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Carbamoylation of Glutathione Reductase by *N,N-Bis*(2-chloroethyl)-*N*-nitrosourea Associated with Inhibition of Multidrug Resistance Protein (MRP) Function

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ABSTRACT. Intracellular glutathione (GSH) concentrations have been implicated recently as a regulatory determinant of multidrug resistance protein (MRP)-mediated drug efflux. Inhibition of glutathione reductase (GR) activity of *N,N*-bis(2-chloroethyl)-*N*-nitrosourea (BCNU) has been employed extensively to investigate the role of GSH redox cycle in cellular function. The present study examined the effect of BCNU on the MRP-mediated efflux of doxorubicin in the multidrug-resistant human fibrosarcoma cell line HT1080/DR4 overexpressing MRP. No significant difference in GR activity between HT1080 (parental) and multidrug-resistant HT1080/DR4 cells was detected (38.6 ± 2.2 and 37.8 ± 5.28 nmol/min/10^6 cells, respectively). Exposure of HT1080 and HT1080/DR4 cells to 100–500 μM BCNU decreased GR activity concentration dependently with subsequent reduction in cellular GSH pools in both cell lines. Inhibition of GSH biosynthesis by D,L-buthionine-(*S,R*)-sulfoximine (D,L-BSO), a specific inhibitor of γ-glutamylcysteine synthetase, significantly reduced MRP-mediated drug efflux and potentiated the cytotoxicity of doxorubicin in MRP-expressing HT1080/DR4 cells (dose modifying factor 20.8). While equally effective inhibition of GR activity by BCNU was observed in parental and resistant cells, a significant increase in intracellular retention of doxorubicin was only achieved in MRP-expressing HT1080/DR4 cells. Furthermore, inhibition of MRP function following treatment with BCNU or D,L-BSO was directly related to the degree of GSH depletion in MRP-expressing tumor cells [r = 0.94 ($P < 0.001$) and 0.99 ($P < 0.001$), respectively]. Based on northern blot analysis of MRP mRNA levels, exposure of HT1080/DR4 cells to BCNU did not produce down-regulation of MRP gene expression. The results reported herein indicate that derivatives of nitrosourea with carbamoylating properties are potent inhibitors of MRP function. Depletion of intracellular GSH pools by inhibition of the GSH redox cycle or GSH *de novo* biosynthesis significantly inhibited MRP-mediated doxorubicin transport and restored intracellular drug concentrations *in vitro*. BIOCHEM PHARMACOL 53;6:801–809, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. multidrug resistance protein; BCNU; glutathione-reductase; glutathione; glutathione S-transferase; ethacrynic acid

MDR§ has been associated recently with overexpression of a 190-kDa membrane phosphoglycoprotein [1–5], designated MRP [1]. MRP belongs to the ATP-binding cassette transmembrane transporter superfamily and is involved in transmembrane drug transport processes [1, 2, 4, 5]. Transfection of an MRP cDNA expression vector into drug-sensitive cells confers an MDR phenotype accompanied by alterations in cellular drug accumulation and retention [6–9].

Recently, MRP has been identified as a multispecific organic anion transporter (MOAT) [10], and it was demonstrated that the endogenous GSH S-conjugate leukotriene C_4 and anionic amphiphilic conjugates are substrates for MRP [10–12]. Furthermore, conjugates of xenobiotics (i.e. glucuronosyl-etoposide and glutathionyl-melphalan) were implicated in MRP-mediated drug transport [13]. Loe

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§ *Abbreviations:* MDR, multidrug resistance; MRP, multidrug-resistance protein; BCNU, *N,N*-bis(2-chloroethyl)-*N*-nitrosourea; CEIC, 2-chloroethyl isocyanate; GR, glutathione reductase (EC 1.6.4.2); D,L-BSO, D,L-buthionine (*S,R*)-sulfoximine; LRP, lung resistance-related protein; PCR, polymerase chain reaction; GSH, glutathione; IC_{50}, drug concentration that inhibits cell growth by 50%; resistance factor, (IC_{50} resistant/IC_{50} parental cell line); G3PDH, glycerol 3-phosphate dehydrogenase; and GST, glutathione S-transferase (EC 2.5.1.18).

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and colleagues [12] and Jedlitschky and co-workers [13] reported that unmodified anthracyclines and *Vinca* alkaloids were not substrates for MRP, results contrasting with the findings reported by Paul *et al.* [14]. Inhibition of intracellular GSH *de novo* biosynthesis by D,L-BSO, a potent inhibitor of γ-glutamylcysteine synthetase [15], results in increased drug accumulation and retention of anthracyclines [16–18], vincristine [19], etoposide [19], and rhodamine [16] in MRP- but not in *MDR1*-mediated MDR [16–18]. Although these findings indicate that intracellular GSH is a regulatory determinant of MRP function, the role of the GSH pathway in MRP-mediated drug transport is not fully understood.

Carbamoylation of GR by BCNU and its breakdown products (i.e. CEIC [20]), results in a specific and irreversible inactivation of the enzyme *in vitro* [21–24] and *in vivo* [25]. Since GR plays a key role in the maintenance of GSH homeostasis, it is possible that inhibition of GR by BCNU may also result in the inhibition of MRP function.

To test this hypothesis we investigated *in vitro* the effect of BCNU on MRP function in the parental human fibrosarcoma cell line HT1080 and the multidrug-resistant HT1080/DR4 cell line expressing MRP [3].

MATERIALS AND METHODS

Chemicals

Doxorubicin HCl, USP, was obtained from Farmitalia Carla Erba S.P.A. (Milan, Italy). BCNU was provided by Bristol Myers Squibb (Princeton, NJ) and dissolved in ethanol and diluted with sterile water to a final concentration of 10 mM (stock solution). D,L-BSO was obtained from the Sigma Chemical Co. (St. Louis, MO) and dissolved in sterile water (stock solution 20 mM). All solutions were prepared fresh and used immediately. The highest concentration of ethanol (0.1%) used in the assays was found to be non-cytotoxic and without effect on drug resistance.

Cell Lines

The characteristics of the human fibrosarcoma cell line HT1080 (parental) and the MDR subline HT1080/DR4 (MRP and LRP positive, P-glycoprotein negative) have been described previously [3, 26, 27]. HT1080 and HT1080/DR4 cells were grown as monolayers in Eagle's minimum essential medium supplemented with Earle's balanced salt solution and 10% heat-inactivated fetal bovine serum (FBS), non-essential amino acids, and L-glutamine.

Cell cultures were kept under exponential growth conditions in a humidified atmosphere of 5% CO_2 in air at 37°.

Cytotoxicity Evaluation

In vitro drug sensitivity of the HT1080 cell lines was assessed by the sulforhodamine B assay [28]. In brief, cells in exponential growth were seeded at a density of 1000 cells/well in 96-well microtitre plates (Falcon, Becton Dickinson Labware, Plymouth, U.K.) and allowed to attach overnight. After a 2-hr drug exposure, cells were washed and reincubated in drug-free medium. At four cell doubling times after drug treatment, cells were fixed with trichloroacetic acid, washed, and stained with sulforhodamine B as originally described [28]. In a second protocol, HT1080 and HT1080/DR4 cells were seeded and grown under the same conditions, but preincubated for 24 hr with nontoxic concentrations of D,L-BSO (0.3 to 3.0 μM), then exposed for 2 hr to doxorubicin, washed, and reincubated in medium with and without D,L-BSO. Cells were fixed at four doubling times after doxorubicin exposure, washed, and stained with sulforhodamine B as described above.

Absorbance was measured at 570 nm using a 96-well plate reader (340 EL BIO Kinetics Reader, BIO-TEK Instruments Inc., Winooski, VT). Drug concentration that inhibited cell growth by 50% (IC_{50}) was determined from semilogarithmic concentration–response plots.

Enzyme Activity of GR (EC 1.6.4.2) and Intracellular GSH Content

Cells were harvested with 1 mM EDTA in PBS, washed twice with ice-cold PBS, and lysed by sonication in 5 mM dipotassium hydrogen phosphate buffer (pH 7.5). Enzyme activity of GR was determined at 37° in the 120,000 *g* supernatant as described previously [29] and results are expressed as nanomoles GSH formed per minute per 10^6 cells.

Intracellular GSH concentrations were determined using the GR recycling method of Griffith [30] using a final concentration of 2% (w/v) salicylic acid (Sigma) for deproteinization. Absorbance was measured at 412 nm for 5 min, and GSH concentrations were calculated by reference to a standard curve that was run with each batch of samples. Results are expressed as nanomoles GSH per 10^6 cells.

Cellular Drug Accumulation and Retention

To determine cellular drug retention in exponentially growth HT1080 and HT1080/DR4 cells, cells were exposed first to either 100–500 μM BCNU (1 hr) or 1–100 μM, D,L-BSO (24 hr) followed by a 2-hr exposure to 2.0 μM doxorubicin at 37°. Samples were taken immediately after doxorubicin exposure (0 time, drug accumulation) and after a 4-hr reincubation in drug-free medium (drug retention) as described previously [31, 32]. Cellular drug concentrations were determined by analyzing cells with a FACScan (Becton Dickinson, San Jose, CA). A linear relationship between the average cellular fluorescence intensity and cellular doxorubicin concentration was demonstrated [33, 34]. Excitation wavelength was 488 nm, and emitted light was captured using a 650 long-pass filter. Results were calculated and analyzed with Win List software (Verity Software House, Tonshaw, ME). Cellular drug concentrations were expressed as a percentage of mean Arbitrary Fluorescence Units (A.F.U.).

Reverse Transcriptase-Polymerase Chain Reaction Amplification and Northern Blot Hybridization

Total RNA was isolated by a single-step guanidine isothiocyanate–phenol–chloroform extraction [35]. One microgram of total RNA of HT1080/DR4 cells was used for MRP cDNA probe synthesis with an RNA PCR kit (Perkin–Elmer, Branchburg, NJ). Reverse transcription was performed using random hexamers as primer according to the instructions of the manufacturer. The PCR reaction was carried out in a GeneAmp 2400 PCR system (Perkin–Elmer) for 30 cycles of denaturation (95°, 60 sec), annealing (60°, 60 sec), and extension (72°, 90 sec). The MRP-specific PCR primers (position 2540–2865, [1]) were as follows: sense 5′-CGTGTACTCCAACGCTGAC-3′ and antisense 5′-CTGGACCGCTGACGCCCGTGAC-3′, respectively. The PCR products were gel purified, and the band of the expected size of 326 bp was isolated.

To determine the effect of BCNU treatment on MRP mRNA expression, HT1080 and HT1080/DR4 cells were exposed for 1 hr to 250 μM BCNU, washed twice with PBS, and reincubated with drug-free medium for 6 hr. Northern blots containing 15 μg of total RNA were prehybridized at 65° (1 hr) in 0.5 M dipotassium hydrogen phosphate buffer containing 7% (w/v) SDS, 1% (w/v) BSA, and 1 mM EDTA. Hybridization was performed overnight at 65° with a random primed, [α-^{32}P]dCTP-labeled MRP cDNA fragment corresponding to nucleotides 2540–2865 of MRP cDNA [1]. After autoradiography, blots were stripped and reprobed with a [^{32}P]-labeled cDNA probe for G3PDH (Clontech Laboratories Inc., Palo Alto, CA) to determine variation in RNA loading.

Statistical Analysis

The difference between the mean values was analyzed for significance using the unpaired two-tailed Student's *t*-test for independent samples; P values < 0.05 were considered to be statistically significant.

RESULTS

Inhibition of GR Activity

Inhibition of GR activity following a 1-hr exposure to BCNU in HT1080 and HT1080/DR4 cells was investigated, and the results are summarized in Table 1. It has been shown previously that a 30–60 min exposure to BCNU results in maximum inhibition of GR activity [23]. No significant difference in GR activity was observed between the untreated parental HT1080 and the untreated drug-resistant HT1080/DR4 cells. Although drug exposure to 100 μM BCNU caused a significant ($P < 0.01$) inhibition of enzyme activity in both parental and resistant cells, greater than 90% inhibition of GR activity in HT1080/DR4 cells was achieved only at BCNU concentrations greater than 100 μM, a cytotoxic concentration in both cell lines (IC_{50} of BCNU in HT1080 and HT1080/DR4 cells was 45.5 ± 6.5 and 67.0 ± 4.2 μM, respectively; see Table 3).

GSH Concentrations

Intracellular GSH concentrations were 1.4-fold higher in resistant HT1080/DR4 cells than in parental HT1080 cells (11.9 ± 1.0 vs 8.2 ± 0.49 nmol/10^6 cells, respectively). BCNU at a concentration greater than 100 μM produced a significant decrease in intracellular GSH (Fig. 1). Exposure to 500 μM BCNU did not produce any further depletion of GSH in parental and resistant HT1080 cells, results that may be related to the remaining enzyme activity of GR in HT1080 and HT1080/DR4 cells (2.56 ± 0.45 and 1.15 ± 0.18 nmol/min/10^6 cells, respectively) or up-regulated *de novo* biosynthesis of GSH.

In addition, the effect of D,L-BSO on GSH biosynthesis was investigated, and the results are shown in Table 2. D,L-BSO at concentrations of 10 and 100 μM produced a significant concentration-dependent depletion of intracellular GSH pools in both parental HT1080 and resistant HT1080/DR4 cells.

Effect of BCNU on MRP-Mediated Drug Efflux

The effect of BCNU-induced inhibition of GR activity on MRP-mediated doxorubicin efflux in HT1080/DR4 cells was investigated, and the results are shown in Fig. 2. While equally effective inhibition of GR activity in HT1080 and HT1080/DR4 cells was achieved at BCNU concentrations

TABLE 1. *In vitro* inhibition of GR activity by BCNU

	GR activity* (nmol GSH formed/min/10^6 cells)			
	BCNU			
	No drug	100 μM	250 μM	500 μM
HT1080	38.6 ± 2.20 (100%)	3.18 ± 1.82 (8.2%)	2.96 ± 1.65 (7.7%)	2.56 ± 0.45 (6.6%)
HT1080/DR4	37.8 ± 5.28 (100%)	12.0 ± 0.55† (32%)	2.16 ± 1.34 (5.7%)	1.15 ± 0.18 (3.0%)

* Cells were exposed to BCNU (1 hr) or drug-free medium. GR activity was measured as described in Materials and Methods. The results are presented as mean values ± SD of three independent experiments in duplicate; enzyme activity as a percentage of untreated cells is shown in parentheses.

† $P < 0.01$ between parental and resistant cells.

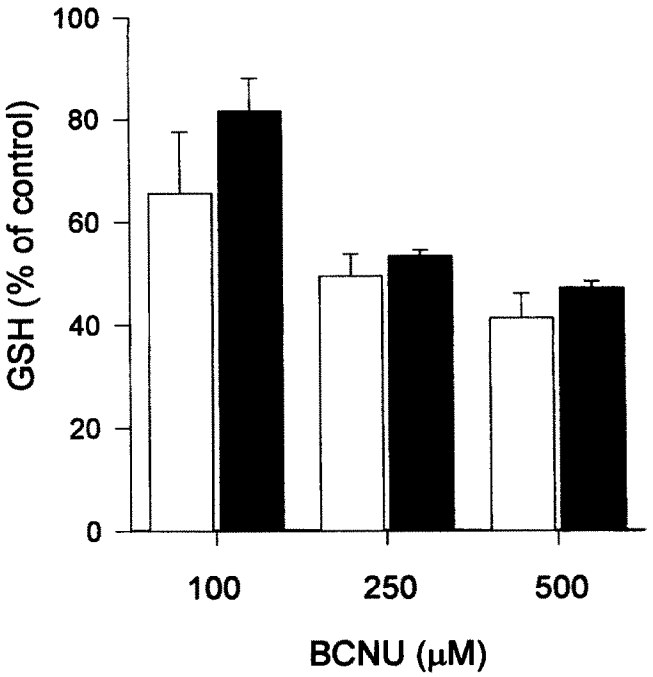

FIG. 1. Concentration-dependent depletion of GSH in parental HT1080 cells (open bars) and MRP-expressing HT1080/DR4 cells (closed bars) by BCNU. Cells were preincubated for 1 hr with 100–500 μM BCNU or drug-free medium, and intracellular GSH concentrations were determined as described in Materials and Methods. Glutathione levels in untreated HT1080 and HT1080/DR4 cells were 8.23 ± 0.49 and 11.9 ± 1.0 nmol GSH/10^6 cells, respectively. The results are presented as mean values ± SD of three independent experiments. $P < 0.01$ and $P < 0.001$ for untreated HT1080 cells vs 100 or 250 μM BCNU treatment, respectively. $P < 0.05$ and $P < 0.001$ for untreated HT1080/DR4 cells vs 100 or 250 μM BCNU treatment, respectively.

greater than 100 μM (Table 1), restoration of cellular doxorubicin concentrations was achieved only in MRP-expressing tumor cells (Fig. 2). Depletion of GSH pools by D,L-BSO (Table 2) produced a similar increase in cellular drug concentrations in HT1080/DR4 cells, with no alterations of doxorubicin retention in parental cells (Fig. 3). Furthermore, inhibition of MRP-mediated drug efflux by BCNU and D,L-BSO was directly related to the degree of GSH depletion [Fig. 4; inverse linear correlation between intracellular GSH concentrations and doxorubicin retention in HT1080/DR4 cells following treatment with BCNU or D,L-BSO with $r = 0.94$ ($P < 0.001$, slope: −6.12) and $r = 0.99$ ($P < 0.001$, slope: −3.78), respectively]. These data demonstrate that inhibition of MRP-mediated drug efflux from resistant HT1080/DR4 cells is achievable by inhibition of the GSH redox cycle, resulting as a consequence of BCNU-induced impairment of GR activity or inhibition of *de novo* biosynthesis of GSH caused by D,L-BSO.

Effect of BCNU on MRP Gene Expression

The levels of MRP mRNA after treatment of HT1080/DR4 cells with BCNU (1 hr) were determined using a 326 bp MRP cDNA fragment (Fig. 5). While parental HT1080 cells had no detectable amounts of MRP mRNA, expression of MRP mRNA was observed in resistant HT1080/DR4 cells, confirming the MDR phenotype as previously reported [3]. Under the conditions used, exposure to BCNU did not alter MRP mRNA levels in HT1080/DR4 cells, suggesting that inhibition of MRP-mediated drug transport by BCNU was not related to (a) down-regulated *MRP* gene expression due to DNA-alkylating properties of BCNU and its breakdown product, or (b) alterations of *MRP* gene expression by oxidative stress induced by GSH depletion.

In Vitro *Drug Sensitivity and D,L-BSO-Induced Reversal of Drug Resistance*

The cytotoxicity of doxorubicin, cisplatin, melphalan, and BCNU was determined in HT1080 and HT1080/DR4 cells, and the IC_{50} values are shown in Table 3. HT1080/DR4 cells were 255-fold resistant against doxorubicin, consistent with reported results by Slovak *et al.* [26]. While no cross-resistance was detected for cisplatin and melphalan, a mod-

TABLE 2. *In vitro* depletion of intracellular GSH by D,L-BSO in HT1080 and HT1080-DR4 cells*

	GSH (nmol/10^6 cells)			
	D,L-BSO			
	No drug	1 μM	10 μM	100 μM
HT1080	8.23 ± 0.49 (100%)	6.26 ± 1.29 (76%)	2.69 ± 0.84 (32%)	0.51 ± 0.1 (6.1%)
HT1080/DR4	11.9 ± 1.0† (100%)	10.7 ± 0.35‡ (89%)	3.95 ± 0.53 (33%)	ND§

* Cells were exposed to D,L-BSO (24 hr) or drug-free medium. GSH content was determined as described in Materials and Methods. The results are presented as mean values ± SD of three independent experiments in duplicate; GSH levels as a percentage of untreated cells is shown in parentheses.

† $P < 0.05$ between parental and resistant cells.

‡ $P < 0.01$ between parental and resistant cells.

§ Not detectable.

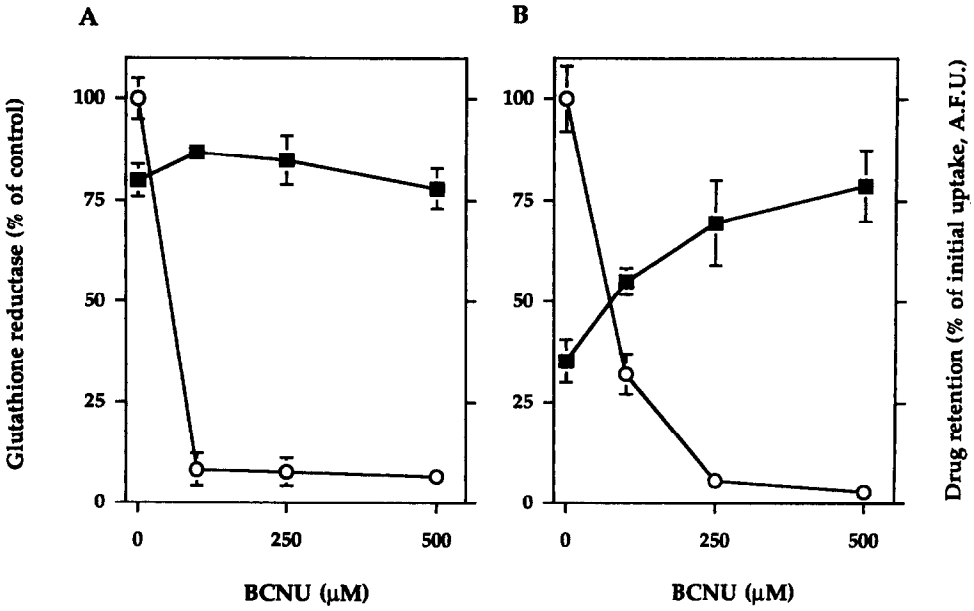

FIG. 2. Effect of GR activity (open circles) on drug retention of doxorubicin (closed squares) in parental HT1080 (A) and resistant HT1080/DR4 (B) cells. Cells were preincubated for 1 hr with 100–500 μM BCNU or drug-free medium, washed, and exposed for 2 hr to 2.0 μM doxorubicin. Drug retention was determined as described in Materials and Methods. Doxorubicin concentrations are expressed as a percentage of initial drug uptake calculated as mean Arbitrary Fluorescence Units (A.F.U.); GR activity is expressed as a percentage of untreated controls. The results are presented as the mean values ± SD of at least three experiments. The A.F.U. of HT1080 and HT1080/DR4 cells without an exposure to BCNU were 28.1 ± 0.3 and 16.4 ± 0.9 ($P < 0.001$), respectively. The cellular GR activities of HT1080 and HT1080/DR4 cells were 38.6 ± 2.2 and 37.8 ± 5.3 nmol GSH formed/min/10^6 cells, respectively.

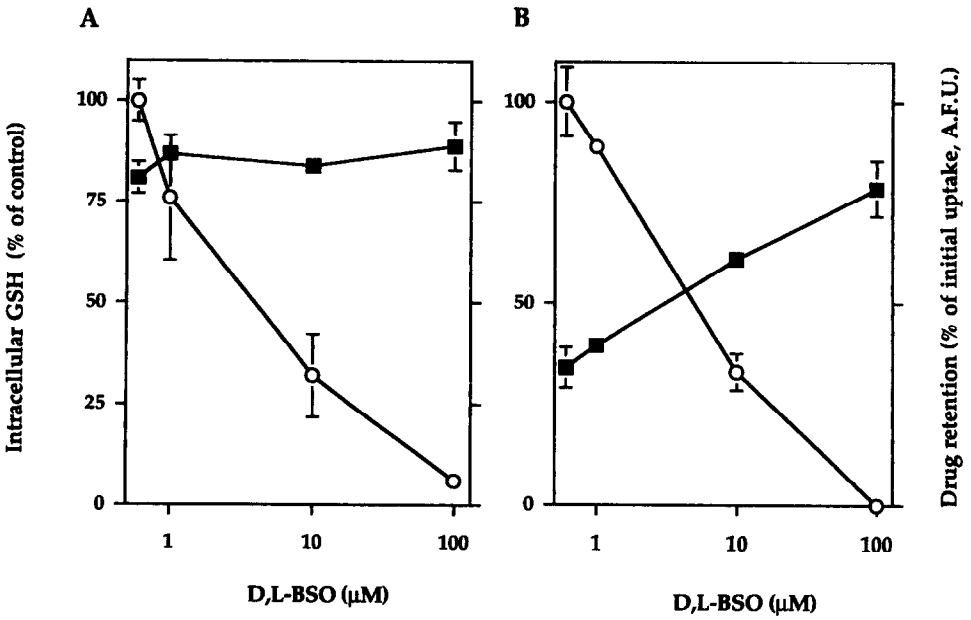

FIG. 3. Effect of intracellular GSH pools (open circles) on drug retention of doxorubicin (closed squares) in parental HT1080 (A) and resistant HT1080/DR4 (B) cells. Cells were preincubated for 24 hr with 1.0–100 μM D,L-BSO or drug-free medium, washed, and exposed for 2 hr to 2.0 μM doxorubicin. Drug retention was determined as described in Materials and Methods. Doxorubicin concentrations are expressed as the percentage of initial drug uptake calculated as mean Arbitrary Fluorescence Units (A.F.U.); intracellular GSH content is expressed as a percentage of untreated controls. The results are presented as the mean value ± SD of at least three experiments. The A.F.U. values of HT1080 and HT1080/DR4 cells without exposure to D,L-BSO were 26.2 ± 0.4 and 18.5 ± 1.4, respectively. The intracellular GSH levels of HT1080 and HT1080/DR4 cells were 8.23 ± 0.5 and 11.9 ± 1.0 nmol GSH/10^6 cells, respectively.

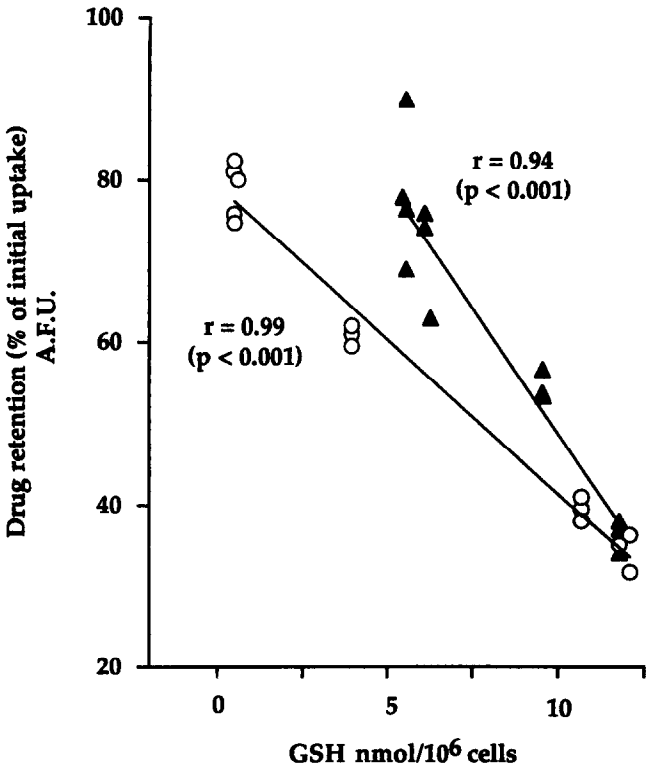

FIG. 4. Inverse relationship between intracellular GSH concentrations with drug retention of doxorubicin in MRP-expressing HT1080/DR4 cells. Cells were preincubated either for 1 hr to 100–500 μM BCNU (closed triangles) or for 24 hr to 1.0–100 μM D,L-BSO (open circles), washed, and then exposed for 2 hr to 2.0 μM doxorubicin. Drug retention was determined as described in Materials and Methods. Doxorubicin concentrations are expressed as a percentage of initial doxorubicin uptake calculated as mean Arbitrary Fluorescence Units (A.F.U.); GSH concentrations are expressed as nmol/10^6 cells. The points represent independent experiments; the plotted lines show a linear fit of the data. The A.F.U. values of HT1080/DR4 cells without exposure to BCNU or D,L-BSO were 16.4 ± 0.9 and 18.5 ± 1.4, respectively.

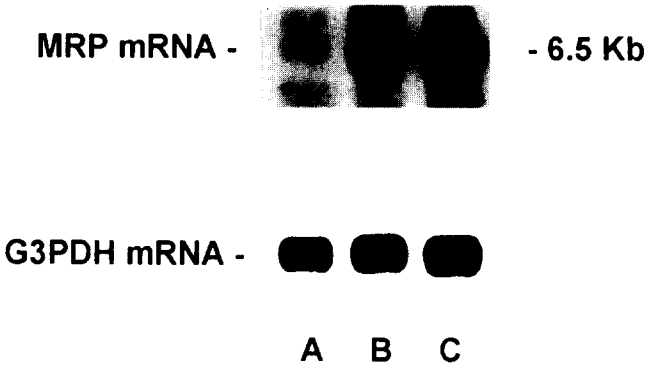

FIG. 5. Northern blot analysis of MRP mRNA in HT1080/DR4 cells after exposure to BCNU using a random primed, [α-^{32}P]dCTP-labeled 326 bp MRP cDNA fragment corresponding to nucleotides 2540–2865 of MRP cDNA [1]. Parental HT1080 cells, no drug (A); resistant HT1080/DR4 cells, no drug (B); HT1080/DR4 cells, 250 μM BCNU (C). HT1080/DR4 cells were exposed for 1 hr to 250 μM BCNU or drug-free medium; MRP mRNA analysis was performed after cells were reincubated for 6 hr in drug-free medium. Variation in RNA loading was determined with a [^{32}P]-labeled cDNA probe for G3PDH.

est resistance to BCNU was observed in HT1080/DR4 cells (drug resistance factor 0.79, 0.96, and 1.6, respectively).

Using a 24-hr preincubation and a continuous exposure to non-cytotoxic concentrations of D,L-BSO (0.3 to 3.0 μM), while no modulatory effect was observed in parental HT1080 cells, cytotoxicity of doxorubicin in HT1080/DR4 cells was increased significantly ($P < 0.001$, dose-modifying factor 10.2) (Fig. 6A). Increasing the duration of cellular exposure to D,L-BSO produced greater potentiation of doxorubicin cytotoxicity, from 10- to 20-fold (Fig. 6B). In contrast, recent experiments from our laboratory have shown that exposure to D,L-BSO had no effect on the cytotoxicity of doxorubicin in MDR-1 cDNA transfected KB-TX 641 cells and nude mice bearing P-glycoprotein-expressing A2780/Dx5 xenografts,* results which suggest that P-glycoprotein-mediated drug transport is not GSH dependent and further underscore the specificity of D,L-BSO-induced GSH depletion on MRP function.

DISCUSSION

Intracellular GSH concentrations have been implicated recently as a regulatory determinant of MRP-mediated drug efflux [12, 16, 18]. The results of the present study demonstrate a link between the functional status of GSH redox cycle and MRP function in multidrug-resistant HT1080/DR4 cells. BCNU significantly inhibited GR activity, the enzyme required for the maintenance of cellular GSH homeostasis. BCNU [21, 23, 25] and other carbamoylating nitrosoureas [i.e., *N,N'*-bis(*trans*-4-hydroxycyclohexyl)-*N'*-nitrosourea (BCyNU) [36, 37], *N*-(2-chloroethyl)-*N'*-cyclohexyl-*N*-nitrosourea (CCNU) [38], and CEIC [23, 24, 38] have been employed extensively as potent and specific inhibitors of GR. In view of the present

TABLE 3. Cytotoxicity of doxorubicin, cisplatin, melphalan, and BCNU in the human sarcoma cell lines HT1080 and HT1080/DR4 overexpressing MRP*

Drug	IC_{50} (μM) HT1080	IC_{50} (μM) HT1080/DR4	RF
Doxorubicin	0.22 ± 0.02	55.0 ± 5.0	255
Cisplatin	12.1 ± 1.3	9.5 ± 1.6	<1
Melphalan	13.0 ± 1.4	12.5 ± 0.7	<1
BCNU	45.5 ± 6.5	67.0 ± 4.2	1.6

* Cytotoxicity was determined using the sulforhodamine assay. Drug exposure (2 hr) was performed as described in Materials and Methods. The results are presented as mean values ± SD of at least four independent experiments. RF, resistance factor: IC_{50} resistant/IC_{50} parental cell line.

* Rustum YM and Skenderis BS, unpublished results.

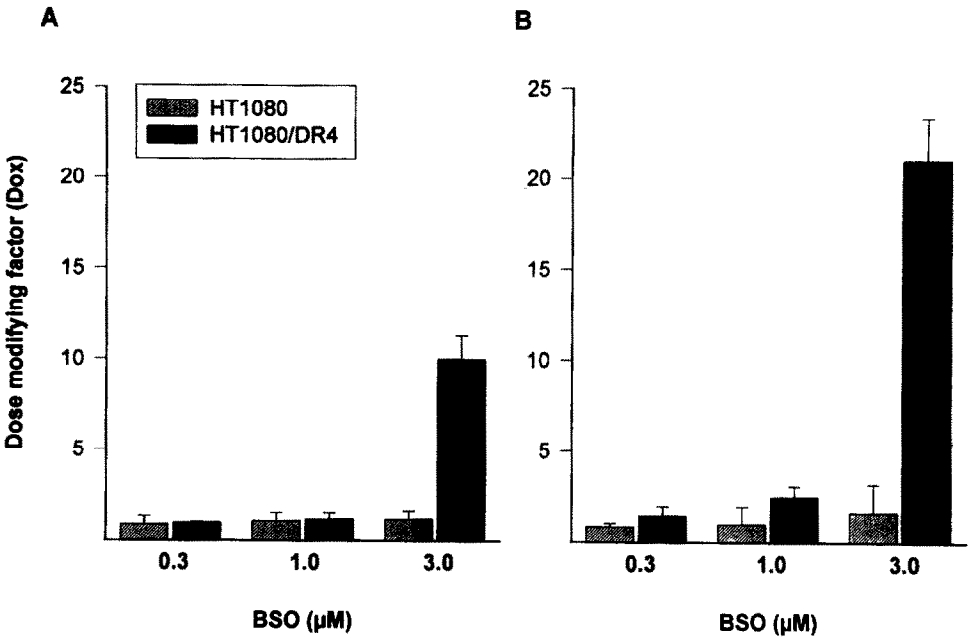

FIG. 6. Modulation of MRP-mediated doxorubicin resistance by D,L-BSO in parental HT1080 (shaded bars) and resistant HT1080/DR4 cells (closed bars). Cells were preincubated for 24 hr (A) or continuously exposed (B) to a non-cytotoxic concentration ($<IC_{10}$) of D,L-BSO or drug-free medium. Cytotoxicity was performed by the sulforhodamine B assay as described in Materials and Methods; the dose-modifying factor is the ratio of IC_{50} drug/IC_{50} [drug + D,L-BSO]. The IC_{50} values of doxorubicin in parental HT1080 and MRP-expressing HT1080/DR4 cells were 0.22 ± 0.02 and 55.0 ± 5.0 μM, respectively.

study, inhibition of GR by BCNU was associated with restoration of cellular drug concentration in MRP-expressing HT1080/DR4 cells, with no apparent effect on parental cells lacking MRP expression (Fig. 2).

Previous experiments from our laboratory have shown that in MRP-expressing HT1080/DR4 cells reversal of doxorubicin resistance by the pyridine analogue PAK-104P, a potent inhibitor of P-glycoprotein- and MRP-mediated MDR function, is related to increased cellular drug concentrations [31]. BCNU produced a significant depletion of intracellular GSH (Fig. 1), results that could be explained by the mechanisms of BCNU action on GSH levels: (a) specific inhibition of GR activity with subsequent inhibition of the GSH redox cycle [21, 24, 38], and (b) GSH depletion due, at least partially, to conjugation of GSH with BCNU breakdown products (i.e. CEIC [23]). The reduction of intracellular GSH by BCNU specifically inhibited MRP function, findings that are consistent with recent studies applying D,L-BSO, a specific inhibitor of the GSH biosynthesis, as a drug-resistance modifier of MRP-mediated MDR *in vitro* [12, 16–19, 39] and *in vivo* [40]. In contrast, recent studies have shown that cellular GSH did not play an important role in *MDR1*-mediated drug transport and resistance [16–18], data that support the specificity of D,L-BSO-induced GSH depletion on MRP function. However, since BCNU produces carbamoylation of some macromolecules, BCNU may, in part, also directly interact with MRP.

Although intracellular GSH appears to be a regulatory determinant of MRP function (Fig. 4), the role of the GSH pathway in MRP-mediated drug transport is still poorly understood. MRP has been related to the multispecific organic anion transporter (MOAT) [10], and it has been speculated that positively charged and neutral xenobiotics may be transported by MRP after conjugation or complex formation with GSH. However, since GSH conjugation has not been shown to be a substantial pathway for the biotransformation of xenobiotics to which MRP and P_{170}-glycoprotein (*MDR1*) confer drug resistance [41], its role in MRP function remains uncertain.

Conjugation of xenobiotics to GSH has been related to GST, and GST activity is 1.6-fold increased in resistant HT1080/DR4 cells [42]. Thus, it may be speculated that GST is involved in MRP-mediated drug efflux and drug resistance. However, using different schedules of exposure to ethacrynic acid, a potent inhibitor of GST activity [41, 43], no inhibition of MRP-mediated drug transport of doxorubicin and rhodamine was observed in HT1080/DR4 cells (data not shown). Furthermore, cisplatin [41] and melphalan [41, 44] serve as substrate for GSH conjugation, and glutathionyl-melphalan has been proposed recently as a substrate for MRP [13]. In the present study, no cross-resistance to cisplatin or melphalan (Table 3) was observed, results which may suggest that at least GST-mediated conjugation to xenobiotics is not involved in drug resistance of MRP-expressing HT1080/DR4 cells.

It is interesting to note that MRP has been associated recently with the ATP-dependent cellular export of glutathione disulfide (GSSG) [45]. Thus, the role of a competitive inhibition of MRP-mediated drug transport by increased concentrations of GSSG due to inhibition of GSH redox cycle (BCNU) or GSH *de novo* biosynthesis (D,L-BSO) needs to be evaluated.

Since oxidative stress has been reported to alter expression of xenobiotic-detoxifying enzymes (i.e. GST and chloramphenicol acetyltransferase [46], the effect of BCNU on MRP mRNA levels was investigated. Exposure to BCNU did not induce down-regulation of MRP gene expression in HT1080/DR4 cells, findings that are consistent with reported results on COR-LR23/R cells lacking alter-

ations of *MRP* gene and protein expression after D,L-BSO-induced reversal of MRP-mediated MDR [16].

In conclusion, the results of the present study implicate intracellular GSH concentration as a determinant for MRP-mediated drug transport. Impairment of GR activity by BCNU resulted in a significant inhibition of MRP-mediated drug transport of doxorubicin in HT1080/DR4 cells but not in parental cells, indicating that the maintenance of the glutathione redox cycle is required for MRP-mediated MDR function *in vitro*.

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