Glutathione and the response of malignant cells to chemotherapy

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In the past, the role of glutathione (GSH) in the production of drug resistance was assumed to be limited to electrophilic agents such as the bifunctional alkylating agents and cisplatin. Recently, however, evidence has emerged that links GSH homeostasis with regulation of the multidrug-resistance-related protein and the control of apoptotic cell death. These findings open up new avenues for the discovery of drugs that augment anticancer agents already n use or that have cytotoxic potential in their own right

Although glutathione (γ-glutamylcysteinylglycine, GSH) was first discovered over 100 years ago, determination of the correct structure of the tripeptide (Figure 1) was not completed until 1935 (Ref. 1). It is now known that GSH is the most abundant nonprotein intracellular thiol and that it is present in concentrations of 1–5 mM in most of the cell types analysed[2]. Three distinct intracellular pools of GSH have been described: cytosolic, nuclear and mitochondrial. Of these, homeostasis of GSH within the cytosolic and mitochondrial pools is best characterized.

GSH is synthesized within the cytosol by a two-step, energy-dependent process. The enzyme γ-glutamylcysteine synthetase catalyses the formation of γ-glutamylcysteine from glutamate and cysteine. Glutathione synthetase catalyses a further peptide linkage with glycine to form the tripeptide. GSH may be depleted in the cytosol by oxidation or export across the cell membrane. Oxidation of GSH results in the formation of glutathione disulphide (GSSG), but this is rapidly returned to the reduced state by glutathione reductase, thus maintaining the GSH:GSSG ratio at around 99:1. The glutamate from GSH exported from the cell is salvaged by the action of the membrane-bound enzyme γ-glutamyltranspeptidase, which transfers the γ-glutamyl group to the α-amino group of an acceptor amino acid. The residual cysteinylglycine may also pass back into the cell and be available to re-enter the GSH synthetic pathway[3]. The GSH levels within the nucleus and mitochondria appear to be regulated independently from the cytosolic compartment. This is clearly demonstrated by comparing studies using buthionine sulphoximine (BSO – a potent inhibitor of γ-glutamylcysteine synthetase) and BCNU [1,3-bis(2-chloroethyl)-1-nitrosourea – an inhibitor of glutathione reductase] to lower GSH levels in the cytosol and nucleus. BSO can effectively diminish total cellular GSH by 90% and has been shown to potentiate the cytotoxicity of the bifunctional alkylating agents melphalan and chlorambucil. However, a more detailed study has revealed that both nuclear and mitochondrial GSH pools are relatively resistant to BSO-mediated GSH depletion[4]. BSO depletes the cytosolic pool within 8 h, compared with 24 h for the nuclear pool. Conversely, a study of a melanoma cell line demonstrates rapid depletion of both nuclear and cytosolic GSH by BCNU, resulting in enhanced sensitivity to the topoisomerase II inhibitor adriamycin (doxorubicin), a drug that is known also to generate oxygen free radicals[5]. BCNU selectively inhibits glutathione reductase, preventing reduction of GSSG and resulting in GSH depletion in the presence of oxidative stress. Recovery of GSH levels occurs six times slower in the nucleus than in the cytosol. Notably, the enhancement of melphalan cytotoxicity by the combination

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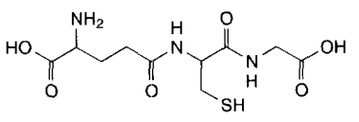

Figure 1. *Structure of glutathione (γ-glutamylcysteinyl-glycine).*

of adriamycin and BCNU is up to ten times greater than the enhancement produced by BSO. The differences in the kinetics of GSH homeostasis in the nuclear compartment could provide a useful target for increasing the effectiveness of genotoxic drugs.

Control of GSH levels in mitochondria also appears to be distinct from the cytosolic pool. Mitochondria lack the enzymes required to synthesize GSH *de novo* but can effectively import GSH from the cytosol via both high- and low-affinity transporters[6]. Mitochondria have high levels of glutathione reductase and are therefore able to efficiently recycle GSSG to GSH. As a result of oxidative respiration, mitochondria are the major endogenous source of reactive oxygen species (ROS), which, if allowed to accumulate, would be toxic not only to the mitochondria but also to other intracellular targets. GSH appears to play an important role in maintaining the mitochondrial redox potential[7]. There is increasing evidence that mitochondria are involved in the regulation of programmed cell death (apoptosis)[8]. In particular, preservation of the mitochondrial transmembrane potential appears to be crucial in preventing apoptosis induced by certain stimuli, including steroids, DNA damage and Fas (CD95) crosslinking[7,9]. Evidence that the control of the redox status of mitochondrial thiols may be important in maintaining this balance[10] is discussed in greater detail below. Research directed at the development of drugs targeted to modify this balance could provide a useful mechanism for enhancing the propensity of cells to undergo apoptosis and therefore potentiate the activity of cytotoxic drugs.

Biochemical reactions involving GSH

The thiol group of GSH can participate in two main types of reaction, involving either a one- or a two-electron transfer. These reactions allow GSH to perform key roles within a normal cell, including conservation of the redox status of a cell and participation in certain detoxification processes.

GSH as an antioxidant

In one-electron transfer reactions, hydrogen atoms are donated to carbon-, oxygen- or nitrogen-centred free radicals. The resultant GS^{-} dimerizes to form GSSG, which is rapidly reduced by the action of glutathione reductase[11]. By this mechanism, GSH detoxifies oxygen free radicals, the inevitable by-product of aerobic respiration, and an intracellular environment is generated that maintains most protein thiol groups in a reduced state. The prevention of intramolecular disulphide linkage can affect the tertiary structure of many proteins, and hence their function. Subtle changes in GSH levels could therefore provide a mechanism for post-translational modification of proteins through which their activity can be regulated.

GSH conjugate formation: the role of glutathione S-transferases

Transfer of two electrons occurs in electrophile–nucleophile reactions with the formation of GSH conjugates. In these reactions, GSH will react with electrophilic substrates after the formation of thiolate ions. Conjugate formation may occur spontaneously or through the action of glutathione *S*-transferases (GSTs). Catalysis of such reactions by GSTs is believed to occur via promotion of thiolate ion formation and juxtaposition of this reactive species with electrophilic substrates[12].

The GSTs form a multigene family of enzymes, divided into four classes – α (GSTA), μ (GSTM), π (GSTP) and θ (GSTT) – on the basis of sequence homology and immunological crossreactivity[13]. In the native form, GSTs are active as homo- or heterodimers. The latter may only be formed with members of the same class. All GSTs are highly specific with regard to GSH, but electrophile specificity varies considerably between the classes. Furthermore, within each class, the isoforms exhibit significant differences in substrate specificity, in spite of having considerable sequence homology[12,13]. For example, the human μ-class isoenzyme, GSTM1, can catalyse the formation of GSH conjugates with *trans*-stilbene oxide, a capability not shared by other GST classes or even other GSTM isoforms[13].

The level expressed of the different classes of GST is also tissue specific; in human liver the GSTA class forms 80% of the total GST expressed, and the GSTA1-1 isoform predominates. In contrast, human colonic tissue expresses GSTP as the major class of GST. Differential expression also occurs within an organ. For example, in the kidney, GSTAs predominate in the proximal tubules, whereas GSTPs and GSTMs are the major isoforms in the thin loop of Henle, the distal tubules and the collecting ducts[14].

In the case of GSTM1 and GSTTs, inter-individual differences in expression can arise from genetic

polymorphism[15,16]. In approximately 50% of the Caucasian population the gene encoding GSTM1 is deleted from both alleles on chromosome 1. Several studies have emphasized the importance of this genetic polymorphism in susceptibility to carcinogenesis, particularly in smokers[17], but no correlation with drug resistance has been reported.

Differences in expression both at the tissue level and between individuals is of particular significance when considering the role of GSTs in detoxifying xenobiotics. Furthermore, it is important to consider differences in GST isoform substrate specificity in the interpretation of studies measuring GST enzyme activity. This is exemplified in a study by Evans and coworkers[18] in which GST denitrozation of BCNU was studied in two rat glioma cell lines with differing drug sensitivity. The resistant line had lower GST activity than the sensitive cell line when the total enzyme activity was measured with the most commonly used GST substrate, 1-chloro-2,4-dinitrobenzene (CDNB). However, in a subsequent study[19], denitrozation was measured directly, and the resistant cell line was shown to have an elevation in a specific μ-class isoenzyme responsible for inactivation of BCNU. This was associated with a decrease in expression of other GST isoforms with a high specific activity against CDNB. This emphasizes the importance of establishing the exact pattern of isoform expression and the substrate specificity of each isoform before valid interpretation can be made from studies of GST-mediated drug resistance.

GSH and cytotoxic drugs

Correlation between GSH, GST levels and cellular resistance

Over the past three decades, increasing evidence has accumulated supporting the role of GSH and GSTs in cellular resistance to cytotoxic drugs. Studies have used cell lines, with comparisons between drug-sensitive and drug-resistant clones, and samples obtained from a variety of tumour types. The majority of human tumours display increased levels of certain GST isoenzymes, in particular those of the GSTM class[20]. Many, but not all, studies have demonstrated an elevation of GSH levels and GST activity in sublines resistant to bifunctional alkylating agents or cisplatin[20–22]. For example, analysis of the chlorambucil-resistant Chinese hamster ovarian carcinoma cell line (CHO-Chl^r) showed a 1.8-fold increase in GSH levels and expression of an α-form of GST that was not detectable in parental CHO-K1 cells[23]. In another study, a breast cell line, MCF7, exhibiting adriamycin resistance *in vitro*, demonstrated a 45-fold increase in GST activity compared with the parent line[24]. GST and GSH levels have also been compared in cell lines derived from the ovarian adenocarcinoma of a patient before and after treatment with cisplatin, chlorambucil and 5-fluorouracil. Elevation of both parameters was associated with the development of clinical and *in vitro* resistance[25].

Further evidence that alterations in GSH and GST levels are related not only to *in vitro* drug resistance but also to clinical response to chemotherapy, comes from a study in which GST expression in lymphoblasts was examined in 70 children with acute lymphoblastic leukaemia[26]. A clear relationship was demonstrated between expression of GSTMs and disease-free survival. More recently, in a study of patients with advanced ovarian carcinoma, GSH levels in peripheral blood lymphocytes have been found to be suppressed following ifosfamide therapy, and the degree of suppression appears to correlate with clinical outcome[27].

Evidence correlating increased levels of GSH and GST with drug resistance is not necessarily evidence of a causal relationship but may simply represent coexpression with other proteins responsible for drug resistance. More-direct evidence for the involvement of GSH/GST in drug resistance has been obtained from experiments in which either GST expression is increased by transfection of cDNA or in which drug sensitivity is assessed following exposure to agents that reduce GSH levels or inhibit GST activity.

Several groups have successfully transfected plasmids carrying specific GST genes into several cell lines but have produced conflicting results. For example, Miyazaki and coworkers[28] have demonstrated that transfection of human GSTP into CHO cells is associated with a 2–3-fold increase in resistance to cisplatin and carboplatin. They also demonstrated that transfection of GSTA increases resistance to bleomycin. Conversely, Leyland Jones and coworkers[29] have reported that MCF7 breast carcinoma cells, also transfected with α- and π-class genes, do not develop resistance to cisplatin or bifunctional alkylating agents. These results imply that transfection of GST alone is inadequate to convey drug resistance in some cell types and that usually the development of drug resistance arises from several coordinated changes in both this and other enzyme systems.

Further information on the specific involvement of GSTs in drug resistance is derived from inhibition studies. This has been achieved through the use of agents such as ethacrynic acid and indomethacin, although interpretation of the results of these studies is complicated by the fact that these agents may also affect GSH levels. In a study of CHO-Chl^r

cells, known to have increased expression of an α-form of GST, it was demonstrated that 1 mM indomethacin causes a partial, but significant, reversal of chlorambucil resistance[30]. In both rat and human tumour cell lines, sensitization to alkylating agents has been reported following exposure to ethacrynic acid[22,31]. This was associated with decreased GST activity towards CDNB and a fall in intracellular GSH (Ref. 32). Using immunodeficient mice bearing human colonic carcinoma, it was shown that the combination of melphalan and ethacrynic acid enhanced tumour growth delay[33]. Clinical trials of ethacrynic acid in combination with alkylating agents have been carried out but unfortunately were complicated by systemic toxicity. Notably, ethacrynic acid is a potent diuretic and its use resulted in significant depression of blood pressure. There was also an increase in systemic toxicity induced by the alkylating agents. The anti-GST activity of ethacrynic acid is not specific to tumour cells and results in increased myelosuppression, which proved to be the most dose-limiting toxicity[34].

Figure 2. *X-ray crystallography of glutathione-S-transferase structure (from Web: http://molbio.info.nih.gov/cgi-bin/moldraw?1GSF).*

X-ray crystallography studies of GSTs have clarified their structure and have advanced the understanding of the mechanism of their catalytic activity[35,36]. This in turn has made the development of more isoform-specific GST modulators possible. The GST protein exists in dimers, comprising two 24–26-kDa subunits (206–221 amino acids) (Figure 2). Each monomer contributes a kinetically independent active site that has two binding regions: a G site for binding GSH and an H site for binding the hydrophobic electrophile. The p*K* for the cysteine thiol in GSH is around 9.6; however, the local environment of the active site favours a more neutral p*K*, facilitating GSH conjugation with the electrophile. Studies involving site-directed mutagenesis of key amino acids within these regions have provided important information about the mechanism of catalysis and the way the GST isoforms achieve their different substrate specificities (reviewed in Ref. 12). If the isoform profile of tumour cells could be identified and shown to be different from normal proliferating cells, it may then be possible to use isoform-specific GST inhibitors with alkylating agents to enhance tumour-specific cytotoxicity. Studies are currently being carried out to identify isoform-specific inhibitors of GSTs. Examination of GST enzyme activity using a series of GSH analogues has shown that the binding-site shape and lipophilicity are key determinants of GST isozyme selectivity[37]. This information, and improved knowledge of GST isozyme crystal structure, has contributed to the development of isozyme-selective GST inhibitors that could prove useful adjuvants to chemotherapy. The best characterized of these inhibitors is the GSH analogue γ-glutamyl-*S*-(benzyl)cysteinyl-*R*(−)-phenylglycine diethyl ester (TER199)[38]. TER199 is a GSTP-specific inhibitor[39] and has been shown to potentiate the cytotoxicity of chlorambucil in several carcinoma cell lines and, in combination with melphalan, to enhance the growth delay of xenograft tumours in severe combined immunodeficient (SCID) mice[40]. In both these respects, TER199 seems similar to ethacrynic

acid as a modulator of GST activity; however, it also appears to stimulate cytokine production and growth of bone marrow stem cells[38]. If this proves to be true in a clinical setting, TER199 could play an important role in potentiating the cytotoxicity of alkylating agents without dose-limiting myelosuppression.

The specific overexpression of GSTs in tumour cells has also been exploited in the development of prodrugs activated by GST. Several compounds have been identified that are activated on reaction with GSH to form cytotoxic alkylating species: for example, 1,2-dibromoethane and 1-benzoyl-1,2-bis(methylsulphonyl)-2-(2-chloroethyl)-hydrazine[12]. These reactions are not isoform specific, however, and in clinical use are likely to be associated with limiting systemic toxicity. If tumour cells were to overexpress specific GST isozymes, this would provide a mechanism by which a drug could be designed to be selectively activated by that isoform, hence targeting the cytotoxic effect to tumour cells. It is well established that GSTP is frequently overexpressed in tumour cells, and TER286 [γ-glutamyl-α-amino-β(2-chloroethyl)phosphodiamidate sulphonylpropionyl-(*R*)-(−)-phenylglycine] is a prodrug that comprises a GSTP-specific GSH analogue linked to an analogue of cyclophosphamide. The cytotoxicity of the drug is masked until the alkylating component is cleaved from the compound by intracellular GSTP activity. *In vitro* studies using recombinant GST proteins showed GSTA to have similar efficiency as GSTP in cleaving this compound[38]. Thus, although TER286 is not exclusively activated by GSTP, in view of the high expression of GSTP in tumour cells, this compound could prove to be clinically advantageous.

Direct evidence for the spontaneous formation of drug–GSH conjugates has been shown for cisplatin and the bifunctional alkylating agents melphalan[41] and chlorambucil. The reaction of GSH with bifunctional alkylating agents is accelerated by some forms of GST. This substrate specificity has also been demonstrated for melphalan. In a study using GSTA purified from melphalan-resistant CHO-Chl^r cells, the rate of formation of GSH conjugates was increased in the presence of the enzyme. However, addition of GSTP purified from drug-sensitive CHO-K1 cells had little effect (Figure 3). Both isoforms were active against CDNB (Ref. 42).

In addition to possessing GSH (G)- and electrophile (H)-binding sites, GSTAs have a region, believed to lie in the cleft between the monomers, that is capable of binding a range of anionic molecules such as bilirubin, steroids, azo

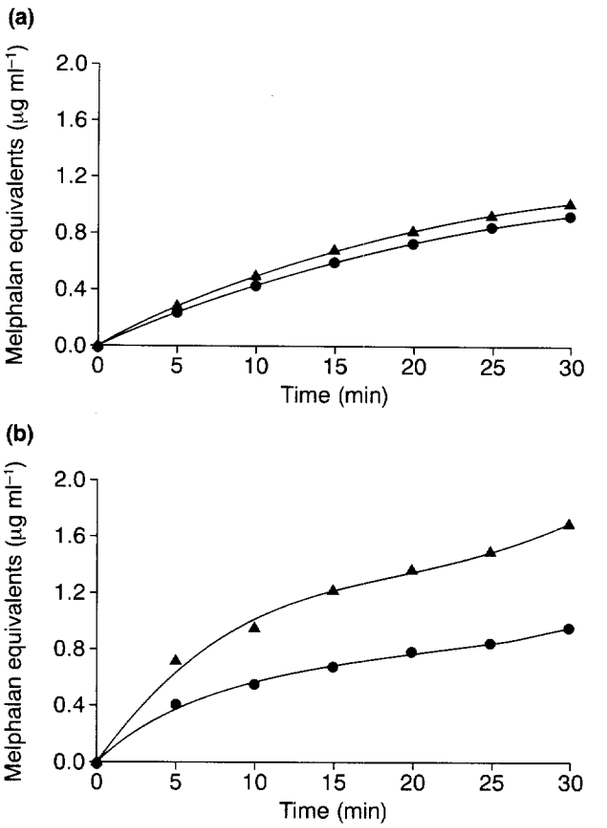

Figure 3. *Rate of glutathione conjugation with melphalan, enhanced with glutathione-S-transferase isolated from either (a) the melphalan-sensitive CHO-K1 cell line or (b) the melphalan-resistant CHO-Chl^r cell line. Triangles and circles show the conjugation rate with and without enzyme, respectively.*

compounds and certain carcinogens. For this reason, these forms of GST, which are highly expressed in the liver, were originally described as ligandins[43]. Recently, an additional mechanism for substrate binding has been described that appears to involve the G and H sites rather than the ligandin site and that may not be restricted to the α-form of the enzyme[44]. In this mechanism, substrates bind to the H site after GSH binding to the G site, but substrate release is very slow. Under normal circumstances, such a reaction would result in inhibition of enzyme activity and would not affect the level of the substrate involved. In the case of the GSTs, however, the level of expression of the enzyme is such that the sequestration of substrate may have a significant impact on the intracellular level of a toxic compound. Meyer and coworkers[44] have provided evidence that binding of

chlorambucil to GST may occur at the H site and suggest that this may be an important factor in the development of drug resistance. In the study described in Figure 3, measurement of melphalan–GSH conjugates was performed after protein precipitation with trifluoroacetic acid, a process that may liberate sequestered molecules.

Elevation of intracellular GSH is most frequently associated with development of resistance to bifunctional alkylating agents and anthracyclines. The importance of the intracellular concentration of GSH to cancer therapy was first recognized over 40 years ago when GSH depletion was shown to sensitize cells to ionizing radiation[45]. Subsequently, studies using the murine leukaemic cell line L1210 demonstrated that melphalan cytotoxicity could be modified by changes in GSH levels[46]. This was supported by evidence from studies of human tumour cell lines and patient samples. Increased GSH levels were shown to be associated with decreased sensitivity to both platinum-based drugs and alkylating agents in ovarian, prostatic and gastric carcinomas[45,47–50].

As described above, depletion of GSH levels can be achieved by inhibition of γ-glutamylcysteine synthetase activity by BSO. Treatment with BSO has been shown to restore sensitivity to alkylating agents, anthracyclines[51] and platinum-based drugs in resistant cell lines[52]. Furthermore, *in vivo* studies in athymic nude mice bearing the human ovarian cancer cell line NIH:OVCAR-3 intraperitoneally demonstrated that the combination of BSO and melphalan increased survival compared with melphalan alone[53], suggesting that GSH depletion may prove to be an effective adjuvant treatment in resistant malignancies. Phase I clinical trials have reported that BSO in combination with melphalan is well tolerated, the main side effect being mild nausea and vomiting[54,55]. Treatment proved possible to reduce GSH levels in peripheral mononuclear cells to 10% of control levels. In serial biopsies of ovarian, lung and breast tumours, similar reductions in GSH levels in the tumour tissues were revealed. Phase II clinical trials using combinations of BSO with melphalan or platinum-based drugs in ovarian carcinoma and in melanoma are in progress.

Studies investigating the mechanism by which GSH could modify drug sensitivity have led to the suggestion that intracellular GSH levels may be an important trigger in apoptosis. In steroid-induced apoptosis in murine thymocytes, the percentage of cells undergoing apoptosis could be modified by altering the GSH levels. Increased levels of reduced GSH inhibited dexamethasone-induced cell death, whereas treatment with oxidized GSH increased the occurrence of apoptosis[56]. It was suggested that the ratio of reduced and oxidized GSH may be of particular importance in determining the sensitivity of the cell to steroid-induced apoptosis. Sensitivity to glucocorticoids has been well established as an important indicator of prognosis in childhood acute lymphoblastic leukaemia. A recent study of 19 children and 13 adults with this disease demonstrated that higher GSH levels in lymphoblasts were significantly correlated with a decrease in sensitivity to prednisolone, daunorubicin and melphalan *in vitro*[57]. Thus, although GSH appears to play some role in steroid resistance, the mechanism requires further investigation.

GSH, the multidrug-resistance phenotype and the multidrug-resistance-associated protein

Alkylating agents and platinum drugs are strong electrophiles that exert their cytotoxic effect through the formation of DNA adducts. GSH has a higher affinity for these electrophiles than the nucleophilic sites on target nucleotides and therefore GSH conjugates that have reduced cytotoxicity are formed. Glutathione conjugates are extruded from the cell by a range of specific pumps[58]. Sequential removal of the glutamyl and glycine groups by γ-glutamyltranspeptidase and surface-bound dipeptidases yields cysteine conjugates that are further processed to give mercapuric acids, which can be excreted in the urine. Glutamate and glycine are reabsorbed across the cell membrane to be resynthesized into GSH as part of the γ-glutamyl cycle, as described above (Figure 4).

Recently there has been a resurgence of interest in the role of GSH in drug resistance through the recognition that there are links between the γ-glutamyl cycle and the regulation of the activity of the multidrug-resistance-associated protein (MRP). The MRP is a 190-kDa glycoprotein expressed at the cell surface and on vaculolar membranes. It is a member of the ATP-binding cassette (ABC) superfamily of transport proteins and has a 15% amino acid sequence homology with p-glycoprotein (Pgp)[59]. MRP expression was first described in the Pgp-negative multidrug-resistant cell line H69AR, a small-cell lung cancer cell line, and has subsequently been identified in a number of other types of tumour tissue, including leukaemias, nonsmall-cell lung, breast, cervix, prostate and bladder carcinomas (reviewed in Ref. 59). As with Pgp expression, MRP expression confers a multidrug-resistance phenotype with decreased sensitivity to certain groups of cytotoxic drugs, including anthracyclines,

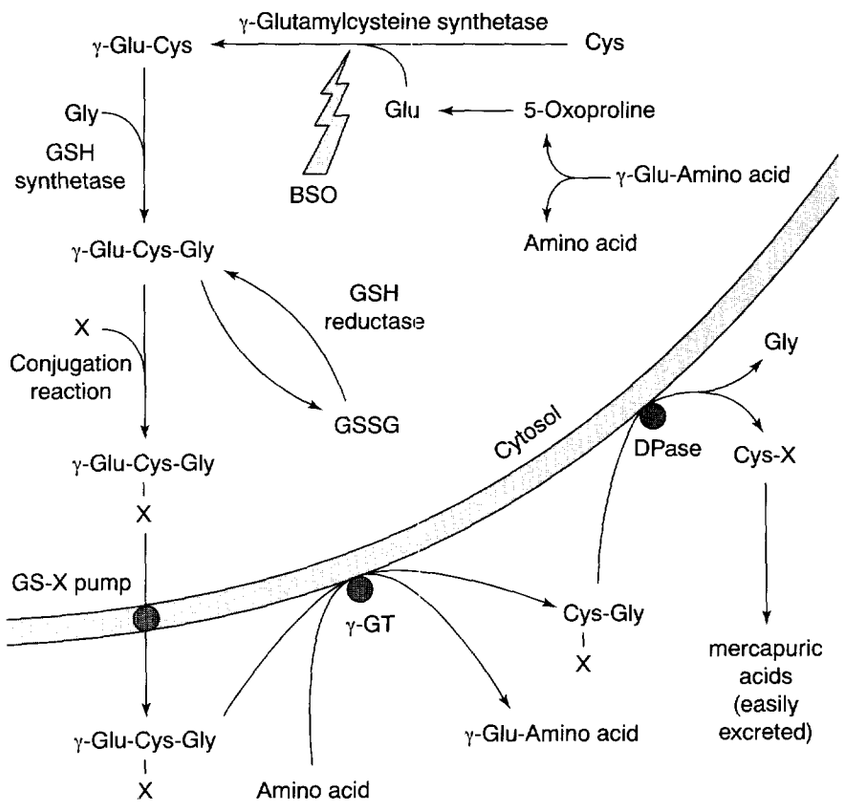

Figure 4. *Glutathione homeostasis. Synthesis of GSH occurs in a two-step process in the cytosol from its component amino acids (shown to the upper right hand side). It is then available for either one-electron transfer reactions leading to the formation of GSSG (i.e. antioxidant activity) or two-electron transfer reactions involving conjugation with a xenobiotic (i.e. detoxification activity). A membrane GS-X pump exports conjugates from the cell, where they are sequentially processed by γGT and DPase to release components of GSH (which can be recycled after cell uptake) and cysteine conjugates (which are further processed to mercapuric acids and excreted). BSO, buthionine sulphoximine; DPase, dipeptidase; γ-GT, γ-glutamyltranspeptidase; GSH, glutathione; GSSG, glutathione disulphide; GS-X, glutathione–xenobiotic conjugate; X, xenobiotic.*

epidophyllotoxins and *Vinca* alkaloids. As with Pgp, the MRP phenotype is associated with reduced intracellular drug accumulation and enhanced drug efflux.

The modification of Pgp-mediated drug resistance by compounds such as verapamil and cyclosporin A is well documented and has been applied in clinical trials[60]. In contrast, MRP-mediated drug resistance is unaffected by these drugs but can be reversed by manipulation of GSH levels. GSH depletion following exposure to BSO can restore drug sensitivity in MRP-expressing cell lines. In studies of the mechanism of MRP transport, GSH depletion resulted in a daunorubicin accumulation defect that was reversible on restoration of GSH levels[61,62]. Furthermore, plasma membrane vesicle studies have demonstrated that MRP activity is associated with transport of drugs and GSH conjugates, including anthracyclines, etoposide and vincristine[58,63]. GSH–melphalan conjugates are well described[41] and have been shown to be substrates for MRP transport[58]; however, resistance to alkylating agents is not part of the MRP phenotype. Furthermore, no stable conjugates of GSH with anthracyclines, *Vinca* alkaloids or epidophyllotoxins have been demonstrated. There is evidence to suggest that GSH is required only for transport of positively charged or neutral drugs (e.g. daunorubicin and vincristine) because transport of anionic molecules is not influenced by GSH depletion[64].

Although GSH homeostasis and MRP function are clearly linked, the exact mechanism of interaction remains unclear. It has been speculated that GSH depletion simply leads to oxidative damage of plasma membranes and subsequent leakage of cytotoxic drugs; however, this would not account for the specificity of the effect of GSH depletion on drug resistance in cell lines overexpressing MRP. GSH may provide a gating mechanism to enable MRP to transport nonanionic molecules, either by the reduction of regulatory thiols on the MRP molecule itself or on other intracellular proteins that might be involved in regulation of MRP activity. Thus, GSH depletion might result in inhibition of MRP transport activity and increased intracellular drug accumulation by indirect, rather than direct, mechanisms.

GSH and the control of apoptosis

Apoptosis is now generally believed to be the common pathway by which most cytotoxic drugs kill cells[65].

Understanding the control of apoptosis is therefore crucial to elucidate the many mechanisms of drug resistance. Although several studies support the notion that cellular GSH levels correlate to cytotoxic drug resistance, identifying the specific role played by GSH in apoptosis is complicated by the current incomplete knowledge of the mechanisms regulating entry into the apoptotic pathway. Oxidative stress has been proposed as a final common trigger for apoptosis[66]. Evidence supporting the importance of ROS to apoptosis include the observations that addition of hydrogen peroxide or lipid peroxides, or the depletion of antioxidants such as GSH, can induce apoptosis[67,68]. Conversely, elevation of levels of antioxidants[68–70] or overexpression of GSH-dependent enzymes (e.g. glutathione peroxidase)[71] can inhibit apoptosis. Several studies have clearly demonstrated that the onset of apoptosis is associated with a fall in GSH levels and, in some cases, an increase in ROS (Refs 72–74).

It has also been argued that ROS are not an essential trigger for apoptosis because it can be induced in anaerobic conditions, when the formation of ROS is minimized[75]. Studies of the Jurkatt leukaemia cell line demonstrated that Fas/Fas-ligand-induced apoptosis was associated with specific transport of GSH from the cell rather than an increase in ROS – thus, there is a loss of protection against oxidants[76,77]. It is possible that the mitochondrial dysfunction produced by culturing cells in low oxygen environments results in increased cell susceptibility to oxidants, but it is more probable that oxidative stress is not the final trigger but only one of many different points of entry into the apoptotic pathway.

GSH is frequently dismissed as a nonspecific antioxidant that protects the cell by neutralizing potentially damaging ROS. There is, however, compelling evidence to suggest that GSH homeostasis has a more fundamental role in the activation or inhibition of proteins crucial to cell survival[76]. Two major groups of proteins have been defined as having key roles in regulating apoptosis: the BCL-2 protein family (for a recent review see Ref. 78) and the cell signalling cascade[79]. Many proteins have thiol moieties whose redox status is crucial to their function[80,81], and there is evidence that the DNA-binding capacity of some transcription factors is redox status dependent with sulphydryl groups on cysteine residues as the target of regulation[82]. This may have important implications for the regulation of cell signalling pathways. Studies have shown that the DNA-binding function of AP-1 (the Fos–Jun heterodimeric complex) is inhibited by diamide and *N*-ethylmaleimide, both of which are agents that specifically modify sulphydryl groups[83]. The influence of oxidants and antioxidants on the function of AP-1 and the nuclear factor (NF-κB) has been the subject of several studies. Interestingly, there is evidence of opposing effects of redox status on induction of NF-κB compared to AP-1. Meyer and coworkers[81] demonstrated that NF-κB activation was promoted by peroxides and hydrogen peroxide, whereas antioxidants, including thiols, were strong inhibitors[84]. Conversely, antioxidants (*N*-acetylcysteine and dithiocarbamates) efficiently induced AP-1 DNA binding and hence transactivation. The AP-1 heterodimer appears to be less sensitive to inhibition by oxidants. Droge and coworkers[84] have demonstrated that increasing intracellular GSSG can selectively inhibit DNA binding by NF-κB without affecting AP-1 binding. Thiols, however, could enhance DNA binding of both transcription factors. Thiol-induced AP-1 transactivation is dependent on induction of its component proteins, Fos and Jun (Ref. 81), and reduction of a single conserved cysteine residue in their DNA-binding zone[83]. The AP-1 complex is localized in the nucleus, in contrast to NF-κB, which is primarily located in the cytosol. NF-κB activation requires dissociation of an inhibitory protein IκB, followed by translocation of the transcription factor into the nucleus. It is interesting to note that although oxidants promote the translocation activity of NF-κB, NF-κB DNA binding is enhanced by peroxides[81] and inhibition by GSSG[84]. As discussed previously, there are important kinetic differences in the maintenance of GSH homeostasis in the nuclear and cytosolic pools. This may have significant implications for the response of many cell signalling proteins and transcription factors to the redox status within the different intracellular compartments and may also prove to be cell-type specific. Further evaluation of these pathways could lead to more-focused targets in modulating apoptosis.

Clearly, the intracellular response to ROS and redox homeostasis can no longer be considered in terms of nonspecific stress responses but appears to constitute important mediators in the control of cell proliferation and cell survival. Further understanding of the activity of GSH and associated enzymes within this framework may reveal valuable targets by which cell survival could be manipulated.

Conclusion

The involvement of GSH in detoxification of toxic compounds by conjugation reactions and in the inactivation of ROS is well established. However, recent advances in the understanding of the importance of redox homeostasis to

cell survival has led to renewed interest in the role of GSH, as it appears to be important in both the control of apoptosis and the regulation of drug extrusion. Thus, after 40 years, the GSH pathway continues to offer attractive targets for drug discovery.

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