

# Overexpression of Glutathione S-Transferase and Elevation of Thiol Pools in a Multidrug-Resistant Human Colon Cancer Cell Line

CHUCK C.-K. CHAO, YING-TANG HUANG, CHANG M. MA, WEI-YUAN CHOU, and SUE LIN-CHAO

Tumor Biology Laboratory, Department of Biochemistry, Chang Gung Medical College, Taoyuan, Taiwan 33332 (C.C.-K.C., Y.-T.H., C.M.M.), Medical Laboratories, National Defense Medical Center, Taipei, Taiwan (Y.-T.H., W.-Y.C.), and Institute of Molecular Biology, Academia Sinica, Taipei 11529, Taiwan (S.L.-C.), Republic of China

Received May 6, 1991; Accepted October 7, 1991

## SUMMARY

A human colon cancer cell line with acquired multidrug resistance (MDR) was assayed for the intracellular GSH level and the activity of GSH-S-transferase (GST), which catalyzes the conjugation reaction of electrophilic drugs with GSH. The GSH level and GST activity (as measured with 1-chloro-2,4-dinitrobenzene) were elevated in the resistant cells by 1.7-fold and 2-fold, respectively. This elevated catalytic activity of the resistant cells was reflected in a 2-fold increase in GST- $\pi$  mRNA, which was not the result of gene amplification. In addition, buthionine sulfoximine, a specific inhibitor of GSH synthesis, significantly increased Adriamycin sensitivity in both the MDR and the parental cells, affecting the former more than the latter. The effects seen with buthionine

sulfoximine were not seen with puromycin and actinomycin D. A dramatic overexpression of *mdr1*, a P-glycoprotein gene responsible for the MDR phenotype, was also observed in the MDR cells. In contrast, none of these products (i.e., *mdr* P-glycoprotein, GSH level, total GST activity, GST- $\pi$  gene copy, and GST- $\pi$  mRNA level) was elevated in HeLa cells resistant to cisplatin and some alkylating agents, supporting the notion that the acquisition of cisplatin resistance differs from the mechanism of MDR. These results indicate that the intrinsic GSH level and GST- $\pi$  activity affect anthracycline resistance *per se* and not MDR in the human colon cancer cells.

GSH (L- $\gamma$ -glutamyl-L-cysteinylglycine) accounts for the majority of the intracellular nonprotein sulfhydryl or thiol content. It participates in many important cellular functions, including protection from free radical damage, detoxification of xenobiotics, and synthesis of DNA precursors (for review, see Refs. 1 and 2). Evidence has accumulated concerning the role of intrinsic GSH as a modulator of cellular responses to drugs and irradiation (3-9). This was confirmed by studies showing that the elevation of cellular GSH is associated with the development of induced resistance to Melphalan, nitrogen mustard, or *cis*-diamminedichloroplatinum(II) (cisplatin) (10-15). In addition, depletion of intracellular GSH by treatment with BSO, a potent inhibitor of  $\gamma$ -glutamylcysteine synthetase (16, 17), renders both normal and cancer cells more sensitive to drugs and/or radiation (7, 12, 18-22). GSTs catalyze the coupling reaction of intracellular electrophiles with GSH (for reviews, see Refs. 23 and 23a). The multifunctional isozymes of GST, which are

found in virtually all tissues, are presumed to be involved in the detoxification of a variety of noxious xenobiotics, including certain anticancer drugs and their metabolites. Elevation of GSTs is associated with the acquired resistance of cells to certain anticancer drugs (24). A number of studies have confirmed the generality of this phenotype (11, 25-28). In the majority of cases, the elevated expression of GST- $\alpha$  has been firmly correlated with increased resistance to alkylating agents. This was directly supported by studies in which the transfection of a GST- $\alpha$  cDNA into COS cells led to an unstable overexpression of GST- $\alpha$ , resulting in an increased resistance of cells to alkylating agent (29). Thus, the GSH/GST levels in cancer cells may be of particular relevance to the emergence of resistance to alkylating agents. However, the role of GSH/GSTs in resistance to other drugs, such as doxorubicin, is less clear.

We have previously reported an ADR human colon cancer cell line that had developed an enhanced P-glycoprotein-mediated efflux mechanism, showing a typical MDR phenotype (30). This cell line is cross-resistant to a variety of drugs, including puromycin and actinomycin D. Although it is logical to assume that P-glycoprotein plays a vital role in determining

This work was supported in part by grants from Chang Gung Medical College (CMRP318) and the National Science Council, R.O.C. (NSC79-0412-B182-50), to C.C.-K.C., and from the National Science Council, R.O.C. (NSC78-0203-B001-15), to S.L.-C.

**ABBREVIATIONS:** GST, glutathione-S-transferase; BSO, DL-buthionine-S,R-sulfoximine; MDR, multidrug resistance (resistant); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ADR, Adriamycin-resistant; CPR, cisplatin-resistant.

the MDR phenotype (for review, see Ref. 31), quantitatively the drug efflux did not correlate with drug sensitivity in this MDR cell line. Therefore, we attempted to look at the other possible mechanisms that may mediate the MDR phenotype. Of great interest, as first reported by Batist *et al.* (10), is the overexpression of GST- $\pi$  in MDR human breast cancer cells, although those authors did not measure other potentially important products. Nakagawa *et al.* (32) have recently demonstrated that overexpression of human GST- $\pi$  by transfection of the gene into transformed NIH3T3 cells conferred resistance to Adriamycin and ethacrynic acid but not to cisplatin and the alkylating agent Melphalan. *mdr* P-glycoprotein overexpression was not detected in these resistant transfectants. These results suggest a potential role for GSH and GST- $\pi$  in the development of MDR. In this report, we show evidence that confirms part of this hypothesis. Because GSH/GSTs affect only Adriamycin resistance in our MDR cells, the results suggest that the level of GSH/GSTs is important in anthracycline resistance *per se* and not MDR. The results also reveal an overexpression of GST- $\pi$  and *mdr* P-glycoprotein in the MDR cells.

## Materials and Methods

**Chemicals and medium.** Dulbecco's modified Eagle's medium, fetal bovine serum, and penicillin/streptomycin were obtained from GIBCO (Gaithersburg, MD). Platamine (cisplatin) was purchased from Farmitalia Carloerba Ltd. BSO and Adriamycin were purchased from Sigma (St. Louis, MO). Other chemicals and suppliers are indicated below.

**Cell cultures and determination of resistance.** Human colon cancer SW620 parental and MDR cells (30) and HeLa parental and CPR cells (33) were maintained as previously described. The acquired resistance of the cells to drug is defined as the ratio of  $ID_{50}$  (the drug concentration inhibiting cell survival by 50%) of resistant cells to that of parental cells. Cytotoxicity was assayed by the MTT colorimetric method (34).

**Isolation of membrane proteins and SDS-PAGE.** Cellular membrane proteins were isolated according to the method described by Riordan and Ling (35). In brief, cells were washed with phosphate-buffered saline and disrupted using a Dounce homogenizer. The extent of cell breakage was checked under the phase-contrast microscope. The following differential centrifugation was applied: nuclear spin,  $300 \times g$  for 10 min; mitochondrial spin,  $4,000 \times g$  for 10 min; and membrane spin,  $35,000 \times g$  for 30 min. The membrane pellet was resuspended in 0.5% Triton X-100 at  $4^\circ$  for 30 min and separated on 10% SDS-PAGE.

**Cellular GSH content.** Total cellular GSH content was measured using the enzyme recycling assay (36). After  $5 \times 10^6$  cells were suspended in 300  $\mu$ l of phosphate-EDTA solution (125 mM  $KH_2PO_4$ , 6.3 mM EDTA, pH 7.5), cells were sonicated at  $4^\circ$  (120 W, 30 sec) and then 100  $\mu$ l of 12% 5-sulfosalicylic acid were added to each sample tube. The solution was mixed and allowed to precipitate for 2–3 hr, on ice. After centrifugation at  $10,000 \times g$  for 15 min, protein-free lysates were obtained. The enzyme reaction was carried out in a 1-ml cuvette, in 175 mM  $KH_2PO_4$ , 6.3 mM EDTA, 0.21 mM NADPH, 0.6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) (Sigma), 0.5 unit/ml glutathione reductase (Sigma), at  $25^\circ$ . The absorbance at 412 nm ( $A_{412}$ ) was monitored for 3 min at 20-sec intervals, and the GSH content was calculated from the rate of change in  $A_{412}$  on the basis of the standard curve in each experiment.

**GST activity.** Cells ( $1 \times 10^7$ ) were washed with phosphate-buffered saline and resuspended in 500  $\mu$ l of 10 mM Tris·HCl, pH 7.8, containing 0.2 M NaCl. After sonication at  $4^\circ$  (120 W, 30 sec), they were centrifuged in a microfuge at 12,000 rpm for 30 min. The supernatant was assayed for total GST activity using 1-chloro-2,4-dinitrobenzene (Sigma), according to the method of Habig and Jakoby (37), and for GST- $\alpha$

activity using cumene hydroperoxide (Sigma), according to the method of Stockman *et al.* (38).

**Hybridization probes.** A 0.75-kilobase *EcoRI* fragment was cut out from pGPi-2 (39) (a kind gift from Dr. M. Muramatsu, University of Tokyo, Japan) and used as a probe for GST- $\pi$  gene. The human *mdr* gene probe was a full-length human *mdr1* cDNA clone cut with *Bam*HI and *Sal*II from pGEM3Zf(-)-*mdr1* (a generous gift from Dr. P. Borst, Netherlands Cancer Institute). Rat  $\beta$ -actin cDNA (a gift from Dr. S. N. Cohen, Stanford University) was used to probe the human gene, as an internal control. Probes were purified by electrophoresis on NA45 membranes (Schleicher & Schuell) and radiolabeled by the random priming method, with [ $\alpha$ - $^{32}P$ ]dCTP (40), to a specific activity of  $10^6$  cpm/ $\mu$ g of DNA.

**RNA and DNA blot hybridizations.** All solutions and procedures involving the isolation of DNA and RNA, electrophoresis, blot transfers, and hybridizations were carried out by standard methods (41). For Northern RNA blots, 10  $\mu$ g of total RNA from the cell lines were fractionated by electrophoresis on 1% agarose containing 6.7% formaldehyde. RNA was transferred to Hybond-N filters (Amersham), UV-cross-linked with Stratalinker (Stratagene), and hybridized at  $42^\circ$  for 16 hr in hybridization buffer ( $6 \times$  standard saline citrate, 50% deionized formamide,  $10 \times$  Denhardt's solution, 10 mM EDTA, 0.1% SDS) containing  $5 \times 10^5$  cpm of probe/ml. The filter was then washed at  $65^\circ$  in  $2 \times$  standard saline citrate, 0.1% SDS, followed by exposure on X-ray film, with an intensifying screen, at  $-80^\circ$  for 3 days or as otherwise indicated. The X-ray film was scanned in a densitometer to estimate the density of the bands. Before blotting, genomic DNAs were digested with the restriction enzymes *Hind*III and *Eco*RI (New England Biolabs) and checked in a 1% agarose gel. For slot blot, amounts of RNA or DNA were loaded according to the manufacturer's instruction (Schleicher & Schuell) and processed for hybridization with probes. The filter was then washed, followed by exposure on X-ray film, with an intensifying screen, for 3 days at  $-80^\circ$ .

## Results

**Characteristics of the sensitive and resistant cells.** An ADR human colon cancer cell line (SW620-ADR) that was showing a typical MDR phenotype was maintained as previously described (32). A typical dose-response pattern of Adriamycin sensitivity for SW620 parental and ADR cells is presented in Fig. 1. For example, surviving colonies were barely detected in parental cells treated with  $1 \times 10^3$  ng/ml Adriamycin, whereas the majority of the ADR cells survived this or even a higher drug concentration. The  $ID_{50}$  values (the drug concentration causing 50% inhibition of cell growth) are shown in Table 1. The  $ID_{50}$  of Adriamycin for SW620-ADR cells was 45  $\mu$ g/ml, compared with 0.6  $\mu$ g/ml for the parental SW620 cells. The calculated fold resistance of ADR cells to Adriamycin was 75. In contrast, ADR cells showed a 1.2-fold resistance to cisplatin, compared with SW620 parental cells, indicating that the SW620-ADR cells were as sensitive to cisplatin as the parental cells. For comparison, the drug sensitivity of a CPR HeLa cell line (HeLa-CPR) (33) was also included. HeLa-CPR cells exhibited an 18-fold resistance to cisplatin, but both HeLa lines showed similar sensitivity to Adriamycin.

**Overexpression of *mdr1* transcripts and ~170-kDa membrane proteins in ADR cells.** We have previously shown that SW620-ADR cells have acquired the ability to decrease drug accumulation inside the cell, and this characteristic holds true for a variety of hydrophobic drugs, which is typical of the MDR phenotype (30). It is reasonable to speculate, then, that the ADR cells overexpress P-glycoprotein, a ~170-kDa membrane protein that is believed to be responsible for the MDR phenotype (for review, see Ref. 31). As expected,

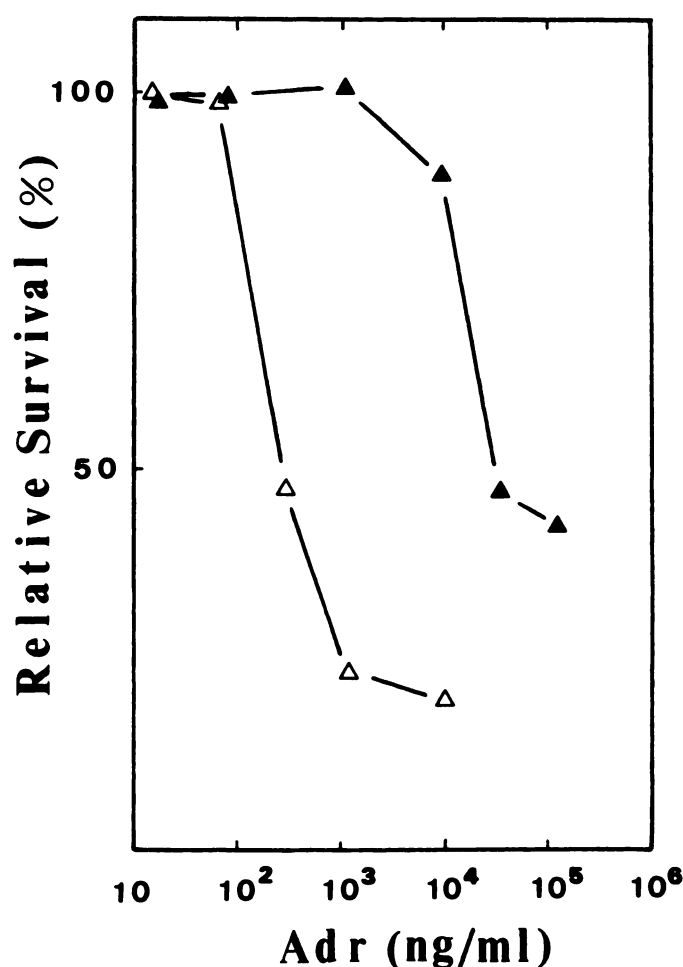


Fig. 1. Relative sensitivity of SW620 parental and ADR cells to Adriamycin (Adr).  $\Delta$ , SW620 parental cells;  $\blacktriangle$ , SW620-ADR cells.

TABLE 1

**ID<sub>50</sub> values of anticancer agents for the parental and drug-resistant cells**

ID<sub>50</sub> is the drug concentration effective in inhibiting 50% of the cell growth, measured after 4 days of continuous exposure to the drug. Values in parentheses are fold resistance.

Cell line	ID <sub>50</sub>	
	Adriamycin	Cisplatin
	$\mu\text{g/ml}$	
SW620	0.6	3.0
SW620-ADR	45.0 (75)	3.5 (1.2)
HeLa	0.8	0.1
HeLa-CPR	0.8 (1)	1.8 (18)

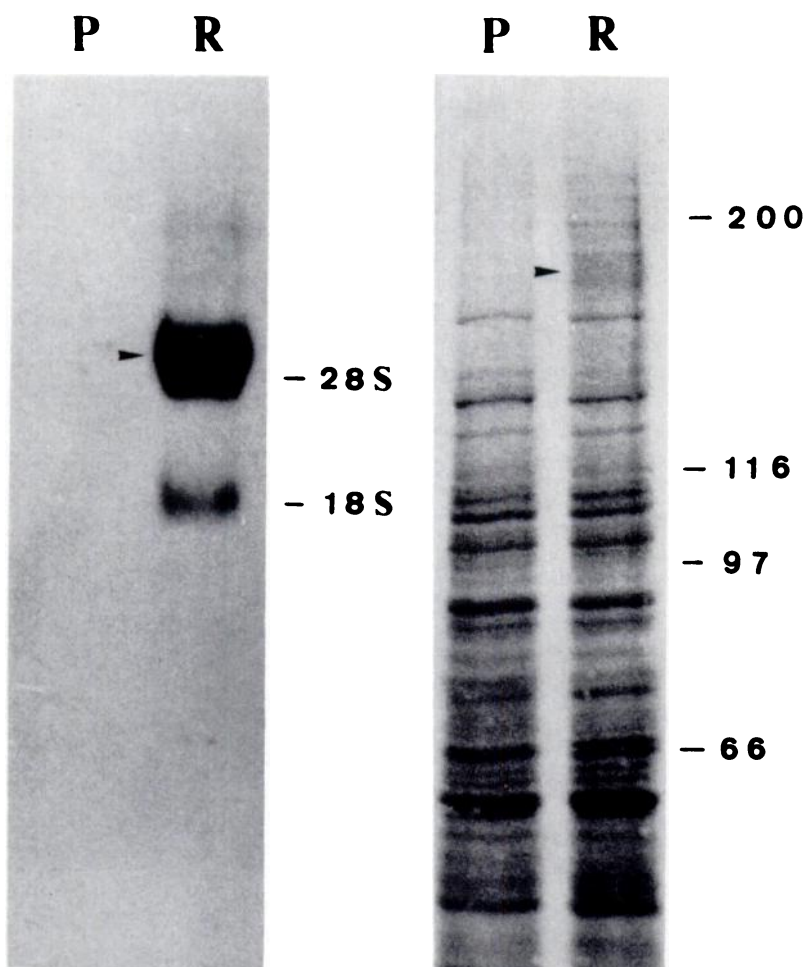
the steady state level of *mdr1* mRNA was dramatically increased in SW620-ADR cells (Fig. 2, lane R, indicated with arrowhead at left), in contrast to parental cells (Fig. 2, lane P). However, the observed intensity of *mdr1* may be overestimated because of a nonspecific hybridization band around the 18 S rRNA. Membrane proteins of SW620 parental and ADR cells were isolated and separated on 10% SDS-PAGE. A protein band around 170 kDa (indicated with arrowhead at right in Fig. 2) was consistently overexpressed in ADR cells (Fig. 2, lane R) but was not detected in parental cells (Fig. 2, lane P). The ~170-kDa membrane protein overexpressed in ADR cells is likely to be P-glycoprotein. Together, the increased drug pump-

ing and cross-resistance to hydrophobic drugs in ADR cells (30) and the results presented here indicate that ADR cells have acquired a typical MDR phenotype.

**Increment of GSH levels and the GST enzymatic activity in ADR cells.** The GSH level and GST activity were measured in drug-resistant cells. The level of GSH in the cells was normalized to the amount of cellular proteins and is shown in Table 2. SW620-ADR cells contained 250 nmol/mg of protein, compared with 151 nmol/mg of protein in parental cells. There was a 1.7-fold increase, which is significantly different between the two cell lines ( $p < 0.01$ ). On the other hand, the GST enzymatic activity for parental and ADR cells was 128 and 321 nmol/min/mg of protein, respectively. The difference is also statistically significant ( $p < 0.01$ ). In contrast, HeLa and HeLa-CPR cells showed similar levels of GSH. The GST enzymatic activity for HeLa and CPR cells was 43 and 23 nmol/min/mg of protein. The difference is significant ( $p < 0.02$ ). The biological significance of the reduced total GST activity in the CPR cells, compared with the parental cells, is not clear. The results of the measurements of GSH and GST by biochemical means in ADR cells were supported by a functional analysis, i.e., BSO inhibition (see below). GST- $\alpha$  enzymatic activity was also included for reference. As shown in Table 3, the parental and the ADR cells showed no difference in GST- $\alpha$  activity. In contrast, HeLa-CPR and parental cells contained 3.99 and 0.68 nmol/min/mg of protein of the enzymatic activity, respectively. There was an approximately 6-fold enhancement in GST- $\alpha$  activity in HeLa-CPR cells, compared with their parental cells.

**Drug sensitization by BSO in ADR cells and in parental cells to a lesser extent.** The role of GSH in drug sensitivity was tested in the cells by BSO inhibition. A sublethal concentration of BSO (50  $\mu\text{M}$ ) was used to test its cytotoxic effect. The ID<sub>50</sub> of Adriamycin, with or without BSO, is shown in Table 4. The ID<sub>50</sub> of Adriamycin for SW620 and ADR cells was 0.56 and 40, respectively. When BSO was added, the ID<sub>50</sub> for the two cell lines was 0.4 and 22  $\mu\text{g/ml}$ , respectively. There was a 1.4- and 1.8-fold increase in drug sensitivity for the parental and ADR cells, respectively. The data indicate that inhibition of GSH synthesis by BSO renders both the MDR and the parental cells sensitive to the drug, affecting the former more than the latter. However, the same BSO effects were not seen with drugs such as puromycin and actinomycin D (which also produce MDR) (data not shown), suggesting that the BSO effects are probably only seen in drugs that form free radicals.

**Overexpression of the GST- $\pi$  gene in ADR cells.** Mammalian cells have evolved a family of GST enzymes used for coupling with GSH in the process of quenching free radicals. Among these, overexpression of GST- $\pi$ , as a consequence of transfection of the gene, is correlated with the development of resistance of the cells to Adriamycin (32). Here, we are reporting that human cells with the acquired MDR phenotype also expressed a high level of GST- $\pi$ . As shown in Fig. 3, both HeLa parental and HeLa-CPR cells expressed a relatively low level of GST- $\pi$ , as opposed to SW620 parental and SW620-ADR cells. Quantitation by scanning densitometry showed a 2-fold increase of GST- $\pi$  mRNA in ADR cells. For comparison, mRNA levels of *mdr1* are also shown (see Fig. 3, MDR). However, the intensity of *mdr* mRNA is probably overrepresented, because of the relatively high background of the non-specific hybridization. The gene probe was retrieved, and the



**Fig. 2.** Northern blot hybridization of *mdr1* gene and SDS-PAGE of membrane proteins in SW620 parental and ADR cells. *Left*, Northern hybridization. Ten micrograms of total cellular RNA were loaded. *Right*, SDS-PAGE of cell membrane proteins. Ten micrograms of proteins were loaded, after which the gel was separated and visualized by silver-staining. *P*, SW620 parental cells; *R*, SW620-ADR cells. The location of 28 S and 18 S rRNA is indicated for Northern hybridization. Protein size markers are also shown at the right.

**TABLE 2**

**Intracellular GSH levels and GST activities in the parental and drug-resistant cell lines**

Values are mean  $\pm$  standard deviation of three or four determinations.

Cell line	GSH level	GST activity
	nmol/mg of protein	nmol/min/mg of protein
SW620	151 $\pm$ 22.5	128 $\pm$ 9.1
SW620-ADR	250 $\pm$ 39.9 (< 0.01) <sup>a</sup>	321 $\pm$ 31.9 (< 0.01) <sup>a</sup>
HeLa	89 $\pm$ 13.5	43 $\pm$ 6.5
HeLa-CPR	94 $\pm$ 37.7 (NS) <sup>b</sup>	23 $\pm$ 4.8 (< 0.02) <sup>a</sup>

<sup>a</sup> These probability values (indicated in parentheses), as determined by the two-tailed *t* test, show the significance of the difference in GSH and GST between SW620 and SW620-ADR or between HeLa and HeLa-CPR cells.

<sup>b</sup> NS, not significant ( $p > 0.2$ ); there is no significant difference in GSH and GST between HeLa and HeLa-CPR cells.

same blot was used to hybridize with the  $\beta$ -actin probe (Fig. 3, *ACT*). The actin mRNA was similar in the SW620 and ADR cells. In fact, a slightly greater amount of actin mRNA was detected in HeLa parental and CPR cells, indicating that the overexpression of GST- $\pi$  found in ADR cells is not an artifact of sample loading.

**Normal GST- $\pi$  gene copy in ADR cells.** The gene copy and the mRNA level of GST- $\pi$  in the cells were further quantitated using slot blotting (Fig. 4). Indicated amounts of DNA or RNA of parental or resistant SW620 cells (Fig. 4A) or HeLa cells (Fig. 4B) were used for the assay. The same DNA level of GST- $\pi$  gene was found in SW620-ADR and parental cells, indicating that there was no gene amplification of GST- $\pi$  in

**TABLE 3**

**Intracellular GST- $\alpha$  activities in the parental and drug-resistant cell lines**

Values are mean  $\pm$  standard deviation of three determinations.

Cell line	GST- $\alpha$ activity
	nmol/min/mg of protein
SW620	3.64 $\pm$ 0.56
SW620-ADR	3.03 $\pm$ 0.47 (NS) <sup>a</sup>
HeLa	0.68 $\pm$ 0.13
HeLa-CPR	3.99 $\pm$ 0.56 (< 0.01) <sup>b</sup>

<sup>a</sup> NA, not significant ( $p > 0.2$ ); there is no significant difference in GST- $\alpha$  between SW620 and SW620-ADR cells.

<sup>b</sup> This probability value (indicated in parenthesis), as determined by the two-tailed *t* test, shows the significance of the difference in GST- $\alpha$  between HeLa and HeLa-CPR cells.

ADR cells. There was 2-fold amplification of this gene in HeLa-CPR cells, compared with the HeLa parental cells. By quantitation of the mRNA level of GST- $\pi$ , a 2-fold increase was detected in ADR cells, whereas in HeLa cells there was a much lower GST- $\pi$  mRNA level. Prolonged exposure of the X-ray film did not show GST- $\pi$  overexpression (data not shown). The slot blotting results are consistent with the Northern blotting.

## Discussion

As previously reported, the resistance of the ADR colon cancer cells used in this study is associated with a decrease in drug accumulation, relative to the parental line (32). Although

**TABLE 4**  
**Effect of BSO on Adriamycin toxicity**

Cells were incubated in medium containing Adriamycin, in the presence or absence of 50  $\mu$ M BSO, for 4 days and were measured for inhibition of cell growth. Values are mean  $\pm$  standard deviation of three or four determinations. Numbers in parentheses are fold sensitization, as calculated from the ratio of  $ID_{50}$  of cells without BSO to that of cells treated with BSO.

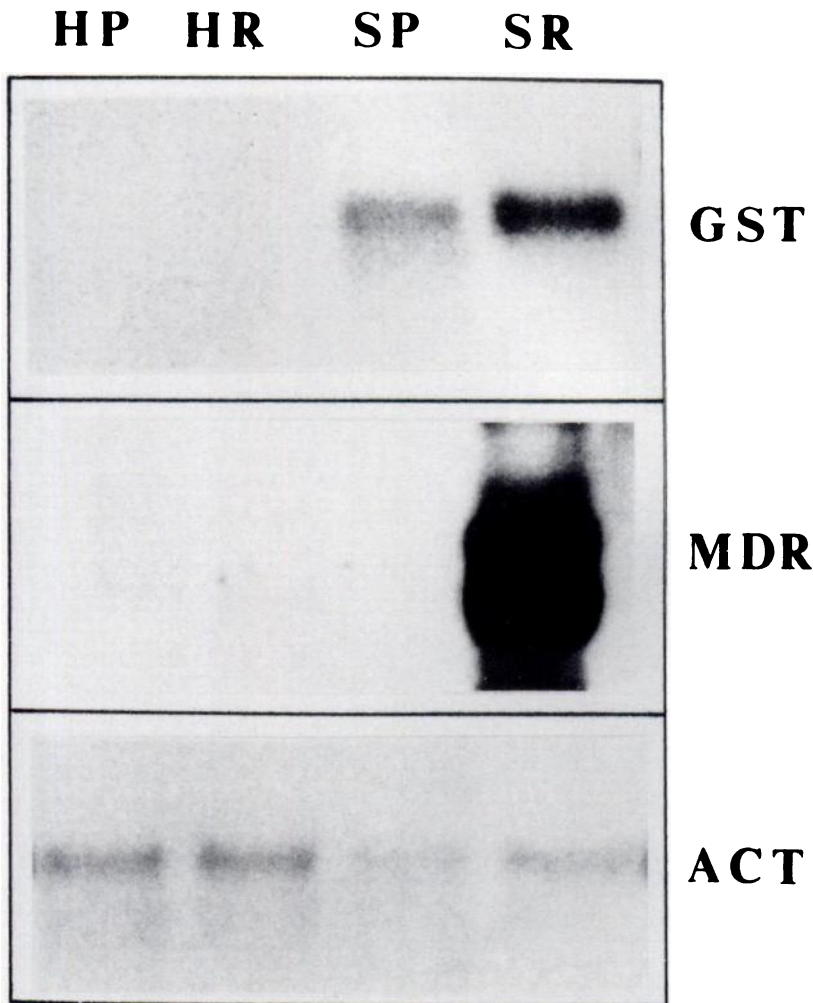
Cell line	$ID_{50}$		$p^*$
	-BSO	+BSO	
		$\mu$ g/ml	
SW620	0.56 $\pm$ 0.07	0.4 $\pm$ 0.05 (1.4)	<0.005
SW620-ADR	40 $\pm$ 3.5	22 $\pm$ 2.0 (1.8)	<0.01

\*These probability values, as determined by the two-tailed *t* test, show the significance of the difference between cells with and without BSO treatment.

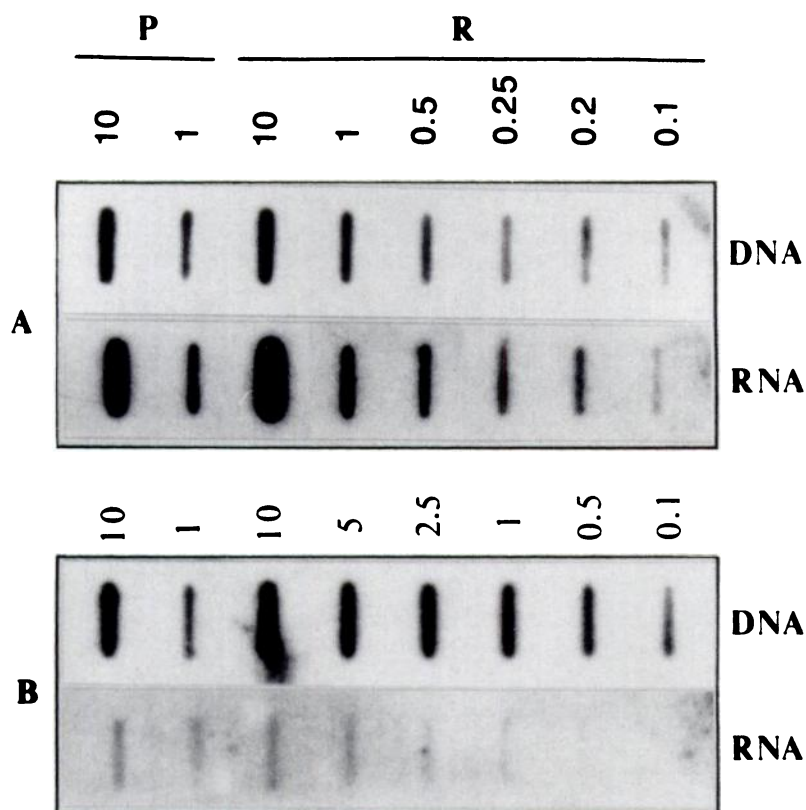
selected by exposure only to Adriamycin, the ADR cells have developed a phenotype that is characteristic of MDR. Because the magnitude of this alteration (2–3-fold) is insufficient to account for the overall level of drug resistance in these cells (75-fold), we have investigated other potential mechanisms that might be associated with MDR. In this report, we have observed a 2-fold overexpression of GSH level and GST enzymatic activity in the ADR cells. In addition, BSO increased the cellular sensitivity to Adriamycin in both the ADR and the parental cells, affecting the former more than the latter. The results indicate that the level of GSH/GSTs in these cells

affects anthracycline resistance. Because the effects seen with BSO are not observed with puromycin and actinomycin D, to which the ADR cells are cross-resistant, the effects are probably specific for anthracycline resistance *per se* and not for MDR in general. Because our ADR cells possess features typical of the MDR phenotype, the overexpression of P-glycoprotein in MDR cells, as frequently found, may not account for all of the MDR mechanism. Other investigations have also shown that BSO treatment can sensitize MDR MCF-7 breast cancer cells and P388 leukemia cells to doxorubicin toxicity (42–43). In contrast, a HeLa cell line resistant to cisplatin, the resistance of which was not mediated by the MDR mechanism, did not show any detectable increase in the level of GSH.

Resistant cells selected by prolonged drug exposure often exhibit multiple genetic and biochemical differences from the parental cell lines. These changes may affect the expression of many gene products associated with drug resistance, such as P-glycoprotein, GSTs, and metallothionein. Our data reveal the coordinated overexpression of P-glycoprotein and GST- $\pi$  genes in the ADR cells. A 2-fold increase of GST- $\pi$  mRNA was measured in ADR cells. This may reflect, although it does not prove, the overexpressed total GST activity. The results are consistent with the observations of Nakagawa *et al.* (32), that transfection of *c-H-ras*-transformed NIH-3T3 cells with human GST- $\pi$  cDNA conferred a 2–3-fold rise in Adriamycin



**Fig. 3.** Northern blot hybridization of GST- $\pi$  gene in SW620 parental, SW620-ADR, HeLa parental, and HeLa-CPR cells. Ten micrograms of total cellular RNA were loaded in each lane. The same blot was probed with gene-specific probe (indicated at the right), retrieved, and reprobed with the second probe. HP, HeLa parental; HR, HeLa-CPR; SP, SW620 parental; SR, SW620-ADR cells. GST, GST- $\pi$ ; MDR, *mdr1*; ACT,  $\beta$ -actin.



**Fig. 4.** Slot blot hybridization of GST- $\pi$  in DNA or RNA of SW620 parental, SW620-ADR, HeLa parental, and HeLa-CPR cells. The amounts of DNA or total RNA loaded on each slot are indicated at the top of each panel. P, parental cells; R, resistant cells. A, SW620 cells; B, HeLa cells.

resistance at the  $IC_{37}$  drug concentration. Interestingly, protection was observed only at low drug concentrations; at  $IC_{50}$  concentrations and above, the resistant patterns of the transfected and parental cells were identical. However, other gene transfer experiments yielded conflicting results. As reported by Moscow *et al.* (44), expression of various levels of GST- $\pi$  in different transfected MCF-7 clones was associated with resistance to known substrates of GST- $\pi$  (i.e., ethracrynic acid, BPDE). No consistent alteration in the  $IC_{50}$  of doxorubicin was observed when these transfected clones were compared with parental cells. Their subsequent experiments suggest that overexpression of GST- $\pi$ , either alone or in concert with P-glycoprotein, does not appear to influence MDR significantly. Therefore, the level of GSTs in the cells is not necessarily associated with anthracycline resistance. Because MCF-7 cells contain minimal GST activity and no detectable GST- $\pi$ , the conflict between these studies may be due to cell specificity. In addition, we showed an increase in GST- $\alpha$  activity in CPR cells but not in ADR cells, compared with their parental lines. These findings confirm the idea that GST- $\alpha$  is important for cellular resistance to alkylating agents. We also found that the overexpression of GST- $\pi$  in our ADR cells is not because of gene amplification, suggesting that the mechanism of GST- $\pi$  overexpression is different from that of *mdr* P-glycoprotein, whose overexpression in the ADR cells is dictated by gene amplification (30). The influence of GSH/GSTs on Adriamycin cytotoxicity indicates a potential role of GSH metabolism in the development of anthracycline resistance *per se* and not MDR. In any case, the ADR cells are extremely resistant to Adriamycin. Given the marginal level of GSH-related resistance in these cells, it seems doubtful that such a mechanism would be elicited at the levels of resistance shown in patients. Because

the BSO effect on drug resistance is often seen at low drug concentrations, the GSH-related resistance might be relevant in the early stages of the development of drug resistance in clinical practice.

#### Acknowledgments

We thank Professors Masami Muramatsu (University of Tokyo, Japan) and Piet Borst (Netherlands Cancer Institute) for pGPi2 and pGEM3Zf(-)-*mdr*1, respectively. We are also grateful to Dr. Mary Helen Lu Young and the *Molecular Pharmacology* reviewers for helpful comments.

#### References

- Meister, A., and M. E. Anderson. Glutathione. *Annu. Rev. Biochem.* **52**:711-760 (1983).
- Arrick, B. A., and C. F. Nathan. Glutathione metabolism as a determinant of therapeutic efficiency: a review. *Cancer Res.* **44**:4224-4232 (1984).
- Lafleur, M. V. M., J. Woldhuis, and H. Loman. Effects of sulphhydryl compounds on the radiation damage in biologically active DNA. *Int. J. Radiat. Biol.* **37**:493-498 (1980).
- Micetich, K., L. A. Zwelling, and K. W. Kohn. Quenching of DNA: platinum(II) monoadducts as a possible mechanism of resistance to cis-diamminedichloroplatinum(II) in L1210 cells. *Cancer Res.* **43**:3609-3613 (1983).
- O'Neill, P. Pulse radiolytic study of the interaction of thiols and ascorbate with OH adducts of dGMP and dG: implications for DNA repair process. *Radiat. Res.* **96**:198-210 (1983).
- Revesz, L. The role of endogenous thiols in intrinsic radioprotection. *Int. J. Radiat. Biol.* **47**:361-368 (1985).
- Russo, A., and J. B. Mitchell. Potentiation and protection of doxorubicin cytotoxicity by cellular glutathione modulation. *Cancer Treat. Rep.* **69**:1293-1296 (1985).
- Eastman, A. Cross-linking of glutathione to DNA by cancer chemotherapeutic platinum coordination complexes. *Chem. Biol. Interact.* **61**:241-248 (1987).
- Ali-Osman, F., and K. Srievengopal. Evidence that glutathione quenches chloroethylated DNA and attenuates DNA interstrand cross-link induction by 1,2-bis(2-chloroethyl)-1-nitrosourea. *Proc. Am. Assoc. Cancer Res.* **29**:1064 (1988).
- Batist, G., A. Tulpule, 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).
- Buller, A. L., M. L. Clapper, and K. D. Tew. Glutathione S-transferase in

- nitrogen mustard-resistant and -sensitive cell lines. *Mol. Pharmacol.* **31**:575-578 (1987).
12. Richon, V. M., N. Schulte, and A. Eastman. Multiple mechanisms of resistance to *cis*-diamminedichloroplatinum(II) in murine leukemia L1210 cells. *Cancer Res.* **47**:2056-2061 (1987).
  13. Wolf, C. R., I. P. Hayward, S. S. Lawrie, K. Buckton, M. A. McIntyre, D. J. Adams, A. D. Lewis, A. A. R. Scott, and J. F. Smyth. Cellular heterogeneity and drug resistance in two ovarian adenocarcinoma cell lines derived from a single patient. *Int. J. Cancer* **39**:695-702 (1987).
  14. Hospers, G. A. P., N. H. Mulder, and E. G. E. De Vries. Mechanisms of cellular resistance to cisplatin. *Med. Oncol. Tumor Pharmacother.* **5**:145-151 (1988).
  15. Ozols, R. F., H. Masuda, and T. C. Hamilton. Mechanisms of cross-resistance between radiation and antineoplastic drugs. *Natl. Cancer Inst. Monogr.* **6**:159-165 (1988).
  16. Griffith, O. W., and A. Meister. Potent and specific inhibition of glutathione synthesis by buthionine sulfoximine (*S*-*n*-butyl homocysteine sulfoximine). *J. Biol. Chem.* **254**:7558-7560 (1979).
  17. Griffith, O. W. Mechanism of action, metabolism, and toxicity to buthionine sulfoximine and its higher homologs, potent inhibitors of glutathione synthesis. *J. Biol. Chem.* **257**:13704-13712 (1982).
  18. 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).
  19. Mitchell, J. B., A. Russo, J. E. Biaglow, and S. McPherson. Cellular glutathione depletion by diethyl maleate or buthionine sulfoximine: no effect of glutathione depletion on the oxygen enhancement ratio. *Radiat. Res.* **96**:422-428 (1983).
  20. Hamilton, T. C., M. A. Winker, K. G. Louie, B. Batist, B. C. Behrens, T. Tsuruo, K. R. Grotzinger, W. M. McKoy, R. C. Young, and R. F. Ozols. Augmentation of Adriamycin, Melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian carcinoma cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem. Pharmacol.* **34**:2583-2586 (1985).
  21. 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 modulation by buthionine sulfoximine. *Cancer Res.* **45**:2110-2115 (1985).
  22. Ozols, R. F., K. G. Louie, J. Plowman, *et al.* Enhanced Melphalan cytotoxicity in human ovarian cancer *in vitro* and in tumor-bearing nude mice by buthionine sulfoximine depletion of glutathione. *Biochem. Pharmacol.* **36**:147-153 (1987).
  23. Mannervik, B. The isozymes of glutathione transferase. *Adv. Enzymol.* **57**:357-417 (1985).
  - 23a. Ketterer, B. Detoxification reactions of glutathione and glutathione transferases. *Xenobiotica* **16**:957-973 (1986).
  24. 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).
  25. McGown, A. T., and B. W. Fox. A proposed mechanism of resistance to cyclophosphamide and phosphoramidate mustard in a Yoshida cell line *in vitro*. *Cancer Chemother. Pharmacol.* **17**:223-227 (1986).
  26. Taylor, C. W., M. G. Brattain, and L. C. Yeoman. Occurrence of cytosolic protein and phosphoprotein changes in human colon tumor cells with the development of resistance to mitomycin C. *Cancer Res.* **45**:4422-4427 (1985).
  27. Evans, C. G., W. G. Bodell, K. Takuda, 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).
  28. Robson, C. N., A. D. Lewis, C. R. Wolf, J. D. Hayes, A. Hall, S. J. 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).
  29. Manoharan, T. H., R. B. Puchalski, J. A. Burgess, C. B. Pickett, and W. E. Fahl. Promoter-glutathione *S*-transferase  $\gamma$  cDNA hybrid genes expression and conferred resistance to an alkylating molecule in mammalian cells. *J. Biol. Chem.* **262**:3739-3754 (1987).
  30. Chao, C. C.-K., C. M. Ma, P.-W. Cheng, and S. Lin-Chao. Increased *mdrg* gene expression and decreased drug accumulation in a human colonic cancer cell line resistant to hydrophobic drugs. *Biochem. Biophys. Res. Commun.* **172**:842-849 (1990).
  31. Endicott, J. A., and V. Ling. The biochemistry of P-glycoprotein-mediated multidrug resistance. *Annu. Rev. Biochem.* **58**:137-171 (1989).
  32. Nakagawa, K., N. Saijo, S. Tsuchida, M. Saka, Y. Tsunokawa, J. Yokota, M. Muramatsu, K. Sato, M. Terada, and K. D. Tew. Glutathione *S*-transferase  $\pi$  as a determinant of drug resistance in transfectant cell lines. *J. Biol. Chem.* **265**:4296-4301 (1990).
  33. Chao, C. C.-K., Y.-L. Lee, and S. Lin-Chao. Enhanced host cell reactivation of damaged plasmid DNA in HeLa cells resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res.* **51**:601-605 (1991).
  34. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **65**:55-63 (1983).
  35. Riordan, J. R., and V. Ling. Purification of P-glycoprotein from plasma membrane vesicles of Chinese hamster cell mutants with reduced colchicine permeability. *J. Biol. Chem.* **254**:12701-12705 (1979).
  36. Griffith, O. W. Determination of glutathione and glutathione disulfide using glutathione reductase and 2-vinyl pyridine. *Anal. Biochem.* **106**:207-212 (1980).
  37. Habig, W. H., and W. B. Jakoby. Glutathione *S*-transferase (rat and human). *Methods Enzymol.* **77**:218-230 (1981).
  38. Kano, T., M. Sakai, and M. Muramatsu. Structure and expression of a human class II glutathione *S*-transferase messenger RNA. *Cancer Res.* **47**:5626-5630 (1987).
  39. Stockman, P. K., L. I. McLellan, and J. D. Hayes. Characterization of the basic glutathione *S*-transferase B1 and B2 subunits from human liver. *Biochem. J.* **244**:55-61 (1987).
  40. Fernberg, A. P., and B. Vogelstein. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* **132**:6-13 (1983).
  41. Sambrook, J., E. F. Fritsch, and T. Maniatis. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
  42. Kramer, R. A., J. Zakher, and G. Kim. Role of glutathione redox cycle in acquired and *de novo* multidrug resistance. *Science (Washington D. C.)* **241**:694-697 (1988).
  43. Dusre, L., E. G. Mimnaugh, C. E. Meyers, and B. K. Sinha. Potentiation of doxorubicin cytotoxicity by buthionine sulfoximine in multidrug-resistant human breast cancer cell lines. *Cancer Res.* **49**:511-515 (1989).
  44. Moscow, J. A., A. J. Townsend, and K. H. Cowan. Elevation of  $\pi$  class glutathione *S*-transferase activity in human breast cancer cells by transfection of the GST  $\pi$  gene and its effect on sensitivity to toxins. *Mol. Pharmacol.* **36**:22-28 (1989).

Send reprint requests to: Chuck C.-K. Chao, Tumor Biology Laboratory, Departments of Biochemistry and Medicine, Chang Gung Medical College, Tao-yuan, Taiwan 33332, Republic of China.