Glutathione-associated Enzymes in Anticancer Drug Resistance

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Abstract

The importance of thiol-mediated detoxification of anticancer drugs that produce toxic electrophiles has been of considerable interest to many investigators. Glutathione and glutathione S-transferases (GST) are the focus of much attention in characterizing drug resistant cells. However, ambiguous and sometimes conflicting data have complicated the field. This article attempts to clarify some of the confusion. The following observations are well established: (a) tumors express high levels of GST, especially GSTT, although the isozyme components vary quite markedly between tissues and the isozymes are inducible; (b) nitrogen mustards are good substrates for the GSTp family of isozymes which are frequently overexpressed in cells with acquired resistance to these drugs; (c) most drugs of the multidrug-resistant phenotype have not been shown to be GST substrates and although GST is frequently overexpressed in multidrug-resistant cells, most indications are that this is an accompaniment to, rather than a cause of, the resistant phenotype; (d) transfection of GST complementary DNAs has produced some lines with increased resistance to alkylating agents. Most studies of the relationships between GST and resistance have overlooked the potential importance of other enzymes involved in the maintenance of cellular glutathione homeostasis, and this has complicated data interpretation. Translational research aimed at applying our knowledge of glutathione pathways has produced preclinical and clinical testing of some glutathione and GST inhibitors, with some encouraging preliminary results. In brief, GSTs are important determinants of drug response for some, not all, anticancer drugs. Caution should be encouraged in assessing cause/effect relationships between GST overexpression and resistance mechanisms.

Introduction

Enzymes involved in the metabolism of drugs and xenobiotics are subdivided into two major categories. Phase I, exemplified by the P-450 isozyme family, renders "small" changes onto drugs, sometimes creating reactive sites which can covalently bind with other intracellular molecules. Phase II reactions, exemplified by the GST isozyme family, involve the addition of relatively "bulky" groups onto the drug, most often creating a more water-soluble conjugate, which may be less toxic and more readily excretable. Thus, phase II reactions are generally cytoprotective. GSH, first described in 1888 as philothion, is the most ubiquitous and abundant non-protein thiol in mammalian cells and serves as a necessary nucleophile in a number of detoxification reactions. Normal levels of GSH in humans are 10–30 μM (plasma), 1–3 μM (urine), 3 mM (kidney proximal tubule), and 1–10 mM (tumors of various organ sites) (1). Maintenance of a homeostatic GSH content is achieved by both de novo synthesis and salvage synthesis and a number of interrelated pathways are involved (Fig. 1). The suggestion that GSH is a universal protectant against electrophilic challenge is supported by evolutionary considerations. Most indications point to the emergence of life on Earth in the pre-Cambrian period some 4 billion years ago. Given that the standard rules of natural selection have been in place for this period, time and success have honed many biological traits. Arguably, one of the most critical adaptations has been the capacity to survive a hostile environment. Environmental stresses can take many forms and even humans, despite, or perhaps because of, our propensity to manipulate the forces of natural selection, are subject to a multitude of toxic challenges. Paradoxically, molecular oxygen, while providing an efficient energy production from ingested food, results in free radical and peroxide by-products which have high intrinsic toxicity. Indeed, the emergence of oxygen-liberating phytoplankton and blue-green algae began an era in Earth's development that must have severely stressed existing and emerging life forms. In more contemporary times, especially post-industrial revolution, environmental stresses have multiplied disproportionately. The air of the planet is suffused with both natural and man-made chemicals. Plants, even those propagated by humans, produce toxic components as a strategy to reduce grazing; many of the by-products of food production are mutagenic or carcinogenic. Even natural radiation and sunlight have the capacity to produce toxic insults. It is likely that such hostile and competitive surroundings provided the need for the buildup and refinement of intracellular protective arsenals. Molecular evolution has contributed to the conveyor belt of selected chemical detoxification systems. At the present time, mammals have a cellular detoxification system of protein proportions, with considerable redundancy of function. Through divergent and convergent evolution, organisms are constantly "refining" the form and function of detoxification gene products. The MDR P-glycoprotein is simply part of a large family of so-called ATP-binding cassette "membrane pumps." Moreover, specific GST isozymes serve detoxification, structural, and transport functions in many phyla. For example, certain bacteria overexpress GST to develop resistance to the antibiotic fosfomycin (2); many insects and their larvae use GST to express resistance to insecticides (3); squid and other cephalopods utilize GST as structural lens crystallins (4); mammals use GST for detoxification purposes and to bind and transport molecules like heme and bilirubin (hence the early GST nomenclature of ligandin). Indeed, there is ample evidence that species from every documented phylum utilize GST-mediated detoxification mechanisms.

The suggestion that GST is a universal detoxification system is their capacity to recognize diverse chemical structures. From a teleological standpoint this would seem to make sense. For example, an organism may not be able to predict when, which, or how much of a toxin it will encounter. Thus, the question arises, "What would constitute the most energy-efficient response to an acute stress stimulus?" If exposure to a specific toxin recurs in a chronic fashion, there may be value in the development of an enzyme detoxification system specific for that molecule. However, given the vagaries of exposure, natural selection would more likely favor a broad-based recognition system, where comparatively low avidity, recognition, or binding is compensated by the potential to detoxify a wide range of chemical structures. For an acute toxin exposure to remain sublethal, a cell or organism may have but one chance to prevent terminal damage. Therefore, the evolution of systems such as the GST isozyme families, in which relatively low...
binding affinities are offset by broad substrate specificities would seem to make biological sense.

With this background, there appears to be a sound rationale for considering the role that GSH-associated enzymes play in determining sensitivity to anticancer drugs. While most investigators accept that GSH has a role in drug resistance, the involvement of GSH-associated enzymes (especially the GSTs) has been more contentious. Recent reports have helped to clarify the situation. At least to some extent, the confusion has resulted from the merging of a number of overlapping enzymes (especially the GSTs) has been more contentious. Recent reports have helped to clarify the situation. At least to some extent, the confusion has resulted from the merging of a number of overlapping enzymes (especially the GSTs), whichconstitute the MDR phenotype. Over the last 10 years, a large proportion of the resistance literature has focused on the MDR phenotype. This is especially true with studies of electrophile-producing alkylating agents and those drugs which constitute the MDR phenotype. As shown in Fig. 1, a number of enzymes contribute to the main steps in interrelated GSH-producing and -utilizing pathways. GSTTr is the most prevalent isozyme in cell lines and this has disproportionately focused attention on this area, overshadowing other reports where GST overexpression can be linked with drug resistance.

The remainder of this article will present the substantial evidence favoring the direct involvement of GSH and GST in resistance to a number of cancer drugs used in clinical treatment today.

**Enzymes of GSH Homeostasis**

As shown in Fig. 1, a number of enzymes contribute to the maintenance of intracellular levels of reduced glutathione. The recent cloning and characterization of GCS, the rate-limiting enzyme in the glutathione synthesis of GSH via the γ-glutamyl cycle, has permitted studies which show that increased expression of this enzyme along with GSTTr is a major cause of resistance to drugs such as Adriamycin, just as increased expression of the MDR-1 gene product is frequently not the sole cause of resistance to natural products. GSTTr is the most prevalent isozyme in cell lines and this has disproportionately focused attention on this area, overshadowing other reports where GST overexpression can be linked with drug resistance. The remainder of this article will present the substantial evidence favoring the direct involvement of GSH and GST in resistance to a number of cancer drugs used in clinical treatment today.

**Fig. 1. Summary schema of interrelated GSH-producing and -utilizing pathways.** GSSG, reduced glutathione; DDT, bis(P-chlorophenyl)-trichloroethane.

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As shown in Fig. 1, a number of enzymes contribute to the maintenance of intracellular levels of reduced glutathione. The recent cloning and characterization of GCS, the rate-limiting enzyme in the production of GSH via the γ-glutamyl cycle, has permitted studies which show that increased expression of this enzyme along with GSTTr may be causally linked to resistance to alkylating agents and platinum-based anticancer drugs. Increasing the intracellular capacity for de novo synthesis of GSH could presumably impact upon other enzymes (including GSTTr) which use GSH as a cofactor. Intracellular GSH levels are most often within a range of 0.1 to 10 mM. Micromolar concentrations of BSO can produce a 90% or greater reduction of intracellular GSH over a 24-h period, thus producing intracellular concentrations in the high micromolar range. The values for most of the GST-catalyzed conjugations of alkylating agents with GSH are in the low micromolar range. However, direct extrapolation must be applied with caution, since intracellular com-
partmentalization and a variety of endogenous GSH functions may restrict the availability of GSH for detoxification reactions.

The capacity of a tumor cell to maintain GSH is determined by a number of interacting pathways which are shown in Fig. 1. Many of the enzymes involved in these pathways have been targeted for therapeutic intervention by modulators of anticancer drug resistance. Perhaps the most widely studied is BSO, an inhibitor of γ-GCS, the rate-limiting step of GSH synthesis via the γ-glutamyl cycle. A large number of in vitro and in vivo reports have shown that BSO can enhance tumor cell susceptibility to alkylating agents and reverse some acquired resistant phenotypes. Because BSO is a specific inhibitor of γ-GCS, reversal of resistance can reasonably be attributed to the resultant depletion of intracellular GSH. In addition, a number of drugs can cause an increased expression of mRNA for γ-GCS transcript, illustrating the propensity of the cell to provide a compensatory response to a drug-induced depletion. Thus, interference with de novo synthesis of GSH appears to be an effective means of modulating resistance to certain drugs and regulation of γ-GCS is a critical control point for cellular protection. It will be of considerable interest to determine if cotransfection of γ-GCS with other GSH-utilizing enzymes such as GST can confer greater levels of resistance to alkylating agents. Relevant to this is a recent observation that life span extension in Drosophila melanogaster is achieved only when cDNAs for both superoxide dismutase and catalase are cotransfected, not with either alone (6). This suggests a cooperativity of the free radical detoxification systems in this species. Such a principle could readily be extrapolated to the GSH-based detoxification pathways. With so many interacting and mutually dependent biochemical pathways, the question arises as to how critical coregulation of the enzymes of GSH homeostasis may be in controlling the overall cellular protective thiol environment. In addition to the many reports of elevated GST, increased expression of both γ-GGT and glutathione peroxidase have been implicated in drug resistance (7). The fact that at least three distinct human peroxidase genes have been identified suggests that care will be needed in analyzing differential expression and regulation of these enzymes and their potential role in drug resistance.

Since recycling of the constituent amino acids of GSH is an important factor in GSH homeostasis, membrane-associated γ-GGT might logically be expected to be critical in allowing the "salvage synthesis" of the tripeptide. Whether or not enhanced γ-GGT will produce an overall increase in intracellular GSH is, once again, dependent upon GSH-mediated feedback responses, most of which have yet to be elucidated. In addition to γ-GGT, glutathione reductase and the glyoxalase system function to recycle GSH (Fig. 1). Under conditions of extreme oxidative stress, it is possible that glutathione reductase could become saturated. By extrapolation, increased reductase activity could enhance the ability of a cell to survive such conditions. In mammalian cells, this correlation has not been reported, perhaps reflecting either the relative uninducibility of the gene or the efficient extrusion of oxidized glutathione from the cellular milieu. Often overlooked in terms of GSH metabolism is the glyoxalase system. Methylglyoxal is a by-product of intermediary metabolism and can be present in millimolar concentrations in rapidly growing tissues. Indeed, increased glyoxalase I protein and transcript levels are found in tumor tissue compared to the normal tissue counterpart (8). Since methylglyoxal is toxic, its rapid and continual conversion to D-lactate is a necessary requisite for cell survival. Glyoxalase II serves to recycle the GSH used in the initial thioether reaction (Fig. 1). Thus, the overall net use of GSH should be zero. However, with such potentially high levels of methylglyoxal, the net commitment and flux of GSH through this pathway may be quite significant. Mammalian glyoxalase II has yet to be cloned and little is presently known of the regulation of the gene or its coordination with glyoxalase I. Future investigations should probably consider how much of a "GSH sink" this pathway represents.

While GST represents a primary focus for many drug resistance studies, numerous other enzymes which either produce, recycle, or use GSH can also be involved in determining cellular response to certain anticancer drugs. The cloning and characterization of coding and regulatory sequences of many of the genes, the products of which are illustrated in Fig. 1, lag behind those of GST genes. The next few years may help to establish the importance to the resistant phenotype of coordinated expression and regulation of these enzymes.

Glutathione S-Transferases

Many excellent review articles have detailed the nomenclature and biological importance of the GST supergene family (e.g., Ref. 9). An in depth discussion of GST nomenclature is outside of the scope of this article. However, in mammalian species the four major cytosolic families have been designated α, μ, π, and θ. At least one microsomal form has also been described. The cytosolic forms share homology and probably have evolved from a common ancestral gene. Most of the published material linking GST to anticancer drug resistance has focused on the cytosolic families, although this does not preclude a role for microsomal GST. Indeed, the importance of membrane-associated GST in drug metabolism has not been studied in any great detail and could prove to be a fertile area for future research.

The cytosolic GSTs exist as monomeric subunits which have catalytic activities either as homo- or heterodimers. Xenobiotics, including anticancer drugs, can influence the transcriptional regulation of GST genes, primarily through effects mediated by AP1-like regulatory elements, which for the rat α GST family have been characterized as antioxidant response elements (10). GSTπ is also inducible and frequently overexpressed in neoplastic tissues when compared to their normal counterparts. Indeed, GSTπ is a tumor marker for a number of different cancers. The fact that it is usually the most common of the GST in both tumor tissues and cell lines and therefore the easiest to detect experimentally contributes to the prevalence of literature references for this isozyme. GSTμ is variably expressed in human tissues or cell lines. This is because approximately 40–50% of the human population has a null phenotype for GSTμ and thus have no constitutive expression of the isozyme. This has elicited interest in the relationship between cancer incidence and GST expression and large scale population studies are currently under way to correlate susceptibility to lung cancer in smokers who have the GSTμ null phenotype (11). These studies are predicated on the hypothesis that lack of expression of GST is related to increased DNA damage by mutagens in smoke. This rationale parallels the hypothesis of altered drug metabolism by GST in resistant cells and illustrates one example of potential overlap between drug resistance and chemoprevention.

GST Studies in Cell Lines

Of most significance to this article are the following general associations between increased GST expression and drug resistance: (a) GSTα isozymes and nitrogen mustards; (b) GST μ and nitrosoureas; (c) GSTπ and the MDR phenotype. The evidence for a causal link for point a and point b is good. For point c, most of the published data would support the principle that increased GSTπ is more likely a consequence of a pleiotropic stress response. As discussed below, if GSTπ is to be unambiguously defined as a causative factor in MDR, better evidence is required.

Empiric assessment of GST involvement in anticancer drug resistance has come primarily from work with cultured mammalian cell lines. It has been standard operating procedure to develop a resistant cell line by selection with the drug of choice and then to analyze a
number of cellular properties to achieve a correlation between a particular trait and drug resistance. Such studies have generated extensive correlative data and have provided many tumor cell lines with comparatively high levels of GST expression. Inherently disadvantageous in this approach is the natural tendency to overinterpret the cause/effect relationship of GST overexpression and drug response (a problem not unique to GST). This is especially true for GSTπ. In most instances, the data which link GSTπ to enhanced cellular detoxification of anticancer drugs, and by extrapolation to drug resistance, is not completely convincing. For example, chlorambucil conjugation with GSH can be catalyzed by human GSTπ, but the catalytic efficiency is some 40- to 50-fold less than for human GSTα (Fig. 2; Ref. 12). In a cell line with high GSTπ and no GSTα, it has not been conclusively demonstrated that GST catalysis would be a major detoxification pathway. As will be discussed, there is presently little direct evidence that any anticancer drugs are direct substrates for GSTπ.

Selected cells with an acquired resistance phenotype are often not cloned from a single progenitor and are represented by a heterogeneous population; this may serve to compound interpretation problems. In some instances, authors may state that because cell line A has 10-fold the GST activity of cell line B, it is 10-fold resistant to a particular drug. Notwithstanding the heterogeneity problem, the relationship between steady-state GST levels and drug response may be linear, but this must be demonstrated, not assumed. GST activity measurements in cell lines vary from as low as 4 nmol/min/mg (MCF7 human breast) to >800 (numerous hepatomas). Because GSTs have numerous endogenous metabolic roles (i.e., ligand binding/transport, eicosanoid synthesis, etc.; see Ref. 13) in addition to their detoxification functions, there is no straightforward way to make a direct quantitative comparison between enzyme amount or activity and drug response. In addition, there are other factors which must also be considered in such an analysis. For example, the turnover of both the enzyme and transcript will influence the ability of a cell to adjust the steady-state levels. There is evidence in drug-resistant cells that the half-lives of GSTπ and its mRNA are doubled in comparison to wild type cells (14). This propensity to “recognize” a toxic challenge by altering protein or transcript half-life has also been identified for other cellular proteins such as tubulin, where cells resistant to antimetabolite drugs accumulate higher levels of transcript for β-tubulin (15).

Thus, the intrinsic steady-state levels of GST, the transcriptional and translational rates, and the stability and turnover of both protein and transcript should all be factored into any analysis of response to chemotherapy. Moreover, binding affinities and relative amounts of different GST isozymes, as well as competing reactions and relative intracellular content of substrate and substrate-GSH conjugate, will be important. Since GSTs use GSH as a cofactor in the formation of a thioether bond with its drug substrate, these same concerns will relate to the enzymes of GSH biosynthesis. As indicated above, GSH homeostasis is itself influenced by a multiplicity of cellular controls and feedback mechanisms. While concentrations of reduced glutathione in tumor cells often approach millimolar, this does not automatically imply a surplus of available GSH. Compartmentalization and competing reactions will play an important role in determining availability. In addition, there is now significant evidence that drug-GSH conjugates are removed from the interior of the cell by an energy-dependent membrane transporter. Although the precise identity of the pump in tumor cells is not known, a potentially important report has recently appeared which identifies a Mr 38,000 membrane protein which can efflux both GSH conjugates and drugs such as daunomycin, daunorubicin, and vinblastine (16). Partial homology with other members of the ATP-binding cassette family members is reported. Thus, it may be that certain membrane proteins can serve to recognize both hydrophobic drugs and other phase I metabolites such as GSH conjugates. This could provide a possible link between MDR and GSH metabolism. It appears likely that other GSH-conjugate membrane pumps will eventually be identified. Obviously, differences in expression of such proteins may influence the apparent intracellular concentrations of drug metabolites and this could produce potential differences in cytotoxicity. There would seem to be value in pursuing this avenue of research investigation.

Any listing of drug-resistant tumor cells with elevated GST will frequently include a number of MDR lines. With the exception of the mouse Friend erythroleukemia cell line, the MDR lines (or more specifically those selected with drugs which are part of the MDR...
phenotype) primarily overexpress GST\(\pi\) (1). The prevalence of GST\(\pi\) as a marker of transformation and carcinogen exposure suggests that the enhanced enzyme levels may be an effect of the selection process, rather than a cause of the MDR-resistant phenotype. Indeed, there is presently little indication that any of the classical MDR drugs are substrates for this isozyme. Most do not form reactive electrophilic species. One potential caveat is Adriamycin. While the debate over the precise cytotoxic mechanism of the anthracyclines has become less contentious since the identification of topoisomerase II as a drug target, there is little doubt that quinone-mediated free radicals formed from Adriamycin have the capacity to cause oxidative damage and cytotoxicity (7). Lipid peroxidation is a frequent cellular result of Adriamycin exposure. Such peroxides may further break down to yield hydroxylalkenals, which have been shown to be substrates for GST isozymes (17). It is possible to hypothesize that these hydroxylalkenals can induce, or select for, increased GST expression and that their detoxification by GST can act as a cellular protective effect. Such a mechanism has been invoked previously for the low level of resistance to Adriamycin in a GST-\(\pi\)-transfected pT 22-3 cell line (18). Because of the extensive data to support the role of the P-glycoprotein and more recently the MRP gene product in resistance to MDR drugs, it would seem reasonable to conclude that if GST\(\pi\) has a role in determining the MDR response it may be minor. Increased levels of GST\(\pi\) in MDR cells may prove merely to be the consequence of a coordinate up-regulation of a number of stress-inducible genes.

**Anticancer Drugs as Substrates for GST**

When designing antimitabolite anticancer drugs, tight binding to the target enzyme (frequently involved in some aspect of DNA synthesis) is desirable. For GST to function as efficient detoxifiers of a wide range of chemical structures, such tight binding could prove to be problematic and indeed for those alkylating agents which have been shown to be substrates, the binding constants suggest affinities in the micromolar range. What seems to be gained from the reduced affinity for an individual chemical is promiscuity of substrate recognition. Those anticancer drugs which have been definitively identified as GST substrates are listed in Table 1. Perhaps of equal significance are those drugs for which there is no convincing evidence of GST-mediated catalysis (Table 1). It is apparent that many, if not all, of the MDR drugs fall into this latter category. An additional consideration is that while no positive substrate specificity results have been published, no specific negative data have been generated for the drugs in this column. Anthracyclines are known to produce reactive quinone moieties, as does the antibiotic fosfomycin (2). Since this antibiotic does undergo GST-mediated catalysis, it would not be unreasonable to suggest that the quinone species of Adriamycin may be subject to catalyzed conjugation with GSH. Furthermore, mitomycin C toxicity is reduced by the presence of increased cellular GSH, although it is not clear if this is a function of an altered reducing environment or the propensity of the drug to form GSH conjugates. Even if the latter is true, the value of GST in enhancing the spontaneous reaction has not yet been determined. It may also be possible that glutathione peroxidases may mediate detoxification of Adriamycin and associated free radical species (7).

A review of the chemical structures of those drugs which are GST substrates shows that a common characteristic is the electrophilic nature of their active cytotoxic moieties. In every case the drug can interact with the thiol of reduced glutathione in a spontaneous manner, creating a thioether product which is characteristically less toxic and more water soluble. The nitrogen mustards have bifunctionality as a result of the two chloroethyl arms. Because of this, there is the possibility that a second reactive site could produce a diglutathionyl product. This bifunctionality is important to the overall cytotoxic properties of the drug, since there is potential to form cross-links within DNA. Such lesions can make the drug 1 to 2 logs more cytotoxic than an equivalent monofunctional mustard. Thus, in a simplistic way, the initial conjugation with GSH serves to reduce the potential cytotoxic threat by turning the bifunctional property into monofunctionality (see Fig. 2). A similar principle can be applied to emphasize the importance of GST catalysis in determining the rate of GSH conjugation. The spontaneous reaction of the nitrogen mustard aziridinium ion with the sulfhydryl of GSH is determined by the potential energy states of the drug electrophile and the macromolecular target nucleophile, which have been defined as "hard" or "soft" based upon the polarization of their reactive centers (19). The aziridinium ion of an activated nitrogen mustard is highly polarized, carrying a high positive charge density at the electrophilic center and is thus defined as a hard electrophile. These will most favorably react with hard nucleophiles, where the high energy transition state of the reaction is most favorable. Within these guidelines, the alkylating species of, e.g., chlorambucil will react with nucleophiles in the following order: oxygen of phosphates > oxygen of bases > amino groups of purines > amino groups of proteins > thiol group of methionine > thiol group of the cysteine in GSH. The less favorable reactions still occur spontaneously, but nucleophilic selectivity may not favor such reactions in a mixed nucleophile environment. To this end, GST catalyzes the conjugation of mustards with GSH by bringing the two into close proximity, creating a conducive hydrophobic environment and reducing the apparent pK of the cysteine thiol from 9.6 to a more neutral value. All of these factors serve to increase both the rate and extent of the conjugation when compared to the spontaneous reaction.

Because nitrogen mustards undergo spontaneous decomposition to produce active alkylating species, it is particularly significant that the aziridinium intermediate of melphalan has been reported as the true substrate for the GST-catalyzed reaction (20). Since all bifunctional nitrogen mustards have the potential to form two aziridinium ions, a significant role for GST-mediated catalysis may be possible. Intracellular chloride concentrations of 8 \(\mu\)M predict that aziridinium ion formation is essentially irreversible. The reactivity of this species with cellular nucleophiles such as phosphate and water is approximately equivalent and assuming GSH concentrations of 2.5–5.0 \(\mu\)M and a pK of 7.4, Bolton et al. (20) calculated that the reaction of GSH with the aziridinium will be 5.5 times greater than hydrolysis. However, tumor microenvironments frequently produce a more acidic pH. At pH 6.5 the contribution of GSH to melphalan detoxification was only equivalent to water. Because of this, the spontaneous GSH reaction may prove to be limiting in terms of how much and how quickly detoxification occurs.
GSH, GST, AND DRUG RESISTANCE

Enhancement of drug resistance can be achieved. The glutathionylation of melphalan catalyzed by GST\(\alpha\) was 2.5- and 9-fold (pH 7.4 and 6.5, respectively) greater than the spontaneous rate, values which correspond well with the comparative rates and extents of chlorambucil conjugation shown in Fig. 2. Thus, the GST-catalyzed rate of both chlorambucil and melphalan conjugation with GSH will be significantly greater than the spontaneous rate (12, 20). The cytoprotective consequences of this enhancement could prove to be the difference between drug-induced sublethal and lethal damage.

Transfection of GST

It is often considered that enhanced resistance to a particular drug must be shown in a transfected cell line in order for the gene product to be causatively linked to the resistant phenotype. This one area has provided substantial controversy for the GST field. A number of transfection studies have been reported and some disparity is apparent. The lack of anticancer drug resistance in MCF7 cells transfected with GST\(\pi\), \(-\alpha\), or \(-\mu\) isozymes (21) has led many to conclude that the link between enhanced GST and anticancer drug resistance is tenuous. This concept was given further credence by data showing that only low levels of resistance to alkylating agents and anthracyclines were found in NIH 3T3, C3H 10T\(\frac{1}{2}\), or monkey COS cells transfected with rat or human GSTs (1). The significance of low levels of resistance has been the subject of much debate. Degrees of selected resistance in MDR cell lines frequently reach the 1,000-10,000-fold range. Yet when MDR-1 recipient transfectant cells are tested, they usually display 5- to 20-fold resistance, with maximal degrees of resistance approaching only 30-fold. For alkylating agent-resistant cell lines, the degree of selected resistance rarely exceeds 20-fold; therefore, it could be argued that the 1.5- to 3-fold resistance reported in the GST transfectants is relatively consistent when comparing the ratio of selected/transfected resistance for alkylating agents/GST and MDR drugs/P-glycoprotein.

Of further relevance is the potential clinical meaning of low levels of resistance. There is little doubt that such levels are experimentally less easy to assay and to analyze statistically. A number of GST transfection studies have characterized low levels of resistance (i.e., 1.5- to 3-fold) to numerous anticancer drugs (reviewed in Ref. 1). Understandably, these levels of resistance have not been convincing. Alkylating agents and electrophile-producing drugs as a group tend to have survival curves characterized by a sublethal damage shoulder and steep kill curve. Given the nature of drug dose escalations in clinical protocols, a 2-fold shift in in vitro sensitivity may translate into a meaningful difference in therapeutic efficacy and patient response. However, it is likely that the debate over how to measure response and what constitutes a “meaningful” degree of resistance will continue. Notwithstanding these quantitative issues, a number of possible factors could help to explain why GST transfection in some cell lines does not produce significantly enhanced drug resistance: (a) intrinsic GSH/GST levels; (b) transcriptional and/or translational rate and turnover of GSH/GST synthesis (enzyme or transcript half-life, etc.); (c) feedback control mechanisms; (d) presence and/or efficiency of a GSH-conjugate membrane efflux pump; (e) effective compartmentalization of GSH/GST; (f) competing detoxification reactions; (g) proven substrate specificity of the transfected GST for the anticancer drug(s) tested.

The uncertainty of these earlier data in proving a direct role for GST in determining drug sensitivity has been largely alleviated by several recent reports. The first is in Saccharomyces cerevisiae where significant resistance to chlorambucil and Adriamycin (maximum resistance, 8- and 16-fold, respectively) was reported in cells transfected with mammalian GST isozymes (22). Because of the yeast cell wall, high concentrations of drug were required to achieve cell kill, leading to some debate as to the relevance of yeast as a test organism. Since yeasts do express endogenous GST that have detoxification functions, the addition of heterologous GST would seem to be a legitimate, reasonable approach to answering specific functional questions.

In mammalian cells, Schecter et al. (23) recently transfected the rat \(Y\)\(\gamma\) gene (\(\alpha\) family) into rat mammary tumor cells and showed high levels of induced resistance to a number of nitrogen mustards. The degree of resistance conferred ranged from 6- to 30-fold for chlorambucil, melphalan, and nitrogen mustard. These resistance factors are equal to, or exceed, those of most acquired resistant cell lines. The results are perhaps more convincing, since overexpression of the \(Y\), GST subunit has been circumstantially linked to mustard resistance by nature of increased expression in rat cell lines (1, 24). In addition, the mouse and human \(\alpha\)-GST family show the best catalytic constants for the conversion of chlorambucil and melphalan to their respective monoglutathionyl derivatives (12, 20).

Two other studies have transfected Chinese hamster ovary cells with GST\(\pi\) (25) or GST\(\alpha\) (26). GST\(\pi\) increased resistance to cisplatin and carboplatin by 2- to 3-fold, while GST\(\alpha\) afforded protection against bleomycin. The latter study also quantitatively correlated GST\(\pi\) expression with bleomycin resistance. Although both papers implicate GST-catalyzed detoxification of the parent drugs or their metabolites as a mechanism of increased resistance, neither of them included such data. These drugs are known to form conjugates with GSH and it is therefore feasible that they may be substrates for GST catalysis. They are included in the category of “indirect evidence” in Table 1, indicating that they are implicated as but not proved to be GST substrates. One feasible explanation for the occurrence of low levels of resistance in transfectants is that the increased GST provides additional thiol groups to act as a drug sink. Some drugs (e.g., ethacrynic acid (27)) can bind directly to GST isozymes. The physical presence of additional GST in transfected cells could subsequently reduce the effective intracellular concentrations of some drugs.

It would seem reasonable to suggest that the positive transfection data should satisfy the question as to whether GSTs have a direct role in some types of drug resistance. However, it is of importance to interpret the clearly positive data in the context of the more equivocal or negative results. There are several possible explanations for the less convincing or negative data. One is that GSTs are not in of themselves sufficient to convey resistance. This would require the existence of putative complimentary “factors” in cells which yielded positive correlates. For example, the requirement for GSH as a cosubstrate may be limiting in some target cells. As discussed elsewhere in this article, the maintenance of intracellular levels of reduced glutathione is controlled by a complex, interrelated series of pathways. The transfection and ultimate overexpression of one enzyme within this pathway may not be the optimal way to achieve or maintain a GSH homeostasis. Interestingly, the negative transfection data have primarily come from MCF7 cells. The wild type cells have a GST specific activity of 4 mmol/min/mg protein; one of the lowest activities, if not the lowest activity, measured in tumor cell lines. In addition, transcript levels for \(\gamma\)-GCS are also extremely low in MCF7 cells, suggesting a limited capacity for de novo GSH synthesis via the \(\gamma\)-glutamyl cycle. Perhaps MCF7 cells, with such low intrinsic steady-state levels of GST and capacity for GSH synthesis, utilize other detoxification pathways more efficiently.

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4 Unpublished observation.
Therapeutic Correlates of GST Expression

Despite a profusion of recent literature reports cataloguing GST expression in many tumor and normal tissues from human biopsies, actual attempts at therapeutic manipulation through drug intervention have been more limited. Much effort has been focused on seeking correlates between patient treatment failures and enhanced expression of genes involved in drug resistance. To date such correlates have been detailed in only a portion of trials involving MDR-1, \(\gamma\)-glutamyltransferase (nitrosoarene resistance), GSH, and GST (1). For all of these systems, negative results may have been compromised by the limited sensitivity of the methodology used and the fact that low levels of resistance may be clinically important but difficult to show experimentally. Limited availability of tissue (especially tumor) that is amenable to sequential biopsy during and/or following chemotherapy also limits the feasibility of this approach. However, there are at least two examples in which enhanced expression of GST has been found in tumor samples from patients who have become refractory to drug treatment.

In the first, two cell lines were established from an ovarian cancer patient with abdominal ascites (28). PEO1 was established from cells that were harvested before starting treatment. PEO4 was established following the acquisition of resistance to a regimen of cis-platinum, chlorambucil, and 5-fluorouracil. When comparing a variety of parameters of glutathione metabolism, increases in \(\gamma\)-GPT (6.5-fold) glutathione peroxidase (2.3), and GST (2.9) were found. The issues which were of most concern in this study were the fact that the tumor cells were cultured (albeit a short time) prior to analysis, perhaps allowing drift in sensitivity. Furthermore, a drug combination was used in the patient with the result that definitive proof that chlorambucil was the causative agent in the overexpression of these enzymes could not be shown. Because of the easy access to sequential tumor biopsy, CLL provides a good model system to study clinical resistance. Analysis of lymphocytes from CLL patients using the general GST substrate 1,2-chlorodinitrobenzene showed a statistically significant 2-fold increase in cells from chlorambucil-resistant patients compared to untreated patients or normal individuals (29). Furthermore, chlorambucil therapy caused a 1.5-fold elevation in enzyme activity in three previously drug-naive patients. These two clinical reports have provided inferential correlational data for patient response and drug sensitivity. Clearly, such studies are not conclusive and more work will be required; but in an area beset by practical limitations, these correlations are encouraging.

The emergence of ethacrynic acid as an effective inhibitor of GST has led to its consideration as a modulator of GST-mediated drug resistance. Preclinical studies have demonstrated that reversal of resistance in cell lines may be achieved at low concentrations of ethacrynic acid. A Phase I trial suggested that drug-induced diuresis with resultant fluid imbalance was the major dose-limiting toxicity (30), but this was not serious enough to prevent the formulation of a Phase II study of ethacrynic acid and chlorambucil in drug-resistant CLL. Because of the low number of such patients and the emergence of fludarabine as a viable treatment for chlorambucil-refractory CLL, the protocol is accruing patients at a slow rate in the United States. Its potential, therefore, remains largely unknown. However, one case report (31) details a partial reversal of chlorambucil resistance in a B-CLL patient treated simultaneously with ethacrynic acid and chlorambucil. Thus, while the approach has merit, large scale studies are needed. Design, synthesis, and testing of novel GST inhibitors are presently in progress (32) and may well yield new compounds worthy of clinical testing.

Assessment of the quantitative aspects of drug resistance is also critical in appreciating the value of a resistance reversal approach. The measurement of drug synergy is usually achieved by isobologram analysis. Although there is some debate over the best mathematical approach to define synergy, it is generally agreed that their combined effects must be greater than their composite additive cytotoxicities. For modulators of resistance, there is frequently little or no intrinsic toxicity at the concentrations required to reverse resistance; therefore, synergy can be readily shown. More problematic, however, is the extrapolation of preclinical synergy to human trials. For example in MDR cell lines, conversion of a 1000-fold resistant phenotype to 20-fold would apparently represent a gain worthy of clinical study based upon published and ongoing clinical trials with MDR-1 modulators. For alkylating agents, such a quantitative in vitro enhancement may be difficult to achieve experimentally, since 10–20-fold resistance is apparently maximal for this class of drugs. These comparisons are further confounded by the clinical consideration that low level resistance may be quite critical in determining success or failure to therapy. It may be necessary to apply a dual standard for judging efficacy. Modulators of alkylating agents may not need to be as quantitatively effective in vitro as those of MDR yet may still be therapeutically valuable.

Conclusions and Perspectives

There is a growing body of evidence that the phenotype of drug resistance may result from reduction in the ability of a cell to recognize and/or instigate programmed cell death as a consequence of exposure to drug. For example, the adenovirus gene EIA has been shown to sensitize cells to a range of anticancer drugs. In this system a functional p53 tumor suppressor gene is required to allow programmed cell death (33). Similarly, bcl-2 seems to prevent cell death by protecting cells from oxidative stress. Moreover, if the bcl-2 gene expression is suppressed, free radical damage accumulates sufficiently to activate the apoptotic pathway (34). These findings could prove to have enormous significance with regard to how drug resistance is viewed and perhaps manipulated. However, in the absence of genetic changes which could create an "apoptotic null phenotype," they may be of more limited relevance to the role of detoxification in determining cell response. As stated earlier, cells have a characteristic survival pattern when treated with alkylating agents. The short half-life, electrophilic reactivity, and covalent nature of these drugs ensures that a damage threshold is reached in an acute, rather than a chronic, manner. Most clinically used alkylating agents have half-lives of less than 1 h. Although there may be latent damage which can lead to cellular toxicity, generally if a cell can minimize the early lesions and maintain damage below a specific threshold, it will survive. The quantitative definition of this threshold is offered in an arbitrary fashion for the purpose of this discussion. However, there is reason to believe that this value will vary both within and between tumor cell populations. In any case, the importance of GSH and associated enzymes lies in the provision of an enhanced rate and extent of detoxification, helping to maintain the amount of damage below that which may trigger any apoptotic cascade. Even in the absence of definitive information as to which drug lesions (and how many) may induce this pathway, the principle of detoxification maintaining damage control would be of significance. Although there is some debate as to the precise role of free radical damage in mediating the initiation of apoptosis, it could be argued that an enhanced cellular capacity to maintain a reduced thiol status may even contribute directly to an interference in the early stages of this process. Indeed, free radical-mediated damage appears to be of primary significance in aging, cancer, and many other human conditions.

What are the future therapeutic possibilities involving GSH/GST? Presently, serum GST levels are being tested as potential markers for cancer detection and even as markers for organ rejection in liver.
transplants. GST\textsuperscript{\textgamma} is up-regulated in a large proportion of the human malignancies thus far analyzed. Certain produgs are under development to take advantage of this observation. An inactive drug which upon catalysis by GST\textsuperscript{\textgamma} becomes cytotoxic, offers a nonempiric rationale for gaining an enhanced therapeutic index. Use of inhibitors of GST have, to this time, focused upon small chemical moieties with little tissue or isozyme specificity. Current and future endeavours are aimed at targeting the inhibitor against specific isozymes. If successful, this could achieve a directed inhibition of GST\textsuperscript{\textalpha} which would yield the best chance of reversing alkylating agent resistance (32). The obverse of this approach is exemplified by ongoing chemoprevention trials with oltipraz. Chronic low level doses of oltipraz are known to induce GST (and other Phase II detoxification enzymes) expression in colon epithelium. The efficacy of this approach in preventing cancer incidence in high risk populations is under intensive study.

One of the more recent in vague efforts at therapeutic management involves the transfer of drug resistance genes or cDNAs into normal tissues, such as bone marrow. Theoretically, this approach should increase the normal tissue tolerance and enhance the therapeutic index of a drug for which marrow is the dose-limiting tissue. This paradigm if applied to alkylating agents and GST and GST enzymes may allow protection of bone marrow stem cells at drug levels sufficient to kill tumor cells. Some consideration of existing data may suggest at least two adaptations which may enhance the chances for success: (a) transfer GST\textgamma together with a GST\textalpha family member. This will increase the normal tissue tolerance and enhance the therapeutic index of a drug for which marrow is the dose-limiting tissue. This paradigm if applied to alkylating agents and GST and GST enzymes may allow protection of bone marrow stem cells at drug levels sufficient to kill tumor cells. Some consideration of existing data may suggest at least two adaptations which may enhance the chances for success: (a) transfer GST\textgamma together with a GST\textalpha family member. This will reduce the chances that GST may be limiting as a cofactor for GST; (b) the rat Y\textgamma, is the most inducible of all the \alpha isozymes by nitrogen mustards (human GST \alpha genes do not have the same 5 regulatory elements as those of the rat). Transfection of the Y\textgamma cDNA has yielded the most mustard-resistant clones. Preliminary substrate specificity data indicate that Y\textgamma isozymes have meaningful catalytic constants for detoxification of the nitrogen mustards (data not shown). These observations suggest that in humans, transgenic Y\textgamma transfers may be more effective than using human \alpha isozyme constructs. Whether this is combined with a rat or human GST\textgamma is less important, since there is no present indication that the two enzymes differ significantly in their capacity for de novo synthesis of GST.

For as long as toxic chemicals with electrophilic centers are used in cancer chemotherapy, there will be a sound rationale for studying GST and the enzymes that produce it and use it. At least for the short term, such drugs should continue to play a significant role in patient treatment.

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References
