

Glutathione Metabolism as a Determinant of Therapeutic Efficacy: A Review

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Abstract

Glutathione, as the chief nonprotein intracellular sulfhydryl, affects the efficacy and interactions of a variety of antineoplastic interventions, mainly through nucleophilic thioether formation or oxidation-reduction reactions. Thus, glutathione plays a role in the detoxification and repair of cellular injury by such diverse agents as mechlorethamine, melphalan, cyclophosphamide, nitrosoureas, 6-thiopurine, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide, the quinone antibiotics (including Adriamycin, daunorubicin, and mitomycin C), the sesquiterpene lactones (such as vernolepin), and other sulfhydryl-reactive diterpenes (like jatrophone). Glutathione may play a similar role in host and tumor cell responses to radiation, hyperthermia, and the reactive reduction products of oxygen secreted by inflammatory cells. Further, glutathione participates in the formation of toxic metabolites of such chemotherapeutics as azathioprine and bleomycin and may affect the cellular uptake of other agents, such as methotrexate. It seems likely that alterations in glutathione metabolism of tumor or host as a result of one therapeutic intervention may affect the outcome of concurrent treatments. Knowledge of these interactions may be useful in designing combination therapy for neoplastic disease.

Introduction

For a variety of antineoplastic interventions, GSH¹ metabolism helps to determine the degree of toxicity to the tumor and/or the host. The importance of GSH in the toxicology and metabolism of several toxins and drugs has been reviewed (65, 84, 99, 103). Our purpose here is to offer a broad overview of the influence of GSH metabolism on therapeutic intervention in neoplastic diseases.

Biochemistry of GSH

Aspects of GSH biochemistry are briefly outlined as background for a consideration of the importance of GSH metabolism in antineoplastic therapy. A more detailed view of GSH metabolism is available in several monographs and review articles (3, 40, 71, 76, 78, 112).

GSH (Chart 1) is the predominant intracellular nonprotein sulfhydryl (NPSH) in a wide range of cells, both prokaryotic and eukaryotic, plant and animal. Biosynthesis of GSH occurs in 2 successive ATP-requiring steps. First, γ -glutamylcysteine synthetase catalyzes the formation of an amide linkage between cysteine and the γ -carboxyl of glutamate. GSH synthetase then

mediates the reaction of glycine with the cysteine carboxyl of γ -glutamylcysteine to form the tripeptide γ -glutamylcysteinylglycine.

In mammalian cells, GSH exists as at least 3, possibly 4, dynamically interchanging metabolites. Under normal steady-state conditions, the majority of GSH exists in the reduced form (0.5 to 10 mM). Oxidation of reduced GSH, either nonenzymatically or by the action of glutathione peroxidase (GPO), yields GSSG. NADPH-dependent reduction of GSSG by GR, as well as efflux, effectively maintains the intracellular concentration of GSSG at very low levels (usually 5 to 50 μ M). The extent to which GSH oxidation is occurring under normal conditions, *i.e.*, the flux rate of GSH through the oxidation-reduction cycle in the absence of an imposed oxidant stress, has not been determined either in tissue culture or *in vivo*.

In a third form, cellular GSH participates in mixed disulfides with both protein and nonprotein sulfhydryls. For example, in an Ehrlich ascites tumor cell line, approximately 35% of total cellular GSH was in disulfide linkage to protein (85).

Thiol esters of GSH constitute yet another potentially significant form of cellular GSH. Although the occurrence and metabolism of such thiol esters have not been adequately studied, the identification by Uotila (121) of at least 3 distinct GSH thiol esterases in human liver suggests functional significance.

The numerous reactions of GSH in mammalian cells can be divided into those involving principally the γ -glutamyl portion of the tripeptide *versus* those of the sulfhydryl moiety. Sulfhydryl-dependent metabolism of GSH can be further subdivided into oxidation-reduction reactions *versus* nucleophilic reactions in which the reduced sulfhydryl reacts with an electrophile to form a thioether (Chart 2). By forming such thioethers, the cell may detoxify potentially harmful compounds.

An alternative scheme by which GSH metabolism can be categorized distinguishes between reversible and irreversible loss of reduced GSH. Reactions involving the formation of GSSG, mixed disulfides, or thiol esters result in reversible loss since resynthesis of GSH from its amino acid constituents is not required for maintenance of cellular content of GSH in the reduced form. In contrast, irreversible loss of GSH is the consequence of reactions resulting in thiol conjugate formation, such as the mercapturic acid pathway, or efflux of GSH from the cell, where it may be degraded by the externally disposed enzyme γ -glutamyl transpeptidase as part of the γ -glutamyl cycle. Synthesis is required for restoration of cellular GSH levels in response to irreversible loss.

GSH has been shown to play a critical role in cellular defense against a variety of injurious agents. In this regard, the sulfhydryl group assumes primary importance. Thus, in many cell types, the GSH oxidation-reduction cycle helps maintain structural and functional viability in spite of endogenous production of reactive oxygen intermediates, an apparently unavoidable consequence

¹ The abbreviations used are: GSH, glutathione; NPSH, nonprotein sulfhydryl; GPO, glutathione peroxidase; GSSG, glutathione disulfide; GR, glutathione reductase; BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AMSA, 4'-(9-acridinylamino)methanesulfon-*m*-anisidide; ROI, reactive oxygen intermediates.

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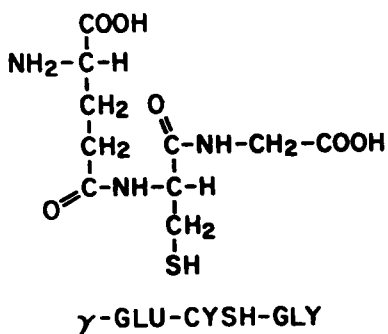


Chart 1. Structure of GSH.

GSH Metabolism as Determinant of Therapeutic Efficacy

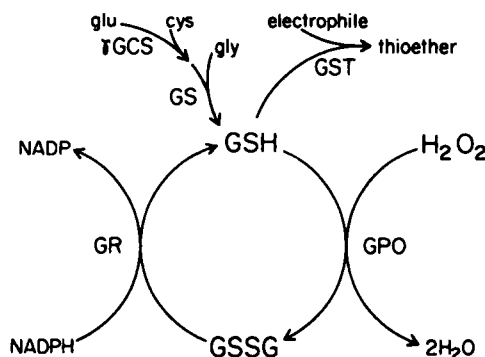


Chart 2. Aspects of sulfhydryl-dependent GSH metabolism. Oxidation-reduction cycle and thioether formation. The enzymes shown are: GPO; GR; γ -glutamyl-cysteine synthetase (γ GCS); glutathione synthetase (GS); glutathione S-transferase (GST).

of aerobic metabolism, or during acute oxidant injury. The capacity of the oxidation-reduction cycle may be related to the cellular content of GSH, GR, and/or GPO, as well as to the ability of the cell to regenerate NADPH via the pentose phosphate shunt. Similarly, as the NPSH of greatest abundance, GSH protects against toxic electrophiles by thioether formation. The effectiveness of this pathway may depend upon the intracellular concentration of GSH, the presence of GSH S-transferases of appropriate specificity, and/or the capacity of the cell for rapid resynthesis of GSH.

Influence of GSH on Antineoplastic Therapy

The various ways in which GSH metabolism may be of importance in antineoplastic therapy can be considered in terms of 4 determinants of therapeutic efficacy.

Detoxification. GSH may protect cells via detoxification of active species and/or repair of injury.

Toxicification. GSH may be involved in the formation of active species, within either the tumor or normal cells of the host.

Delivery. Drug targeting and uptake, and other aspects of delivery of the toxic agent to its site of action, may be influenced by GSH metabolism.

Therapeutic Interactions. An alteration in the GSH status of tumor or host may influence the effectiveness or toxicity of a concurrent or subsequent therapy.

Specific examples of each of these are discussed below.

Detoxification

Chemotherapeutic Agents

Mechlorethamine and Melphalan. The ability of alkylating agents such as the nitrogen mustards to react with a variety of cellular nucleophiles, including sulfhydryl groups, has long been recognized. The toxicity of mechlorethamine in experimental animals was shown to be diminished by appropriately timed administration of cysteine (19) or cysteamine (100). *In vitro* exposure of cells to cysteamine can result in a significant increase in intracellular GSH, presumably by release of GSH from mixed disulfides with proteins (106).

Analyses of the sulfhydryl content of murine tumors of varying sensitivity to mechlorethamine and its *N*-oxide derivative have revealed potentially significant correlations. Hirono (60) generated resistant variants of 3 ascites tumors by repeated passage through treated animals and found in all 3 cases that the NPSH content of the tumors was increased. Among the original cell lines, relative drug susceptibility did not correlate with the content

of NPSH, suggesting a mechanistic distinction between innate *versus* acquired resistance to this agent. Similarly, Goldenberg (45) demonstrated increased NPSH content in 2 variants of a lymphoblast cell line selected *in vitro* for resistance to mechlorethamine.

Comparing 6 murine tumors of varying sensitivity to melphalan, an isomer of melphalan, Calcutt and Connors (22) demonstrated a relationship between inhibition of tumor growth by this agent and the ratio of protein sulfhydryl to NPSH. Ball *et al.* (12) evaluated the content of protein, DNA, RNA, nucleotides, and NPSH in a melphalan-resistant Yoshida sarcoma relative to the sensitive parental cell line growing in the contralateral limb and found the only difference to be an increased level of NPSH in the resistant tumors. More recently, Suzukake *et al.* (116) have shown that an L1210 leukemia cell line made resistant to melphalan contained a 2-fold greater content of GSH and could be resensitized by lowering its GSH content. This was achieved by incubation of the cells in medium deficient in cystine for 24 hr prior to exposure to melphalan. Further, comparing 3 L1210 cell lines, with and without cystine deprivation, resistance to melphalan correlated with both GSH content and enhanced conversion of melphalan to an inactive metabolite (117). A 2-fold increase in NPSH content has also been documented for a melphalan-resistant subline of Chinese hamster ovary cells (14). Seagrave *et al.* (108) have reported that treatment of Chinese hamster ovary cell lines with zinc resulted in increased GSH content and GSH S-transferase activity as well as resistance to melphalan toxicity.

Cyclophosphamide. Cyclophosphamide, a widely used nitrogen mustard, undergoes metabolic activation by hepatic mixed-function oxidases. The generally accepted mechanism for the generation of active metabolites involves oxidation to 4-hydroxycyclophosphamide, which would be in tautomeric equilibrium with aldophosphamide. Nonenzymatic cleavage of aldophosphamide results in the formation of 2 toxic species, phosphoramidate mustard and acrolein. Phosphoramidate mustard is believed to be the therapeutically active species while acrolein is held to be responsible for some aspects of host toxicity, such as hemorrhagic cystitis. An alternative metabolic pathway for 4-hydroxycyclophosphamide, involving loss of water to form a sulfhydryl-reactive species, has been suggested (24, 38). Such sulfhydryl derivatives of cyclophosphamide, themselves inactive, may serve as chemically stable prodrugs, able to release by hydrolysis an active cyclophosphamide metabolite (101).

Gurtoo *et al.* (51) have demonstrated significant dose-dependent depletion of hepatic NPSH in mice by both cyclophosphamide and acrolein, but not phosphoramidate mustard. They further showed that cyclophosphamide-induced inhibition of weight gain in rats was enhanced by coadministration of a GSH depletor, diethyl maleate, and diminished by prior injections of cysteine. Cysteine also delayed the onset of hematuric cystitis in cyclophosphamide-treated rats. Injection of cysteine or GSH protected against cyclophosphamide-induced teratogenesis in pregnant rats (7). The antitumor activity of cyclophosphamide appears not to be inhibited by cysteine administration (51). In a similar manner, *N*-acetylcysteine protected mice from the toxic effect of isophosphamide, an analogue of cyclophosphamide, without interfering with its antitumor activity against L1210. This allowed for the safe administration of higher doses of isophosphamide and enhanced therapeutic efficacy (123).

Nitrosoureas. Inhibition of GR in the erythrocytes of patients receiving therapeutic doses of BCNU was first reported by Frischer and Ahmad (43). Babson and Reed (10) have demonstrated that the isocyanates derived from the breakdown of BCNU and other nitrosoureas are responsible for the irreversible inactivation of GR. They further suggest a correlation between nitrosourea-induced myelosuppression and the inhibition of GR. There is, however, disagreement about the ability of nitrosoureas other than BCNU to inactivate GR (43, 109). The relevance of this finding to the therapeutic action or toxicity of BCNU is not clear. Administration of BCNU to tumor-bearing mice resulted in a significant decrease in hepatic GSH content, accounting for greater than 50% of the dose of BCNU, assuming a one-to-one reaction stoichiometry (75). It has been suggested by Nathan *et al.* (90) that inhibition of GR activity in alveolar macrophages and other pulmonary cells may play a role in the development of pulmonary toxicity after repeated administration of BCNU.

6-Thiopurine. The cytochrome P-450 system activates 6-thiopurine, *in vivo* or *in vitro*, to a reactive intermediate which binds irreversibly through disulfide linkage to protein (62, 63). In the presence of GSH, protein binding was diminished and GSSG was formed. It was proposed that GSH competes with protein sulfhydryls for reaction with the drug metabolite, purine-6-sulfenic acid, forming thiopurine-GSH mixed disulfide. This then reacts with another GSH molecule to regenerate 6-thiopurine and GSSG. Depletion of hepatic GSH concentration by feeding rats a protein-free diet prior to injection of radiolabeled 6-thiopurine resulted in increased protein binding (63). It is not yet clear whether these findings are relevant to the toxicity of 6-thiopurine.

AMSA (NSC 141549). Cysyk *et al.* (30) reported a 40% drop in hepatic GSH content within 2 to 3 hr after *i.p.* injection (100 mg/kg) of this acridine derivative in mice. These authors proposed that excretion in the bile of a GSH-acridine thioether conjugate was the predominant pathway of detoxification. Subsequently, Shoemaker *et al.* (111) demonstrated that biliary excretion of AMSA, administered *i.v.* to rats (10 mg/kg), was markedly diminished by pretreatment with diethyl maleate, which itself resulted in a 90% depletion of hepatic GSH. A role for the mixed-function oxidase system was also suggested by the observation that excretion was enhanced by pretreatment with phenobarbital and diminished by pretreatment with metyrapone (111). Careful analysis of the fate of radiolabeled AMSA in rats has confirmed the principal biliary metabolite to be a GSH

thioether, with GSH linked to the 5' position of the anisidine ring. Conjugation with GSH presumably occurs after oxidation of AMSA by the mixed-function oxidase system to a quinone-like diimine (110). An analogous mechanism, oxidation to a GSH-reactive quinone imine, has been proposed for Celiptium (NSC 264137), an ellipticine derivative (74, 86).

Although the importance of similar metabolic transformations of AMSA within tumors is not known, it is clear that the levels of both GSH and mixed-function oxidase are important determinants of drug plasma levels (111).

Quinone-containing Drugs. Antineoplastic agents which contain a quinone moiety are believed to undergo metabolic activation by microsomal enzymes to free radicals, themselves toxic, which can interact with oxygen to generate ROI via superoxide anion (11). Examples of such drugs include Adriamycin and daunorubicin (benzantraquinones), mitomycin C and streptonigrin (*N*-heterocyclic quinones), and lapachol (a naphthoquinone). While it is believed that such oxygen-dependent metabolism of streptonigrin forms the basis of its antitumor activity, the observation by Teicher *et al.* (120) that mitomycin C and Adriamycin are preferentially toxic to hypoxic tumor cells suggests the existence of oxygen-independent or -inhibitable pathways of metabolic activation. Nevertheless, the generation of free radicals and ROI by these agents may be an important determinant of drug toxicity.

In particular, substantial evidence implicates GSH metabolism as a major determinant of cardiac damage by Adriamycin. Analysis of the antioxidant defenses of mouse heart has identified selenium-dependent GPO as a major pathway for the detoxification of ROI generated by Adriamycin (37). Doroshow *et al.* (37) have further demonstrated that administration of Adriamycin to mice resulted in a dose-dependent decrease in the activity of cardiac GPO, with no effect on the hepatic enzyme. Obtaining similar results with Adriamycin-treated rabbits, Revis and Marusic (107) also noted a significant decrease in the cardiac content of selenium after drug treatment. GSH content was diminished slightly in both liver and heart after injection of mice with Adriamycin (35, 97). The lethal toxicity of Adriamycin to mice could be markedly increased by first depleting GSH with diethyl maleate (97). Working with isolated hepatocytes, Babson *et al.* (9) have shown that treatment with BCNU, resulting in a >90% inhibition of GR and a 70% depletion of GSH, augmented the toxicity of Adriamycin and increased the degree of lipid peroxidation. In contrast, 75% GSH depletion by diethyl maleate, without inhibition of GR, had no effect. Meredith and Reed (79) have recently reported that, while BCNU depletes only cytoplasmic GSH, the hepatotoxic combination of BCNU and Adriamycin depletes both cytoplasmic and mitochondrial GSH. Administration of various antioxidants to mice, such as tocopherol, cysteamine, and *N*-acetylcysteine, diminished the toxicity of Adriamycin, without interference with its antitumor activity, resulting in an increase in the therapeutic index (36, 42, 88).

Sulfhydryl-reactive Antineoplastic Agents. A high degree of sulfhydryl reactivity is a shared feature of a variety of experimental antineoplastic agents, many of them isolated from natural sources. Fujita and Nagao (44) have reviewed the sulfhydryl reactivity of such antitumor alkylating agents in cell-free systems. However, until recently, little information was available concerning the interaction of such compounds with intracellular sulfhydryl groups, such as GSH. We have studied the effect of 4 sesqui-

terpene lactones and the related diterpene, jatrophone, on tumor cell GSH content (5). We have found these agents to be potent depletors of cellular GSH. In addition, lysis of P815 mastocytoma cells upon incubation with any of these sulfhydryl-reactive agents was greatly enhanced by concurrent inhibition of GSH biosynthesis with buthionine sulfoximine, itself a nontoxic agent. This synergy was most evident with jatrophone; in the presence of buthionine sulfoximine, the concentration of jatrophone required to achieve 50% cytotoxicity in 18 hr was approximately 21-fold less than that necessary in the absence of buthionine sulfoximine (5). As illustrated in Chart 3, buthionine sulfoximine consistently reduced the concentration of lytic agent causing 50% specific release of ^{51}Cr label from the cells for all 4 sesquiterpene lactones. Jackson *et al.* (64) have reported that incubation of Yoshida sarcoma or L1210 cells with cytembena (*cis*- β -4-methoxybenzoyl- β -bromoacrylate), an anticancer drug susceptible to nucleophilic addition by sulfhydryls, resulted in substantial depletion of GSH. Perhaps the therapeutic effectiveness of cytembena and other sulfhydryl-reactive agents could be improved by the use of inhibitors of GSH biosynthesis.

Radiation

The attenuated radiosensitivity of cells in an oxygen-deficient atmosphere compared to irradiation in air has been termed the "oxygen effect." Neither nucleic acids nor enzymes in aqueous suspension exhibit such an effect. Howard-Flanders (59) showed that the sensitivity of T-2 bacteriophage to inactivation by irradiation was independent of the concentration of oxygen. The addition of cysteamine decreased the extent of inactivation when irradiation was conducted in a nitrogen atmosphere but had no effect in the presence of oxygen. Similar studies concerning inactivation of DNA and trypsin by irradiation in the presence and absence of GSH helped to focus attention on cellular thiols as mediators of the oxygen effect (61).

The notion that cellular NPSH might be responsible for anoxic radioresistance has been evaluated in a variety of settings. Revesz *et al.* (105) studied a subline of an Ehrlich ascites tumor obtained by sublethal *in vitro* irradiation between passages, for 23 passages, as well as a series of single-cell clones from this subline. In comparison to the parental cell line, the cell populations thus derived, with one exception, exhibited both enhanced anoxic radioresistance and increased NPSH content. In the presence of oxygen, all sublines tested were as susceptible to the effects of radiation as was the parental line.

The role of GSH in cellular response to irradiation has been further evaluated by experiments with cultures of fibroblasts from a child suffering from 5-oxoprolinuria due to homozygous GSH synthetase deficiency and from his healthy brother (32, 33, 80). The deficient cells (containing approximately 6% of control GSH) and normal cells were of equivalent sensitivity when irradiated in an atmosphere of 100% oxygen. However, with radiation exposure under anoxic conditions, damage to the normal cells was diminished, while the susceptibility of GSH-deficient cells was unchanged. Radiosensitization of hypoxic cells by removal of GSH has been demonstrated with diamide (56, 57), although doses in excess of that necessary to oxidize GSH to GSSG are required, and with diethyl maleate (21), which resulted in a greater than 95% depletion of GSH. Dethmers and Meister (34) have examined the euoxic radiosensitivity of a human T-cell

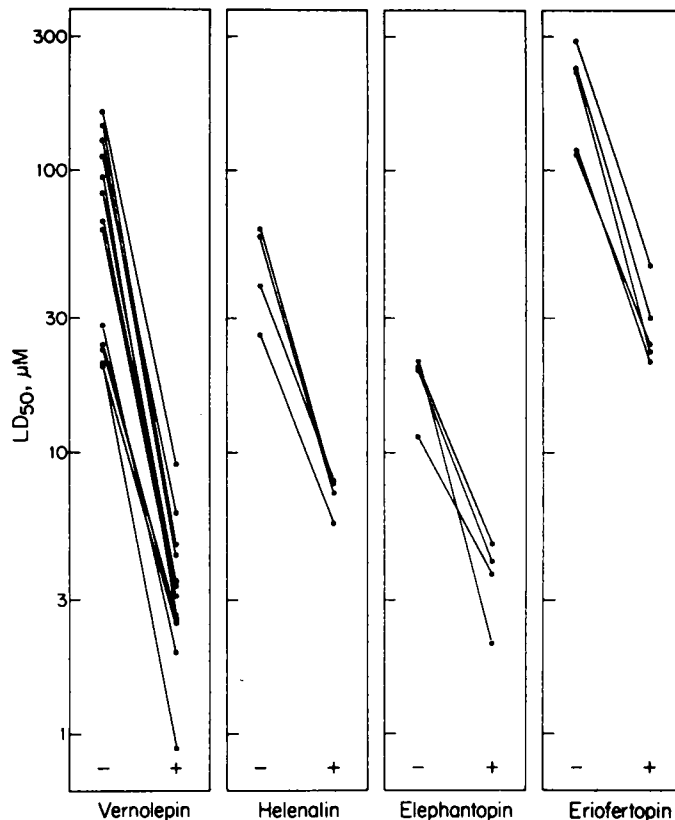


Chart 3. Effect of buthionine sulfoximine on sensitivity of P815 mastocytoma cells to lysis by sesquiterpene lactones. Lysis of ^{51}Cr -labeled tumor cells after an 18-hr incubation with the indicated sesquiterpene lactones was evaluated as described in Ref. 5. Values for concentration of lytic agent causing 50% specific release of ^{51}Cr label from the cells (LD_{50}) were calculated from dose-response curves in the absence and presence of buthionine sulfoximine, as indicated by the - and + at the bottom of each panel. Each pair of connected points represents an experiment. Average spontaneous ^{51}Cr releases in the presence and absence of buthionine sulfoximine were 26.6 ± 4.4 (S.E.) and $25.7 \pm 4.3\%$, respectively ($n = 26$).

line after a 24-hr incubation with buthionine sulfoximine, an inhibitor of GSH biosynthesis, which results in approximately 95% depletion of GSH content. Compared to control cells, these GSH-deficient cells were more radiosensitive, although radiation survival curves were not generated (the highest dose resulted in a survival fraction of 0.5). More recently, Biaglow *et al.* (15) and Mitchell *et al.* (82) have demonstrated that depletion of GSH by inhibition of synthesis with buthionine sulfoximine augmented both hypoxic and euoxic radiosensitivity of A549 human lung carcinoma cells and Chinese hamster V79 cells. Much of this work has been reviewed in greater detail by Biaglow *et al.* (17).

Theoretically, the importance of GSH as a determinant of radiosensitivity could result from a role in the detoxification of radiation-induced toxins, such as free radicals, and/or the repair of critically damaged cell structures. GSH can engage in one-electron reactions with potentially harmful free radicals by hydrogen atom donation, thus forming the relatively stable thiyl radical, and ultimately GSSG [reviewed by Kosower and Kosower (70)]. Analysis of the rate at which irradiation-induced single-strand DNA breaks were repaired by the GSH-deficient human fibroblasts described earlier failed to demonstrate a role for GSH under conditions of anoxia (39). In contrast, GSH content was a determinant of the repair capacity of cells following aerobic

irradiation (39). Still, the precise mechanism(s) by which GSH is involved in hypoxic or euoxic cell defense against radiation damage have not been determined. It is clear, however, that for some cell types, the presence of oxygen serves to change the kinetics, magnitude, and/or nature of the injury such that GSH is no longer protective.

Radiosensitizers

Hypoxic, poorly vascularized cells within solid tumors and their metastases limit the efficacy of both radiotherapy and blood-borne chemotherapy (69). One approach to the problem of hypoxic cell radioresistance has been the development of chemical radiosensitizers, which by virtue of their affinity for electrons serve to mimic the effects of oxygen. Recently, it has been reported that incubation of cells under hypoxic conditions with a variety of nitroimidazole sensitizers, such as misonidazole, resulted in significant loss of GSH (8, 16, 55, 122). Furthermore, radiosensitization of hypoxic Chinese hamster ovary cells by misonidazole was markedly enhanced by inhibition of GSH biosynthesis with buthionine sulfoximine (58). Taylor and Rauth (119) have suggested that misonidazole is metabolized by an oxygen-inhibited pathway, resulting in the generation of cytotoxic species. Others have demonstrated that the toxicity of misonidazole itself toward hypoxic Chinese hamster cells could be augmented by prior depletion of GSH with diethyl maleate (20). Analysis of the significance of GSH depletion to either the mechanism(s) of radiosensitivity or the differential toxicity of misonidazole and related compounds towards hypoxic cells is an important topic for future research.

Hyperthermia

Recent evidence from Mitchell *et al.* (81, 83) has implicated GSH content as a determinant of thermosensitivity and increased GSH synthesis as an early and perhaps critical cellular response to hyperthermia. Working with Chinese hamster V79 cell cultures, they have reported that inhibition of GSH biosynthesis resulted in depletion of GSH and enhanced susceptibility to hyperthermic injury. Development of thermotolerance in these cells was associated with a rapid increase in GSH content, followed by the synthesis of heat shock proteins. Both events were prevented by inhibition of GSH synthesis. Interestingly, a role for an increased content of GSH in the development of thermotolerance may be restricted to the first hr of heating, since a 1-hr delay in inhibition of GSH synthesis allowed for the eventual development of thermotolerance (81). While much remains to be learned concerning the nature of hyperthermic injury and the basis of thermotolerance, this work suggests that GSH metabolism may constitute a profitable target for optimal hyperthermia therapy.

Cells of the Host Immune System

Various cells of the host immune system have the capacity to lyse tumor cells. An understanding of the biochemical mechanisms underlying cell-mediated cytotoxicity could be of considerable value for the development of effective immunotherapy. Over the past 8 years, numerous reports have described oxygen-dependent mechanisms of cytolysis by granulocytes and acti-

vated macrophages (18, 26–29, 52, 92, 94, 96, 114, 115, 124–126). These effector cells can be stimulated to release H₂O₂ and other ROI at substantial rates, resulting in concentrations sufficient to lyse target cells (91, 95, 104). Indeed, exposure to H₂O₂ appears to be the principal injurious event resulting in tumor cell lysis by phorbol myristate acetate-triggered granulocytes and macrophages, by macrophages in the presence of antitumor antibody, and by activated macrophages without the addition of triggering agents if the tumor cells are first exposed to the cytophilic peroxidase of eosinophils (89). The susceptibility of various murine tumor cells to cell-mediated lysis in these settings is largely a measure of the antioxidant capabilities of the target cell, chiefly the GSH oxidation-reduction cycle. The relative susceptibility of 6 tumors to lysis by H₂O₂ (log of H₂O₂ flux in nmol/min necessary to cause 50% lysis in 4.5 hr) correlated with cellular GSH content ($r = 0.91$). Further, depletion of GSH, or interference with its utilization in the GSH oxidation-reduction cycle by inhibition of GPO or GR, sensitized tumor cells to various forms of peroxide-dependent lysis (6, 90).

It should be noted that self-protection of granulocytes and macrophages from the H₂O₂ and other ROI they themselves generate is probably a function of GSH-dependent detoxification as well. Basford (13) has recently reviewed the numerous reports of autooxidative damage in leukocytes with enzyme defects affecting the GSH oxidation-reduction cycle, such as inherited deficiencies in GR or GSH synthetase.

Toxicification

Azathioprine. Azathioprine is a prodrug which is converted into 6-thiopurine and various methylnitroimidazole metabolites. Thiolysis by GSH, with the formation of the GSH-imidazole adduct, is believed to be the major route of azathioprine toxicification (31). Chalmers (25) has shown that the GSH-imidazole adduct is further metabolized to 5-thio-1-methyl-4-nitroimidazole. This compound, itself without activity as an immunosuppressant or stimulant, significantly diminished the degree of immunosuppression obtained with a concurrent injection of 6-thiopurine (46).

Kaplowitz and Kuhlkamp (66) have implicated GSH S-transferases in the thiolysis of azathioprine; both the depletion of hepatic GSH and the formation of 6-thiopurine observed after azathioprine administration were inhibited by administration of probenecid, an inhibitor of GSH S-transferases. Thus, GSH metabolism is potentially important for the toxicification of azathioprine as well as modulation of the therapeutic and toxic effects of the resulting 6-thiopurine (see above).

Bleomycin. Bleomycin, a mixture of 2 structurally similar glycopeptides, is believed to act as an antitumor agent via an oxygen-dependent mechanism of DNA cleavage. Specifically, a ferrous iron-bleomycin complex reacts with oxygen to generate superoxide anion, and the iron-bleomycin complex itself is oxidized to the ferric state. Caspary *et al.* (23) have recently reported that GSH is able to bind to this ferric iron-bleomycin complex and reduce it to the active ferrous-iron complex. They therefore proposed a catalytic oxidation-reduction cycle in which reducing equivalents from GSH are ultimately transferred to oxygen resulting in the formation of superoxide anion and other ROI. Other investigators have not detected significant interaction of GSH with the ferric iron-bleomycin complex, while confirming en-

hanced DNA strand scission by ferrous iron-bleomycin in the presence of GSH (2). Copper(II)-bleomycin is active *in vivo* yet lacks activity in cell-free systems. Freedman *et al.* (41) suggest that metal-free bleomycin chelates copper(II), forming an inactive complex, which upon entry into cells is converted back into metal-free bleomycin by the action of GSH, via a copper(I) intermediate. Metal-free bleomycin then binds available iron(II), forming the active species.

Involvement of GSH in intracellular reductive toxification, as illustrated by the iron-bleomycin catalytic cycle, may be characteristic of other drug interactions. Neocarzinostatin is another antitumor agent for which cleavage of DNA is dependent upon both oxygen and thiols. Evidence has been presented both for (113) and against (67, 68) a role for copper(II) in this process. Recently, Myers *et al.* (87) have reported that the oxidative destruction of erythrocyte ghost membranes by an iron-Adriamycin complex is dependent upon the presence of GSH.

Delivery

Methotrexate. The uptake of methotrexate by cultured rat hepatocytes is accelerated by the addition of GSH to the medium (73). A role for externally disposed membrane sulfhydryl groups was indicated by the demonstration that methotrexate uptake was inhibited by exposure of the cells to an impermeant analogue of diamide (the bis-*N'*-methyl quaternary salt of diazenedicarboxylic acid bis(*N*-methylpiperazide)), which is believed to oxidize only extracellular sulfhydryl groups. GSH reversed this inhibition, presumably by regeneration of critical sulfhydryl groups. In an analogous study with rifamycin SV, Leszczynska (72) has reported that coadministration of GSH, but not GSSG, enhanced drug uptake by the lung, while reducing drug levels in the liver.

Obviously, GSH-dependent metabolism and excretion of systemically administered anticancer agents, as discussed for AMSA and azathioprine, indirectly but profoundly affects the delivery of active drug to its target. The influence of GSH on drug uptake by cells, as suggested for methotrexate, represents an additional type of GSH-drug interaction.

γ -Glutamyl Derivatives as Possible Prodrugs. It has been suggested by Meister and Griffith (77) that " γ -glutamyl derivatives of some chemotherapeutic agents might be transported into certain tumors more readily than the agents themselves" since many tumors exhibit increased γ -glutamyl transpeptidase activity. The feasibility of this approach to prodrug targeting has been demonstrated for agents directed to the kidney (98, 127).

Therapeutic Interactions

From the discussion above, it is evident that many antineoplastic agents alter the GSH status of host and/or tumor by depletion of cellular GSH content and/or inhibition of associated enzymes. Conceivably, this could influence both the toxicity and the therapeutic efficacy of concurrent therapeutic interventions. In other words, changes in GSH metabolism may constitute the biochemical basis of some therapeutic interactions. Examples of several such interactions, both actual and theoretical, will illustrate this point.

Inhibition of GR by BCNU, as noted above, augmented macrophage-mediated tumor cell lysis (90). Similarly, the antitumor activity of H₂O₂ *in vivo* was enhanced by BCNU at subtherapeutic

yet GR-inhibiting doses (93). In view of the role of the GSH oxidation-reduction cycle in the antioxidant defenses of the heart, BCNU might serve to increase the cardiotoxicity of Adriamycin. Another possibility is that the effectiveness of bleomycin may be dependent upon a properly functioning GSH oxidation-reduction cycle within the tumor target, and thus BCNU might interfere with the action of bleomycin. Inhibition of GR could result in increased protein binding of 6-thiopurine metabolites, leading to enhanced toxicity. Finally, inactivation of the GSH oxidation-reduction cycle by BCNU could impair cellular response to radiation damage.

It has been pointed out previously that alterations in GSH content can affect the detoxification and effectiveness of a variety of antineoplastics, including nitrogen mustards, cyclophosphamide, and AMSA. Such alterations in GSH content can arise as a result of the concurrent use of agents which deplete cellular GSH, such as misonidazole, BCNU, and sesquiterpene lactones. GSH is translocated out of cells, and thus intracellular GSH may be the major source of reducing equivalents for the external surface of cell membranes (47). This raises the possibility that GSH depletion may result in diminished permeability of cells to methotrexate. Griffith and Meister (49) have reported that systemic administration to mice of an inhibitor of γ -glutamyl transpeptidase resulted in a 3-fold increase in plasma GSH. Thus, the pharmacokinetics and distribution of methotrexate may also be altered by agents which inhibit human γ -glutamyl transpeptidase, such as the antineoplastic glutamine antagonists 6-diazo-5-oxo-L-norleucine (118) and α -amino-3-chloro-2-isoxazoline-5-acetic acid (1). Finally, resistance to Adriamycin, which has been reported to develop concurrently with thermotolerance following thermic stress (53, 54), may be due in part to the associated elevation in GSH content.

Conclusion

In summary, a role for GSH metabolism as a determinant of therapeutic efficacy has been suggested for a wide variety of antineoplastic interventions (Table 1). Further, alteration of GSH metabolism by one agent might affect the outcome of concurrent or subsequent therapy. *In vivo* efficacy of buthionine sulfoximine as an inhibitor of GSH biosynthesis (4, 48-50), BCNU as an inhibitor of the GSH oxidation-reduction cycle (93) and GSH

Table 1
Examples of antineoplastic interventions for which GSH metabolism may influence therapeutic efficacy

A. Detoxification
1. Chemotherapeutic agents
a. Mechlorethamine and melphalan
b. Cyclophosphamide
c. Nitrosoureas
d. 6-Thiopurine
e. AMSA
f. Quinone-containing drugs
g. Sulfhydryl-reactive agents
2. Radiation
3. Radiosensitizers
4. Hyperthermia
5. Cells of the host immune system
B. Toxification
1. Azathioprine
2. Bleomycin
C. Delivery
1. Methotrexate
2. γ -Glutamyl derivatives as possible prodrugs

monoesters and L-2-oxothiazolidine-4-carboxylate as agents which result in increased GSH content (102, 128) have been reported in a number of murine systems. Thus, manipulation of GSH metabolism in ways suggested by current knowledge may be a rewarding therapeutic strategy to help combat resistance, avoid enhanced host toxicity, and foster antitumor synergy.

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