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# Role of Glutathione and Glutathione S-Transferase in Chlorambucil Resistance

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## SUMMARY

A chlorambucil (CLB)-resistant cell line, N50–4, was developed from the established mouse fibroblast cell line NIH 3T3, by multistep drug selection. The mutant cells exhibited >10-fold resistance to CLB. Alterations in GSH and glutathione S-transferase (GST) were found in CLB-resistant variants. A 7–10-fold increase in cellular GSH content and a 3-fold increase in GST activity were detected in N50–4 cells, compared with parental cells, as determined by enzymatic assays. An increase in steady state levels of the GST-$\alpha$ isozyme mRNA was found in the CLB-resistant cells, as analyzed by Northern blotting. No GST gene amplification or rearrangement was shown by Southern blot analysis. To test the relative roles of GSH and GST in CLB resistance, a number of GSH- and GST-blocking agents were used. The CLB toxicity was significantly enhanced in N50–4 cells by administration of either the GSH-depleting agent buthionine sulfoximine or the GST inhibitors ethacrynic acid or indomethacin. The resistance to CLB cytotoxicity in N50–4 cells, however, was still significantly higher than that of parental cells. The resistance of N50–4 cells to CLB was almost completely abolished by combination pretreatment yielding both GSH depletion and GST inhibition. The results indicate that both increased cellular GSH content and increased GST activity play major roles in CLB resistance in N50–4 mutant cells.

CLB is a bifunctional alkylating agent that has been widely used for the treatment of neoplastic diseases such as CLL, Hodgkin's disease, ovarian carcinoma, and non-Hodgkin's lymphomas (1–3). The clinical application of CLB is, however, limited by acquired drug resistance (4, 5). Mechanisms of CLB cytotoxicity have been extensively investigated for the past 20 years but remain unclear. CLB is taken up into cells by passive diffusion (6–8), and the major pathways of CLB metabolism have been found to involve hydrolysis of the mustard group (8, 9). Chlorambucil can bind to DNA and induce DNA cross-links (8, 10), a process that may prevent DNA replication and cause cell death (11, 12).

Several mechanisms of CLB resistance have been proposed, but the importance of each is not fully defined at this time. No difference in drug uptake was observed between CLB-sensitive and -resistant CLL cells (8, 10). Harrap and Hill (7) found that the hydrolysis of the mustard group of CLB was faster in CLB-resistant Yoshida sarcoma cells. In contrast, Bank *et al.* (8) showed no correlation between cellular metabolism of CLB and sensitivity to CLB in CLL cells. Reduced levels of DNA cross-links have been found in CLB-resistant CHO (13, 14), Yoshida sarcoma (15), and CLL cells (16). The reduction of DNA cross-linking appeared to be due to an increase in the rate of removal of interstrand DNA cross-links in CLB-resistant Yoshida sarcoma (15) and CLL cells (16). In contrast, no difference in DNA repair was detected between CLB-sensitive and -resistant CHO cells (13, 14). However, an inverse correlation was observed between GSH content, GST activity, and CLB-induced DNA cross-links in cultured CHO cells (13) and CLL cells (17).

GSH is an intracellular cysteine-containing tripeptide and is present at high concentrations in most mammalian cells (18). It has been suggested that GSH plays a critical role in cellular defense against a variety of injurious agents, including antineoplastic alkylating agents such as nitrogen mustard (19) and its derivatives Melphalan (20) and chlorambucil (21). GSTs (EC 2.5.1.18) are a family of isozymes that catalyze the conjugation of the electrophilic compounds with GSH. Three structurally distinct gene families of cytosolic GSTs have been identified in rat, as well as in mouse and human, and have been termed $\alpha$ (basic), $\mu$ (neutral), and $\pi$ (acidic) (22).

The involvement of GSH and GST in CLB resistance was suggested by the observations that the gene encoding the GST-$\alpha$ isozyme was amplified in CLB-resistant CHO cells (23). mRNA and protein of $\alpha$-subclass GST in the CLB-resistant cells were also shown to be overexpressed (13, 24). Increased

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**ABBREVIATIONS:** CLB, chlorambucil; GST, glutathione S-transferase; CLL, chronic lymphocytic leukemia; BSO, L-buthionine-SR-sulfoximine; CDNB, 1-chloro-2,4-dinitrochlorobenzene; CHO, Chinese hamster ovary; bp, base pairs.

levels of cellular GSH content, GST gene expression, and GST enzyme activity were also detected in CLB-resistant Walker tumor (25, 26), CHO (13, 21, 24), and CLL (27) cells. More direct evidence supporting the role of GSH and GST in CLB resistance came from enzyme inhibition studies and gene transfer experiments. Robson *et al.* (21) demonstrated a significant enhancement of CLB cytotoxicity in CHO cells by pretreatment of the cells with the GSH synthesis inhibitor BSO. Tew *et al.* (26) observed an increase in CLB sensitivity in rat and human tumor cell lines after pretreatment of the cells with the GST inhibitor ethacrynic acid, and Hall *et al.* (24) showed a partial reversal of CLB resistance in CHO cells by preincubation of the cells with the GST inhibitor indomethacin. Black *et al.* (28) showed that transfection of $\alpha$-class GST recombinants induced 3–16-fold resistance to CLB in yeast cells. However, Puchalski *et al.* (29) showed induction of 1.3-fold resistance to CLB in monkey kidney cells after GST-$\alpha$ gene transfection, which did not correlate well with the expression of the GST-$\alpha$ gene in the transfectants, and Leyland-Jones *et al.* (30) failed to induce CLB resistance in GST-$\alpha$ gene-transfected human breast cancer cells. These studies indicate that a number of mechanisms may contribute to CLB resistance *in vitro* and *in vivo* and that the relative contributions of these mechanisms may vary considerably.

In the present study, we have established a CLB-resistant cell line from NIH 3T3 mouse fibroblast cells. This CLB-resistant cell line is characterized by increased intracellular GSH content and elevated GST enzyme activity. We have investigated the relative roles that GSH and GST play in this CLB-resistant cell line, by altering the intracellular GSH pool and GST activity using known GSH- and GST-inhibitory agents. These studies indicate that both increased GSH content and GST activity are major factors involved in CLB resistance in this cell line.

## Materials and Methods

**Cells and cell culture.** CLB-resistant cell lines were developed from the NIH 3T3 mouse fibroblast cell line by stepwise drug selection. NIH 3T3 cells were exposed to increasing concentrations of CLB, from 30 $\mu$M to 100 $\mu$M, over 6 months. The degree of CLB resistance was monitored every 2 months by clonogenic assay. The CLB-resistant cell line N50–4 was cloned by the glass ring cloning technique (31). All cells were grown as monolayers in $\alpha$-minimal essential medium, supplemented with 10% (v/v) fetal bovine serum and antibiotics (penicillin, 100 units/ml; streptomycin, 100 $\mu$g/ml), at 37° in a humidified atmosphere containing 5% $CO_2$.

**Cell survival and CLB cytotoxicity.** Cells (ranging from 500 to 5000) were plated on 100-mm$^2$ Petri dishes and incubated for 4 hr at 37°, to allow cell adhesion. For cell survival measurements, CLB was dissolved in acidified ethanol, added to the cell culture medium for 3 hr, and then replaced with fresh drug-free medium. For BSO-mediated GSH depletion experiments, cells were pretreated with BSO for 18 hr and then treated with CLB for 3 hr. For GST inhibitor experiments, cells were preincubated with ethacrynic acid for 10 min or with indomethacin for 1 hr, followed by treatment with CLB for 3 hr. After 1-week incubation, medium was aspirated and surviving cells were visualized by methylene blue staining. Colonies of 50 cells or more were scored. Results are expressed as percentage of survival of CLB-treated cells, compared with CLB-untreated cells. In drug combination studies involving BSO, ethacrynic acid, or indomethacin with CLB, relative survival was determined by the percentage of survival of CLB-treated cells, compared with cells treated with these agents without CLB.

**GSH measurement.** Total cellular GSH content was measured by using the enzyme-recycling assay (32). Cells were lysed by sonication, proteins were precipitated with 4% 5-sulfosalicyclic acid, and GSH content in the supernatant was measured in the presence of 0.5 unit/ml glutathione reductase, at 25°. Protein content was determined by the method of Bradford (33). GSH is expressed as nanomoles per milligram of protein or nanomoles per $10^6$ cells. For the BSO-mediated GSH depletion assay, cells were treated with BSO for 18 hr and were harvested by rubber policeman for GSH analysis.

**GST measurement.** GST activity was measured by the method of Habig *et al.* (34). Cells were harvested, sonicated, and centrifuged at 12,000 × *g* for 10 min at 4°. The supernatant was assayed for GST activity by using 1 mM CDNB as the electrophilic substrate. GST activity is expressed as nanomoles of GSH-CDNB conjugates formed per minute per milligram of protein or nanomoles per minute per $10^6$ cells.

**Northern and Southern blot analyses.** Cytoplasmic RNA was isolated by the method of Gough (35). RNA samples were electrophoresed in 1.0% agarose containing 2.2 M formaldehyde and were transferred to nylon membrane in 20× SSPE (1× SSPE is 3 M NaCl, 0.2 M $NaH_2PO_4$, 20 mM EDTA) (36).

Genomic DNA was extracted from the cells by the method of Maniatis *et al.* (36) and was digested to completion with the appropriate restriction endonucleases, at 37°. After electrophoresis in 1.0% agarose, the gel was treated with 0.25 M HCl and rinsed with water, and the DNA samples were blotted to nylon membranes in 0.4 M NaOH, 0.6 M NaCl (37).

The membranes of RNA or DNA blots were rinsed with 2× SSPE, air dried, and fixed by baking at 80° for 2 hr. Prehybridization and hybridization were carried out in 50% formamide, 4× SSPE, 5× Denhardt's solution (50X Denhardt's solution is 5 g Ficoll, 5 g polyvinylpyrrolidone, 5 g bovine serum albumin per 500 ml $H_2O$) (36), 1% SDS, at 42°. cDNA probes for GST Ya (pGTB 38) and GST Yb (pGTA/C48) were obtained from Dr. C. B. Pickett (38), and GST Yp (pTSS1–2) was obtained from Dr. W. D. Henner (39). cDNA fragments, namely, a 521-bp *Pst*I fragment from pGTB 38, a 845-bp *Pst*I fragment from pGTA/C48, and a 334-bp *Eco*RI/*Sau*3a fragment from pTSS1–2, were labeled with [$\alpha$-$^{32}$P]dCTP to a specific activity of $2 \times 10^8$ cpm/$\mu$g DNA, by the random primer method (36). After hybridization, the filters were washed twice at room temperature in 2× SSPE, 0.1% SDS, for 0.5 hr and twice at 65° in 0.1× SSPE, 0.1% SDS, for 0.5 hr. Filters were then exposed to Kodak XAR-5 film at −70°, with intensify screens.

**Statistical analysis.** The changes in CLB cytotoxicity in cells either with or without pretreatment were compared by analysis of covariance. Values at $p < 0.05$ were deemed statistically significant.

## Results

In order to study the mechanisms of CLB resistance, a CLB-resistant cell line, N50–4, was selected from NIH 3T3 mouse cells, as described in Materials and Methods. The CLB concentrations required to kill 90% of the cells were 13 $\mu$M for NIH 3T3 cells and 140 $\mu$M for N50–4 cells; thus, the resistance of N50–4 cells to CLB was about 10-fold, compared with the parental cells (Table 1; Fig. 1). The resistant phenotype of N50–4 cells was very stable and has been maintained in continuous culture in the absence of CLB for more than 12 months. A 7–10-fold increase in intracellular GSH content and a 3-fold increase in GST activity were detected in the CLB-resistant cells, by enzymatic assays (Table 1). The growth rates of the two cell lines were similar, as determined by cell-doubling time (Table 1). No difference in cell volume was detected between N50–4 and parental NIH 3T3 cells, as measured by a Coulter counter (Coulter JT2; Coulter Electronics of Canada, Ltd).

To understand the mechanism for the increase in GST activity, we investigated the expression of the GST genes, by

TABLE 1

**Comparison of CLB sensitivity, GSH content, and GST activity in NIH 3T3 and N50-4 cell lines**

Values are mean ± standard error of the number of determinations in parentheses.

| | NIH 3T3 | N50-4 |
|---|---|---|
| Doubling time (hr) | 17.5 ± 0.1 (4) | 17.4 ± 0.4 (4) |
| Chlorambucil $LD_{90}$ ($\mu$M)* | 13 | 140 |
| Fold resistance (at $LD_{90}$) | | 10.8 |
| GSH content (nmol/mg of protein) | 6.1 ± 1.1 (5) | 57.9 ± 5.3 (6) |
| GSH content (nmol/$10^6$ cells) | 1.9 ± 0.3 (5) | 14.7 ± 1.7 (6) |
| GST activity (nmol/min·mg of protein) | 29.8 ± 1.3 (4) | 108.6 ± 2.1 (4) |
| GST activity (nmol/min·$10^6$ cells) | 10.2 ± 1.1 (4) | 36.7 ± 4.0 (4) |

* Concentrations of CLB that kills 90% of the cells.

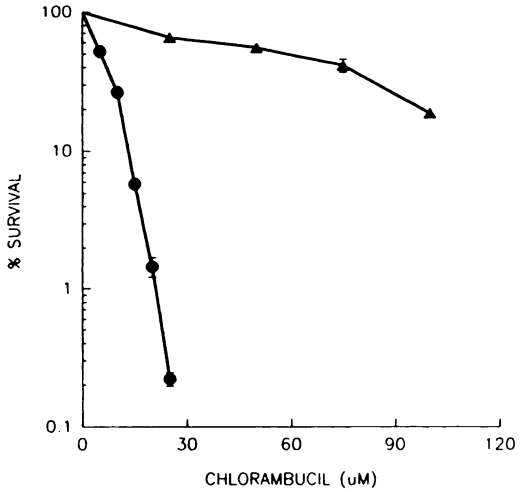



**Fig. 1.** Dose-response curves of NIH 3T3 and N50–4 cells to CLB. NIH 3T3 (●) or N50–4 (▲) cells were exposed for 3 hr to various concentrations of CLB. Cell survival was determined as described in Materials and Methods. *Points*, mean of four determinations; *bars*, standard error. On occasion, the confidence intervals were too small to be shown.

Northern blot analysis. A steady state accumulation of GST $\alpha$-class mRNA was found in CLB-resistant N50–4 cells, and the GST $\alpha$-class mRNA was not readily detected in NIH 3T3 cells (Fig. 2A). No significant differences were observed in mRNA levels of GST $\mu$- and $\pi$-class genes between the two cell lines. We further screened the genomic DNA of these cell lines by Southern blot analysis after *Pst*I restriction endonuclease digestion, using cloned cDNA fragments of GST Ya ($\alpha$), Yb ($\mu$), and Yp ($\pi$) genes as probes. No evidence of amplification or rearrangement of the GST genes was found in CLB-resistant cells (Fig. 2B). Similar results were obtained by digestion of the genomic DNA with other restriction enzymes, such as *Hind*III and *Eco*RI (data not shown).

To test the relative contribution of GSH and GST to CLB resistance, we altered the intracellular GSH content or GST activity, using known GSH or GST enzyme inhibitors (23, 25, 40–44). BSO, a potent GSH synthesis inhibitor (40), was used to deplete GSH from the cells. With BSO preincubation for 18 hr, a dramatic decrease in intracellular GSH content was detected in both CLB-sensitive and -resistant cells (Fig. 3). By treating the CLB-resistant N50–4 cells with 50 $\mu$M BSO, which resulted in a 72% reduction in intracellular GSH content and a 18.8% cytotoxicity, the sensitivity to CLB was significantly increased (Fig. 4). However, the resistance of the N50–4 cells


**Fig. 2.** Northern and Southern blot analyses of CLB-sensitive and -resistant cell lines. A, Cytoplasmic RNA (20 $\mu$g) from NIH 3T3 and N50–4 cell lines was separated in agarose gels containing formaldehyde and was blotted onto nylon filters. The filters were probed with radioactively labeled cDNA to the Ya ($\alpha$), Yb ($\mu$), and Yp ($\pi$) GST genes and the $\beta$-actin gene. B, Genomic DNA (20 $\mu$g) was digested with the restriction enzyme *Pst*I, separated in 1.0% agarose gels, and blotted. The filters were hybridized to the GST cDNA fragment probes as described in Materials and Methods.

to CLB with BSO pretreatment was still greater than that of wild-type cells. This difference in CLB sensitivity was not due to higher intracellular GSH contents in CLB-resistant variants, because similar results were obtained with pretreatment with a higher dose of BSO (100 $\mu$M).

The inhibition of GST by ethacrynic acid and indomethacin was demonstrated using cell lysates from N50–4 cells (data not shown). Pretreatment of CLB-resistant cells with 50 $\mu$M ethacrynic acid and 100 $\mu$M indomethacin produced 5.3% and 6.3% cytotoxicity, respectively, and these concentrations were used as pretreatment doses. The CLB cytotoxicity was significantly increased in N50–4 cells with either ethacrynic acid or indomethacin pretreatment (Fig. 5). However, under these condi-

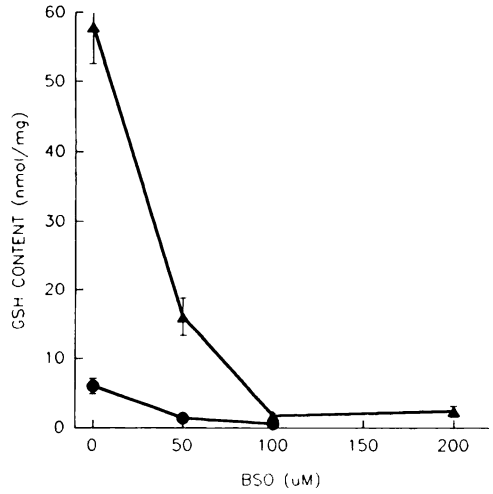



**Fig. 3.** Effect of BSO on intracellular GSH content. Cells growing in monolayers were treated with BSO for 18 hr. The cells were then harvested and GSH content was measured by the enzyme-recycling assay. ●, NIH 3T3 cells; ▲, N50–4 cells. *Points*, mean of four determinations; *bars*, standard error. On occasion, the confidence intervals were too small to be shown.

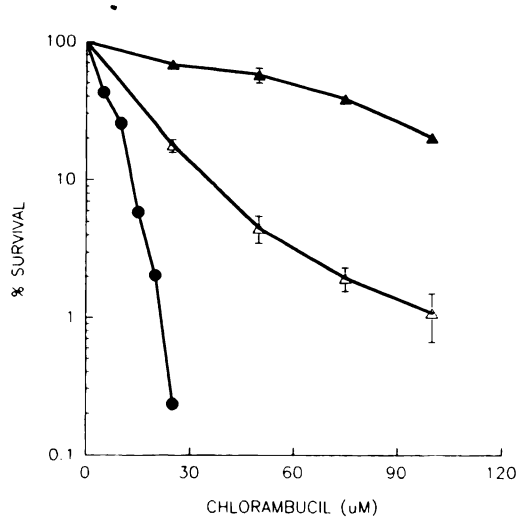
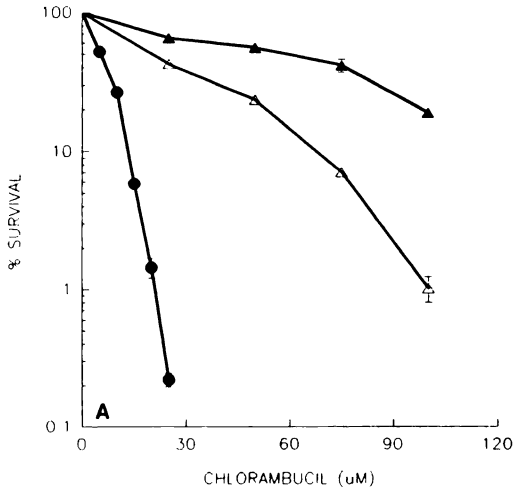



**Fig. 4.** Effect of BSO-mediated GSH depletion on CLB cytotoxicity. N50–4 (△) cells were pretreated with BSO (50 μM) for 18 hr and then exposed to CLB for 3 hr. Cell survival with BSO and CLB treatment is expressed as a percent of the number of cells surviving in the presence of BSO (50 μM) alone. NIH 3T3 (●) and N50–4 (▲) cells were exposed to CLB for 3 hr as non-BSO-treated controls. *Points*, mean of four determinations; *bars*, standard error. On occasion, the confidence intervals were too small to be shown.

tions N50–4 cells were still more resistant to CLB than the parental cells (Fig. 5).

These results suggested that both increased intracellular GSH content and GST activity may play important roles in CLB resistance. We further tested this hypothesis by a combination pretreatment of the CLB-resistant cells with both BSO and GST inhibitors. Pretreatment of the N50–4 cells with BSO plus ethacrynic acid or BSO plus indomethacin produced 26% and 28% cytotoxicity, respectively. The resistance to CLB in N50–4 cells was almost fully reversed to the phenotype of the parental cells by combination treatment with the GSH-depleting agent BSO and the GST inhibitors ethacrynic acid or indomethacin (Fig. 6).



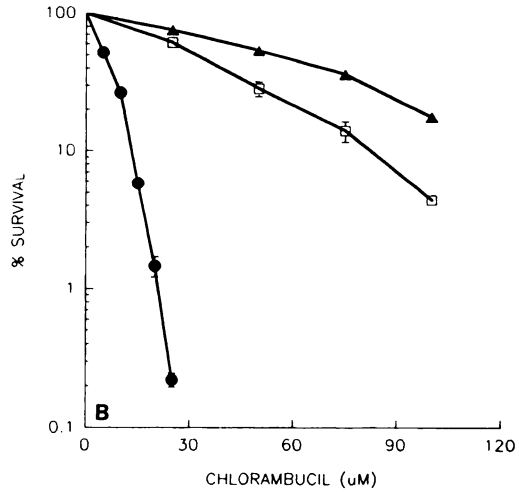




**Fig. 5.** Effect of ethacrynic acid (A) or indomethacin (B) on CLB cytotoxicity. N50–4 cells were pretreated with the GST inhibitors ethacrynic acid (50 μM), for 10 min (△), or indomethacin (100 μM), for 1 hr (□), and were then exposed to CLB for an additional 3 hr. Percentage of cell survival with both a GST inhibitor and CLB is based on the survival of the cells treated with GST inhibitor alone. NIH 3T3 (●) and N50–4 (▲) cells were exposed to CLB for 3 hr without GST inhibitor, as untreated controls. *Points*, mean of four determinations; *bars*, standard error. On occasion, confidence intervals were too small to be shown.

## Discussion

In the present study, we have observed a significant increase in intracellular GSH content and GST activity in the CLB-resistant N50–4 cell line, compared with the parental NIH 3T3 cells. Also, we have assessed the role of GSH and GST in CLB resistance by using known GSH- and GST-inhibitory agents to reduce the intracellular GSH content or to inhibit the GST activity in the CLB-resistant cell line.

Pretreatment with BSO to deplete intracellular GSH resulted in a significant enhancement of CLB cytotoxicity in CLB-resistant cells. The resistance of the N50–4 cells to CLB, however, was still much higher than that of wild-type cells. Treatment of N50–4 cells with a higher concentration of BSO (100 μM) did not abolish the CLB resistance, indicating that the intracellular GSH content is not the only factor responsible for the CLB resistance.

The role of GST in CLB resistance was studied using the GST inhibitors ethacrynic acid and indomethacin. Pretreat-

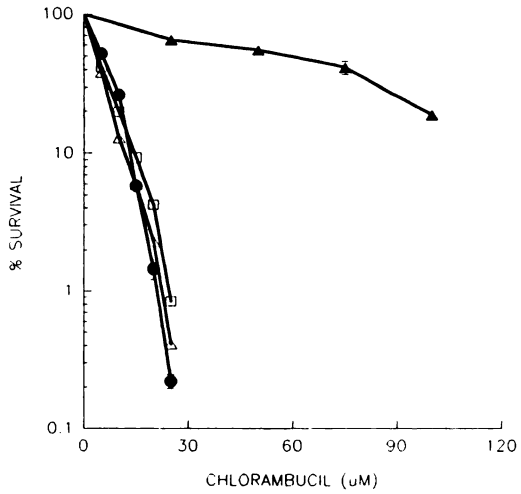



**Fig. 6.** Effect of GSH depletion and GST inhibition on CLB resistance. N50–4 cells were pretreated with BSO (50 μM) for 18 hr, followed by pretreatment with ethacrynic acid (50 μM) for 10 min (△) or indomethacin (100 μM) for 1 hr (□); cells were then exposed to CLB for 3 hr. The percentage of cell survival with the combination treatment is based on the number of surviving cells pretreated with BSO and GST inhibitor alone. NIH 3T3 (●) and N50–4 (▲) cells exposed only to CLB, without any pretreatment, served as control. *Points*, mean of four determinations; *bars*, standard error. On occasion, the confidence intervals were too small to be shown.

ment with ethacrynic acid or indomethacin increased the sensitivity to CLB in N50–4 cells, but these cells were still significantly more resistant to CLB than were the wild-type cells. Pretreatment of the CLB-resistant cell line with both a GSH-depleting agent (BSO) and a GST inhibitor (ethacrynic acid or indomethacin) resulted in the N50–4 cells being almost as sensitive to CLB as the parental cells, suggesting that both GSH and GST play important roles in CLB resistance in this cell line.

These findings support the previous observations that CLB can react with GSH both nonenzymatically and enzymatically (45, 46). Dulik *et al.* (45) reported that CLB can conjugate with GSH nonenzymatically but at levels 2–5-fold lower than microsomal GST-mediated conjugation. Ciaccio *et al.* (46) demonstrated a significant increase in CLB conjugation with GSH by a GST-mediated process, compared with spontaneous CLB-GSH conjugation. Our results showing residual resistance after GST activity was decreased with ethacrynic acid or indomethacin may be due to nonenzymatic conjugation of CLB with the elevated GSH levels found in the resistant cells.

Observations from Northern blot analysis showed that the mRNA level of the GST-α gene in the CLB-resistant cells was greatly increased, which would explain the increased intracellular GST activity. No significant differences in mRNA of GST π- and μ-class genes were found in the two cell lines, which is consistent with the findings in CHO cells (23, 24). Our results support the findings that the GST-α isozyme is more efficient in conjugating CLB than are other GST isozymes (46). Robson *et al.* (13) showed that expression of GST α- and π-class proteins was significantly increased in CLB-resistant CHO cells. Schisselbauer *et al.* (27) found a 2-fold increase in GST activity in CLB-resistant CLL cells, compared with nonresistant CLL cells and cells from normal individuals, but no obvious correlation between different isozymes of GST expressed and degree of CLB resistance in CLL cells. Ciaccio *et al.* (46) showed that GST-α was more efficient than GST-π and -μ isozymes purified from mouse liver cytosol for GSH conjugation with CLB. Black *et al.* (28) demonstrated that expression of both GST-α and -π in yeast cells could confer resistance to CLB. These observations suggest that GST-α, as well as π and μ isozymes, may play a role in CLB resistance. In the present study, we found that GST α-class gene expression was predominantly increased in CLB-resistant cells, as is found in other CLB-resistant cell lines established *in vitro* (13, 23). These results indicate that GST-α protein may be most important for CLB resistance *in vivo*.

By using genomic DNA analysis, we found no evidence of GST gene amplification or rearrangement in the CLB-resistant N50–4 cells. In other studies, Robson *et al.* (21) were also unable to detect GST gene amplification in the CLB-resistant CHO cells but found an abnormal karyotype with a modal chromosome number of 29 in the resistant cells, compared with 22 for the parental cells, which appears to have resulted largely from chromosome rearrangements. Lewis *et al.* (23), however, found that the genes coding for GST α-class proteins were amplified 4–8-fold in CLB-resistant CHO cells by Southern blot analysis. Our study indicates that mechanisms other than gene amplification can produce an increase in GST mRNA level. Experiments are now underway to determine whether the increased GST-α gene expression is occurring at the transcriptional or post-transcriptional level of control.

In summary, this study gives strong evidence that both increased intracellular GSH content and GST activity, mainly α-class isozyme, are major mechanisms of resistance to CLB in the N50–4 mouse fibroblast cell line. In addition, because these two mechanisms appear to be responsible for most of the resistance to CLB in these cells, the N50–4 cell line may serve as a valuable model for studies investigating methods for reversing alkylating agent resistance.

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**References**

1. McElwain, T. J., J. Toy, E. Smith, M. J. Peckham, and D. E. Austin. A combination of chlorambucil, vinblastine, procarbazine and prednisolone for treatment of Hodgkin's disease. *Br. J. Cancer* **36**:276–280 (1977).
2. Knospe, W. H., V. Loeb, and C. M. Huguley. Bi-weekly chlorambucil treatment of chronic lymphocytic leukemia. *Cancer (Phila.)* **33**:555–562 (1974).
3. Gale, R. P., and K. A. Foon. Chronic lymphocytic leukemia: recent advances in biology and treatment. *Ann. Intern. Med.* **103**:101–120 (1985).
4. Wheeler, G. P. Studies related to mechanisms of resistance to biological alkylating agents. *Cancer Res.* **23**:1334–1349 (1963).
5. Harrap, K. R., A. Jeney, P. J. Thraves, P. G. Riches, and R. Wilkinson. Macromolecular targets and metabolic properties of alkylating agents, in *Molecular Actions and Targets for Cancer Chemotherapeutic Agents* (A. Sartorelli, ed.), Vol. 2. Academic Press, New York, 45–84 (1980).
6. Begleiter, A., and G. J. Goldenberg. Uptake and decomposition of chlorambucil by L5178Y lymphoblasts *in vitro*. *Biochem. Pharmacol.* **32**:535–539 (1983).
7. Harrap, K. R., and B. T. Hill. The selectivity of action of alkylating agents and drug resistance. III. The uptake and degradation of alkylating drugs by Yoshida ascites sarcoma cells *in vitro*. *Biochem. Pharmacol.* **19**:209–217 (1970).
8. Bank, B. B., D. Kanganis, L. F. Liebes, and R. Silber. Chlorambucil pharmacokinetics and DNA binding in chronic lymphocytic leukemia lymphocytes. *Cancer Res.* **49**:554–559 (1989).
9. Hill, B. T., and K. R. Harrap. The uptake and utilization of chlorambucil by lymphocytes from patients with chronic lymphocytic leukemia. *Br. J. Cancer* **26**:439–443 (1972).
10. Silber, R., M. Potmesil, and B. B. Bank. Studies on drug resistance in chronic lymphocytic leukemia. *Adv. Enzyme Regul.* **29**:267–276 (1989).
11. Farmer, B. P. Metabolism and reactions of alkylating agents. *Pharmacol. Ther.* **35**:301–358 (1987).
12. Foon, K. A., K. R. Rai, and R. P. Gale. Chronic lymphocytic leukemia: new insights into biology and therapy. *Ann. Intern. Med.* **113**:525–539 (1990).

13. Robson, C. N., A. D. Lewis, C. R. Wolf, J. D. Hayes, A. Hall, S. Proctor, A. L. Harris, and I. D. Hickson. Reduced levels of drug-induced DNA cross-linking in nitrogen mustard-resistant Chinese hamster ovary cells expressing elevated glutathione *S*-transferase activity. *Cancer Res.* **47**:6022–6027 (1987).
14. Jiang, B.-Z., B. B. Bank, Y.-H. Hsiang, T. Shen, M. Potmesil, and R. Silber. Lack of drug-induced DNA cross-links in chlorambucil-resistant Chinese hamster ovary cells. *Cancer Res.* **49**:5514–5517 (1989).
15. Harrap, K. R., and E. W. Gascoigne. The interaction of bifunctional alkylating agents with the DNA of tumor cells. *Eur. J. Cancer* **12**:53–59 (1976).
16. Panasci, L., D. Henderson, S. J. Torres-Garcia, V. Skalski, S. Caplan, and M. Hutchinson. Transport, metabolism, and DNA interaction of Melphalan in lymphocytes from patients with chronic lymphocytic leukemia. *Cancer Res.* **48**:1972–1976 (1988).
17. Johnston, J. B., L. G. Israels, G. J. Goldenberg, C. D. Anhalt, L. Verburg, M. R. Mowat, and A. Begleiter. Glutathione *S*-transferase activity, sulfhydryl group and glutathione levels, and DNA cross-linking activity with chlorambucil in chronic lymphocytic leukemia. *J. Natl. Cancer Inst.* **82**:776–779 (1990).
18. Arrick, B. A., and C. F. Nathan. Glutathione metabolism as a determinant of therapeutic efficacy: a review. *Cancer Res.* **44**:4224–4232 (1984).
19. Evans, C. G., W. J. Bodell, K. Tokuda, P. Doane-Setzer, and M. T. Smith. Glutathione and related enzymes in rat brain tumor cell resistance to 1,3-bis(2-chloroethyl)-1-nitrosourea and nitrogen mustard. *Cancer Res.* **47**:2525–2530 (1987).
20. Suzukake, K., B. P. Vistica, and D. T. Vistica. Dechlorination of L-phenylalanine mustard by sensitive and resistant tumour cells and its relationship to intracellular glutathione content. *Biochem. Pharmacol.* **32**:165–167 (1983).
21. Robson, C. N., J. Alexander, A. L. Harris, and I. Hickson. Isolation and characterization of a Chinese hamster ovary cell line resistant to bifunctional nitrogen mustards. *Cancer Res.* **46**:6290–6294 (1986).
22. Mannervik, B., P. Alin, C. Guthenberg, H. Jennson, M. K. Tahir, M. Warholm, and H. Jornvall. Identification of three classes of cytosolic glutathione transferase common to several mammalian species: correlation between structural data and enzymic properties. *Proc. Natl. Acad. Sci. USA* **82**:7202–7206 (1985).
23. Lewis, A. D., I. D. Hickson, C. N. Robson, A. L. Harris, J. D. Hayes, S. A. Griffiths, M. M. Manson, A. E. Hall, J. E. Moss, and C. R. Wolf. Amplification and increased expression of *alpha* class glutathione *S*-transferase-encoding genes associated with resistance to nitrogen mustards. *Proc. Natl. Acad. Sci. USA* **85**:8511–8515 (1988).
24. Hall, A., C. N. Robson, I. D. Hickson, A. L. Harris, S. J. Proctor, and A. R. Cattan. Possible role of inhibition of glutathione *S*-transferase in the partial reversal of chlorambucil resistance by indomethacin in a Chinese hamster ovary cell line. *Cancer Res.* **49**:6265–6268 (1989).
25. Wang, A. L., and K. D. Tew. Increased glutathione *S*-transferase activity in a cell line with acquired resistance to nitrogen mustards. *Cancer Treat. Rep.* **69**:677–682 (1985).
26. Tew, K. D., A. M. Bomber, and S. J. Hoffman. Ethacrynic acid and piriprost as enhancers of cytotoxicity in drug resistant and sensitive cell lines. *Cancer Res.* **48**:3622–3625 (1988).
27. Schisselbauer, J. C., R. Silber, E. Papadopoulos, K. Abrams, F. P. LaCreta, and K. D. Tew. Characterization of glutathione *S*-transferase expression in lymphocytes from chronic lymphocytic leukemia patients. *Cancer Res.* **50**:3562–3568 (1990).
28. Black, S. M., J. D. Beggs, J. D. Hayes, A. Bartoszek, M. Muramatsu, M. Sakai, and C. R. Wolf. Expression of human glutathione *S*-transferases in *Saccharomyces cerevisiae* confers resistance to the anticancer drugs Adriamycin and chlorambucil. *Biochem. J.* **268**:309–315 (1990).
29. Puchalski, R. B., and W. E. Fahl. Expression of recombinant glutathione *S*-transferase $\pi$, Ya, or $Yb_1$ confers resistance to alkylating agents. *Proc. Natl. Acad. Sci. USA* **87**:2443–2447 (1990).
30. Leyland-Jones, B. R., A. J. Townsend, C.-P. D. Tu, K. Cowan, and M. E. Goldsmith. Antineoplastic drug sensitivity of human MCF-7 breast cancer cells stably transfected with a human $\alpha$ class glutathione *S*-transferase gene. *Cancer Res.* **51**:587–594 (1991).
31. Freshney, R. I. *Culture of Animal Cells: A Manual of Basic Technique.* Alan R. Liss, Inc., New York, 136 (1983).
32. Griffith, O. W. Determination of glutathione and glutathione-disulfide using glutathione reductase and 2-vinylpyridine. *Anal. Biochem.* **106**:207–212 (1980).
33. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
34. Habig, W. H., M. J. Pabst, and W. B. Jakoby. Glutathione-*S*-transferase: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**:7130–7139 (1974).
35. Gough, N. M. Rapid and quantitative preparation of cytoplasmic RNA from small numbers of cells. *Anal. Biochem.* **173**:93–95 (1988).
36. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1982).
37. Reed, K. C., and D. A. Mann. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13**:7207–7220 (1985).
38. Pickett, C. B. Structure and regulation of glutathione *S*-transferase genes. *Essays Biochem.* **23**:116–143 (1987).
39. Wang, Y., B. A. Teicher, T. C. Shea, S. A. Holden, K. W. Rosbe, A. Aiachi, and W. D. Henner. Cross-resistance and glutathione-*S*-transferase-$\pi$ levels among four human melanoma cell lines selected for alkylating agent resistance. *Cancer Res.* **49**:6185–6192 (1989).
40. Griffith, O. W., and A. Meister. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine. *J. Biol. Chem.* **254**:7558–7560 (1979).
41. Ahokas, J. T., F. A. Nicholls, P. J. Ravenscroft, and B. T. Emmerson. Inhibition of purified rat liver glutathione *S*-transferase isozymes by diuretic drugs. *Biochem. Pharmacol.* **34**:2157–2161 (1985).
42. Ploemen, J. H. T. M., B. Van Ommen, and P. J. Van Bladeren. Inhibition of rat and human glutathione *S*-transferase isoenzymes by ethacrynic acid and its glutathione conjugate. *Biochem. Pharmacol.* **40**:1631–1635 (1990).
43. Wu, C., and K. P. Mathews. Indomethacin inhibition of glutathione *S*-transferases. *Biochem. Biophys. Res. Commun.* **112**:980–985 (1983).
44. Nicholls, F. A., and J. T. Ahokas. Inhibition of purified glutathione *S*-transferases by indomethacin. *Biochem. Biophys. Res. Commun.* **119**:1034–1038 (1984).
45. Dulik, D. M., O. M. Colvin, and C. Fenselau. Characterization of glutathione conjugates of chlorambucil by fast atom bombardment and thermospray liquid chromatography/mass spectrometry. *Biomed. Environ. Mass Spectrom.* **19**:248–252 (1990).
46. Ciaccio, P. J., K. D. Tew, and F. P. LaCreta. The spontaneous and glutathione *S*-transferase-mediated reaction of chlorambucil with glutathione. *Cancer Commun.* **2**:279–286 (1990).

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