ± 9.82 (49%)
100	9.30 ± 0.76 (75%)	26.40 ± 1.86 (71%)

[a] Values determined by Lowry et al. [27] were 0.17 and 0.15 mg protein/10^6 cells in WT amd MLNr cells, respectively
[b] Cells were treated for 18 h and immediately prepared for GSH assay Numbers in parentheses represent the percentage of GSH depletion relative to that in untreated cells

Table 4. Effect of EA and BSO on MLN cytotoxicity in WT and MLNr cells

	IC_{50} (μM)		DMF	
	WT	MLNr	WT	MLNr
MLN	5.6	70	–	–
MLN + 50 μM EA[a]	3.9	41	1.44	1.71
MLN + 100 μM BSO[b]	4.2	40	1.33	1.75

[a] Cells were preincubated with EA for 18 h and MLN was added in the presence of EA in continuous exposure
[b] Cells were preincubated with 100 μM BSO for 18 h. BSO was then removed and cells were immediately treated with MLN in continuous exposure
[c] DMF (dose modifying factor): IC_{50} melphalan/IC_{50} melphalan + modulator

Table 5. Effect of BSO and EA on MLN-induced DNA-DNA cross-links in MLNr cells

Time (h)	DNA-DNA cross-link index		
	MLN[a]	MLN + BSO[b]	MLN + EA[c]
6	0.05 ± 0.02	0.12 ± 0.02*	0.10 ± 0.01*
20	0.03 ± 0.01	0.05 ± 0.01	0.05 ± 0.01

[a] MatB-MLNr cells were treated with 30 μM MLN for 1 h. Cross-links were estimated at 6 and 20 h post-incubation in drug-free medium
[b] MatB-MLNr cells were treated with 100 μM BSO for 18 h. BSO was then removed, cells were treated with 30 μM MLN for 1 h, and cross-links were determined at 6 and 20 h post-incubation in drug-free medium
[c] MatB-MLNr cells were treated with 50 μM EA for 18 h. MLN was added at 30 μM in the presence of EA for 1 h. Cross-links were determined at 6 and 20 h post-incubation in drug-free medium
* $P < 0.05$

(total [^{14}C]-MLN activity, 1,585 ± 149 and 1,472 ± 137 cpm/10^5 nuclei in WT and MLNr cells, respectively).

Thin-layer chromatographic analysis was carried out on the pellet collected from the 60-min accumulation experiments. MLN was extensively metabolized by both sublines, and no significant difference between WT and MLNr was detected (Fig. 3). As previously reported [10], the mono- and dihydroxy-MLN metabolites could not be distinguished; thus only the data for dihydroxy-MLN are reported.

Effect of GSH and GST depletion on cell survival and DNA cross-links

Since MLNr cells showed increased intracellular GSH concentration and GST activity [33], we investigated the effects of BSO (an inhibitor of the rate-limiting GSH synthetic enzyme γ-glutamylcysteine synthetase [19]) and EA (an inhibitor of GST activity showing some specificity for the Yc/Yp GST subunits [1, 2, 9]) on cell survival and DNA cross-link formation. A 20-h pre-incubation of WT or MLNr cells with BSO at concentrations ranging from 20 to 200 μM showed that BSO was not significantly toxic to either WT or MLN cells (data not shown). In contrast to BSO, EA was found to be more cytotoxic to cells [after 18 h exposure, the concentration that inhibited the growth of 10% of the cell population (IC_{10}) as 48 and 62 μM and the IC_{50} was 80 and 140 μM for WT and MLNr cells, respectively]. Table 3 shows that at nontoxic concentrations, BSO was very effective in depleting GSH in MatB cells. Treatment with 20, 50, and 100 μM depleted GSH levels by approximately 40%, 50%, and 70% in both WT and MLNr cells. A nontoxic dose of EA (10–50 μM) failed to inhibit GST activity under the conditions of the GST assay using CDNB as a substrate. Preincubation of cells with 100 and 200 μM EA for 18 h inhibited GST activity by approximately 9% and 18%, respectively, in both WT and MLNr cells. However, these concentration were found to inhibit cell growth as determined by the MTT assay. Since EA is a competitive of GST, the failure of nontoxic concentrations to inhibit GST activity may be explained by the possibility that supersaturation levels of CDNB used as a substrate in the GST assay may have overcome the remaining intracellular concentration of EA. Using minimally toxic concentrations, we found that both BSO (100 μM) and EA (50 μM) sensitized MLNr cells (the magnitude of resistance of MLNr cells decreased to approximately 7-fold to that of WT cells that had been treated with MLN alone; Table 4). Significant sensitization was also observed in WT cells.

The induction of cross-links observed in MLNr cells in the presence of 30 μM MLN in combination with 100 μM BSO and 50 μM EA is shown in Table 5. Neither BSO nor EA alone had any effect on cross-link formation at the concentrations tested (data not shown). Pretreatment of MLNr cells with either 100 μM BSO or 50 μM EA increased the cross-link formation induced by MLN in MLNr cells as measured at 6 h after treatment ($P < 0.05$). After a 20-h incubation in drug-free medium, no significant difference was detected between MLN alone and MLN used in combination with EA or BSO ($P > 0.5$). In WT cells, no significant difference in cross-link formation was detected between MLN alone and MLN used in combination with EA or BSO at either 6 or 20 h post-incubation in drug-free medium (data not shown). In the presence of both modulators, significant differences were found between the amounts of DNA-DNA cross-links that were induced in MLNr cells and those that were formed in WT cells (cross-link index obtained after 1 h exposure to 30 μM MLN followed by 6 h post-incubation in drug-free medium was 0.30 ± 0.02).

Discussion

Nitrogen mustards are bifunctional alkylating agents that are used as cytostatic and cytotoxic drugs. Their toxic effect is related at least in part to their ability to produce DNA cross-links via the formation of drug-DNA monoadducts, mainly at the N7 atom of guanine residues [18, 31, 40]. Alterations in drug-DNA interactions have been found to be associated with resistance to alkylating agents in many animal- and human-derived tumor cell lines [5, 15, 32].

MatB rat mammary carcinoma cells represent a model in which a number of apparently distinct mechanisms of DNA "defense" are co-selected or co-regulated by continuous exposure to MLN (MLNr cells) and thus constitute a useful tool for the investigation of modulation strategies in resistant tumors. MLNr cells are cross-resistant to most known alkylating drugs and to X-rays, but they are also moderately cross-resistant to so-called natural products (doxorubicin, vincristine; Table 1). Given the cross-resistance of these cells to CLB and HN2 and the similarity of the chemical structures of these drugs in terms of reactive groups (all possess an identical reactive chloroethyl group), we expected to find a common mechanism for the limitation of DNA damage. We demonstrated quite distinct alterations in the kinetics of drug-DNA interactions for these three agents. Whereas no significant cross-link formation was induced by MLN or CLB for up to 24 h, cross-link accumulation occurred immediately following the exposure of MLNr cells to NH2, but at an incidence lower than that observed in WT cells.

The low level of cross-links obtained using MLN and CLB in MLNr cells suggests either that drug accumulation in the nucleus is decreased or absent or that some inhibition processes of cross-link formation exist. The approximately 35% decrease in MLN accumulation is a contributory factor to the resistance, but it cannot explain the almost total absence of cross-links. Thin-layer chromatographic analysis revealed that both WT and MLNr cells contained significant intracellular concentrations of MLN and metabolized it to the same degree, and radiolabeled MLN was found in nuclei isolated from pretreated WT and MLNr cells. Furthermore, the cross-resistance to X-rays and the failure of CLB to induce cross-links argue against the cell membrane's being the principal site of alteration in these cells. In contrast to MLN, CLB is passively diffused and does not use specific transport mechanisms [10]. A Chinese hamster ovary (CHO) cell line that accumulates CLB to the same extent as its corresponding WT cells has previously been reported to lack CLB-induced cross-link formation [22].

Both elevated GSH levels and increased GST activity have been found in MLNr cells [33]. GST catalysis of GSH conjugation with MLN and CLB has previously been reported [11, 29]. Our results showed that BSO significantly depleted intracellular GSH, sensitized MLNr cells to MLN, and moderately increased cross-link formation. EA also sensitized MLNr cells to MLN and significantly increased cross-link formation. As compared with WT cells, the increase in MLN toxicity to MLNr cells that had been pretreated with BSO was only partial, in spite of the 70% GSH depletion obtained using 100 μM BSO (which reduces the GSH level to that seen in WT cells). This underlines the multifactorial nature of resistance, in which GSH is only partially involved. The sensitization of both WT and MLNr cells by EA in the present study is consistent with previous investigations using rat and human tumor cells [36]. The increased cross-link formation observed in MLNr cells in the presence of BSO or EA without significant effect at 20 h post-incubation suggests that the role of GSH and GST lies in the initial formation of cross-links. The partial increase in DNA-DNA cross-links obtained in MLNr cells in the presence of BSO or EA could be explained by differences in the cellular distribution of these modulators and in their effectiveness in different cellular compartments.

It has been reported that GSH may act as a quenching agent of monoadducts [4]. As such a reaction would take place in the nucleus rather than in the cytoplasm, the intranuclear levels of GSH may be a determinant factor in this process. BSO has been found to be relatively inefficient at reducing intranuclear levels of GSH [13]. This might explain the slight effect for BSO on cross-links, even at a concentration of 200 μM (data not shown), and may also be the case for EA.

The enhanced GST activity seen in the MLNr cells may have included a nuclear pool that contributed to the lack of cross-link formation. Previous findings suggest that specific nuclear GSTs may be involved in the DNA-repair process [23]. Little is known about the isoenzyme distribution of nuclear GSTs, but it may be quite distinct from that in the cytosol. The minimal effect of EA on MLNr cells may also be explained by either a very low K_m value for the nuclear GST or an insensitivity of the nuclear-pool GST to EA such as that seen for BSO [13]. More effective inhibitors of GST may be even more efficient. The absence of cross-links was observed for drugs whose action involves a delayed process between drug-DNA monoadducts and cross-link formation (MLN and CLB), in contrast to HN2, whose action involves instantaneous cross-link formation. This suggests that an efficient process of monoadduct repair is involved in the case of MLN and CLB, whereas this process seems to be overwhelmed in the case of HN2. Such a system may act independently or may be related to nuclear GST, but this has yet to be demonstrated. Finally, only DNA-DNA cross-links were used as endpoints in the present study, and we therefore cannot rule out the occurrence of other alterations such as DNA intrastrand and DNA-protein cross-links. A comparison of different types of DNA damage certainly merits future investigation.

In conclusion, both BSO and EA are effective modulators of resistance to alkylating drugs. The increased incidence of cross-links in the presence of these modulators suggests that both GSH and GST may be involved in cross-link formation through drug inactivation as well as repair of drug-DNA monoadducts. Investigations in progress will establish the effect of these modulators on drug-DNA interaction and repair.

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