Cancer Chemother Pharmacol (1996) 38: 45–51 

ORIGINAL ARTICLE

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# Glutathione S-transferase and glutathione peroxidase are essential in the early stage of Adriamycin resistance before P-glycoprotein overexpression in HOB1 lymphoma cells

Received: 8 March 1995/Accepted: 4 August 1995

**Abstract** We have previously established Adriamycin-resistant HOB1 cell lines showing the multidrug resistance (MDR) phenotype. For further study, we analyzed the free-radical scavengers glutathione S-transferase (GST) and glutathione peroxidase (GPX) by enzyme assays and Northern blots. Three cell lines, HOB1/ADR0.1, HOB1/ADR1.0, and HOB1/ADR5.0, represented HOB1 cells resistant to 0.1, 1.0, and 5.0 μ*M* Adriamycin, respectively. The *mdr*1 transcript was overexpressed in HOB1/ADR0.1 cells, and the amount of its expression reached a maximum between HOB1/ADR1.0 and HOB1/ADR5.0 cell lines. The increases in GST activity and GST-π expression were observed only in high-level-resistant cell lines (HOB1/ADR1.0 and HOB1/ADR5.0), which also showed increased GPX activity and expression. For investigation of the cytotoxic effect of Adriamycin on HOB1 cells prior to the *mdr*1 overexpression, an appropriate number of parental HOB1 cells were treated with 0.1 μ*M* Adriamycin for 7 days, and the viable cells (HOB1/ADR) were isolated and subjected to analyses for *mdr*1, GST-π, and GPX expression and for GST and GPX activity. In comparison with HOB1/ADR0.1 cells, HOB1/ADR cells did not show *mdr*1 overexpression but had significant increases in the activity and expression of GST and GPX. The current study suggests that in the early phase of Adriamycin treatment, GST and GPX are more important than P-glycoprotein for the development in HOB1 cells of resistance against Adriamycin.

**Key words** MDR · P-glycoprotein · GSH · GST · GPX

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**Abbreviations** *MDR* Multidrug resistance · *SSC* Standard saline citrate (0.15 *M* NaCl, 0.015 *M* sodium citrate, pH 7.4); *PBS* phosphate-buffered saline (136 m*M* NaCl, 2.5 m*M* KCl, 6.6 m*M* $Na_2HPO_4$, 1.5 m*M* $KH_2PO_4$) · *MTT* 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) · *GSH* glutathione · *GST* glutathione S-transferase · *GPX* glutathione peroxidase · *BSO* DL-buthionine-S,R-sulfoximine

## Introduction

Adriamycin is a quinone anticancer agent. The quinone group appears frequently in the structure of cancer chemotherapeutic agents. The importance of this functional group to the cytotoxicity of these agents remains not entirely clear. The quinone groups are enzymatically bioactivated through oxidation-reduction reactions to free-radical intermediates, with subsequent production of oxygen radicals in the presence of $O_2$ [1, 2] leading to DNA breaks [3, 4] and lipid peroxidation [5, 6]. Tritton and Yee [7] have reported that Adriamycin can exert its cytotoxic action solely by interaction at the cell membrane.

Alterations in glutathione (GSH) metabolism can have an important impact on the cytotoxicity of various free-radical-producing anticancer drugs [8]. GSH accounts for the majority of the cellular nonprotein thiol content. It participates in many important cellular functions, including protection from free-radical damage, detoxication of xenobiotics, and synthesis of DNA precursors [9]. Exposure of tumor cells to quinone anticancer agents has induced increases in GSH content [10–12] and in glutathione S-transferase (GST) activity [10–14]. The likely interaction may be brought about between GSH and the electrophilic moiety. This reaction may occur directly with GSH or can be catalyzed by the enzyme GST [15]. Glutathione peroxidase (GPX) plays a key role in cellular

detoxification by catalyzing the reaction of GSH with hydrogen peroxide and other organic peroxides [8]. Enhanced GPX activity has a protective role in Adriamycin resistance [10, 12, 16].

The exposure of tumor cells to hydrophobic cytotoxic agents can lead to selection of multidrug-resistant (MDR) cell lines. These resistant mutants are resistant not only to the selecting agent but also to a wide range of structurally unrelated compounds, such as colchicine, vincristine, Adriamycin, and actinomycin D [17]. MDR is at least in part the result of a decrease in the intracellular drug concentration [18, 19] mediated by a membrane glycoprotein of 170 kDa referred to as the P-glycoprotein [17], and it can be reversed by compounds that competitively inhibit the drug-efflux action of the P-glycoprotein, including the calcium channel blocker verapamil and the calmodulin antagonist trifluoperazine [20–22]. Transfection of *mdr*1 cDNA has established a strong correlation between MDR and the overexpression of P-glycoprotein [23, 24].

Although the importance of P-glycoprotein is supported by the evidence accumulated from numerous Adriamycin-selected drug-resistant cell lines, this protein is not always detectable in MDR cell lines [25, 26]. Non-P-glycoprotein-mediated MDR cell lines have been reported [27–32], some of which overexpress multidrug-resistance protein (MRP) of 190 kDa [30–32]. Mechanisms other than P-glycoprotein may play even more important parts in the development of resistance to Adriamycin for certain cell lines. These include reduced expression of topoisomerase II [14, 33, 34] and increased expression of GSH-related enzymes [10–16, 35–38], although not every resistant cell line has been demonstrated to show these enzyme alterations.

In the current paper, we report that persistent overexpression of GST and GPX was observed at a resistance level at which P-glycoprotein had been overproduced to a maximum and that the activity and expression of these enzymes were found to be transiently increased in cells undergoing early Adriamycin treatment but not exhibiting P-glycoprotein overexpression.

## Materials and methods

### Materials

DTNB (5,5′-dithiobis[2-nitrobenzoic acid]), RPMI 1640 medium and Adriamycin were purchased from Sigma (St. Louis, Mo.). α-[$^{32}$P] – Deoxycytidine triphosphate (dCTP) was obtained from Amersham Life Science (UK). The Klenow fragment of *Escherchia coli* DNA polymerase, *Taq* DNA polymerase, *Eco*R I, GSH, NADPH (nicotinamide adenine dinucleotide phosphate, reduced form), and GSH reductase were supplied by Boehringer Mannheim (Germany). Ficoll-Paque was obtained from Pharmacia (Sweden).

### Cell lines and cell culture

Adriamycin-resistant HOB1 lymphoma cell lines (human) showing the MDR phenotype have previously been established [39]. HOB1 cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum as described elsewhere [40]. For investigation of the short-term effect of Adriamycin on HOB1 cells, more than $1 \times 10^9$ cells in multiple tissue-culture flasks were treated with 0.1 μ*M* Adriamycin for 7 days. More than 95% of the treated cells were killed, and the viable cells (designated HOB1/ADR) were isolated by Ficoll-Paque gradient centrifugation [41]. HOB1/ADR0.1, HOB1/ADR1.0, and HOB1/ADR5.0 represented HOB1 cells resistant to 0.1, 1.0, and 5.0 μ*M* Adriamycin, respectively.

### Northern blots

GST-π [42] and *mdr*1 cDNAs were kind gifts from Dr. C-K Chao, Chang Gung Medical College, Taiwan. *mdr*1 cDNA was originally cloned by Dr. P. Borst (Netherlands Cancer Institute). *mdr*1 and GST-π cDNAs were isolated from plasmids by *Eco*R I. GPX cDNA was synthesized by polymerase chain reaction (PCR) using the primers 5′-CAGTCGGTGTATGCCTTCTC and 5′-ATGTCA GGCTCGATGTCAAT, synthesized according to the reported sequence (43). After the PCR, the 536-bp DNA was resolved by agarose gel electrophoresis. The correct band was excised and eluted from the gel. The cDNAs of *mdr*1, GST-π, and GPX were labeled by random primer extension labeling [44]. Total RNA was purified using the guanidium thiocyanate-phenol/chloroform-extraction method [45]; 20 μg total RNA was separated by electrophoresis in a 1% formaldehyde-agarose gel. Detailed procedures for Northern blotting have been described elsewhere [39].

### GST assay

GST activity was measured according to the method described by Habig et al. [46]. Exponentially growing cells were harvested, washed twice with PBS (phosphate-buffered saline, 136 m*M* NaCl, 2.5 m*M* KCl, 6.6 m*M* $Na_2HPO_4$, 1.5 m*M* $KH_2PO_4$), and then lysed by three episodes of sonication for 10 s each in distilled water. The cell lysate was centrifuged at 10,000 *g* for 15 min, and the supernatant was used for the enzyme assay. To a 1-ml cuvette were added 0.1 ml of 10 m*M* GSH (in 1 *M* potassium phosphate buffer, pH 6.5), 20 μ l of 50 m*M* 1-chloro-2,4-dinitrobenzene (in absolute ethanol), and 100 μl of the above-mentioned supernatant (as milligrams of protein). The protein concentration was determined by the method of Bradford [47]. The final volume was made up to 1 ml with distilled water. The absorbance at 340 nm was recorded for 5 min at 25°C. The enzyme activity was expressed in nanomoles per minute per milligram of protein.

### GPX assay

GPX activity in parental and resistant cells was measured in supernatant prepared as described above using the method of Paglia and Valentine [48]. To a 1-ml cuvette were added 500 μl of 0.1 *M* sodium phosphate (pH 7.0) containing 1.0 m*M* ethylenediaminetetraacetic acid (EDTA), 100 μl of GSH reductase (2.4 units/ml), 100 μl of 10 m*M* GSH, and 100 μl of the above-mentioned sample. The mixture was incubated at 37°C for 10 min, after which 100 μl of 12 m*M* *t*-butyl hydroperoxide and 100 μl of 1.5 m*M* NADPH (dissolved in 0.1 *M* sodium phosphate buffer containing 0.1% sodium bicarbonate, pH 7.0) were added. The absorbance at 340 nm ($A_{340}$ value) was recorded for 5 min at 37°C. The $A_{340}$ values decreased with time. The nonenzymatic activity was assessed using 0.1 *M*

sodium phosphate buffer (pH 7.0) to replace the sample. The enzyme activity was expressed in nanomoles per minute per milligram of protein.

### Determination of GSH content

GSH content was measured by the method of Suzukake et al. [49]. In brief, 0.25 ml of 12% sulfosalicylic acid was added to 0.75 ml of supernatant prepared as described above. The mixture was incubated at 0°C for 30 min and then centrifuged at 10,000 *g* for 20 min. The protein-free supernatant was collected for the GSH assay. To a 1-ml cuvette were added 700 µl of 0.3 m*M* NADPH (dissolved in 0.1 *M* sodium phosphate buffer containing 1.0 m*M* EDTA, pH 7.2), 0.6 µmol of DTNB (5,5'-dithiobis [2-nitrobenzoic acid]) prepared as previously described [50], and 100 µl of the above-mentioned supernatant; distilled water was added for a final volume of 1 ml. Finally 0.48 units of GSH reductase was added to the reaction mixture. The GSH level was measured by recording the absorbance at 412 nm at 30°C for 6 min and was expressed in nanomoles per milligram of protein.

### In vitro cytotoxicity assay

Exponentially growing cells were harvested, washed, and resuspended in appropriate amounts of medium. Cells were seeded into 96-well microtiter plates at 20,000 cells/well in 0.1 ml of medium containing increasing concentrations of Adriamycin in replicates of eight. After incubation at 37°C for 96 h, the treated cells were processed for MTT assay [51]. For examination of effect of BSO (butathionine sulfoximine) and verapamil on the cytotoxicity of Adriamycin, 50 µ*M* BSO or 10 µ*M* verapamil was added to the assay mixtures.

## Results

### Expression of the *mdr*1 transcript

Overproduction of P-glycoprotein has been shown in Adriamycin-resistant HOB1 cells [39]. In the present study we examined the amount of *mdr*1 transcript in cell lines displaying varying degrees of resistance. Figure 1 shows the overexpression of the *mdr*1 transcript. The ratio of HOB1/ADR5.0 to HOB1/ADR1.0 in the density of the band was 1.4 as measured using a scanning densitometer; the ratio of HOB1/ADR1.0 to HOB1/ADR0.1 was 4.6. The increase in resistance from 1.0 to 5.0 µ*M* did not cause a significant enhancement of expression, indicating that a plateau in expression had been reached. HOB1/ADR0.1 cells could overexpress the *mdr*1 transcript; however, HOB1/ADR cells that had undergone 0.1 µ*M* Adriamycin treatment for 7 days and were also resistant to the treated agent did not show detectable *mdr*1 expression as observed in parental cells.

### Expression of GST-π and GPX

Northern blots were used to quantitate the amounts of GST-π (probed by *Eco*R I-generated cDNA) and GPX mRNA (probed by PCR-generated cDNA) transcripts in the parental and resistant cell lines. Figure 2A shows the expression of GST-π. Low-level-resistant HOB1 cells (HOB1/ADR0.1 cells) did not exhibit increased expression of the enzyme; HOB1/ADR1.0 and HOB1ADR5.0 cells displayed an approximately 2.6- and 3.4-fold increase, respectively, in expression relative to the parental cells. Figure 3A shows the expression of GPX. As found for the expression of GST-π, no enhanced band was observed in HOB1/ADR0.1 cells in comparison with parental cells. Significant increases in expression of the enzyme were observed in HOB1/ADR1.0 (2.3-fold increase) and HOB1/ADR5.0 (3.4-fold increase) cells. Figures 2B and 3B show that both GST-π and GPX transcripts increased in HOB1/ADR cells (2.1-fold increase in GST-π and

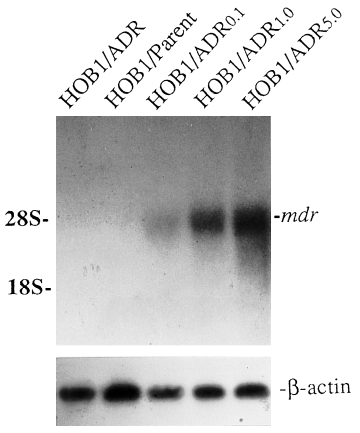


**Fig. 1** Northern blot showing overexpression of the *mdr*1 transcript in Adriamycin-resistant HOB1 cells; 20 µg total RNA was loaded in each lane. After autoradiography, the *mdr* blot was washed in a heated solution of 0.1 × SSC and 0.1% sodium dodecyl sulfate and reprobed with $^{32}$P-labeled human β-actin cDNA. The intensities of the bands were measured using a scanning densitometer

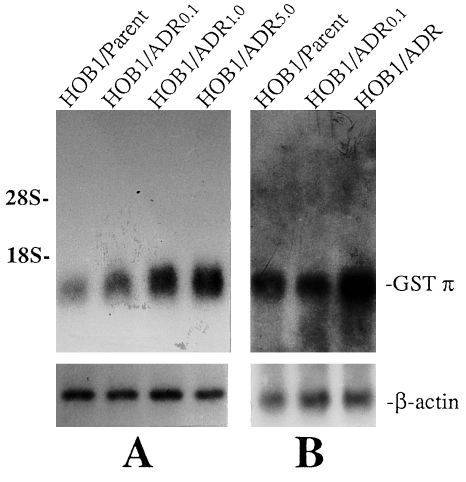


**Fig. 2** Northern blot showing overexpression of GST-π in Adriamycin-resistant HOB1 cells. The experimental procedures were the same as those described for Fig. 1.

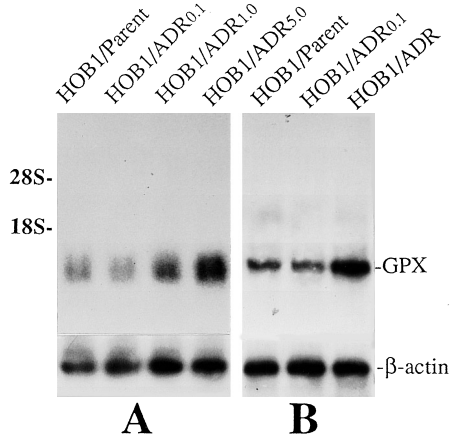


**Fig. 3** Northern blot showing overexpression of GPX in Adriamycin-resistant HOB1 cells. The experimental procedures were the same as those described for Fig. 1.

2.2-fold increase in GPX) as compared with parental cells.

## Assays for GST, GPX and GSH

Table 1 shows the activity of GST and GPX and the content of GSH in the parental and resistant cell lines. The factor of resistance was determined by dividing the activity of the resistant cell line by that of the parental cell line. The GST activity was not significantly enhanced in the low-level-resistant HOB1/ADR0.1 cells. A considerable (3.2-fold) increase was observed in HOB1/ADR5.0 cells. HOB1/ADR1.0 cells displayed a 2.3-fold increase in GST activity. The activity of GPX was 2.2-fold higher in HOB1/ADR1.0 cells and 3.5-fold higher in HOB1/ADR5.0 cells than in the parental cells. The results of the assays for GST and GPX were consistent with those obtained in Northern blots. The GSH content also increased in HOB1/ADR1.0 (1.9-fold) and HOB1/ADR5.0 (2.8-fold) cells. As had been noted in the Northern blot, HOB1/ADR cells showed a 2.0-fold increase in GST activity, a 2.1-fold increase in GPX activity, and a 1.8-fold increase in GSH content.

## Servoeffect of BSO and verapamil on the cytotoxicity of Adriamycin

BSO can inhibit $\delta$-glu-cys synthetase, an enzyme essential for the synthesis of GSH, leading to GSH depletion [52]. In vitro depletion of GSH with BSO has increased the cytotoxicity of a number of chemotherapeutic agents, including Adriamycin, mitomycin, melphalan, and nitrogen mustard [53]. As previously reported [52, 53], the resistance of HOB1 cells to Adriamycin could also be circumvented by BSO (Table 2), indicating a concerted contribution of P-glycoprotein and GSH-related enzymes to drug resistance. Verapamil reversed the resistance of HOB1/ADR0.1 cells almost to the level of parental cells in comparison with the effect of BSO, suggesting that P-glycoprotein is essential for HOB1/ADR0.1 cells. On the other hand, the effect of BSO was much greater in HOB1/ADR1.0 and HOB1/ADR5.0 cells than in HOB1/ADR0.1 cells, suggesting that GSH-related enzymes are essential to the two high-level-resistant cell lines. An interesting finding was that HOB1/ADR cells were less responsive to BSO and verapamil and showed an extraordinary resistance to Adriamycin (Table 2).

**Table 1** Comparison of the intracellular activity of GST and GPX and the content of GSH in parental and Adriamycin-resistant HOB1 cells. Data are expressed as mean values ± SD for three or four determinations (separate drug-selected cell batches). The results were evaluated statistically by two-tailed *t*-tests comparing the significance of the differences between the means of the resistant cells with those of the parental cells ($P < 0.001$ for the values obtained for GST, GPX, and GSH in HOB1/ADR, HOB1/ADR1.0, and HOB1/ADR5.0 cells as compared with those found for parental cells. The differences noted between parental cells and HOB1/ADR0.1 cells in terms of GST, GPX, and GSH values were insignificant.)

| Cell lines | GST (nmol $min^{-1}$ mg $protein^{-1}$)[a] | GPX (nmol/mg protein)[a] | GSH |
|---|---|---|---|
| HOB1/Parent | 22 ± 2.4 | 0.47 ± 0.05 | 126 ± 16 |
| HOB1/ADR | 45 ± 5.5[b] | 0.98 ± 0.08[b] | 227 ± 22[b] |
| HOB1/ADR0.1 | 26 ± 3.7 | 0.44 ± 0.05 | 131 ± 14 |
| HOB1/ADR1.0 | 51 ± 5.2 | 1.03 ± 0.09 | 239 ± 31 |
| HOB1/ADR5.0 | 71 ± 6.6 | 1.65 ± 0.14 | 353 ± 38 |

[a] Protein concentration was determined using the supernatant of 10,000-*g* centrifugation
[b] Unique data

## Discussion

Adriamycin is one of the anthracycline antibiotic anticancer agents, the chemistry of which is rich and complicated because of the quinone-hydroquinone structure. The one-electron reduction of anthracyclines results in the formation of semiquinone free radicals, and these intermediate radicals have been proposed to be responsible for the anticancer activities of the parent drugs. The putative agents responsible for Adriamycin-induced DNA damage and lipid peroxidation have been suggested to be superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide ($H_2O_2$), and the highly reactive hydroxyl radical ($^{\cdot}OH$). These radicals may be induced by the reaction of the Adriamycin semiquinone with oxygen. Among these, the hydroxyl free radical is one of the most likely mechanisms for the antitumor activity of the drug (for review, see [1–4]).

The biochemical roles of GSH in drug detoxification, scavenging of free radicals, and subsequent reduction of Adriamycin toxicity in tumors have been well studied

**Table 2** Effect of BSO and verapamil on the cytotoxicity of Adriamycin to HOB1 cells of varying degrees of resistance. Cells were treated with increasing concentrations of Adriamycin for 96 h and then tested by the MTT assay; 50 μ*M* BSO or 10 μ*M* verapamil was added to the assays. Data are expressed as mean values ± SD derived from eight replicates ($IC_{50}$ Adriamycin concentration causing 50% cell death, *ADR* Adriamycin, *VPL* verapamil)

| Cell lines | ADR[a] | ADR + BSO[a] | ADR + VPL[a] |
|---|---|---|---|
| HOB1/Parent | 0.33 ± 0.02 | 0.31 ± 0.03 | 0.36 ± 0.04 |
| HOB1/ADR | 2.8 ± 0.33[b] | 2.3 ± 0.24[b] | 2.5 ± 0.32[b] |
| HOB1/ADR0.1 | 0.63 ± 0.04 | 0.58 ± 0.06 | 0.38 ± 0.04 |
| HOB1/ADR1.0 | 2.4 ± 0.31 | 1.3 ± 0.23 | 0.92 ± 0.07 |
| HOB1/ADR5.0 | 8.8 ± 0.93 | 4.7 ± 0.42 | 2.5 ± 0.37 |

[a] $IC_{50}$ values (μ*M*)
[b] Unique data.

[1–4]. GSH is the essential cofactor for both GST and GPX [2]. The role of the two enzymes is now generally conceived as a mechanism of cellular protection against adriamycin. Russo and Mitchell [52] have reported that depletion of GSH with BSO increases Adriamycin toxicity in Chinese hamster V79 cells. Conversely, increasing the intracellular GSH content by 2-oxothiozolidine-4-carboxylate treatment protects V79 cells from Adriamycin toxicity, suggesting that free radicals account for the cytotoxicity of this drug [52].

In the current study we showed that HOB1 cells surviving a 7-day treatment with 0.1 μ*M* Adriamcyin could tentatively overexpress GST-π and GPX. The viable cells did not constitute a stable resistant cell line until they had overexpressed the drug-efflux pump, P-glycoprotein (that is, HOB1/ADR0.1, cells had established and were capable of actively dividing after a 6-week treatment with 0.1 μ*M* Adriamycin, during which time they did not reveal sensible cell division). It is noteworthy that was no enhancement of the expression or activity of the two enzymes in HOB1/ADR0.1 cells, which were more sensitive to verapamil than to BSO in the presence of Adriamycin (Table 2). The circumvention of GST and GPX protection by BSO was observed in HOB1/ADR1.0 and HOB1/ADR5.0 cells but was not seen in HOB1/ADR cells. In addition, HOB1/ADR cells were highly resistant to Adriamycin (Table 2). The reasons for this may be three-fold.

First, most anticancer drugs can cause cell-cycle arrest; HOB1 cells surviving a 7-day treatment with Adriamycin may be arrested in the $G_0$ or $G_1$ phase of the cell cycle by a low concentraion of Adriamycin; since most normal cells can endure chemotherapy well, HOB1/ADR cells may escape drug injury due to a slow growth rate (refer to the description above about the establishment of the HOB1/ADR0.1 cell line). Second, the most sensitive HOB1 cells have been killed and removed; therefore the remaining cells, stalled in a dormant stage, are less vulnerable to higher concentrations of Adriamycin than are HOB1/ADR0.1 cells, which have expressed sufficient P-glycoprotein and can thus actively divide. Third, HOB1/ADR cells show increases in GST and GPX activity, and GSH content; therefore they may exploit these detoxifying enzymes to protect themselves against a higher concentration of Adriamycin (in verapamil reversal), in contrast to HOB1/ADR0.1 cells, which have retrieved these enzyme activities to the resting levels (probably because the intracellular agent can be reduced to a harmless level by P-glycoprotein).

We observed that HOB1 cells selected by treatment with a low dose of Adriamycin over a short period showed strong resistance to the agent before they became a stable drug-resistant cell line, indicating that drug-induced growth arrest may have a place in mediating resistance. One example would be chronic myelogenous leukemia (CML)-Leukocytosis in CML is attributable to abnormal accumulation rather than accelerated proliferation of granulocytes; thus, conventional chemotherapy is ineffective in eradicating the neoplastic clones due to their slow growth rates [54].

Although similar data on GST and GPX have been reported for Adriamycin-resistant cell lines [10–14, 36–38], our experimental results emphasize that elevated expression of the two enzymes is observed only when P-glycoprotein has not been overproduced (HOB1/ADR) or when cells have been treated with such a high dose of the agent (1 μ*M* in HOB1/ADR1.0, 5 μ*M* in HOB1/ADR5.0) that the P-glycoprotein is overburdened. A concentration of 0.1 μ*M* is within the cytotoxic range of Adriamycin [55]. HOB1/ADR0.1 cells maintained in 0.1 μ*M* Adriamycin can express detectable *mdr*1 transcript but cannot overexpress GST and GPX. Inversely, HOB1/ADR cells treated over a short period with 0.1 μ*M* Adriamycin do not display an increase in the *mdr*1 transcript but do show overexpression of GST and GPX. This may be a major mechanism by which clinical tumor cells acquire resistance to Adriamycin when P-glycoprotein has not been timely induced such that the agent can freely enter cells without encountering the resistance of a drug-efflux pump. Once the P-glycoprotein has been overexpressed and the drug-extrusion action of the protein is sufficient to reduce the concentration of the intracellular agent to a harmless level, the GST and GPX activities revert to the level of parental cells, although they may nonetheless play a role in metabolizing the cytotoxic free radicals.

Adriamycin does not select HOB1 cells that have shown significant overexpression of the P-glycoprotein (Fig. 1, failed detection of the *mdr*1 transcript in HOB1/ADR cells). This may be the reason why HOB1/ADR cells must entail unusual increases in GSH-related enzymes in comparison with HOB1/ADR0.1 cells. A failure to identify the *mdr*1 gene product does not imply that the gene has not been expressed. However, the slight increase in the *mdr*1

transcript that may be transiently expressed in any adriamycin-treated cell line should not be sufficiently effective to reduce the intracellular drug concentration to a level at which cells can survive. Resistant cells selected by prolonged drug treatment usually exhibit multiple genetic and biochemical differences from the parental cell lines. These changes may affect the expression of gene products associated with drug resistance, including P-glycoprotein. It should be stressed that clinically drug-resistant tumor cells are not stabilized in a certain cytotoxic agent. A single course of clinical chemotherapy does not usually exceed 2 weeks, which seems too short a period to cause a gene amplification, especially considering the fluctuation of the agent's concentration in the human body. Therefore, overexpression of the P-glycoprotein and multidrug-resistance protein (MRP) may be a later consequence of drug resistance.

The resistance of tumor cells to Adriamycin is multifactorial. In addition to increased expression of the *mdr*1, GST, and GPX transcripts, resistant cell lines also show decreased susceptibility to drug-induced DNA damage and reduced levels of topoisomerase II [14, 33, 34]. Furthermore, several investigators have reported changes in the activity of UDP-glucuronyl transferase (increased [56]), catalase (increased [57]), and DT-diaphorase (decreased [36]), although the expression of these enzymes is not necessarily altered in other Adriamycin-resistant cell lines. Accumulated data have mainly focused on well-established drug-resistant cell lines, which are not sufficient to reflect the real problems encountered in clinical chemotherapy. The current study gives more consideration to a short-term treatment of tumor cells with Adriamycin. As far as we know, very few, if any, papers have reported resistance of tumor cells to Adriamycin in the early stage before P-glycoprotein has been overexpressed. From the results presented herein, it can be concluded that for HOB1 cells, P-glycoprotein in essence is less important than are the GSH-related enzymes in the initial step of resistance development.

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