0026-895X/90/020192-06$02.00/0

MOLECULAR PHARMACOLOGY, 37:192-197

Alterations in Glutathione and Glutathione-Related Enzymes in a Multidrug-Resistant Small Cell Lung Cancer Cell Line

SUSAN P. C. COLE, HEATHER F. DOWNES, SHELAGH E. L. MIRSKI, and DEBRA J. CLEMENTS

Departments of Oncology (S.P.C.C., H.F.D., S.E.L.M., D.J.C.), Pharmacology and Toxicology, and Pathology (S.P.C.C.), Queen's University, Kingston, Ontario, Canada K7L 3N6

Received August 24, 1989; Accepted October 18, 1989

SUMMARY

H69AR is a multidrug-resistant small cell lung cancer cell line derived from a drug-sensitive cell line, H69, by selection in doxorubicin. It is cross-resistant to a wide variety of natural product-type antineoplastic agents but does not overexpress P-glycoprotein. In the present study, the levels of GSH and GSH-related enzymes in the H69AR cell line were determined and compared with those found in H69 cells. Unlike other drug-resistant cell lines, GSH levels were diminished 6-fold in H69AR cells (0.67 ± 0.28 μg/mg of protein), compared with H69 cells (4.23 ± 1.17 μg/mg of protein) ($p < 0.01$). This unusually low level of GSH may explain the pronounced collateral sensitivity of H69AR cells to buthionine sulfoximine (BSO), an inhibitor of the rate-limiting enzyme in GSH biosynthesis (ID_{50} of 4.4 μM BSO for H69AR cells versus ID_{50} of 300 μM BSO for H69 cells). BSO did not enhance doxorubicin cytotoxicity in the H69AR cell line, despite further depletion of GSH. GSH-reductase (EC 1.6.4.2) activity was elevated 2-fold in H69AR cells, compared with sensitive H69 cells (75.34 ± 14.94 versus 38.62 ± 5.06 nmol of NADPH/min/mg of protein) ($p < 0.05$). Both selenium-dependent and -independent GSH-peroxidase (EC 1.11.1.9) activities were unchanged in the resistant H69AR cell line, compared with its parent cell line. γ-Glutamyl transpeptidase (EC 2.3.2.2) activity was 5-fold elevated in H69AR cells, compared with H69 cells (2.50 ± 0.44 versus 0.46 ± 0.21 nmol of *p*-nitroaniline/min/mg of protein) ($p < 0.01$), whereas GSH-*S*-transferase (EC 2.5.1.18) activity was 10-fold higher (201.98 ± 43.62 versus 19.77 ± 1.72 nmol of 1-chloro-2,4-dinitrobenzene/min/mg of protein in H69AR and H69 cells, respectively) ($p < 0.01$). The GSH-*S*-transferases from both cell lines were purified by affinity chromatography and immunoblot analysis identified the GSH-*S*-transferases as belonging to the anionic π class. GSH-*S*-transferases from the μ or α classes were not detectable in either cell line. In conclusion, marked differences in GSH levels and the activities of three of four GSH-related enzymes were observed between the multidrug-resistant H69AR cell line and its parent cell line. Further study is required to determine whether these changes are causally related to the development of drug resistance in this model system.

SCLC is the most rapidly progressing form of lung cancer and has an extremely poor prognosis. Advances in combination chemotherapy have resulted in improved median survival time, but the five-year survival rate for SCLC patients remains at only 5–10% (1). Although tumor regression is initially observed in 70–90% of patients after chemotherapy, MDR almost invariably develops in patients with this disease.

A variety of biochemical changes in MDR tumor cells have been described but the most consistent alteration has been the increased expression of a high molecular weight plasma membrane glycoprotein termed P-glycoprotein, which is involved in enhanced drug efflux from MDR cells (2, 3) (reviewed in Ref. 4). Recent evidence supports the idea that overexpression of this protein plays a role, at least in some tumors, in clinical drug resistance observed in cancer patients (5, 6). However, there are now reports of MDR cell lines in which P-glycoprotein does not appear to be involved (7–10). In addition, overexpression of P-glycoprotein is detected in only a subset of patients whose tumors have developed drug resistance. Thus, acquired MDR is a complex phenomenon and is likely multifactorial in nature.

To study the MDR phenotype of SCLC, we have developed a drug-resistant SCLC cell lines by selection in DOX (7). This cell line, designated H69AR, possesses the MDR phenotype but does not overexpress P-glycoprotein (7, 11). Thus, it provides a model system for the investigation of non-P-glycoprotein-mediated forms of MDR.

GSH and GSH-related enzymes have long been known to play an important role in drug detoxification reactions. More

This work was supported by a grant from the National Cancer Institute of Canada to S.P.C.C. H.F.D. was the recipient of the Thomas M. and Louise A. Brown Research Scholarship from the Faculty of Medicine, Queen's University.

ABBREVIATIONS: SCLC, small cell lung cancer; MDR, multidrug resistance or multidrug resistant; BSO, DL-buthionine-(*SR*)-sulfoximine; PBS, phosphate-buffered saline; GST, glutathione-*S*-transferase; DOX, doxorubicin; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide; CDNB, 1-chloro-2-,4-dinitrobenzene; NGS, normal goat serum; BSA, bovine serum albumin; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); TBS, Tris-buffered saline.

recently, enhanced levels of GSH and its associated enzymes have been firmly implicated in mechanisms of resistance to alkylating agents (12) and γ-irradiation (13). However, their role in resistance to natural products (the MDR phenotype) is less well established (14–16). The present study was undertaken to determine whether GSH levels and GSH-related enzyme activities are altered in H69AR cells and, thus, provide some insight into the mechanism by which these cells are able to resist the toxicity of natural product-type antineoplastic agents.

Materials and Methods

Sources of materials. DOX was obtained from the Ontario Cancer Foundation, Kingston Regional Cancer Centre. It was reconstituted in PBS at a concentration of 200 μM and stored frozen at −20°. All subsequent dilutions were made with tissue culture medium. BSO, MTT, BSA, nitroblue tetrazolium, bromochloroindolyl phosphate, cumene hydroperoxide, rat liver GST, GSH-reductase, NADPH, oxidized and reduced GSH, *S*-hexyglutathione-agarose, *S*-hexylglutathione, CDNB, and DTNB were obtained from Sigma Chemical Co. (St. Louis, MO). Antibodies to GST μ, α, and π subclasses (Bioprep, Dublin, Ireland) were kindly provided by Drs. K. Tew and M. Clapper (Fox Chase Cancer Center, Fox Chase, PA). Goat anti-rabbit IgG alkaline phosphatase conjugate was obtained from Sigma. NGS was obtained from Rockland Co. (Gilbertsville, PA) and fetal bovine serum was obtained from GIBCO.

Cell culture. The SCLC cell line NCI-H69 (H69) was provided by Dr. J. Minna (National Institutes of Health, Bethesda, MD). A MDR variant of H69, designated H69AR, was obtained by culturing H69 cells in increasing concentrations of DOX up to 0.8 μM and has been described previously (7). The cell lines were routinely maintained in RPMI 1640 medium (GIBCO) supplemented with 5% heat-inactivated fetal bovine serum and L-glutamine (4 mM). H69AR cells were challenged with 0.8 μM DOX every month and were cultured in drug-free medium at least 72 hr before all experiments. Cells were cultured in the absence of antibiotics and were negative for *Mycoplasma* contamination when tested using the 4,6-diamidino-2-phenylindole DNA-binding assay (17).

Chemosensitivity testing. Chemosensitivity testing was done using a slight modification of the colorimetric MTT assay (18). In brief, H69 and H69AR cells were plated at 2.5×10^4 cells/well/100 μl in 96-well microtiter plates. After incubation for 24 hr, drugs were added at 2 times the desired final concentration in a volume of 100 μl/well and the cells were incubated for a further 5 days. Three hours before the end of drug exposure time, 100 μl of medium were removed from each well and 25 μl of MTT (2 mg/ml in PBS) were added. Three hours later, isopropanol/1 N HCl (24:1) was added with thorough mixing, followed by further incubation for 1 hr to ensure complete solubilization of the formazan crystals. The absorbance at 570 nm was determined using a Dynatech MR600 microtiter plate reader. Within each experiment, determinations were done in quadruplicate. Cytotoxicity is expressed as a percentage of control absorbance values obtained from wells containing cells but no drugs. The IC_{50} is defined as the concentration of drug that reduces the absorbance values to 50% of untreated control cell values. The relative resistance is expressed as the ratio of the IC_{50} of the resistant cells to the IC_{50} of the sensitive cells.

In the combination experiments, the cells were either preincubated with BSO for 18 hr before the addition of DOX or, in a separate experiment, DOX and BSO were added together to the cells.

Assays. The levels of total oxidized and reduced GSH were determined by the spectrophotometric assay of Tietze (19), using DTNB as substrate. To measure GSH-reductase activity, a cell sonicate was prepared from H69 and H69AR cells by resuspending the cells at a concentration of 10×10^6 cells/0.5 ml of PBS and sonicating for two 15-sec pulses with a 30-sec interval between each pulse. The sonicate was centrifuged at 100,000 × *g* in a Beckman TL-100 ultracentrifuge, and the supernatant (cytosol) was assayed for enzyme activity by the method of Carlberg and Mannervik (20). GST activity was measured on cell cytosols prepared as described above, using CDNB as substrate (21). To measure GSH-peroxidase activity, cell lysates were prepared by resuspending 100×10^6 cells/ml of PBS and sonicating for three 15-sec pulses with a 10-sec interval between pulse. The resulting sonicate was centrifuged at 3000 × *g* and the supernatant was assayed for enzyme activity at 25°, using both hydrogen peroxide and cumene hydroperoxide as substrates, according to the method of Paglia and Valentine (22) as modified by Reddy *et al.* (23). To measure γ-glutamyl transpeptidase activity, crude solubilized membranes were prepared by resuspending 50×10^6 cells in 0.5 ml of H_2O and were homogenized followed by the addition of 0.5 ml of 0.5% Triton X-100. After remaining on ice for 60 min with intermittent mixing, the solubilized cell preparation was centrifuged at 11,000 × *g*. Enzyme activity of the resulting supernatant was measured using a kit from Sigma based on the method described by Szasz (24).

Spectra were recorded on a Perkin Elmer 552 or Pye Unicam spectrophotometer. All assays were done in duplicate within each experiment and each experiment was performed at least three times. The statistical significance of results obtained was determined using an unpaired Student's *t* test.

The protein concentrations of the cell preparations were determined by the method of Lowry *et al.* as modified by Peterson (25), except for the γ-glutamyl transpeptidase assays, where protein was measured by the Bradford method (Bio-Rad protein assay kit).

Affinity purification of GSTs and immunoblotting. GSTs were purified from H69 and H69AR cells by *S*-hexylglutathione affinity chromatography according to the method of Buller *et al.* (26). Affinity-purified GSTs (2 μg/lane) and rat liver GST (5 μg/lane) were run on a 15% Laemmli gel (27) and transferred to nitrocellulose paper overnight at 60 V, 100 mA (28). After blocking in 3% BSA in TBS, the blots were incubated with anti-GST-μ, -α, or -π antibodies diluted 1:5000 in TBS/3% BSA/5% NGS for 1 hr. Binding of primary antibody was detected with goat anti-rabbit IgG alkaline phosphatase conjugate (diluted 1:500 in TBS/3% BSA/5% NGS), with nitroblue tetrazolium and bromochloroindolyl phosphate as substrates (29). Color development was stopped with 0.05 M citrate buffer, pH 3.5.

Results

GSH. As shown in Table 1, the sensitive H69 cells contained 4.23 ± 1.17 μg/mg of protein oxidized and reduced GSH, which was 6-fold higher than in the resistant H69AR cells (0.670 ± 0.28 μg/mg of protein) ($p < 0.01$).

Chemosensitivity testing. The toxicity of BSO in the H69 and H69AR cell lines was determined using the MTT assay. Interestingly, the sensitive cells exhibited a 68-fold resistance to the cytotoxic effects of BSO, compared with the MDR H69AR cells (Fig. 1). Thus, the ID_{50} in H69 cells was 300 μM BSO, whereas the ID_{50} for H69AR cells was 4.4 μM BSO.

Exposure of H69AR cells to 1 μM BSO for 18 hr further reduced GSH levels, as expected (Table 1). However, as shown in Fig. 2, this depletion did not enhance DOX cytotoxicity in

TABLE 1

Glutathione levels in H69 and H69AR cells

Numbers in parentheses are number of experiments.

Treatment	Total GSH	
	H69	H69AR
	μg/mg of protein	
None	4.23 ± 1.17 (4)	0.670 ± 0.28[a] (5)
BSO, 1 μM, 18 hr	ND[b]	0.030

[a] $p < 0.01$ by *t* test comparison with H69.
[b] ND, not determined.

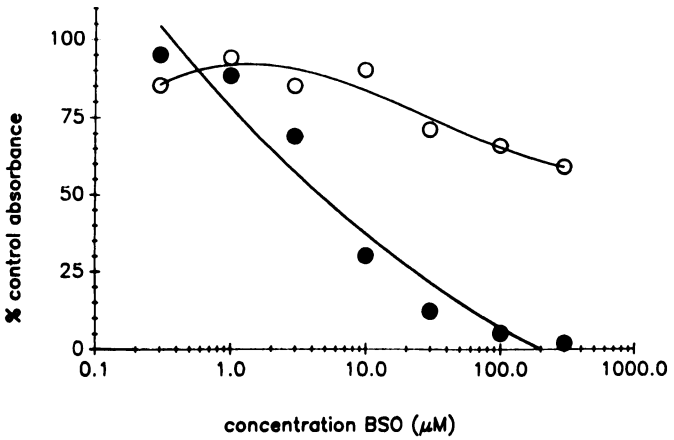

Fig. 1. Effect of BSO on the growth of SCLC cell lines H69 (○) and MDR H69AR (●), as measured by the MTT assay. Cells were plated at 2.5 × 10^4 cells/well on day 0, BSO was added on day 1, and cell viability was measured on day 5. Each *point* represents the mean of four determinations. Standard deviations were generally less than 10% of the mean values and have been omitted for clarity.

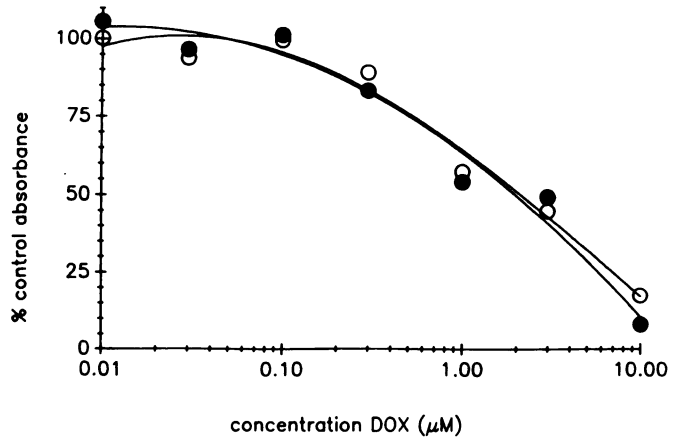

Fig. 2. Effect of BSO on the DOX sensitivity of the MDR SCLC cell line H69AR. Cells were plated on day 0 at 2.5 × 10^4 cells/well in the absence (○) and presence (●) of BSO (1 μM). DOX was added on day 1 and cell viability was measured on day 5. Each *point* represents the mean of four determinations. Standard deviations were less than 10% of the mean values and have been omitted for clarity.

the H69AR cell line. The dose-response curves of H69AR cells to DOX were identical in the presence and absence of BSO (1 μM), whether the cells were incubated for 18 hr with BSO before the addition of DOX (Fig. 2) or whether both drugs were added at the same time (results not shown).

GSH-reductase, GSH-peroxidase, γ-glutamyl transpeptidase, and GST activities. GSH-reductase activity was measured using the spectrophotometric method that monitors the decrease in absorbance at 340 nm caused by the utilization of NADPH by the enzyme. GSH-reductase activity was found to be 2-fold elevated in H69AR cells, compared with sensitive H69 cells (75.34 ± 14.94 versus 38.62 ± 5.06 nmol of NADPH/min/mg of protein) ($p < 0.05$) (Table 2).

GSH-peroxidase activity was measured using the method in which the production of oxidized GSH (GSSG) is coupled to its reduction back to reduced GSH by the addition of exogenous GSH-reductase. Thus, the utilization of NADPH (measured at 340 nm) as the GSSG is converted to its reduced form reflects the GSH-peroxidase activity. Selenium-dependent peroxidase activity was measured using H_2O_2 as substrate and was 6.14 ± 1.42 nmol of NADPH/min/mg of protein in H69 cells, as compared with 6.04 ± 2.40 nmol of NADPH/min/mg of protein in H69AR cells. Selenium-independent organic peroxidase activity was measured using cumene hydroperoxide as substrate and was found to be 5.33 ± 1.77 nmol of NADPH/min/mg of protein in H69AR cells. Thus, neither selenium-dependent nor -independent GSH-peroxidase activities were significantly altered in the resistant cells (Table 2).

TABLE 2

Glutathione-associated enzyme activities in H69 and H69AR cells

Numbers in parentheses are number of experiments.

	H69	H69AR
GSH-reductase (nmol of NADPH/min/mg of protein)	38.62 ± 5.06	75.34 ± 14.94[a]
	(3)	(3)
GSH-peroxidase (nmol of NADPH/min/mg of protein)		
Hydrogen peroxide	6.14 ± 1.42	6.04 ± 2.40
	(4)	(4)
Cumene hydroperoxide	5.33 ± 1.77	6.64 ± 1.91
	(3)	(3)
γ-Glutamyl transpeptidase (nmol/min/mg of protein)	0.46 ± 0.21	2.50 ± 0.44[b]
	(3)	(3)
GSH-S-transferase (nmol of CDNB/min/mg of protein)	19.77 ± 1.72	201.98 ± 43.62[b]
	(3)	(6)

[a] Significantly different from H69 ($p < 0.05$).
[b] Significantly different from H69 ($p < 0.01$).

γ-Glutamyl transpeptidase activity was determined using the method in which γ-glutamyl *p*-nitroanilide and glycylglycine are used as the donor and acceptor substrates, respectively, for the transfer of the γ-glutamyl group. γ-Glutamyl transpeptidase activity was 5-fold elevated in H69AR cells compared with H69 cells (2.50 ± 0.44 versus 0.46 ± 0.21 nmol of *p*-nitroaniline/min/mg of protein) ($p < 0.01$) (Table 2).

Total GST activity was measured using CDNB as substrate, because all forms of the enzyme are active with this substrate. GST activity was 10-fold elevated in H69AR cells, compared with H69 cells (201.98 ± 43.62, versus 19.77 ± 1.72 nmol of CDNB/min/mg of protein) ($p < .01$) (Table 2).

Isoenzyme analysis by immunoblotting. GSTs of cytosolic fractions prepared from 6.78 × 10^8 H69 cells and 2.8 × 10^8 H69AR cells were purified by affinity chromatography using *S*-hexylglutathione-agarose. Enzyme elution was effected with 5 mM *S*-hexylglutathione and the GST activity, measured using CDNB as substrate, eluted as a single peak. This procedure resulted in approximately 300-fold purification of H69 GST activity and 140-fold purification of H69AR GST activity.

Using antibodies against the three major classes of GSTs in an immunoblot analysis, the only detectable isoenzymes found in both the H69 and H69AR purified GSTs belonged to the anionic π-class of enzymes (Fig. 3). No evidence of μ- or α-class GSTs was observed.

Discussion

The H69AR cell line developed in this laboratory, although selected in DOX, is cross-resistant to multiple antineoplastic agents including the *Vinca* alkaloids and epipodophyllotoxins (7). However, overexpression of P-glycoprotein is not detectable

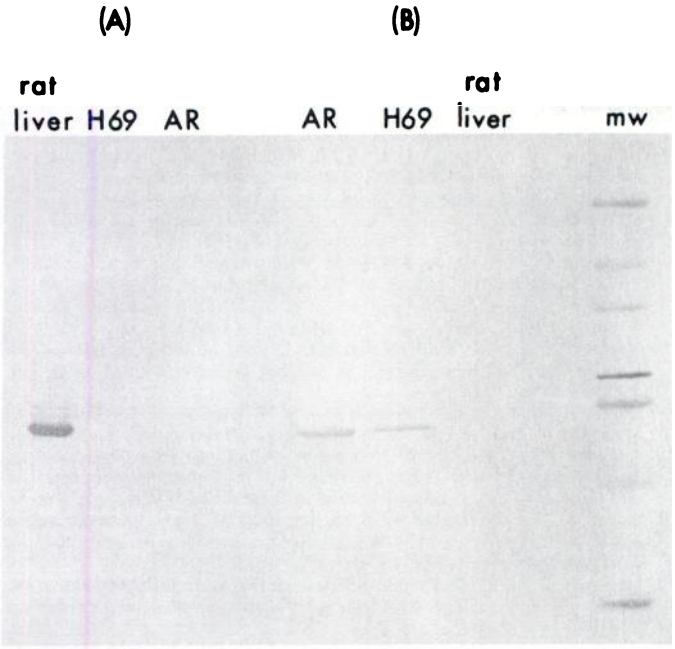

Fig. 3. Immunoblot analysis of affinity-purified GSTs from rat liver (5 μg/lane), H69 cells (2 μg/lane), and H69AR cells (2 μg/lane). Nitrocellulose membranes were incubated with antibodies against human GST μ and α (A) or an antibody against human anionic GST π (B). Further details are given in Materials and Methods. Molecular weight markers (in descending order, *M*, 66,000, 45,000, 36,000, 29,000, 24,000, 20,000, and 14,000) are shown in the *lane* on the *extreme right*.

in H69AR cells at the protein level (7) or at the RNA level (11), even when such sensitive techniques as the polymerase chain reaction are employed.[1] Thus, this cell line provides a unique model in which to study non-P-glycoprotein-mediated mechanisms of MDR.

Elevated levels of GSH have been associated with the development of resistance to alkylating agents (30, 31) and γ-irradiation (32) and are thought to protect the resistant cells from cytotoxic damage. BSO, an irreversible inhibitor of the rate-limiting enzyme of GSH biosynthesis (33), partially reverses this resistance, presumably by removing the protection afforded by GSH. Such reversal has also been observed in some MDR cell lines (15, 34, 35). However, in contrast to many drug-resistant cell lines, the MDR H69AR cells contained markedly lower levels of GSH than the drug-sensitive H69 cells (Table 1). These results indicate that GSH itself does not play a role in the resistance mechanism of H69AR cells. The lower levels of GSH may also explain why the H69AR cells were considerably more sensitive to the cytotoxic effects of BSO by itself (Fig. 1). Such collateral sensitivity has been observed in other MDR cells lines (36), although apparently to a lesser degree. Finally, because the GSH level in H69AR cells is lower than in the H69 cells, it is not surprising that further depletion by BSO does not enhance the DOX sensitivity of this resistant cell line (Fig. 2). Whether BSO treatment alters the sensitivity of H69AR cells to other antineoplastic agents is currently under investigation.

GSH-reductase is an enzyme critical for maintaining the proper proportions of reduced and oxidized GSH in cells. It is, therefore, of interest that this enzyme was significantly elevated in H69AR cells compared with H69 cells (Table 2). Meijer *et al.* (36) also found this enzyme elevated in their DOX-resistant SCLC cell line. These results are in contrast to those of some other MDR cell lines, where no differences in GSH-reductase activity have been found (37). However, H69AR cells are so profoundly reduced in their GSH content that the elevation in GSH-reductase activity may represent an attempt by these cells to replenish their GSH stores.

GSH-peroxidases catalyze the reduction of hydroperoxides by GSH and, thus, play a role in protecting biological tissues from oxidative damage by free radicals and other reactive chemical species. Two major types of GSH-peroxidases have been described (38). One is a selenium-dependent enzyme that is active with both organic hydroperoxides and H_2O_2, whereas the other is selenium independent and has no activity with H_2O_2. The latter activity is mediated by some isoenzymes of GST (38). Elevation of GSH-peroxidase activity, attributable to both forms of the enzyme, has been reported in several MDR cell lines selected in DOX (15, 36, 39). Because at least one mechanism of DOX cytotoxicity appears to be mediated through its enzymatic reduction to a semiquinone free radical metabolite, which in turn generates toxic reactive oxygen species (40), it has been suggested that elevated GSH-peroxidase levels may contribute to the protection of the MDR cells from DOX killing (15, 41). This clearly is not an invariable property of DOX-selected cell lines, because H69AR cells do not differ from H69 cells in either their selenium-dependent or -independent GSH-peroxidase activities (Table 2). Other resistant cell lines selected in DOX that do not have elevated GSH-peroxidase activity have also been described (35, 36, 42).

γ-Glutamyl transpeptidase is a predominantly membrane-bound enzyme that catalyzes the first step in the catabolism of GSH (43). The activity of this enzyme has been found to be elevated in preneoplastic cells (44) and in some drug-resistant cell lines (12, 45). Because elevated GSH levels have been found in association with an increase in γ-glutamyl transpeptidase activity, it has been postulated that the enhancement of this enzyme activity increases the availability of cysteine for GSH synthesis and results in the up-regulation of GSH biosynthesis (12, 45). This seems an unlikely explanation for the 5-fold elevation in γ-glutamyl transpeptidase activity found in H69AR cells (Table 2), because GSH levels are so markedly reduced. On the contrary, it may be that the elevated γ-glutamyl transpeptidase activity is responsible, at least in part, for the diminished GSH levels in H69AR cells. Of interest is the finding of Lutzky *et al.* (35) in a DOX-selected human leukemia cell line that γ-glutamyl transpeptidase was reduced 2-fold in association with a 2-fold reduction in GSH levels. Thus, no consistent pattern linking levels of γ-glutamyl transpeptidase activity and GSH has been observed in drug-resistant cells.

The GSTs are a multigene family of dimeric proteins, whose main detoxification function is to catalyze the conjugation of GSH to electrophilic metabolites, thus rendering them more water soluble. Batist *et al.* (14) were the first to describe markedly elevated levels of GST activity in a MDR human breast carcinoma cell line, MCF-7 ADR, which also overexpresses P-glycoprotein. Elevation of GST in a number of other MDR cell lines has been reported subsequently (16, 46, 47). It has been hypothesized that the expression of P-glycoprotein and GST may be under common regulatory control (48). The

[1] J. H. Gerlach, E. Chanda, M. J. Pinkoski, and S. P. C. Cole, unpublished data.

results of the present study do not support this idea because, although GST activity is elevated in the H69AR cell line (Table 2), it does not overexpress P-glycoprotein. Conversely, the MDR myeloma cell line 8226/DOX40 does overexpress P-glycoprotein but does not have increased GST activity (42). Thus, elevated GST activity is frequently, but not invariably, accompanied by overexpression of P-glycoprotein. Furthermore, elevated GST activity is not uniquely associated with anthracycline resistance, because there are several reports of DOX-selected drug-resistant cell lines in which levels of this enzyme are similar to those of the sensitive parent cell line (35, 42).

Yusa *et al.* (16) have suggested that elevated GST does not confer drug resistance, because a revertant of a resistant cell line in their study contained a level of GST activity similar to that of its corresponding resistant cell line. In the present study, similar indirect evidence suggests that the 10-fold elevation of GST in H69AR cells is not responsible for the observed drug resistance, because the GST activity in a variant H69AR cell line that has regained some drug sensitivity is similar to that found in H69AR cells (data not shown). More direct evidence for this conclusion has been provided recently by Moscow *et al.* (49), who demonstrated that transfection of breast cancer cells with the GST-π gene did not result in a change in the sensitivity of these cells to DOX or other drugs characterizing the MDR phenotype.

The GST isoenzymes are assigned to one of three classes, α, μ, or π, depending on their isoelectric points and immunologic cross-reactivities as well as their amino acid homology (50). In cell lines resistant to alkylating agents, elevated levels of α-class GST isoenzymes have been observed (12, 46). In our cell line, as in other MDR cell lines and human tumors (8, 14, 16, 47), the overexpressed GST belongs to the π-class of isoenzymes (Fig. 3) and in the case of H69AR cells no μ- or α-class isoenzymes could be detected. The absence of GST-α may explain why selenium-independent GSH-peroxidase activity was not elevated in H69AR cells, because this enzyme activity is predominantly associated with the α-class of GSTs (38, 50).

In conclusion, we have found that the activities of three enzymes that play a role in determining intracellular GSH levels are enhanced in the MDR H69AR cell line. The apparent consequence of these changes is a marked reduction in GSH levels. Other MDR cell lines examined in a similar manner have displayed a pattern of alterations different from that described in the present study. The absence of a consistent pattern among the various MDR cell lines makes it unlikely that these changes can be linked to a mechanism of drug resistance. It may be that these alterations in GSH and GSH-related enzymes are all epiphenomena causally unrelated to drug resistance, as suggested by others (16, 42, 49). However, direct evidence from gene expression ablation experiments is required before the possibility that these enzymes contribute to the resistance phenotype of H69AR cells can be unequivocally eliminated.

Acknowledgments

The authors wish to thank Drs. G. Batist, M. Clapper, K. Tew, R. Kramer, B. Bennett, and T. Massey for helpful discussions and advice. The skilful assistance of Bryn Harris in the preparation of this manuscript is gratefully acknowledged.

References

1. Niiranen, A. Long-term survival in small cell carcinoma of the lung. *Eur. J. Cancer Clin. Oncol.* **24**:749–752 (1988).
2. Gerlach, J. H., J. A. Endicott, P. F. Juranka, G. Henderson, F. Sarangi, K. L. Deuchars, and V. Ling. Homology between P-glycoprotein and a bacterial haemolysin transport protein suggests a model for multidrug resistance. *Nature (Lond.)* **324**:485–489 (1986).
3. Gros, P., Y. B. Neriah, J. M. Croop, and D. E. Housman. Isolation and expression of a complementary DNA that confers multidrug resistance. *Nature (Lond.)* **323**:728–731 (1986).
4. Bradley, G., P. K. Juranka, and V. Ling. Mechanism of multidrug resistance. *Biochim. Biophys. Acta* **948**:87–128 (1988).
5. Bell, D. R., J. H. Gerlach, N. Kartner, R. N. Buick, and V. Ling. Detection of P-glycoprotein in ovarian cancer: a molecular marker associated with multidrug resistance. *J. Clin. Oncol.* **3**:311–315 (1985).
6. Goldstein, L. J., H. Galski, A. Fojo, M. Willingham, S.-L. Lai, A. Gazdar, R. Pirker, A. Green, W. Crist, G. M. Brodeur, M. Lieber, J. Cossman, M. M. Gottesman, and I. Pastan. Expression of a multidrug resistance gene in human cancers. *J. Natl. Cancer Inst.* **81**:116–124 (1989).
7. Mirski, S. E. L., J. H. Gerlach, and S. P. C. Cole. Multidrug resistance in a human small cell lung cancer line selected in Adriamycin. *Cancer Res.* **47**:2594–2598 (1987).
8. Deffie, A. M., T. Alam, C. Seneviratne, S. W. Beenken, J. K. Batra, T. C. Shea, W. D. Henner, and G. J. Goldenberg. Multifactorial resistance to Adriamycin: relationship of DNA repair, glutathione transferase activity, drug efflux, and P-glycoprotein in cloned cell lines of Adriamycin-sensitive and -resistant P388 leukemia. *Cancer Res.* **48**:3595–3602 (1988).
9. Slovak, M. L., G. A. Hoeltge, W. S. Dalton, and J. M. Trent. Pharmacological and biological evidence for differing mechanisms of doxorubicin resistance in two human tumor cell lines. *Cancer Res.* **48**:2793–2797 (1988).
10. McGrath, T., and M. S. Center. Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein. *Biochem. Biophys. Res. Commun.* **145**:1171–1176 (1987).
11. Trent, J. M., P. S. Meltzer, M. L. Slovak, A. B. Hill, W. S. Dalton, W. T. Beck, and S. P. C. Cole. Cytogenetic and molecular biological alterations associated with anthracycline resistance, in *Mechanisms of Drug Resistance in Neoplastic Cells* (P. V. Woolley III and K. D. Tew, eds.). Academic Press, San Diego, CA, 259–276 (1988).
12. 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).
13. Moore, W. R., M. E. Anderson, A. Meister, K. Murata, and A. Kimura. Increased capacity for glutathione synthesis enhances resistance to radiation in *Escherichia coli*: a possible model for mammalian cell protection. *Proc. Natl. Acad. Sci. USA* **86**:1461–1464 (1989).
14. Batist, G., A. Tulpuli, B. K. Sinha, A. G. Katki, C. E. Myers, and K. H. Cowan. Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J. Biol. Chem.* **261**:15544–15549 (1986).
15. Kramer, R. A., J. Zakher, and G. Kim. Role of the glutathione redox cycle in acquired and *de novo* multidrug resistance. *Science (Wash. D. C.)* **241**: 694–697 (1988).
16. Yusa, K., H. Hamada, and T. Tsuruo. Comparison of glutathione *S*-transferase activity between drug-resistant and -sensitive human tumor cells: is glutathione *S*-transferase associated with multidrug resistance? *Cancer Chemother. Pharmacol.* **22**:17–20 (1988).
17. Russell, W. C., C. Newman, and D. H. Williamson. A simple cytochemical technique for demonstration of DNA in cells infected with mycoplasmas and viruses. *Nature (Lond.)* **253**:461–462 (1975).
18. Cole, S. P. C. Rapid chemosensitivity testing of human lung tumor cells using the MTT assay. *Cancer Chemother. Pharmacol.* **17**:259–263 (1986).
19. Tietze, F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. *Anal. Biochem.* **27**:502–522 (1969).
20. Carlberg, I., and B. Mannervik. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J. Biol. Chem.* **250**:5475–5480 (1975).
21. Habig, W. H., M. J. Pabst, and W. B. Jakoby. Glutathione *S*-transferases: the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**:7130–7139 (1974).
22. Paglia, D. E., and W. N. Valentine. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J. Lab. Clin. Med.* **70**:158–169 (1967).
23. Reddy, C. C., C.-P. D. Tu, J. R. Burgess, C.-Y. Ho, R. W. Scholz, and E. J. Massaro. Evidence for the occurrence of selenium-independent glutathione peroxidase activity in rat liver microsomes. *Biochem. Biophys. Res. Commun.* **101**:970–978 (1981).
24. Szasz, G. A kinetic photometric method for serum γ-glutamyl transpeptidase. *Clin. Chem.* **15**:124–136 (1969).
25. Peterson, G. L. A simplification of the protein assay method of Lowry *et al.* which is more generally applicable. *Anal. Biochem.* **83**:346–356 (1977).
26. Buller, A. L., M. L. Clapper, and K. D. Tew. Glutathione *S*-transferases in nitrogen mustard-resistant and-sensitive cell lines. *Mol. Pharmacol.* **31**:575–578 (1987).
27. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
28. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins

from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
29. Mierendorf, R. C., C. Percy, and R. A. Young. Gene isolation by screening *lambda* gt11 libraries with antibodies. *Methods Enzymol.* **152**:458–469 (1987).
30. Amad, S., L. Okine, B. Le, P. Najarian, and D. T. Vistica. Elevation of glutathione in phenylalanine mustard-resistant murine L1210 leukemia cells. *J. Biol. Chem.* **262**:15048–15053 (1987).
31. Suzukake, K., B. J. Petro, and D. T. Vistica. Reduction in glutathione content of L-PAM resistant L1210 cells confers drug sensitivity. *Biochem. Pharmacol.* **31**:121–124 (1982).
32. Louie, K. G., B. C. Behrens, T. J. Kinsella, T. C. Hamilton, K. R. Grotzinger, W. M. McKoy, M. A. Winker, and R. F. Ozols. Radiation survival parameters of antineoplastic drug-sensitive and -resistant human ovarian cancer cell lines and their modification by buthionine sulfoximine. *Cancer Res.* **45**:2110–2115 (1985).
33. Griffith, O. W., and A. Meister. Potent and specific inhibition of glutathione synthesis by buthionine sulfoxmine (*S-n*-butyl homocysteine sulfoximine). *J. Biol. Chem.* **254**:7558–7560 (1979).
34. Dusre, L., E. G. Mimnaugh, C. E. Myers, and B. K. Sinha. Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast tumor cells. *Cancer Res.* **49**:511–515 (1989).
35. Lutzky, J., M. B. Astor, R. N. Taub, M. A. Baker, K. Bhalla, J. E. Gervasoni, Jr., M. Rosado, V. Stewart, S. Krishna, and A. A. Hindenburg. Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells. *Cancer Res.* **49**:4120–4125 (1989).
36. Meijer, C., N. H. Mulder, H. Timmer-Bosscha, J. G. Zijlstra, and E. G. E. de Vries. Role of free radicals in an Adriamycin-resistant human small cell lung cancer cell line. *Cancer Res.* **47**:4613–4617 (1987).
37. Lewis, A. D., J. D. Hayes, and C. R. Wolf. Glutathione and glutathione-dependent enzymes in ovarian adenocarcinoma cell lines derived from a patient before and after the onset of drug resistance: intrinsic differences and cell cycle effects. *Carcinogenesis (Lond.)* **9**:1283–1287 (1988).
38. Mannervik, B. Glutathione peroxidase. *Methods Enzymol.* **113**:490–495 (1985).
39. Mimnaugh, E. G., L. Dusre, J. Atwell, and C. E. Myers. Differential oxygen radical susceptibility of Adriamycin-sensitive and -resistant MCF-7 human breast tumor cells. *Cancer Res.* **49**:8–15 (1989).
40. Sinha, B. K., and J. L. Gregory. Role of one-electron and two-electron reduction products of Adriamycin and daunomycin in deoxyribonucleic acid binding. *Biochem. Pharmacol.* **30**:2626–2629 (1981).
41. Sinha, B. K., E. G. Mimnaugh, S. Rajagopalan, and C. E. Myers. Adriamycin activation and oxygen free radical formation in human breast tumor cells: protective role of glutathione peroxidase in Adriamycin resistance. *Cancer Res.* **49**:3844–3848 (1989).
42. Bellamy, W. T., W. S. Dalton, P. Meltzer, and R. T. Dorr. Role of glutathione and its associated enzymes in multidrug-resistant human myeloma cells. *Biochem. Pharmacol.* **38**:787–793 (1989).
43. Meister, A., S. S. Tate, and O. W. Griffith. γ-Glutamyl transpeptidase. *Methods Enzymol.* **77**:237–253 (1981).
44. Hendrich, S., and H. C. Pitot. Enzymes of glutathione metabolism as biochemical markers during hepatocarcinogenesis. *Cancer Metab. Rev.* **6**:155–178 (1987).
45. Ahmad, S., L. Okine, R. Wood, J. Aljian, and D. T. Vistica. γ-Glutamyl transpeptidase (γ-GT) and maintenance of thiol pools in tumor cells resistant to alkylating agents. *J. Cell. Physiol.* **131**:240–246 (1987).
46. Tew, K. D., and M. L. Clapper. Glutathione *S*-transferases and anticancer drug resistance, in *Mechanisms of Drug Resistance in Neoplastic Cells* (P. V. Woolley III and K. D. Tew, eds.) Academic Press, San Diego, CA, 141–159 (1988).
47. Shea, T. C., S. L. Kelley, and W. D. Henner. Identification of an anionic form of glutathione transferase present in many human tumors and human tumor cell lines. *Cancer Res.* **48**:527–533 (1988).
48. Cowan, K. H. Glutathione *S*-transferases and drug resistance. *Proc. Am. Assoc. Cancer Res.* **30**:674 (1989).
49. Moscow, J. A., A. J. Townsend, and K. H. Cowan. Elevation of π class glutathione *S*-transferase activity in human breast cancer cells by transfection of the GST π gene and its effect on sensitivity to toxins. *Mol. Pharmacol.* **36**:22–28 (1989).
50. Mannervik, B., P. Alin, C. Guthenberg, H. Jensson, M. K. Tahir, M. Warholm, and H. Jornvall. Identification of three classes of cytosolic glutathione transferases common to several mammalian species: correlation between structural data and enzymatic properties. *Proc. Natl. Acad. Sci. USA* **82**:7202–7206 (1985).

Send reprint requests to: Dr. Susan P.C. Cole, Department of Oncology, Room 331 Botterell Hall, Queen's University, Kingston, Ontario K7L 3N6, Canada.