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Glutathione-related enzymes, glutathione and multidrug resistance

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Abstract

This review examines the hypothesis that glutathione and its associated enzymes contribute to the overall drug-resistance seen in multidrug resistant cell lines. Reports of 34 cell lines independently selected for resistance to MDR drugs are compared for evidence of consistent changes in activity of glutathione-related enzymes as well as for changes in glutathione content. The role of glutathione S-transferases in MDR is further analyzed by comparing changes in sensitivity to MDR drugs in cell lines selected for resistance to non-MDR drugs that have resulting increases in glutathione S-transferase activity. In addition, results of studies in which genes for glutathione S-transferase isozymes were transfected into drug-sensitive cells are reviewed. The role of the glutathione redox cycle is examined by comparing changes in elements of this cycle in MDR cell lines as well as by analyzing reports of the effects of glutathione depletion on MDR drug sensitivity. Overall, there is no consistent or compelling evidence that glutathione and its associated enzymes augment resistance in multidrug resistant cell lines.

Introduction

An appealing hypothesis emerged from the initial observation of increased glutathione S-transferase (GST) activity in a multidrug resistant cell line [1,2]: that glutathione-based detoxification systems, which were long known to guard normal tissues from chemical injuries, might also protect malignant tissues from chemotherapeutic agents. This idea had a certain historical logic, for it placed these detoxification systems, which were already known to act upon a broad range of substances, into the context of multidrug resistance (MDR). The idea possessed an attractive symmetry, for it appeared possible that glutathione-related metabolic pathways might cooperate with the drug efflux pump P-glycoprotein in drug excretion. And, as antibodies and cDNA probes for glutathione-dependent enzymes were available, the hypothesis was readily accessible to study. As a result, connections between MDR and glutathione-related enzymes have been extensively examined.

This article will review the literature regarding glutathione, glutathione-related enzymes and MDR. It will not address resistance to alkylating agents *per se*, a topic in which the interest in glutathione and its dependent enzymes is equal to that seen in the study of MDR. The review will first discuss GST isozymes, and will then examine the literature as it relates to the components of the glutathione redox cycle, including glutathione peroxidases, glutathione reductase, and glutathione itself.

Cytosolic GSTs and MDR

In the following discussion we will use the nomenclature system for human cytosolic GSTs that was described recently by Mannervik *et al.* [3]. In brief, alpha class GST subunits are identified as GSTA1-

1 and GSTA2-2; mu class GST subunits are identified as GSTM1a-1a, GSTM1b-1b, GSTM2-2 and GSTM3-3; and pi class GST as GSTP1-1.

It is a leap of logic to assume, since GSTs are detoxification enzymes with broad substrate specificity and MDR drugs have a variety of chemical structures, that GSTs must therefore play a role in detoxifying MDR drugs. In fact, though it is now widely assumed that cytosolic GSTs are capable of detoxifying MDR drugs, there is scant biochemical evidence to support these assumptions.

Although the ability to conjugate substrates with glutathione is the defining activity of GSTs, there is no evidence that any of the MDR drugs are conjugated with glutathione *en route* to detoxification and elimination. Specifically, glutathione conjugates of doxorubicin, vincristine, etoposide and actinomycin D have not been described. The single exception may be the participation of microsomal GST, a membrane-bound enzyme which is structurally dissimilar to the cytosolic GSTs, in the metabolism of mitoxantrone [4]. In contrast, cytosolic GST-mediated glutathione conjugation of the non-MDR antineoplastic agents chlorambucil [5,6] and melphalan [7], in addition to the cyclophosphamide metabolite acrolein [8], have been well-documented.

It is possible that GST isozymes could detoxify MDR drugs through pathways other than conjugation with glutathione. GSTs bind hydrophobic substances with high affinity and may participate in the intracellular transport and sequestration of these molecules [9], such as in the intracellular transport of bilirubin in the liver [10]. Thus, these isozymes may protect cells by binding toxins and preventing them from interacting with critical cellular targets. It has therefore been proposed that a possible role for GSTs in MDR may be to bind antineoplastic agents and present them to the P-glycoprotein pump for extrusion from the cell [11]. This hypothesis was supported by comparisons of P-glycoprotein with homologous bacterial transport proteins which require the activity of soluble periplasmic proteins to perform their transport functions [11]. However, Black *et al.* found no evidence that GST isozymes P1-1 and A1-1 could bind doxorubicin [12]. No biochemical evidence has thus far been published which demonstrates direct binding of GST isozymes to MDR drugs or their metabolites.

Since some GSTs, most notably certain alpha class isozymes, possess intrinsic peroxidase activity, it has been postulated that these isozymes could detoxify harmful organic peroxides. As we will discuss later, such toxic peroxide intermediates are created in the metabolism of doxorubicin [13]. However, the GST isozyme that is most frequently found to be elevated in MDR cell lines, GSTP1-1, possesses very low levels of peroxidase activity [14].

Is increased GST activity necessary for MDR?

At least 32 cell lines that were independently selected for resistance to MDR drugs have been described in which GST activity has been characterized [1,15–35]. These cell lines, whose characteristics are summarized in Table 1, were isolated by incubating the parental cell lines with the selecting drug, usually in serial passages of stepwise increasing drug concentrations. Most, but not all, express increased amounts of P-glycoprotein and increased levels of *mdr*-1 RNA.

Table 1 must be interpreted bearing in mind the following caveat. Most GSTs catalyze the conjugation of glutathione to chloro-2,4-dinitrobenzene (CDNB) at an easily measurable rate [9]. The GST activity cited in Table 1 reflects the ability of cell cytosols to catalyze this reaction. However, using this single assay for comparative purposes can be deceptive, as many tissues contain complex mixtures of GSTs and the rate of CDNB conjugation differs between isozymes. Complete characterization of the GST content of tissues requires identification of their subunits. In the studies outlined in Table 1, GST isozyme expression was not always determined. Studies which identified individual GST isozymes may have utilized immunological techniques or analysis of RNA expression.

Table 1 includes summaries of GST changes in 25 cell lines selected for resistance to doxorubicin in which bulk cytosolic GST activity is reported. Only 10 of these 25 resistant cell lines have demonstrated increased GST activity in comparison to

Table 1. Glutathione and glutathione-related enzymes in MDR cell lines

Parental cell line	Tumor origin	Fold resistance	GST fold change	Isozyme class increased	GSH fold change	GPx fold change	GRed fold change	Reference
Selected with doxorubicin								
V-79	Hamster fibroblast	3000	0.75	π	0.78	0.67	1.13	Medh *et al.* [15]
CHO	Hamster ovary	27	1.5	α				Hoban *et al.* [16]
H-134	Ovary	*	nc					Broxterman *et al.* [17]
A2780	Ovary	100	1.25		1.55	0.87		Hamilton *et al.* [18]
A2780	Ovary	1000	nc					Yusa *et al.* [19]
TR170	Ovary	2.3	nc	0.86	0.47	0.84		Hosking *et al.* [20]
Hattori	Breast	3.3	0.4				0.34	Yusa *et al.* [19]
MCF-7	Breast	192	44.7	π	0.9	12.9		Batist *et al.* [1]
MCF-7	Breast	900	0.5		1.07			Chen *et al.* [21]
MCF-7	Breast	2.6	2.6	π	1.11	1.15	1.95	Whelan *et al.* [22]
MCF-7	Breast	300	nc					Yeh *et al.* [23]
MCF-7	Breast	75			0.64			Taylor *et al.* (34)
H69	Lung-small cell ca.	73	10	π	0.1	1.24		Cole *et al.* [24]
SW1573	Lung-squamous	*	2					Cole *et al.* [24]
RPMI8226	Myeloma	40	nc		1.65	0.88		Bellamy *et al.* [25]
P388	Leukemia	37	0.33		1.95	3.33	1.14	Kramer *et al.* [26]
P388	Leukemia	5	1.4	π				Deffie *et al.* [27]
P388	Leukemia	10	1.5	π				Deffie *et al.* [27]
P388	Leukemia	103			0.97	1.36		Nair *et al.* [35]
HL60	Leukemia	111	nc		0.54	0.85	1.38	Lutzky *et al.* [28]
K562	Leukemia	128	nc					Yusa *et al.* [19]
K562	Leukemia	155	nc					Kato *et al.* [29]
FLC	Erythroleukemia	13	nc	α	0.74	1.44		Schisselbauer *et al.* [30]
SW620	Colon	108	0.7		nc			Lai *et al.* [31]
LS180	Colon	7.5	nc		nc			Lai *et al.* [31]
DLD-1	Colon	21	1.9	not π	1.3			Lai *et al.* [31]
SW620	Colon	75	2.5	π	1.65			Chao *et al.* [32]
Selected with etoposide								
MCF-7	Breast	5.1	6.7		1.24	1.45	0.58	Hosking *et al.* [20]
SuSAP	Testicular	5.1	nc		0.89	0.73	1.15	Hosking *et al.* [20]
HN-1P	Head and Neck	4	0.94		0.95	1.44	1.19	Hosking *et al.* [20]
Selected with vincristine								
MCF-7	Breast	13.7	1.3		1.15	5.25	0.58	Hosking *et al.* [20]
MCF-7	Breast	11.1	6.7		1.27	4.6	0.6	Whelan *et al.* [33]
Selected with colchicine								
MCF-7	Breast	24	70					Yusa *et al.* [19]
Selected with vinblastine								
CCRF-CEM	Leukemia	10	nc					Yusa *et al.* [19]

Fold resistance was calculated as the ratio of the IC_{50} of the resistant cell line to that of the parental cell line. GST activity in all cell lines was measured with CDNB as substrate. GPx — glutathione peroxidase activity, measured using either cumene hydroperoxide or hydrogen peroxide as substrate. GRed — glutathione reductase. * indicates MDR phenotype, fold-resistance not specified. nc — no change. Blank spaces indicate variable not determined.

the parental cell line, and only five out of 25 have increases of GST activity of 2-fold or greater. In contrast, 15 out of 25 resistant sublines had either no change of GST activity or decreased levels of GST activity. Thus, contrary to what is frequently stated, GST activity is most often unchanged in doxorubicin-selected cell lines. The isozyme pattern of four of the five cell lines with greater than 2-

fold increase in GST activity was characterized, and in each case demonstrated evidence of an increase in the GSTP1-1 isozyme, an isozyme with little intrinsic peroxidase activity.

There is no evidence, from Table 1, for a dose-response relating GST activity to the level of resistance to doxorubicin; in other words, GST activities were increased in some of the least resistant cell lines, and unchanged or decreased in some of the most resistant cell lines. There is also no evidence that GST changes may be cell line specific. For example, only two of four different MCF-7 human breast cancer cell lines selected for resistance to doxorubicin have shown increased GST activity, again with no direct relation between the degree of resistance and GST activity.

Despite the infrequent association of elevated GST activity and acquired drug resistance *in vitro*, several investigators have looked for associations between the variability of sensitivity towards MDR drugs of independent cell lines not selected for drug resistance and the cell lines' relative amounts of GST activity. These studies are difficult to interpret since different cell lines obviously possess a multitude of different characteristics. Nevertheless, a positive association between GST expression and doxorubicin resistance was reported for a series of colon cancer cell lines [36], lung cancer cell lines [37], but not with breast tumors in short-term culture [38].

Two studies have looked at cell lines established from the same patient before and after therapy, as a method of understanding mechanisms of drug resistance acquired *in vivo*. An increase in GSTP1-1 expression was seen in a neuroblastoma cell line established after chemotherapy in comparison to the pretreatment cell line [39]. GST activity was also increased in a series of small cell lung cancer cell lines established from a single patient whose disease evolved from drug sensitive to drug resistant [40]. Although these associations are provocative they provide neither evidence of a causal relationship nor a mechanism of action for GST expression in MDR.

Is increased GST activity sufficient for MDR?

It is likely that cells possess many alternate routes of detoxification. Although the data in Table 1 indicate that increased GST activity is not necessary for resistance, it does not determine whether increased GST activity does contribute to resistance in the cell lines where it is found to be elevated.

If increased GST activity imparts resistance to MDR drugs, then one would expect that cell lines with increased GST activity, regardless of the derivation of the cell line, would demonstrate cross-resistance to MDR drugs. To address this question, cell lines selected for resistance to non-MDR drugs, and which reported elevation of GST activity, were reviewed for reports of cross-resistance to MDR drugs. Of approximately two dozen reports of resistant cell lines to non-MDR drugs in which GST activity was characterized, half displayed increases in GST activity. Of these, half of the studies reported whether the resistant subline was cross-resistant to doxorubicin. Cell lines fulfilling these criteria are presented in Table 2 [41–44].

All of the seven cell lines in Table 2 have

Table 2. Doxorubicin cross-resistance in cell lines with increased GST activity after selection for resistance to alkylating agents

Parental cell line	Tumor	Selecting agent	Fold resistance	GST fold increase	Isozyme class	Doxorubicin cross-resistance	Reference
G6331	melanoma	cisplatinum	9	5.4	π	no	Wang *et al.* [41]
CHO	ovary	cisplatinum	10	6	π	no	Saburi *et al.* [42]
H69	lung	cisplatinum	11	6.7	π	no	Kasahara *et al.* [43]
SKOV-3	ovary	cisplatinum	23	1.3	π	3.3-fold	Shellard *et al.* [44]
G6331	melanoma	melphalan	4	4.9	π	no	Wang *et al.* [41]
G6331	melanoma	BCNU	4	3.4	π	no	Wang *et al.* [41]
G6331	melanoma	4-NC	11	3.1	π	no	Wang *et al.* [41]

increased GST activity relative to the parental cell line, and in all cases it is the GSTP1-1 isozyme that accounts for the increased activity. This is similar to the increase in GSTP1-1 isozyme expression seen in MDR cell lines with increased GST activity (Table 1). However, only one of the seven cell lines shown in Table 2 displayed cross-resistance to doxorubicin, and this was the cell line with the lowest fold-increase of GST activity. Thus, increased GSTP1-1 activity in non-MDR selected cell lines does not by itself appear to be capable of producing cross-resistance to doxorubicin.

Another clue to the role of increased GST activity in resistant cell lines comes from examination of MDR cell lines which revert to a drug-sensitive phenotype by serial passage in the absence of the selecting agent. For example, the largest increase in GST activity in the MDR cell lines in Table 1 was seen in an MCF-7 human breast cancer cell line selected for resistance to colchicine and which was cross-resistant to doxorubicin [19]. A revertant subline of this resistant cell line completely lost resistance to colchicine and doxorubicin, yet retained its elevated GST activity [19]. Thus, it is unlikely that the elevated GST activity alone could have accounted for the observed drug resistance. In contrast, however, Batist *et al.* did find decreased levels of GSTP1-1 in a drug sensitive cell line that had reverted from one of the drug resistant MCF-7 cells that expressed increased levels of GSTP1-1 [1].

Transfection of cells with GST expression vectors

Cell lines selected for drug resistance often display multiple phenotypic changes, making it difficult to ascribe a causal relationship between a single biochemical variable and the global transformation of the sensitive cell line into a resistant one. In order to examine the isolated effects of increased expression of individual GST isozymes, several laboratories have performed gene transfer experiments in which drug-resistance is determined in cells transfected with genes for the different GST isozymes. Each of the laboratories have used either different techniques (i.e., acute versus stable transfections) or different host models. These studies are summarized in Table 3.

The MCF-7 human breast cancer cell line has been stably transfected with expression vectors that contain the human pi-class GST (GSTP1-1) [14], a mu-class GST (GSTM1-1) [45], and the alpha-class GSTs (GSTA1-1 and GSTA2-2) [45,46]. MCF-7 cells are a good model to examine the effects of GST on drug resistance since the parental cell line has remarkably low levels of GST activity [14]. In each of the clones transfected with individual GST cDNAs, the increased levels of GST isozyme failed to confer significant levels of resistance to any of the antineoplastic drugs tested (Table 3). Although small differences in drug sensitivity were seen in some of the GST transfected MCF-7 cells, these differences were consistent with clonal variation of the parental cell line as has been seen in clonal analysis of other cell lines [47]. In particular, increased levels of all three major classes of GST isozymes did not confer resistance to doxorubicin in MCF-7 cells. Furthermore, when GSTP1-1 was transfected into cells previously transfected with the gene encoding P-glycoprotein there was no change in the sensitivity of the cells to doxorubicin or to other drugs associated with the MDR phenotype [11]. Thus, GSTP1-1 apparently does not interact with the P-glycoprotein drug efflux pump to enhance drug resistance in MCF-7 cells.

Three other studies have used stable transfection of expression vectors in order to examine the effect of increased GST expression on drug sensitivity (Table 3). Transfection of alpha-class GST expression vectors into T47D human breast cancer cells, while producing transfectants that were resistant to cumene hydroperoxide, did not result in resistance to daunorubicin [48]. In transformed mouse NIH3-T3 cells that were transfected with human GSTP1-1, two clones that expressed higher levels of this isozyme showed a 1.8 to 3-fold increase in the IC_{37} value for doxorubicin [49]. However, little or no significant difference was seen in the IC_{50} and IC_{90} values of any of the clones. It was concluded that the change in IC_{37} to doxorubicin in the GSTP1-1 transfected cells reflected an increased ability of these cells to repair sublethal damage in the cells. In another study, a GSTP1-1 expression vector was transfected into CHO cells; at the reported IC_{90}

Table 3. Relative anthracycline resistance of cell lines transfected with GST expression vectors

Cell line	Organism	Transfected subunit[a]	GST activity (CDNB)	Fold change in activity	Fold change resistance	Conclusion	Reference
MCF-7	Human	P1	28	4	0.4(IC_{50})	Not resistant	Moscow *et al.* [14]
			54	7	1.1(IC_{50})		
			91	11	1.3(IC_{50})		
		P1	40	10	1.1(IC_{50})	Not resistant	Fairchild *et al.* [11]
			78	20	1.4(IC_{50})		
			63	16	0.8(IC_{50})		
			50	17	0.6(IC_{50})		
			44	15	0.8(IC_{50})		
		A1	40	4	0.7(IC_{50})	Not resistant	Leyland-Jones *et al.* [46]
			55	6	1.0(IC_{50})		
			22	2	0.9(IC_{50})		
		A2	17	2	0.7(IC_{50})	Not resistant	Townsend *et al.* [45]
			28	3	0.7(IC_{50})		
			52	5	0.7(IC_{50})		
		M1a	56	6	0.8(IC_{50})	Not resistant	Townsend *et al.* [45]
			150	15	0.7(IC_{50})		
			340	35	1.0(IC_{50})		
T47D	Human	Yc	45	3	Nil	Not resistant	Lavoie *et al.* [48]
			32	2	Nil		
pT22-3	Mouse c-H-Ras	P1	360	3	1.8(IC_{37})	Resistant	Nakagawa *et al.* [49]
			594	4	3.0(IC_{37})		
COS	Monkey	P1	Not reported	Not reported	1.3(IC_{90})	Resistant	Puchalski *et al.* [51]
		Ya	Not reported	1.3	1.3(IC_{90})		
		Yb1	Not reported	Not reported	Nil		
CHO	Hamster	P1	Not reported	Not reported	1.1(IC_{90})	No conclusion	Miyazaki *et al.* [50]
			Not reported	Not reported	1.3(IC_{90})		

[a]Subunits are as follows: A1 — human alpha class; A2 — human alpha class; M1a — human mu class; P1 — human pi class; Ya — rat alpha class; Yc — rat alpha class; Yb1 — rat mu class.

value there was little change (1.1 to 1.3-fold) in resistance to doxorubicin in the transfected cells [50].

To avoid some of the problems associated with clonal variation that may occur during analysis of stably transfected cells, monkey kidney COS cells were transiently transfected with GST expression vectors and then selected by flow cytometry using the GST substrate, monochlorobimane [51]. The product of GST-mediated GSH conjugation with this compound is fluorescent and cells that expressed elevated levels of GST were sorted by fluorescence from those in the population that did not. The sensitivity to antineoplastic agents of the sorted cells containing increased GST levels was compared to cells containing lower levels of GST. When IC_{90} values were compared, COS cells expressing increased levels of GSTP1-1 were reported to be 1.3-fold more resistant to doxorubicin relative to controls. No difference was seen between transfected cells and control cells in sensitivity towards vinblastine.

In addition to mammalian cell lines, the effects of transfected GSTP1-1 and human alpha-class GSTA1-1 upon the sensitivities of the yeast *Saccharomyces cerevisiae* to doxorubicin have also been examined [12]. Since yeast can be transfected with great efficiency, the GST-transfected yeast contained very high levels of GST relative to the intracellular concentrations typically seen in human cell lines. Both transfected GST cDNAs conferred resistance to doxorubicin, but the reported level of resistance of the transfected yeast is difficult to compare to other studies. Usually, the level of resistance is calculated by the ratios of drug concentrations that produce a given level of growth

inhibition. In this study, however, the level of resistance seen in the transfected yeast (3 to 10-fold to doxorubicin) was calculated by the ratio of the number of surviving yeast at a pre-selected drug concentration. Thus, this calculation cannot be compared to the levels of resistance described in other studies.

Given the vast differences in the model systems chosen for study, the data from these GST gene transfers into mammalian systems are actually not that dissimilar. Although the data from the transfection studies in Table 3 all show similar results, that GST transfectants with several-fold increases in total GST activity are less than 2-fold resistant to doxorubicin, the interpretation of these studies has widely varied. Some investigators believe that these small differences in resistance (2-fold or less) constitute meaningful drug resistance [49,51]. Others, including ourselves, have argued that such small differences in resistance may occur between subclones of the same cell line whether transfected or not, so that these minor differences may not necessarily be related to changes in GST expression [11,14,45,46]. In any event, the potency of transfected GST genes compares quite unfavorably to transfection of the *mdr*-1 gene, where levels of resistance conferred by gene transfer are considerably greater than control cells, and where the transfected gene confers predictable phenotypic changes [11].

GST and in vivo studies

Two major areas of investigation have dominated the study of the relation of GST expression to MDR in human tumors. The first area examines whether GST isozyme expression is associated with expression of the P-glycoprotein gene, *mdr*-1. The second area addresses the issue of whether altered GST expression in tumors has prognostic significance for patients receiving treatment with MDR drugs.

To address the question of whether GSTs are relevant in clinical MDR, it must first be determined whether these enzymes are expressed in human tumors. Studies from several laboratories have consistently shown that GST expression is often expressed at remarkably high levels in many types of tumor, and these high levels of expression often appear elevated when compared to GST levels in the normal tissues of origin [52–60]. This is particularly true of gastrointestinal malignancies [55–60]. The high levels of GST activity in human tumors are usually attributable to a single isozyme, GSTP1-1 [52–60]. Increased GST expression was not seen in a series of lung tumors, where normal lung parenchyma possesses intrinsically high levels of the GSTP1-1 isozyme [61].

A number of studies have examined human tumors for concomitant expression of GSTP1-1 and *mdr*-1. Direct correlations between *mdr*-1 and GSTP1-1 gene expression were reported for both lung tumors [62] and renal cell carcinoma cell lines [63]. A weak link between expression of the two genes has been suggested in chronic leukemias [64,65], but an association between GSTP1-1 expression and P-glycoprotein could not be demonstrated in acute leukemias [63–65]. A positive correlation between *mdr*-1 gene expression and GSTP1-1 expression in lymphomas was noted in one study [66], but not in another [53]. No association between GSTP1-1 and P-glycoprotein was seen in a study of gastrointestinal cancers [67] or multiple myeloma [68].

Studies relating GST expression to clinical outcome in leukemia have suggested that GST isozyme expression may be a prognostic marker in this heterogeneous group of diseases. Total GST activity [69] and specific GSTP1-1 expression [70] have shown a positive correlation with clinical response in two series of patients with leukemia. In contrast, GST activity and isozyme distribution did not predict response to chemotherapy in two series of patients with ovarian cancer [71,72], which in one study included the use of the MDR drug doxorubicin [72].

In breast cancer, the idea that GSTP1-1 expression might be a useful prognostic marker arose from the observation that GSTP1-1 expression is inversely correlated with estrogen receptor status [73,74]. Immunohistochemical examination of a series of 240 breast cancer specimens confirmed the inverse relationship between GSTP1-1 expression and estrogen receptor content, and furthermore

suggested that GSTP1-1 expression was a strong predictor of relapse and death in the subgroup of women with axillary node-negative breast cancer, none of whom had received chemotherapy prior to relapse [75]. In contrast, women with axillary node-positive breast cancer who had received adjuvant chemotherapy, expression of GSTP1-1 was without predictive value [75,76]. These data suggest a possible relationship between GSTP1-1 expression and prognosis that is independent of any interaction between GSTP1-1 and chemotherapy.

GSTP1-1 expression was positively correlated with pathologically-defined biologically-aggressive features in human soft tissue sarcomas [77], again suggesting that GSTP1-1 expression may be a marker for a phenotype associated with poor clinical outcome. This phenotype may have nothing to do the GST-mediated drug-detoxification *per se* — it may be a phenotype more likely to express other drug resistance genes, to invade and metastasize to other sites or to resist chemotherapy through interaction with its local environment.

GST summary

After almost a decade of study, the hypothesis that GSTs participate in a mechanism of resistance that contributes to MDR has not been proven. In fact, the evidence against the hypothesis has become quite substantial. With the exception of mitoxantrone, enzyme-mediated glutathione conjugation of MDR drugs been described. There is no relation between the development of MDR drug resistance and increased GST expression (Table 1), nor is there any relation between the development of increased GST expression and MDR drug resistance (Table 2). Furthermore, in our opinion, GST transfection studies of drug sensitive cell lines have consistently demonstrated that marked increases in GST expression do not translate into meaningful levels of MDR drug resistance (Table 3).

At times, the study of the relationship of GSTs to MDR seems to have taken on a life of its own; studies supporting a positive association between GSTs and MDR have been frequently cited, while contradictory studies have often been ignored. However, the challenge in science is often to discard one hypothesis and embrace another. The literature regarding expression of GSTs in tumors, while not supporting a role for GSTs in MDR, has demonstrated a striking and consistent finding of the elevation of GSTP1-1 expression in many series of tumors relative to normal tissues. These clinical observations may be related to the laboratory observations that induction of pi-class GST expression is a marker of preneoplastic transformation in rat liver hyperplastic nodules, preneoplastic lesions which also express P-glycoprotein [78-80]. Although extrapolation from rat to human should be treated with caution [81], the mechanism by which GSTP1-1 expression increases during malignant transformation may reveal a step in oncogenesis. Increased GSTP1-1 expression has been related to both v-H-*ras* transformation of rat liver epithelial cells [82] and to the presence of Epstein-Barr virus in lymphoma [66]. So far, however, identification of factors which govern the specificity of GSTP1-1 gene expression has proven to be elusive [81,83,84].

Increased GST activity is observed in many models of drug resistance, but it is not known whether this reflects a nonspecific reaction to stress or whether GSTs have specific defensive roles. GSTs may be important factors in alkylating agent resistance, a topic which was not reviewed here. It is possible that GSTs do interact with MDR drugs, but that GST activity must be coupled with other, as yet unknown enzymes for effective MDR drug detoxification. GSTP1-1 expression may also be a marker for certain phenotypes arising from malignant transformation, such as a biologically aggressive phenotype in which GSTP1-1 acts as a prognostic factor, as suggested in nonmetastatic breast cancer. Clearly, a deeper understanding of the role of GSTP1-1 in malignancy must come through exploration of mechanisms that control its expression and of its role in tumor cell biology.

The glutathione redox cycle and MDR

The components of the glutathione redox cycle include the complementary enzymes glutathione peroxidase and glutathione reductase, and the substrate glutathione. Each component may play a

significant role in the detoxification of hazardous organic peroxides. Several laboratories have demonstrated that at least one group of MDR drugs, the anthracyclines, produce these potentially toxic reactive oxygen intermediates: doxorubicin-mediated membrane lipid peroxidation has been demonstrated in liver and heart microsomes [85–87], in mitochondria [88] and in isolated nuclei [89]. These anthracycline-induced free-radicals may produce DNA strand breaks [90–91].

A biochemical role for glutathione peroxidase (GPx) activity has been suggested in the detoxification of anthracyclines. This activity has been associated with the selenium-dependent GPxs as opposed to the selenium-independent organic peroxidase activity of the GSTs [26]. There is substantial evidence that the glutathione peroxidases contribute to cellular defense against anthracycline-mediated cardiac toxicity [reviewed by Doroshow *et al.* in Ref. 93].

However, the evidence that glutathione and GPx play functional roles in tumor cell resistance is murky. On the one hand, there is ample evidence that anthracyclines form toxic free radical intermediates in tumor cells in tissue culture (94-96), and decreased anthracycline-induced free-radical formation has been documented in a multidrug resistant MCF-7 human breast cancer cell [13,97]. On the other hand, increased GPx activity has not been generally associated with MDR. Of 11 cell lines selected for doxorubicin resistance which report GPx activity (Table 1), only two resistant cell lines have 2-fold or greater increases in GPx activity [1,26]. In the case of intrinsic resistance, a comparison of five breast cancer cell lines showed no relation between resistance to doxorubicin and total GPx activity [98]. Finally, although Chinese hamster ovary cells selected for resistance to high oxygen concentrations developed a 2-fold increase in GPx activity and a 4-fold increase in glutathione content, this resistant cell line remained as sensitive as the parental cell line to equal intracellular concentrations of doxorubicin [99].

In the case of vincristine resistance, the story may be different. Both cell lines selected for vincristine resistance in Table 1 have elevated GPx activity [20,33]. These findings may be related to the demonstration that other peroxidases, horseradish peroxidase [100] and myeloperoxidase [101] catalyze the oxidative breakdown of vincristine. The intrinsic resistance of myeloid leukemias to vincristine may be related to the high levels of myeloperoxidase seen in this disease [101].

Analysis of GPx enzyme expression in cell lines and tumors has focused on the measurement of total peroxidase activity, using either cumene hydroperoxide or hydrogen peroxide as substrate. It has become apparent, however, that there are at least four different selenium-dependent glutathione peroxidase isozymes. The nucleotide sequences of these different GPx isozymes are distinctive in that they each utilize an internal UGA "stop" codon to encode the amino acid selenocysteine. The isozymes include the classic cytosolic GPx, hgpx1, for which cDNAs [102,103] and genomic sequences [104] have been isolated and characterized; a second cytosolic isozyme, GSHPx-GI, which is highly expressed in the gastrointestinal tract [105, 106]; plasma glutathione peroxidase, an isozyme originally isolated from human plasma [107] but also found in liver, kidney, heart, lung, breast and placenta tissues [108]; and a phospholipid hydroperoxide glutathione peroxidase [109,110]. In addition, there is evidence that other GPx isozymes may exist [111,112]. Reagents which could be used to distinguish between these isozymes, such as isozyme-specific substrates, molecular probes, and antibodies, are not yet widely available. Few of the studies of MDR cell lines cited in Table 1 examined expression of individual GPx isozymes, so it is possible that significant changes in particular glutathione peroxidase isozyme expression could be hidden within these data. For example, the change in GPx isozyme expression in a multidrug resistant MCF-7 cell line is complex: while total activity in this cell line is increased [1,26], it is associated with an increase in expression of both hgpx1 [98,104] and plasma glutathione peroxidase [108], while phospholipid hydroperoxide glutathione peroxidase activity is actually decreased in the resistant cell line in the presence of supplemental selenium [109].

In the MDR cell lines in which glutathione reductase has been measured, no trend was ob-

served for altered enzyme activity in the resistant cell lines. Of the 11 MDR cell lines in which glutathione reductase activity was measured in Table 1, none had a greater than 2-fold increase in activity, and one cell line had a greater than 50% decrease in activity. Similarly, there was no trend in glutathione content in the resistant cell lines: none of the 22 MDR cell lines in which glutathione levels were measured in Table 1 had a greater than 2-fold increase in bulk glutathione content, and only one of the 21 cell lines had a greater than 50% decrease in glutathione content.

At present, there are no known specific inhibitors of GPxs, and the only known inhibitor of glutathione reductase, BCNU, is often toxic to cells at inhibitory concentrations. Thus, evaluation of the role of the entire glutathione redox cycle has frequently been made by depleting cells of glutathione, and assuming that decreased intracellular glutathione significantly decreases the capacity of the glutathione redox cycle.

Several studies have examined the effects of buthionine sulfoximine (BSO), a γ-glutamyl cysteine synthetase inhibitor which lowers intracellular glutathione (GSH), on anthracycline cytotoxicity. These studies, which are summarized in Table 4 [18,24–26,28,31,32,35,113–119], demonstrate wide variability in their results, which may reflect different methodologies or different intrinsic biological properties of the different cell lines. The effect of BSO on doxorubicin toxicity is summarized by the calculated Dose Modifying Factor (DMF), which is the ratio of doxorubicin IC_{50} values without and with BSO treatment. When the DMF values of BSO in drug resistant derivatives were compared to the parental cell lines they exhibited little or no difference (n = 3), an increase (n = 6), or a decrease (n = 2). In two xenograft models, growth delay was similar between the parental and resistant sublines after treatment with BSO. In addition, there does not appear to be any direct relationship between the degree of BSO-mediated glutathione depletion, relative to untreated cells, and the resulting DMF. This heterogeneity of findings makes it impossible to reach conclusions about the role of the glutathione concentrations in either intrinsic or acquired resistance. In cell lines where BSO appears to have a marked effect, it is not clear whether this is due to direct inhibition of the glutathione-redox cycle, to other effects of glutathione depletion, or to other toxic effects of BSO. For example, although it was suggested that BSO and verapamil acted synergistically in reversing MDR [26], another study demonstrated that BSO actually appeared to reverse MDR by increasing the cytotoxicity of verapamil [120]. Curiously, BSO treatment of both wild type and GST transfected yeast actually increased the resistance of both to doxorubicin [12].

Studies in which GPx levels have been selectively increased have suggested a role for GPx in MDR. Doroshow *et al.* increased intracellular GPx concentrations in drug sensitive cells by scrape loading, a procedure in which the cell membrane of tissue culture cells is disrupted with a plastic scraper in the presence of purified extracellular enzyme [121]. This technique resulted in a 10- to 20-fold increase in glutathione peroxidase activity in drug-sensitive MCF-7 human breast cancer cells, which was associated with an increase in the cloning efficiency of these cells after exposure to certain concentrations of doxorubicin [121]. In a gene transfer study, a cDNA for the hgpx1 GPx isozyme was transfected into T47D human breast cancer cells [122]. Although resistance to MDR drugs was not examined in this study, the transfected cells did become resistant to the quinone, menadione, which is chemically related to the anthracycline quinoid doxorubicin [122]. The determination of the role of GPxs in MDR awaits more definitive transfection studies with genes encoding all the different GPx isozymes.

Summary — glutathione redox cycle

Anthracyclines can exert toxic effects through the generation of dangerous peroxides and the glutathione redox cycle can detoxify these reactive species. Furthermore, there is significant evidence that the glutathione redox cycle is involved in protection from anthracycline-induced cardiac toxicity. Nevertheless, it is not clear from the current studies whether the glutathione redox cycle is actually utilized by malignant cells for protection

Table 4. Effect of BSO on MDR drug sensitivity

Cell line	Origin	Parental		Resistant		Reference
		Fold dec GSH	DMF*	Fold dec GSH	DMF*	
vs. doxorubicin						
P815	mastocytoma	nr	1.2	na	na	Arrick *et al.* [113]
HEp3	buccal ca.	.45	8	na	na	Lee *et al.* [114]
V79	hamster	.50	9	na	na	Russo & Mitchell [115]
A2780	ovary	.33	11.4	.24	1.5-5	Hamilton *et al.* [18]
SW620	colon	.11	1.5	.18	1.3	Lai *et al.* [31]
DLD-1	colon	.07	2.3	.06	1.1	Lai *et al.* [31]
LS180	colon	.19	1.1	.12	3.0	Lai *et al.* [31]
SW620	colon	nr	1.4	nr	1.8	Chao *et al.* [32]
H69	lung	nr	nr	.05	1	Cole *et al.* [24]
8226	myeloma	nr	1	.06	1	Bellamy *et al.* [25]
P388	leukemia	nr	1	nr	2	Kramer *et al.* [26]
P388	leukemia	.06	1.3	.05	5	Nair *et al.* [35]
MCF-7*	breast	nr	1	nr	4.4	Kramer *et al.* [26]
MCF-7*	breast	nr	nr	.23	5–7	Dusre *et al.* [116]
vs. daunorubicin						
HL60	leukemia	.08	.83	.07	17.5	Lutzky *et al.* [28]
vs. cyanomorpholino doxorubicin						
ES-2	ovary	.18	1	.15	3	Lau *et al.* [117]
Xenografts	Origin	Sensitive		Resistant		Reference
		Fold dec GSH	Growth delay	Fold dec GSH	Growth delay	
vs. doxorubicin						
16C	murine mammary adenocarcinoma	.16	1.3	.21	1.5	Lee *et al.* [114]
vs. vincristine						
TE-671	rhabdomyosarcoma	.15	2.1	.05	2.0	Rosenberg *et al.* [119]

DMF — dose modifying factor, the ratio of IC_{50}s for MDR drugs between cells exposed to BSO and cells exposed to MDR drug alone; nr — not reported; na — not applicable.

from anthracycline cytotoxicity. Certainly, overall levels of the components of this system do not consistently change in MDR cell lines, and the effects of BSO on resistant cell lines are highly variable. However, the reagents utilized in the analysis of the glutathione redox cycle have thus far been relatively nonspecific, and thus can not determine whether the relative expression of specific GPx isozymes is an important parameter in MDR. Comprehensive transfection studies with the various GPx isozyme genes should help to illuminate the role of GPxs in MDR.

Similarly, the measurement of overall glutathione levels may also be deceptive in assessment of the role of glutathione in drug detoxification. The ratio of reduced to oxidized glutathione (GSH to the disulfide GSSG) in cytosol and in organelles such as the endoplasmic reticulum may be important factors in its function [123]. Thus, future study of glutathione and its role in MDR may focus more upon qualitative aspects of its metabolism.

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