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Glutathione and Related Enzymes in Multidrug Resistance

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INTRODUCTION

THE DEVELOPMENT of resistance to chemotherapeutic agents, at concentrations which were once effective for treatment, is a major obstacle in the clinical treatment of cancers. Several mechanisms have been described that mediate such resistance: (i) those which afford increased rates of drug efflux from the cell, so that the drug interacts with its target to a reduced extent (e.g. P-glycoprotein [1], multidrug resistance-associated protein (MRP) [2]); (ii) decreased drug sensitivity, either by elevating levels of the target to overcome drug doses, or mutating the target, thus rendering the drug ineffective (e.g. dihydrofolate reductase) [3]; (iii) increased DNA repair mechanisms as a means to reverse cytotoxicity (e.g. O^6-alkylguanine transferase) [4]; and (iv) altered expression of metabolic and detoxification processes that protect the cell against such damage. This latter mechanism may be mediated by the glutathione/glutathione-S-transferase (GSH/GST) detoxification system and is the focus of the discussion below. Many reports have shown that resistance to alkylating agents is associated with increased GSH levels and GST activity.

GSH and its associated enzymes serve a protective role within the cell. GSH is an important intracellular antioxidant and is the most abundant non-protein thiol present in the cell. GSH's conjugation with a compound, either spontaneously, or when catalysed by GST, renders the compound less toxic against cellular targets, and more hydrophilic and thus more readily excretable. The GSH tripeptide (γ-glutamylcysteinylglycine) is synthesised by cells *de novo* via the γ-glutamyl cycle, salvaged by γ-glutamyl transpeptidase (γGT), or recycled through the sequential action of glyoxalase I (gly I) and glyoxalase II (gly II) (Figure 1). In *de novo* synthesis, γ-glutamylcysteine synthase (γGCS), the rate limiting enzyme, catalyses the peptide linkage between the γ-carboxyl group of glutamate and the amino group of cysteine. GSH synthase then catalyses the condensation of the carboxyl group of cysteine with the amino group of glycine to form the tripeptide. GSH salvage through plasma membrane-bound γGT occurs through the transfer of γ-glutamyl group of extracellular GSH to an α-amino group of an acceptor amino acid, releasing cysteinyl-glycine into the cell cytosol while the γ-glutamyl amino acid remains on the extracellular face of the plasma membrane. The cysteinyl-glycine dipeptide can be used to synthesise GSH. Gly I and gly II are integral to the detoxification to methylglyoxal, a byproduct of aerobic glycolysis. Using GSH as a cofactor, gly I catalyses the formation of *S*-D-lactoyl GSH and gly II catalyses the hydrolysis of the thioester bond to generate D-lactate and GSH. GSH that is oxidised to form dimerised GSH (GSSG) by such agents as hydrogen peroxide in response to oxidant stress can be reduced to monomeric GSH by GSH reductase (GR).

A large body of evidence supporting the role of GSTs in drug resistance has come from their overexpression in cell lines made resistant to certain chemotherapeutic drugs. Many resistant cell lines have a correlative increase in expression or activity of GSH or related enzymes (Table 1). L-Buthionine-*S*-sulphoximine (BSO), a γ-glutamylcysteine synthase inhibitor, which depletes cellular GSH levels, has been used in many studies to demonstrate that a reduced GSH pool sensitises cells to drug treatment [12, 17, 20, 25–34]. Cultured cells from a patient's ovarian adenocarcinoma, obtained after treatment and the development of resistance to chlorambucil, cisplatin and 5-fluoruracil, demonstrated an increase in GSH, and in γ-GT, GSH and GSH peroxidase activities [35]. These data should be interpreted cautiously, because they are correlative, and do not determine if resistance is a cause or an effect of the altered expression of GSH and its related enzymes.

In mammals, GSTs are a phase II metabolism multigene family consisting of the cytosolic isozyme classes designated α, π, μ and θ, which are responsible for the conjugation of reduced GSH to a broad range of electrophilic compounds. A fifth microsomal class also exists, but it has not yet been thoroughly characterised with respect to conjugation of anticancer drugs. The cytosolic enzymes exist as monomeric subunits, having catalytic activities as homo- or heterodimers. Although, in many instances, GSH conjugation can proceed spontaneously, GSTs enhance both the rate and extent of the reaction. GSTπ has been found to be overexpressed in a number of solid tumours (Table 2) and thus, where a particular drug is a substrate for this isozyme, such overexpression may result in more rapid detoxification, thereby diminishing the effectiveness of a treatment. A significant amount of evidence has been compiled in recent years demonstrating the involvement of GSH and associated enzymes in drug resistance. Such conclusions are based upon data from cell lines either with acquired or intrinsic resistance to certain chemotherapeutic agents, or established from a clinical specimen, which is resistant. Transfection studies where GSTs

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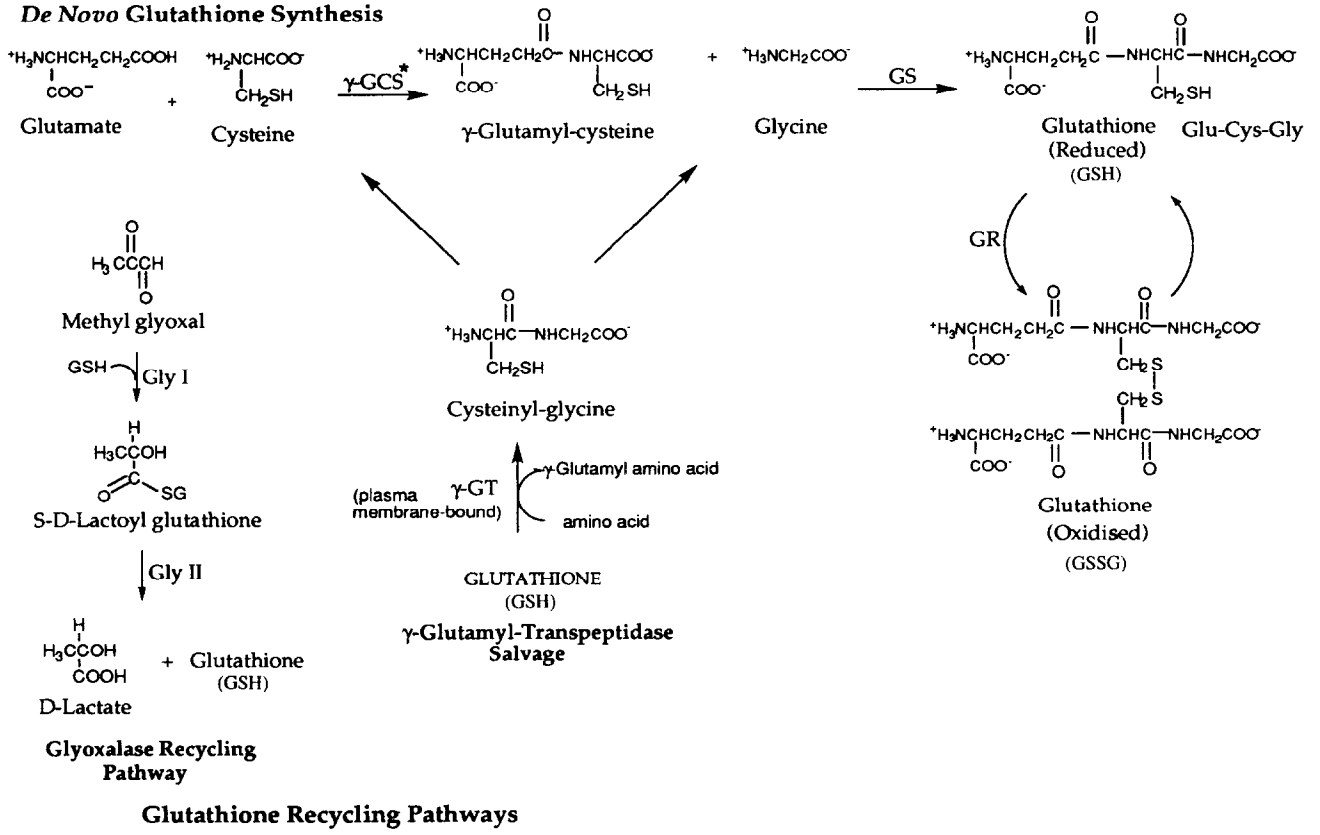

Figure 1. ***De novo*** **synthesis and salvage pathways for glutathione. γ-GCS, γ-glutamylcysteine synthase; GS, glutathione synthase; GR, glutathione reductase; γ-GT, γ-glutamyl-transpeptidase; GSH, glutathione; Gly I, glyoxalase I; Gly II, glyoxalase II. *, rate limiting step and site of BSO inhibition. γ-Glutamyl amino acid remains on extracellular face of plasma membrane.**

have been introduced into naive systems, as well as *in vivo* studies where increased GSH levels and GST activity have been demonstrated in tumour tissue relative to normal tissue, have also provided supportive evidence.

In addition to their GSH conjugation activity, GSTs have organic peroxidase activity. The α isozyme functions in a non-selenium-dependent capacity to reduce organic hydroperoxides [47] and the α and μ isozymes have been shown to be active against 4-hydroxyalkenals, which are produced as a result of free-radical-initiated lipid peroxidation [48]. Base propenals, products of DNA degradation due to hydroxyl radical generation, have been shown to be catalytically conjugated by GSTπ [49]. Because many electrophilic agents produce lipid peroxide byproducts, these reactions may have potential significance to decrease damage induced by anticancer drugs.

GLUTATHIONE-*S*-TRANSFERASE SUBSTRATES

The common characteristic of substrates for GSH-catalysed conjugation to GSH is their electrophilicity. The nucleophilic cysteine of GSH has a pK_a of >9.5. GSTs lower this to approximately 7.0, a more physiologically relevant value to catalyse the formation of a thioether bond with the drug at its electrophilic centre. This electrophilic property is a primary characteristic of most DNA alkylating agents, and many reports have focused on this class of anticancer drugs as substrates for GSTs. Drugs proven to be substrates for GSTs are listed in Table 3. Structures of some of the alkylating agents and a general reaction scheme are illustrated in Figure 2. In general, the α isozyme hase been associated with nitrogen mustard detoxification, and the μ isozyme with nitrosourea detoxification. The increased expression of GSTπ in MDR cell lines made resistant to certain drugs has led to the implicit assumption that it is a causal resistance factor. There is only limited evidence, however, that anticancer drugs are direct substrates for GSTπ, and thus, its increased expression is more likely a consequence of a pleiotropic stress response.

Chlorambucil, melphalan, and cyclophosphamide fall under the nitrogen mustard category of alkylating agents that are substrates for GST-catalysed conjugation. Other substrates for GSTs include (1,3-bis(2-chloroethyl)-1-nitrosourea) BCNU [56, 57], a nitrosourea, and thiotepa and its active metabolite tepa [58], a trifunctional alkylating agent containing three aziridine moieties co-ordinated to a phosphorous atom. Prior to DNA alkylation, these compounds form an aziridinium ion, which is a strong electrophile and believed to be the actual GST substrate for GSH conjugation [55, 58, 61]. Several alkylating agents, because they have the potential to form more than one aziridinium ion intermediate, are said to have multiple functionality. Bifunctional mustards are 10-fold more cytotoxic compared to monofunctional compounds on the basis of their ability to cross-link nucleic acids. Conjugation of an aziridinium ion to GSH removes this bifunctionality, in effect reducing the drug's potency by one log.

TRANSFECTION DATA

Perhaps the most compelling evidence for a protein's role in a biological event comes from transfection studies. Results

Table 1. Altered GSH metabolising enzymes in drug resistant cells

Drug	Cell line	GST activity	Increased isozyme expression	Other altered expression	[Ref.]
Doxorubicin	MCF-7 human breast	+	π		[5, 6]
		+	π, but decreased μ		[7]
	FLC mouse leukaemia	+	α		[8]
BCNU	9L2 rat gliosarcoma	−	μ, but decreased π		[9]
	G3361 human melanoma	+	π		[10]
CDNB	NCI H322 human lung	+	π		[11]
			α		
	SW620 human colon	+	π	+GSH	[12]
Chlorambucil	Walker 256 rat mammary	+	α		[13, 14]
	CHO hamster ovarian	+	α		[15]
Cisplatin	CHO hamster ovarian	+	π		[10, 16]
	NOS2 human ovarian	+	π	− γ-GT activity	[17]
	Human ovarian	Unaffected	n.d.	+GSH +γ-GCS +γ-GT	[1]
Cyclophosphamide	G3361 human melanoma	+	π		[10]
	Yoshida rat sarcoma	+	n.d.	+GSH	[18]
4HC	MCF-7 human breast	+	No change	+GSH	[19]
Ethacrynic Acid	HT29 human colon	+	π	+GSH	[20]
	MCF-7 human breast	n.d.	π		[21]
			μ		
Melphalan	HS-Sultan human myeloma	Unaffected	π		[22]
	G3361 human melanoma	+	π		[10]
Mitomycin C	J82 human bladder	+	n.d.		[23]
Vincristine	MCF-7 human breast	+	π, but decreased μ		[7]

Increased activity or expression is represented by (+) symbol; decreased activity or expression is represented by (−) symbol. BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CDNB, 1-chloro-2,4-dinitrobenzene, a generic GST substrate commonly used in spectrophotometric assays to assess GST activity; n.d., not determined.

Table 2. Altered GSH metabolising enzymes in various tumour types

Tumour type	GST activity	Increased isozyme expression	Other altered expression	[Ref.]
Ovarian		π		[35, 36]
	+			[37]
	+		+GSH	[38]
			+GSH Px activity	
Breast	+		+GSH	[38]
			+GSH Px activity	
	+			[39]
Lymphocytes				
ALL		π		[40]
CLL-chlorambucil resistant	+			[41]
Bladder			+GSH Px activity	[42]
			+Catalase activity	
Lung				
Mixed histologies-cisplatin resistant			+GR	[44]
			+GSH Px activity	
Non-small cell lung carcinoma	+		+GSH	[43]
			+GSH Px activity	
Colorectal	+	π		[45]
	+			[46]

GSH Px, glutathione peroxidase; GR, glutathione reductase. Elevated enzyme activity represented by (+) symbol.

Table 3. Known anticancer drugs and metabolites that are GST substrates

Drug	[Ref.]
Chlorambucil	[50–52]
Melphalan	[53, 54]
Cyclophosphamide	[55]
Acrolein	[55]
BCNU	[56, 57]
Thiotepa	[58]
Ethacrynic Acid	[55]
Base Propenals	[59]
Hydroxyalkenals	[59]
Hydroperoxides	[60]

BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea. Hydroxyalkenals, base propenals, and DNA hydroxyperoxides are generated from DNA free radical damage.

obtained for several GSTs in a number of cell lines have provided evidence that overexpression of GSTs confers a level of resistance to a broad range of primarily alkylating drugs.

Human GSTπ and human GSTα transfected into *S. cerevisiae* [62] resulted in a significant reduction in the cytotoxic effects of chlorambucil and doxorubicin, a member of the anthracycline family of antibiotic anticancer drugs. Maximal resistance in the yeast system was 8-fold for chlorambucil and 16-fold for doxorubicin. Although doxorubicin has not been shown to be a substrate for GST, or to form conjugates with GSH, resistance may be a consequence of protection from indirect doxorubicin damage. Metabolism of doxorubicin generates free radicals that can lead to DNA or lipid peroxides. The peroxidase activity of GSTs could serve to reduce these peroxides and conjugate any metabolites resulting from lipid or DNA peroxidation.

Human GSTπ and GSTα have also been transfected into Chinese hamster ovary (CHO) cells. GSTπ was found to increase resistance to cisplatin and carboplatin 2–3-fold [63] while GSTα dose-dependently increased resistance to bleomycin [64], a drug that causes scission of DNA by an oxidative process of generating hydroxyl radicals [49]. Although they are known to form conjugates with GSH, neither the platinum drugs, nor bleomycin have been shown to be GST substrates. The enzymes may be acting in some way to sequester free drug, or its metabolites, either through non-product binding of the drugs to the enzyme by hydrophobic interactions, or by some other mechanism. If this were the case, increased enzyme concentrations in the transfected CHO cells could serve as a sink for drug binding.

Transfection of *GSTA2* into NIH3T3 mouse fibroblasts conferred 5.8- and 10.8-fold increased resistance to chlorambucil and mechlorethamine [66]. A MatB rat mammary carcinoma cell line also transfected with rat *GSTA2* resulted in a 6–12-fold resistance to melphalan, 10–16-fold resistance to mechlorethamine, and 7–30-fold resistance to chlorambucil [65]. Late passage cells (14 months post transfection) have demonstrated both a diminished enzyme expression, and resistance as assayed by drug cytotoxicity. In addition, mechlorethamine-induced DNA cross-links have been found to decrease in a time-dependent manner following transfection of the *GSTA2* subunits. These studies show not only the correlation of enzyme expression with resistance, but also the correlation of increased enzyme with inhibition of drug activity.

NIH3T3 cells transfected with human *GSTP1* demonstrated resistance to doxorubicin and ethacrynic acid, a known conjugator of GSH and inhibitor of GSTs, but failed to confer resistance to cisplatin, melphalan, and chlorambucil [67]. The fact that resistance to these alkylating agents was not detected is not surprising given the fact that GSTπ has a low K_m/K_{cat} value for such alkylating agents [69]. The low-level resistance afforded to doxorubicin may be a result of increased detoxification of lipid peroxide degradation products [70, 71].

Not all studies of transfected cells have yielded positive data. MCF-7 breast carcinoma cells transfected with human *GSTA2* and genes encoding human GSTπ isozymes showed an increase in GST activity, but no resistance to doxorubicin, cisplatin, melphalan, chlorambucil, BCNU or CDNB [68]. MCF-7 cells have the lowest intrinsic GST activity of the 60 cell lines in the National Cancer Institute's human cell line panel [72]. They also have low GSH levels. It is possible that these cells do not utilise GSH pathways in a manner similar to other human cells. Even in the presence of elevated GSTs, such as the transfected system, a reduced pool of GSH may limit both conjugate formation and protection.

TRANSPORT OF GSH AND ITS CONJUGATES

It is important for the cell to eliminate conjugates efficiently, since many GS–drug conjugates are potential inhibitors of GSTs and other GSH-associated enzymes. In addition, the conjugates may retain some cytotoxicity, so their efficient removal may be beneficial for the survival of the cell. As with many important biological functions, there is a redundancy in function for conjugate removal, and GSH conjugates have been shown to be transported by several systems.

A Na^+-dependent transporter was originally described by Lash and Jones in the mid-1980s [73]. It is localised in the basolateral membrane of the kidney and intestine, and has been shown to transport γ-glutamyl compounds such as the reduced and oxidised form of glutathione, as well as probonecid, an agent used as an inhibitor of tubular secretion [74]. Another GSH transporter has been identified that is Na^+-independent. It is also a carrier of both reduced and oxidised glutathione and it is localised to the brush borders of the intestine and the basolateral and cannicular membranes of liver cells [75, 77].

More recently, an efflux system has been described for GSH/conjugate removal, the ATP-dependent GSH-xenobiotic (GSH-X) pump. Unlike the Na^+-dependent and independent transporters described above, it is found in various organs and cell types [78]. This pump exhibits a broad substrate specificity, and because of its transport activity towards anionic amphiphilic compounds, it is also known as the multispecific organic anion transporter [79]. There is increasing evidence that the multidrug resistance-associated protein (MRP), which is overexpressed in tumour cells resistant to anticancer drugs, may be the same protein as the GSH-X pump. MRP is a member of the ATP-binding cassette-transporter gene family and has been shown to mediate extracellular transport of various natural product drugs such as anthracyclines, vinca alkaloids, and epipodophylotoxins in numerous cell lines, as well as being found to confer a multidrug resistance phenotype in cells transfected with the MRP gene [80, 81].

Recently, ATP-dependent transport has been identified for

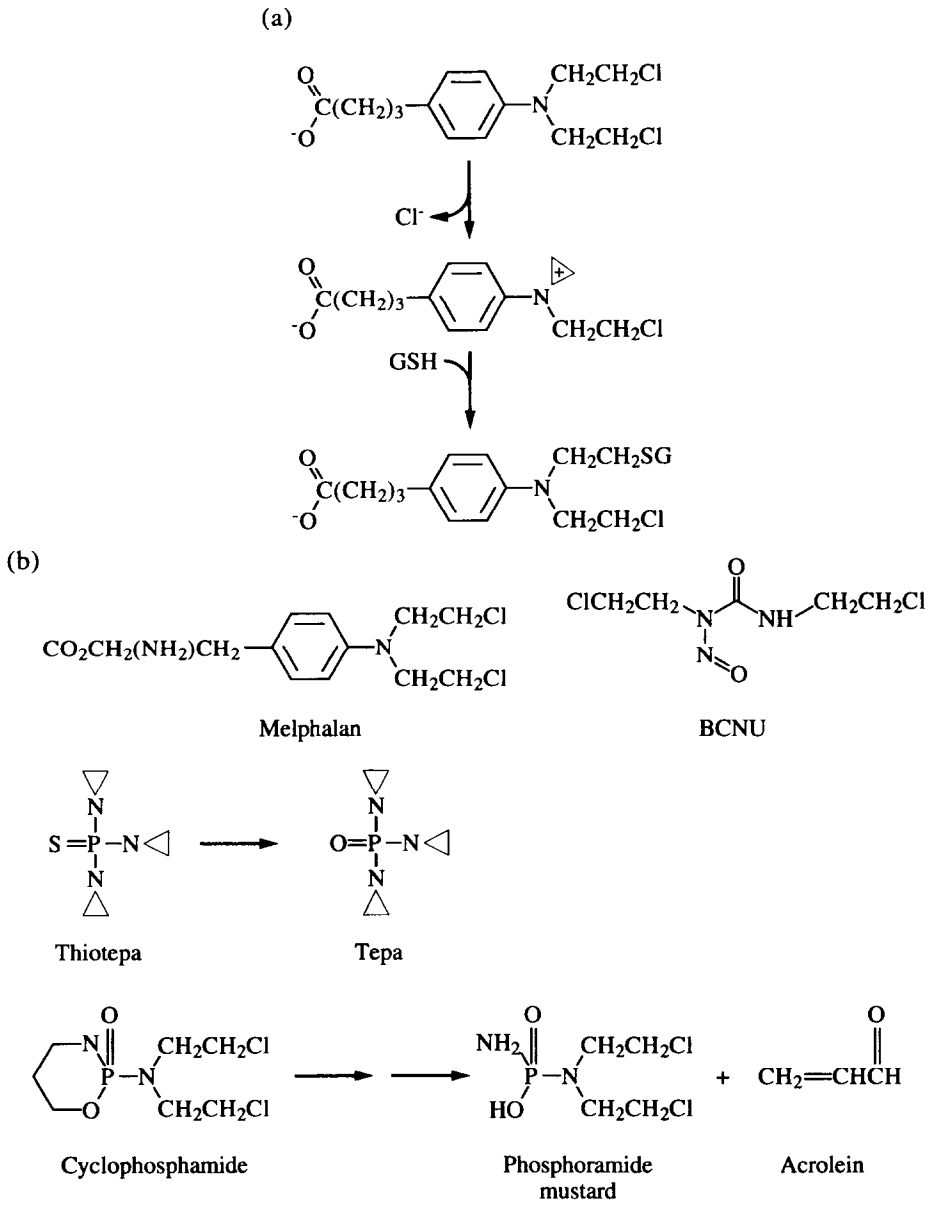

Figure 2. (a) Example of chlorambucil conjugation with GSH by GSTα. Loss of a chlorine atom causes the formation of an aziridinium ion which forms a thioether bond with GSH. (b) Alkylating agents which are known to conjugate with GSH. BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea. Cyclophosphamide is metabolised to phosphoramide mustard and acrolein, degradation products of aldophosphamide. Tepa is a metabolite of thiotepa.

platinum–GSH complexes, *S*-(2,4-dinitrophenyl)GSH, and LTC_4, an endogenous substrate for GSH conjugation and transport, in several cell preparations [80, 82–84]. The evidence that these compounds are transported by MRP is compelling. First, the rates of transport have been correlated with the level of expression of MRP [80]. Second, the transport is inhibited by other GSH-X substrates. Third, transfection of MRP into an intrinsically low MRP-expressing cell line resulted in a dramatic increase in GSH conjugate transport [82, 85].

In addition, MRP has been labelled with a specific photoaffinity analogue of GSH [86]. A number of GSH conjugates and several natural product drugs such as doxorubicin, etoposide and vinblastine compete with MRP for this labelling. The fate of monochlorobimane (MCB), a known conjugator of GSH which fluoresces upon conjugation [87], was followed after exposure of the compound in MRP-overexpressing NIH3T3 cells. The MRP-bearing cells exhibited an increased conjugate efflux compared to control cells. The transfected cells also displayed an altered intracellular pattern of MCB–GSH fluorescence, initially characterised by a migration to perinuclear structures and later an intracellular scattering, which may suggest vesicular sequestration. It is interesting to note that this same pattern of distribution was observed in studies of the fate of daunorubicin in MRP transfectants [88]. These data demonstrate not only an affinity of GSH conjugates for MRP, but also correlate the presence of a GSH conjugate to areas of the cell where MRP has been localised.

Because MRP is usually associated with resistance to natural product drugs, and not with resistance to alkylating agents, the efflux by MRP described above is presumably not the only mechanism by which MRP exerts its multidrug resistance

phenotype, and there is very likely a GSH component of MRP drug efflux. This point is demonstrated by the fact that in studies of cell lines overexpressing MRP, depletion of GSH levels reverses the resistance phenotypes previously observed [29, 31, 89]. In addition, the photoaffinity analogue of GSH competed with natural product drugs that were in an unconjugated state, providing further evidence that MRP may transport both GSH conjugates and non-conjugated anticancer drugs [86, 90].

REGULATION AND INDUCTION OF GLUTATHIONE-*S*-TRANSFERASES

The regulation of GSTs and their induction by various chemicals is a complex and evolving area of research. Multiple factors affect the induction/regulation of these genes. These can include age-, sex-, tissue- and species-specific considerations. The mechanisms by which xenobiotics induce transcription of GSTs has been elucidated by work in rodents, specifically with the rat *GSTA2* gene. Those chemicals that have been identified as inducers of GSTs and other phase II enzymes are diverse. (For an excellent review of these inducers see Hayes and Pulford [91].) However, the inducers can be categorised into four groups that act in distinct manners. These are (1) planar aromatic compounds, (2) phenolic oxidants and Michael acceptors, (3) barbiturates, and (4) glucocorticoids.

Planar aromatic compounds, such as polycyclic aromatic hydrocarbons (PAH), have been designated as bifunctional because they induce both phase I and phase II enzymes [92]. Induction by PAH occurs through the xenobiotic response element (XRE) and is Ah receptor dependent. The XRE was initially described for the inducible phase I *CYP* genes [93–95]. The XRE described in the promotor region of the rat *GSTA2* gene is identical to that in the *CYP1A1* gene [96]. PAH is believed to bind to the Ah receptor present in the cytosol of the cell, causing its dissociation from its HSP 90 chaperone. The ligand–Ah receptor complex displays a higher DNA-binding affinity than its HSP 90 associated form, and interacts with the XRE present in the promotor region of the gene causing its inducible expression [97, 98]. Much of this pathway is derived from knowledge of, and similarity to, XRE-mediated PAH induction of the *CYP1A1* gene. In addition to acting in a direct manner to induce gene expression through interaction with the XRE, a model has been proposed whereby PAH indirectly induces phase II enzymes through induction of the *CYP1* genes [92, 99]. The newly upregulated phase I enzymes can oxidise the inducing compound, creating an electrophilic centre. This may then further enhance the propensity of the agent to enact antioxidant response element (ARE)-mediated induction.

The ARE, a second *cis*-acting regulatory element identified in the rat *GSTA2* gene, mediates the induction of the gene by monofunctional inducers (those compounds that induce phase II enzymes only) [96, 100, 101]. These inducers are either electrophilic Michael acceptors or are metabolised to compounds that become Michael acceptors [92]. In contrast to XRE, ARE-mediated enzyme induction is Ah receptor independent. The ARE located −722 to −682 bp downstream of the transcriptional start site of rat *GSTA2* is responsible for basal as well as inducible activity of the gene, and is very similar to an element called the electrophile response element (EpRE) identified in the mouse *GSTA1* gene [94]. The two sequences, which are 41 bp in length, differ only by two nucleotides. The result of this slight difference is that the EpRE contains two ARE sequences adjacent to one another. The extra enhancer in the EpRE causes the gene to be more responsive to induction by several compounds [91]. It is believed that *trans*-acting factors that bind to the AP-1 sequence (also called the TRE for TPA response element) may also interact with the ARE owing to the high degree of homology of these sequences.

Other GSH homeostasis genes are known to contain AP-1 sites in their promotor regions. These include the genes that encode for rat and human GSTπ, and human γ-GCS. The rat *GSTP1* gene contains one AP-1 site in an enhancer region 2.5 kb upstream from the transcription start site, and another in the proximal promotor region, which regulates basal expression of the gene [102–104]. The human *GSTP1* gene also contains two AP-1 recognition sequences. The first is located −69 to −63 bp upstream from the transcriptional start site [105, 106] and a second site +35 to +41 bp, which lies within a region of the gene that is essential for maximal basal activity of the promoter [107]. Mulcahy and Gipp have recently cloned the 5′-flanking region of the γ-*GCS* gene, and found it to contain a putative ARE, as well as other AP-1 sequence-recognition sites [108]. Similarly, Yao and associates have cloned this region of the human γ-*GCS* gene and identified various AP-1 sites that were shown to have activity by gel shift assays [109].

NF-κB, a member of the Rel family of transcriptional activator proteins, represents another possible mechanism of regulating cellular GSH homeostasis and enzyme induction in a chemically or oxidatively stressful environment [110]. Agents such as peroxides and phorbol esters activate NF-κB by causing its dissociation from inhibitory I-κB subunits [111]. Putative sites for NF-κB binding have been identified in the 5′-upstream sequences of genes involved in GSH homeostasis (γ-*GCS*) and inducible xenobiotic metabolising enzymes (NAD(P)H quinone oxidoreductase [109, 112].) Expression of GST has also been found to be under the control of NF-κB. NF-κB was reported to serve in a negative regulatory capacity for the human *GSTP1* gene [113]. The negative regulation may occur because of the NF-κB recognition sequence's close proximity to the AP-1 response element located at base-pair position −69. Whether any local protein or conformational interactions occur has not been determined.

Gene methylation may also serve to regulate expression of the GSTs. Decreased expression of GSTπ in prostate carcinomas was found to be due to hypermethylation of the *hGSTP1* gene [114]. Gene hypermethylation was observed in all 20 carcinoma specimens studied, and immunohistochemical staining with antiserum against GSTP1 did not detect the enzyme in 88 of 91 prostate carcinomas analysed. Since GSTP1 is frequently found to be overexpressed in other types of tumours, the anomaly of the prostate finding may prove to be sufficient to the aetiology to the disease.

GENETIC POLYMORPHISMS

Over the last two decades, it has become increasingly apparent that individuals differ in their capacity to handle environmental and chemical challenges. Genetic polymorphisms present among populations and individuals in drug metabolising enzymes may determine the risk of developing diseases, depending on ability to process and detoxify carcinogens, as well as the capacity to respond to drug treatment. Humans

are especially likely to encounter small lipophilic carcinogenic chemicals through dietary intake of fresh water invertebrates or fish from contaminated waters, or from livestock exposed to growth-modulating chemicals, which may accumulate in and be slowly released from fatty tissues. Humans also expose themselves to carcinogens through lifestyle choices such as dietary consumption of alcohol, over-cooked or preserved food and smoking. These chemicals can undergo phase I and phase II metabolism for detoxification and clearance. GSH and GSTs, along with other drug metabolising enzymes, are involved in these processes.

Recently, human polymorphisms for the class θ and μ GSTs have been described. The first genetic polymorphism described was for GSTμ [115]. Subsequent studies have shown that the polymorphism was due either to a gene deletion or to specific allelic variation resulting in a catalytically active enzyme with altered charge properties. Three common alleles have been described at the locus for human *GSTM1*, designated *GSTSM1*A, GSTM1*B* and *GSTM1*0* which give rise to the GSTM1A, GSTM1B or the GSTM1 null phenotypes, respectively. The frequency of *GSTM1* alleles shows significant inter-ethnic variation. For example, *GSTM1*0* homozygosity occurs in approximately 45% of Western Europeans, 58% of Chinese and only 22% of Nigerians [116]. In certain Polynesian populations, there is a greater than 90% incidence of the GSTM1 null phenotype [117].

Because GSTM1A-1A and GSTM1B-1B have substrate specificity towards epoxides that results from cigarette smoke, a good deal of attention has been focused on the relevance of the *GSTM1* locus in susceptibility to lung cancer. DNA-adduct formation following exposure to PAH has been found to be higher in cells taken from GSTM1 null phenotype individuals [118], and although there are conflicting data, some groups have reported that the GSTM1 null phenotype is consistently associated with a high susceptibility to developing lung cancer [119]. There is also some evidence that the null phenotype may be associated with other types of cancer. For example, squamous cell carcinoma of the lung has been found in 62% of patients with the GSTM1/0 phenotype [120].

This same genotype has also been linked to the risk of developing bladder cancer, where epidemiological studies have reported that 85% of 53 patients with bladder cancer were GSTM1*0/0 compared with 53–60% in controls [121]. Also, analysis of epidemiological data from various parts of the world has led to the proposition that the GSTM1 null phenotype may be a causative factor in 17% of bladder cancer cases [122]. Similar approaches have suggested that the null phenotype may prove to be a determinant factor in the development of skin cancer and adenocarcinoma of the colon or stomach [123]. In addition to epidemiological analysis of disease susceptibility, there is also some indication that the GSTM1 null phenotype may influence the metabolism of some anticancer drugs and thereby, the response to chemotherapy. Only nitrosoureas have been shown to be direct substrates for the GSTμ family of isozymes, with the catalysed denitrosation resulting in the detoxification of the active therapeutic alkylating species.

In a single clinical trial, the GSTM1 null phenotype appeared to correlate with event-free survival in children with acute lymphoblastic leukaemia (ALL) [124], demonstrating that a null phenotype may be beneficial in certain instances. In 71 ALL patients, 62% were GSTM1 null, of which 82% remained in remission. These authors suggested that possession of either *GSTM1*A* and *GSTM1*B* alleles led to an approximate 3-fold increased risk of relapse. Since none of the drugs used to treat these patients have yet been shown to be substrates for GSTμ isozymes, it is conjectural whether there is a direct cause/effect relationship in this analysis. Whether the μ null phenotype conveys some other gene deficiency has not yet been determined. However, this correlation is interesting and follow-up studies may prove to be enlightening.

A null polymorphism has also been described for the class θ GST at the T1 locus. The frequency of *GSTT1*0* homozygosity also varies amongst ethnic populations, occuring in approximatly 14% of Western Europeans, 38% of Nigerians and 32% of West Indians [116]. When human blood samples were tested for their capacity to conjugate GSH to methylene chloride, a widely distributed industrial solvent which until recently was employed to decaffeinate coffee, a distinct heterogeneity was found, suggestive of a polymorphic expression of the enzyme responsible for the catalytic formation of *S*-chloromethylglutathione [125–127]. In the Ames assay, a rat θ class isozyme, rGSTT1-1, was shown to carry out the conjugation reactions for dibromomethane, ethylene dibromide and with less efficiency, methylene chloride [128]. If the human θ class isozymes are also responsible for these reactions, the previously described θ polymorphism could be important in determining sensitivity to toxification of such halogenated alkanes.

To date, GST θ has not been associated with the direct catalytic detoxification of any of the known anticancer drugs. The overall importance of the θ null phenotype with respect to disease susceptibility for therapeutic response will require further study.

Only limited information about polymorphisms of enzymes involved in the *de novo* or salvage synthesis of GSH is presently available. The glyoxalases (I and II) are responsible for the detoxification and conversion of methylglyoxal to GSH and D-lactate (Figure 1). The gene for glyoxalase I has two alleles, *GLO1* and *GLO2*, which are autosomally inherited in a codominant manner. Recently, the difference between these two alleles has been identified as a single point mutation at amino acid position 111 [129]. This polymorphism appears to be without clinical manifestations since both isoforms have catalytic activity. Although glyoxalases have been implicated in disease states such as *diabetes mellitus*, there is presently no strong indication that polymorphism(s) may be involved with this disease or with any type of cancer. Although a number of methylglyoxal analogues have been tested as anticancer drugs, none have so far made a clinical impact. Thus, the importance of the glyoxalase system in drug response is not yet fully defined [130].

Recent cloning of the catalytic and regulatory subunits of γ-GCS will undoubtedly provide opportunity for the study of potential polymorphisms for these genes. The balance of the salvage enzymes represented by γ-glutamyl transpeptidase, GSH reductase, GSH peroxidase(s) and the glyoxylase system could prove to be an important area of study. The capacity for a particular individual to balance *de novo* and salvage synthesis of GSH may ultimately predict the importance of such potential polymorphisms to therapeutic response to a wide range of electrophile producing anticancer drugs.

IN VIVO AND CLINICAL IMPLICATIONS

Many tumours of leukaemic, ovarian, breast, lung, bladder and colorectal origin have been reported to have increases in

GSH, GST or related enzymes as compared to normal tissues. These are listed in Table 2. An increase in GST activity has been seen in all of these samples, with GSTπ being the most predominant of the isozymes. This correlates with the discovery that 58/60 tumour cell lines, used by the NCI for drug screening purposes, were found to have GSTπ as the most prominent enzyme (Table 4) [72]. Surprisingly, GSTα was expressed only at marginally measurable levels in these cell lines, yet it is a common isozyme in many tumour biopsies [131]. Therefore, the establishment of an immortalised cell line from such tumours as ovarian, colon and prostate, where GSTα levels have been shown to be high, must result in a significant downregulation of the isozyme. This may be a consideration when using cell lines for drug screening. If the detoxification enzymes are not present in the model system tested, the value of the screening results may be compromised.

Table 4. Quantification of normalised mRNA values in cell lines of NCI drug screening programme

Cell line	GSTπ	GSTμ	GSTα	γGCS	Gly-I	GSH
Lung						
A549/ATCC	24.3	0.7	1.0	2.5	2.7	3.6
EKVX	99.9	1.1	0.7	0.9	2.9	6.7
NCI-H522	22.1	1.7	–	0.9	45.2	19.5
HOP-92	14.3	3.8	–	0.8	5.1	8.3
Ovarian						
OVCAR-4	104.4	3.6	0.5	1.7	12.6	13.2
OVCAR-3	21.7	3.0	–	0.7	7.1	9.3
SK-OV-3	26.9	1.0	–	4.3	7.0	13.4
CNS						
SNB 19	34.5	9.9	–	0.8	4.7	8.7
SF-295	12.2	3.1	–	2.8	1.2	13.6
SF-539	7.5	2.2	–	3.1	8.6	13.7
Leukaemia						
K562	26.5	0.9	–	0.3	3.9	25.6
MOLT-4	8.0	4.0	–	0.8	2.5	33.0
HL-60	9.2	0.4	–	0.5	3.2	7
Prostate						
PC3	59.0	1.8	–	0.6	2.1	15.6
DU-145	4.2	3.1	–	3.2	3.6	24
Renal						
ACHN	20.6	1.4	–	0.4	2.6	2.4
UO-31	23.7	2.6	–	0.3	3.2	10.3
786-O	2.1	2.3	0.5	0.5	5.6	0.8
Melanoma						
MALME-3M	30.4	7.2	–	0.9	6.9	10.8
SK-MEL-5	18.9	4.2	0.4	0.9	9.3	33.9
SK-MEL-2	12.0	5.1	–	0.7	12.5	18.6
Breast						
MCF7/ADR-RES	16.8			0.3	2.2	5.1
MCF-7	1.8	0.6	–	0.4	1.3	23.6
T-47D	1.8	2.3	–	1.2	8.1	14.4
Colon						
HCT 15	50.0	1.9	–	0.5	3.0	6.7
HT-29	26.0	–	–	5.1	2.8	7.1
HCT 116	37.6	0.5	–	0.6	2.2	9.4

This is a representative sample of the data from the 60 cell lines analysed. Transcript expression is normalised against the housekeeping gene *36B4*, human oestradiol-independent human acidic ribosomal phosphoprotein PO. (–) designates no measurable transcript levels. Data taken from Tew and associates [72].

Buser and associates, who studied breast and ovarian carcinomas, and Oberli-Schrammli and associates, who studied small and non-small cell lung carcinomas, found significant increases in GSH levels and GSH peroxidase activity in addition to increased GSH activity [38, 42]. Higher intracellular levels of GSH enhance non-enzymatic conjugation rates [132]. This may impart an increased resistance to drugs which conjugate with GSH, even if not a substrate for enzymatic conjugation.

Modulators of GSH levels and of GST enzyme activity are being tested in the clinic as a means of controlling these factors. One such agent is BSO, which inhibits γ-GCS and serves to deplete cellular GSH levels. In the early 1980s, administration of BSO was found to sensitise resistant leukaemias to phenylalanine mustard [133, 134]. Later studies found that co-administration of BSO with melphalan increased the life span of B16 melanoma-bearing mice 1.7-fold [135]. This drug has undergone clinical trials and has been shown to decrease GSH levels in a combination treatment with melphalan [136, 144]. Another sulphur-based compound, sulphasalazine, is also being characterised as a potentially useful modulator of GST activity. This drug is commonly used in the treatment of ulcerative colitis, and only recently has its inhibitory functions of the cytosolic GSTs been discovered and exploited [138]. A promising *in vitro* study demonstrated sulphasalazine's ability to competitively inhibit GSTπ and synergistically enhance cisplatin's cytotoxicity in two small-cell lung cancer cell lines [139]. A recent report, evaluating its efficacy in combination therapy with melphalan in previously treated patients with advanced cancer of differing histology, demonstrated a partial response in 2/4 ovarian patients [140]. Further definitive trials will be required.

Ethacrynic acid is a diuretic agent which has been shown to conjugate with GSH [141] as well as acting as an inhibitor of all three major classes of cytosolic GSTs [142, 143]. It enhances the cytotoxicity of alkylating agents at physiologically relevant concentrations (chlorambucil, melphalan and BCNU in previously resistant cells [144–145]). In the clinic, ethacrynic acid administration significantly reduced the clearance of thiotepa, and in one case study reversed chlorambucil resistance in a CLL (chronic lymphocytic leukaemia) patient [146]. The dose limiting toxicities of ethacrynic acid were attributable to its diuretic properties.

Other strategies are being developed to take advantage of increased GSH/GST activities found in some tumours. For example, since many solid tumours have elevated levels of GSTπ compared with their normal tissue of origin, prodrugs have been designed that are activated by GSTπ. Ter 286 is one such drug. Activation by GSTπ results in proton abstraction by the tyrosine in the active site of the enzyme, thus releasing an active chloroethylating species. Since the drug is inactive prior to GST-mediated cleavage, it is believed that an enhanced therapeutic index may be accomplished. In addition, GSH analogues, which will competitively target specific isoforms of the enzyme, thus reducing their GSH conjugating activities within the cell, are also under development.

CONCLUSION

GSH and its related synthetic and conjugating enzymes play a crucial role in cellular protection against chemical and

oxidative stress. Chemotherapy is an extreme form of such stress. It is not surprising then that the cell has adapted to such cytotoxic insults by altering expression and functioning of detoxification pathways that involve this tripeptide. The many components of these pathways make understanding their interactions in a resistance setting complex and sometimes daunting. In addition to an increased requirement for GSH, which necessitates an increase in either its synthesis or recycling, there is a need for co-ordinate enhancement of the proper transferases, and the activity of efflux pumps such as the GSH-X/MRP pump. The involvement of all of these systems interacting in a very precise manner may be a prerequisite for a resistant phenotype. Studies of tumours and resistant or transfected cells have yielded information and inferences as to the roles that each component may have in the resistant phenotype, but the picture is not yet complete. Further work in defining these interrelated processes will undoubtedly provide an increased understanding of this form of drug resistance and potential targets for the treatment of cancer.

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