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Review

The chemistry and biology of inhibitors and pro-drugs targeted to glutathione S-transferases

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Abstract. The cytosolic glutathione S-transferases are a family of structurally homologous enzymes with multiple functions, including xenobiotic detoxification, clearance of oxidative stress products, and modulation of cell proliferation and apoptosis signaling pathways. This wide-ranging functional repertoire leads to several possible therapeutic uses for isoform-specific GST inhibitors. These inhibitors may be used, in principle, to modulate tumor cell drug resistance, as sensitizers to therapeutically directed oxidative stress, to enhance cell proliferation and to augment anti-malarial drugs. With increasing knowledge of GST structural and function, rational design strategies and mechanism-based inhibitors have been exploited successfully. However, design of isoform specificity remains a significant challenge in GST inhibitor development. Strategies for further inhibitor design and their possible limitations, along with potential therapeutic uses, are summarized.

Key words. Drug design; cancer chemotherapy; anti-malarial drugs; oxidative stress; glutathione analogs.

Introduction

The cytosolic glutathione S-transferases (GSTs) comprise a canonical family of dimeric detoxification enzymes with multiple functions. Mammalian cytosolic GSTs include 7 classes, A, M, P, T, Z, T and O. In addition, the K-class isoforms exist in mitochondria and are structurally similar to the cytosolic forms. Several other classes have been identified and characterized in plants, invertebrates and prokaryotes. Within each class, multiple isoforms may exist. The quaternary structures and canonical subunit folds are compared for the human GST P1-1, A1-1 and M2-2 in figure 1, which depicts the separation of catalytic active sites on each subunit and the pronounced inter-subunit cleft. Historically, GSTs were named according to their ability to catalyze the nucleophilic addition or substitution of glutathione (GSH; γ-glutamyl-cysteinyl-glycine) at electrophilic centers in a wide range of xenobiotic electrophilic substrates. These electrophilic substrates bind to an active site pocket traditionally referred to as the H-site. Typical GST-catalyzed reactions are schematized in figure 2 and include Michael-type addition, nucleophilic aromatic substitution, nucleophilic addition to epoxides, cis-trans double bond isomerization and positional double bond isomerization, and peroxide reduction. Importantly, although many of these reactions are catalyzed by several different GSTs, each isoform exhibits its own substrate selectivity, and the implications of this are summarized below. The structure, function and nomenclature of cytosolic GSTs have been extensively reviewed elsewhere [1–4]. Other recent reviews have summarized several therapeutic or structural aspects of cytosolic GST inhibitors [5–9]. However, in light of the recent increase in our appreciation for possible therapeutic utility of GST inhibitors, an update is appropriate.

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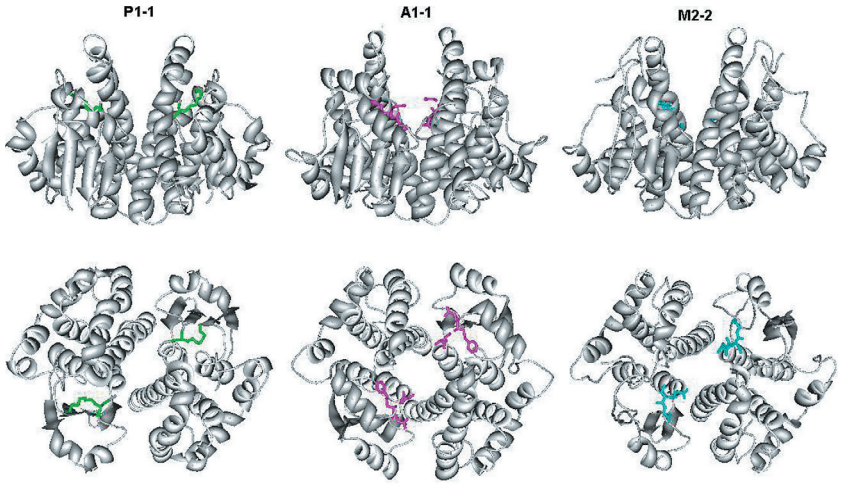

Figure 1. Ribbon diagrams of GSTP1-1, A1-1 and M2-2 with the two-fold axis of symmetry aligned vertically between subunits (top) and looking along the two-fold axis (bottom). For each protein the glutathione moiety or a GSH conjugate is colored in order to demarcate the active site. For GSTP1-1 (pdb 1GSS) the ligand is S-hexyl GSH; for A1-1 (pdb 1GUH) the ligand is S-benzyl GSH; for M2-2 (pdb 1HNB) the ligand is GS-dintrobenzene.

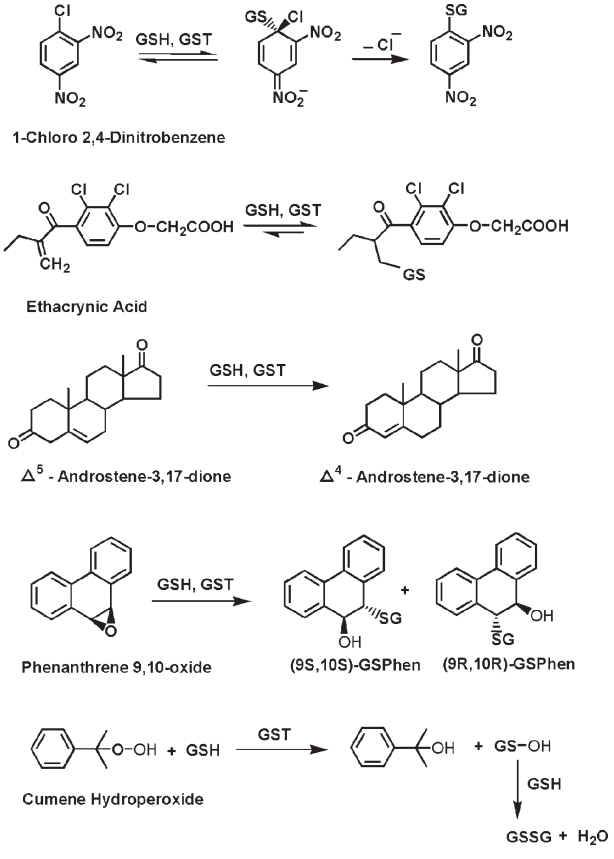

Figure 2. Typical reactions used in in vitro assays to monitor GST activity, including nucleophilic aromatic substitution, with 1-chloro-2, 4-dinitrobenzene (CDNB); Michael-type addition, with ethacrynic acid; double bond isomerization of Δ^5-androstene-3,17-dione; nucleophilic addition to the epoxide phenanthrene 9,10-oxide; hydroperoxide reduction with cumene hydroperoxide.

A structurally unrelated family of proteins with parallel GST-transferase activity is the membrane associated proteins of eicosanoid and glutathione metabolism (MAPEGs). The MAPEG isoforms are likely to contribute significantly to total GSH enzymology and detoxification [10–12]. An additional GST family, which is structurally distinct, is represented by the bacterial FosA and FosB enzymes, which provide antibiotic resistance [13]. Because there have been few explicit attempts to design inhibitors of MAPEG or Fos GST, they are not discussed further here. However, it is reasonable to expect the MAPEGs and Fos GSTs to become important targets of inhibitor design. The structural, functional and evolutionary relationships between these GST classes are summarized elsewhere [12].

In addition to the well-established GSH conjugation activity, several cytosolic GSTs have also been suggested to modulate cellular uptake and distribution of planar aromatic compounds, usually with anionic functional groups [14–16]. This 'ligandin' behavior was first appreciated for the GSTA1-1 and related rat isoform, although other isoforms appear to have a similar ability to bind non-substrate ligands with analogous structures at a site that is not well defined by available structural models. There may be several ligandin sites that overlap with the active site to varying degrees. Although a specific function for this behavior is not clear, high expression levels of GSTs do contribute to cellular uptake and distribution of various ligandin compounds [17–19]. Typical ligandin-type compounds include porphyrins, anionic dyes and steroids and are shown in figure 3. Because ligandin-type compounds usually inhibit GST catalytic activity, examples are included here.

An additional function of GSTs has been appreciated very recently, and is certain to promote further efforts to design

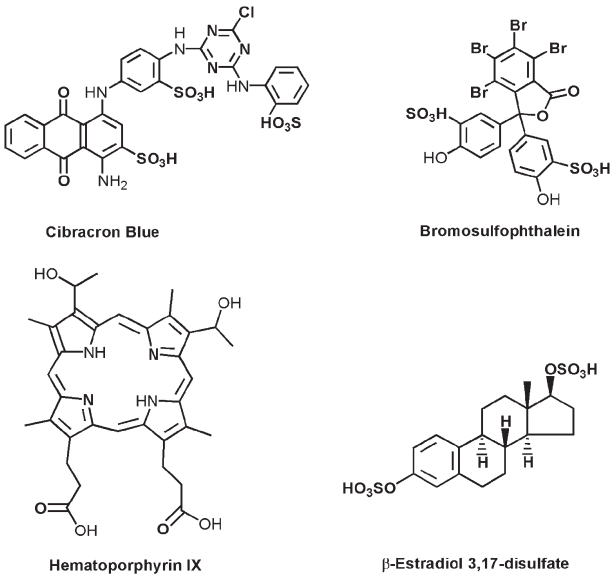

Figure 3. Typical ligandin-type inhibitors. Although ligandin-type inhibitors are structurally diverse, they are often planar aromatic anions.

inhibitors. Specifically, several GST isoforms have been suggested to regulate signal transduction pathways via specific protein-protein interactions with Jun kinase (JunK) or the apoptotic stress kinase, ASK1 [20–23]. The most well documented of these is the inhibitory interaction between GSTP1-1 and JunK, which causes downregulation of the latter, interrupts the cJun/MAPK pathway, and alters the apoptotic and proliferative response. GSTP1-1 also regulates extracellular signal-regulated kinase (ERK). Similarly, GSTM1-1 has been suggested to form inhibitory complexes with ASK1 [22]. This recently discovered aspect of GST function is likely to expand GST inhibitor design into the realm of protein-protein interactions and small molecules that disrupt them.

An interesting aspect of GST inhibitor design emerges from this brief summary of GST functions. It is conceivable, although unproven, that inhibitors may be specifically designed to modulate independently catalysis, ligandin function or signal transduction, inasmuch as the overlap between these binding sites is not well established. In some cases, however, it is possible that inhibition of multiple functions may be advantageous.

Therapeutic utility of GST inhibitors

Cancer drug resistance

Apparently, the first perceived clinical utility of GST inhibitors was as modulators of existing anti-cancer DNA alkylating agents. Because several GST isoforms conjugate GSH to busulfan, melphalan, chlorambucil, thiotepa and other anti-cancer drugs [5–7, 24–27], the over-expression in tumors of GSTs was considered as a possible mechanism of tumor cell drug resistance. It is difficult to quantitatively assess the extent to which different GST isoforms contribute to this resistance, particularly because several transporters, including GS-X pumps, are likely also to contribute to cellular disposition of alkylating agents [27, 28]. The contribution of any GST to drug resistance is likely to vary with cell type and drug, as well as with expression profile of the transporters. However, it is still widely accepted that the GSTs can contribute directly to drug resistance in some cell types via their catalytic activity, so inhibitors of GST catalytic activity remain as a potential therapeutic tool. The 'proof-of-principle' for this strategy was obtained with ethacrynic acid, which is a substrate/inhibitor of several GSTs [29–34]. Although ethacrynic acid effectively increased the sensitivity of cells in model cultures, or even in patients, to melphalan, piriprost or chlorambucil, its potential toxicity and diuretic effects prevented its development for this therapeutic use. Obviously, this therapeutic strategy requires the GST-targeted drug to inhibit catalytic function.

Cell proliferation

More recently, Tew and co-workers demonstrated the potential utility of GST inhibitors as myleoproliferators [35]. This has been prompted by the increasing number of studies documenting the formation of GSTP1-1/JunK complexes, with the functional downregulation of JunK as noted above. Because JunK is a negative regulator of the apoptotic MAPK pathway and cell proliferation, pharmacological inhibition of GSTP1-1/JunK complexation or genetic intervention could lead to cell proliferation [36], and such effects could be useful in immunostimulation. For this therapeutic use, it is essential to disrupt GST-JunK interactions, which may or may not also inhibit catalytic function.

Oxidative stress

There is increasing and compelling evidence that several GST isoforms contribute to a general anti-oxidative stress response, via isoform-dependent mechanisms. In addition to the GSTP1-1/Jun kinase interactions described above, other anti-oxidative stress mechanisms include various GSTs. GSTA4-4, and homologous isoforms, catalyze conjugation of GSH to the lipid peroxidation product 4-hydroxynonenal (4-HNE [37–39]), which in turn is a signaling molecule that modulates stress kinase pathways and an electrophilic intermediate capable of direct protein adduction [40–42]. This apparent preference for 4-HNE by GSTA4-4 is possibly due to a Tyr residue found only in this isoform that is optimally situated to act as a general acid in the protonation of the intermediate enolate that forms, along with the overall shape of the active site [43].

Other anti-oxidative stress responses include the GSTA1-1- and GSTA2-2-catalyzed reduction of lipid peroxides, such as hydroperoxy-phosphatidylcholine [44, 45]. Overexpression of these isoforms in cells provides protection from ultraviolet (UV) light or H_2O_2 [46]. Based on these functions, it is conceivable that isoform-specific GST inhibitors could be used to enhance oxidative stress directed at tumors or infectious agents, as in photodynamic therapy or other radiation therapies.

Infectious disease

Recently, GST activity has been reported in parasites causing diseases such as malaria and schistosomiasis. In malarial parasites, the level of GST catalytic activity varies among different host-specific plasmodia including *Plasmodium berghei* (rodent), *Plasmodium knowlesi* (simian) and *Plasmodium falciparum* (human). The GST levels also vary in the different intraerythrocytic stages of the parasite [47]. Possibly, GST catalysis could contribute to resistance to chloroquin (CQ). GST and GSH levels were found to increase significantly in chloroquine-resistant (CQR) strains as compared with sensitive strains [48]. Interestingly, the increased GST activity was found to be directly proportional to drug pressure. However, the exact role of GST in CQ resistance is still unclear. The malarial parasites are known to detoxify the by-product hemin, which is generated during hemoglobin digestion, by converting it into the insoluble polymer hemozoin. CQ and other aminoquinolones block this polymerization, which results in high levels of free hemin and subsequent death of the parasite due to oxidative stress. An inverse relationship between the hemozoin content and GST levels has been observed in CQR strains [47]. Furthermore, free hemin was also found to be an inhibitor of *P. berghei* and *P. falciparum* GSTs with IC_{50}s (50% inhibitory concentrations) in the 1–50 μM range, suggesting that GST in CQR strains might be responsible for buffering free hemin, possibly by their ligandin function, thereby preventing the activity of chloroquine. However, this mechanism does not account for the simultaneous increase in GSH levels observed in CQR strains. It has been suggested that GSH can degrade hemin in the plasmodia food vacuole via an oxidative pathway [49, 50]. Furthermore, treatment of CQR with buthionine sulfoximine (GSH synthesis inhibitor) partially reversed chloroquine resistance and increased the levels of hemozoin [49]. Whether GST catalyzes any of these GSH mediated reactions is still not known. Apart from this, Dubios et al. [48] have suggested that GST can function as phase II metabolizing enzyme in the parasite and may be responsible for metabolism of chloroquine, giving rise to resistance. This is supported by the fact that CQ and some other antimalarials are inhibitors of GST with IC_{50} values in the low micromolar range [51]. Irrespective of the exact mechanism, it appears that GST plays a significant role in the malarial parasite's life cycle and hence is a potential target for developing a novel class of antimalarials.

More recently, a GST from *P. falciparum* species (pfGST) was cloned, characterized and crystallized [52]. The crystal structure of pfGST resembles other known GSTs, with the homodimeric canonical fold. PfGST, however, has a shorter C-terminus and a more solvent accessible H-site in comparison to other GST classes. Presumably, this H-site can be exploited in rational drug design of inhibitors for pfGST as potential antimalarials. To date, no known inhibitors of pfGST are available, although research groups are working in that direction based on the crystallographic data.

Schistosoma japonicum is the parasite responsible for the deadly tropical disease schistosomiasis. GST from *S. japonicum* [53] has been speculated to act as a primary defense against electrophilic and oxidative damage [53]. Crystal structures of SjGST, with and without the drug praziquantel, are available [54]. The GSH binding site is conserved as in human GSTs; however, there is a distinct difference in the xenobiotic binding site (H-site), providing a possible structural basis for future drug design [54]. Oltipraz, which is a currently used as an anti-schistosomal drug, is known to bind SjGST, indicating that GST might be playing a ligandin type of role similar to the malarial parasite [55, 56]. Recently, this ligandin binding function of SjGST has been studied, and it is suggested that the L-site may not be completely hydrophobic [56]. Apart from being targets for drugs, schistosomal GSTs have also been implicated in vaccine therapy against the disease. Vaccination using *Schistosoma mansoni* GST (Sm28GST), a 28-kDa antigen, has been shown to decrease parasitic egg production and transmission [57].

Design strategies and structural classes of inhibitors

GS-R conjugates

Although all mammalian cytosolic GSTs and the parasite-derived GSTs share a highly conserved glutathione binding site within their canonical fold, the electrophilic substrate binding site, or H-site, varies significantly among different isoforms, and it is reasonably expected to provide a source of isoform specificity among inhibitors. For example, the H-site of the P-class enzymes may have a hydrophilic portion that is not obvious in A-class enzymes [58]. However, it has been difficult to incorporate this strategy into rational drug design because of the uncertainty of structural determinants within the H-site. Possibly, this is due to the existence of multiple distinct, but partially overlapping, hydrophobic sites within a single GST isoform, which together form the large substrate binding H-site. Crystal structures reflect this 'degeneracy' in which different substrates occupy different 'sub-

sites' of the active H-site [59–61], and this is demonstrated in functional binding studies. For example, Colman and co-workers have examined a wide range of affinity reagents with various GSTs, including GSTP1-1 [62, 63]. Taken together, their work suggests that the substrates monobromobimane, 1-chloro-2,4-dinitrobenzene (CDNB), and benzyl isothiocyanate occupy distinct regions of the H-site, with differential proximity to the ligandin site, as probed by 8-anilino-1-naphthalene sulfonate (ANS), which may also have multiple binding sites. Although this is not surprising for a substrate nonselective detoxification enzyme, such degeneracy of a substrate binding site makes rational drug design difficult without frequent iterations of structure determination to ensure that the substrate or inhibitor has not adopted an unexpected orientation in the H-site. In fact, the situation is complicated further by the observation that a single H-site ligand can occupy multiple positions/orientations within a single H-site. For example, the ethacrynic acid moiety of the GSH conjugate formed from it may adopt multiple orientations within the H-site [61].

Even in the absence of detailed structural models for every inhibitor or substrate, the use of the GSH moiety as an anchor to constrain hydrophobic groups with the larger H-site is an obvious and successful approach that has produced biochemically useful probes. Design of many early GST inhibitors involved the use of S-linked GS-R conjugates, with varying R groups [9, 64, 65]. Presumably, this strategy was considered as a result of the observation that GSTs are subject to product inhibition [66]. Product inhibition is apparent with many GS-R conjugates, including, for example, GS-estradiol and GS-aflatoxin conjugates [67, 68]. Conjugates such as S-alkyl and S-benzyl GSH are still commonly used in vitro as biochemical probes, inhibitors and for release of GSTs or GST-fusion proteins from affinity resins.

GSH peptide analogs

The presence of the GSH tripeptide in the structure of GS-R conjugates and its analogs presents problems that limit their clinical use. Specifically, the peptide portion leads to biological instability, degradation by peptidases and isoform nonselectivity. In fact, the enzyme γ-glutamyl transpeptidase specifically hydrolyzes the unusual peptide linkage of glutathione conjugates. Several studies, therefore, have focused on structural modifications of the tripeptide moiety to overcome these limitations. However, the highly conserved and selective GSH binding site in GSTs has made the improvement of physiochemical properties without loss of binding difficult [8]. Replacing GSH amino acids with different residues results in significant loss of activity. The γ-glutamyl portion of GSH is absolutely critical, while changes in glycine and cysteine residues can be tolerated provided they maintain the appropriate charge and hydrophilicity. Burg and Mulder [9] have reviewed the design strategies that enhance the stability of GSH towards the enzyme γ-glutamyl transpeptidase. The main approaches are summarized in figure 4, and include esterification of glutamate α-carboxylic acid and replacing cysteine and glycine moieties with D-aminoadipic acid (**1**), backbone changes such as retro-inverso GSH (r-GSH) (**2**), isosteric substitution of CONH by SO_2NH (**3**), and an internal urea type linkage instead of a peptide bond (**4**). Compounds (**5**) and (**6**) are peptidomimetic GSH analogs in which the sulfhydryl group is attached to hydrophobic groups such as ethacrynic acid or long-chain alkyl groups [9, 64, 65, 58], while the GSH moiety is also modified. In analog **5** the amide is N-methylated to make the peptide bond more resistant to cleavage by peptidases, whereas in **6** a tetrazole is used as an isosteric replacement for the critical glycine acid. These structural modifications within the GSH peptide backbone improved the stability towards peptidases, while esterification of carboxylates introduced lipophilicity and increased membrane permeability of these analogs. Most of these compounds, or their analogs, were also found to be potent inhibitors of human and rat GSTs. However, isoform selectivity was not achieved with these compounds in terms of in vivo efficacy.

TLK S-conjugates, and nonpeptide analogs

Modification of the peptide portion of GSH conjugates has led to many inhibitors. Several examples are shown in figure 5. Telik Technologies (www.telik.com) and others designed a series of GSH analogs with S-functionalized cysteine and variations in the c-terminal glycine based on data reported by Adang et al. and Flatgaard et al. [69, 70]. It is useful to note that Telik compounds, with the 'TLK'

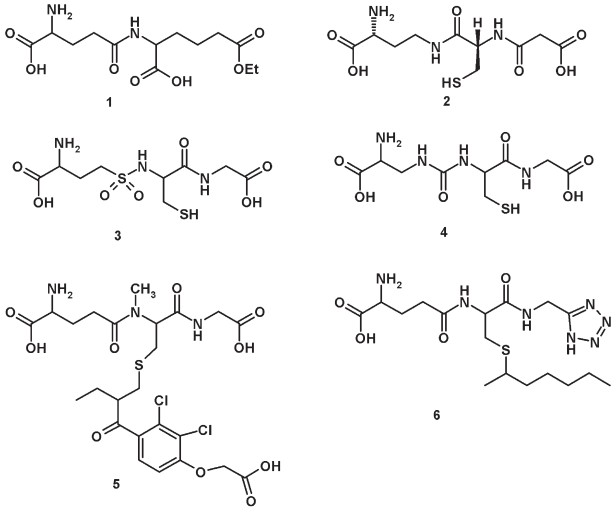

Figure 4. Inhibitors with modifications within the GSH peptide moiety. See text for specific examples.

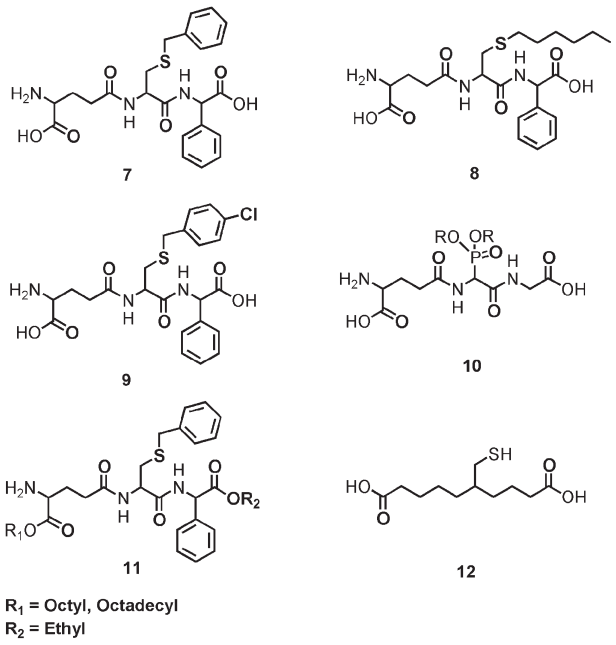

Figure 5. Non-peptide analogs of GSH and GSH conjugates. See text for specific examples.

designation, were in some cases acquired from Terrapin Technologies, which had designated compounds with 'TER' labels. Thus, the older literature refers to compounds with a TER designation that are identical to TLK compounds having the same number. Screening of these compounds against human GSTs resulted in the discovery of highly selective (20-fold for P1-1) compounds **7** and **8.** Structurally, these compounds consist of a α-phenyl glycine residue and a benzyl (**7**) or n-hexyl (**8**) group conjugated at the cysteine sulfur. Further SAR studies led to the identification of a highly potent GST inhibitor with good selectivity for GSTP1-1 (**9**, $K_i = 0.12$ µM) [71]. TLK 199, which is a diethyl ester form of **7**, was found to be a chemosensitizer and to increase the toxicity of several anti-cancer agents. Preclinical data suggest that it is a myeloproliferative agent, and it is currently in phase 2 studies for patients with myelodysplastic syndromes. In another attempt to make isozyme-selective inhibitors, Kunze et al. [72] introduced an O = $P(OR)_2$ moiety in place of the cysteinyl residue CH_2SH (**10**). This resulted in analogs selective for GST M1-1 vs. for P1-1 and more resilient towards degradation by glutamyl peptidase.

An interesting example of the use of a γ-glutamyl-cysteinyl-phenylglycine peptide combined with esterification of the peptide carboxylates explored long-chain esters up to 18 carbons on the phenylglycine carboxylate and 2 carbons on the glutamyl α-carboxylate [73]. In this case (**11**), the inhibitor was used to reverse resistance of cholangiocarcinoma cells to alkylating agents. The longer alkyl chains significantly reduced the rate of ester hydrolysis, and in some cases did not abolish potency for GSTP1-1 inhibition, in crude cell supernatants. It is unclear whether one, both or neither ester must be hydrolyzed to inhibit GSTP1-1, but these results demonstrate the possibility of dramatically altering the peptide portion of an inhibitor in order to optimize pharmacokinetic properties of a presumed pro-drug.

Over the years non-peptide GST inhibitors have also been developed to increase peptidase stability. Klotz et al. [74] made a series of non-peptide glutathione analogs by substituting carbon-carbon single bonds (**12**) or isosteric (**E)** double bonds for the amide bonds. Although the compounds were only moderately potent inhibitors of GST, they provided valuable information about the glutathione binding site (G-site) and serve as good leads for designing non-peptide analogs.

Haloenol lactones have been shown to enhance the cytotoxic effect of cisplatin by acting as GST P1-1inactivators [75]. Several haloenol lactones represented by **13** (figure 6) were developed to identify more potent selective compounds as GST P1-1 inactivators [76–78]. These inhibitors are active site-directed chemical modification reagents that form thioester linkages with Cys-47 of GSTP1-1, with concomitant inhibition of catalytic activity and they are now being further modified to increase their efficacy [77]. A structure-activity study involving 16 analogs suggested that the loss of halide is not the rate-limiting step of enzyme inhibition, but the electronegative halide does stabilize the intermediate enolate.

Bivalent inhibitors

In order to develop selective GST inhibitors, one of our strategies was to exploit the quaternary structure of the enzyme. The crystal structure of GSTs shows a solvent accessible cleft between the two monomers (fig. 1). The nature and identity of residues that line the intersubunit

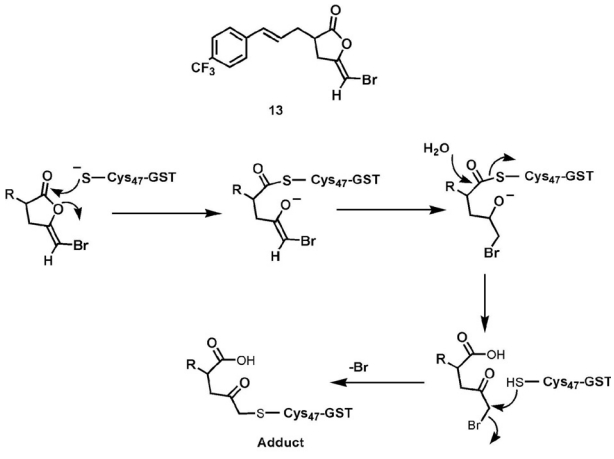

Figure 6. Mechanism of inhibition by haloenol lactones, including 13. Cys-47 of GSTP1-1 reacts to form the thioester and, after hydrolysis, displaces the halide.

cleft varies among different GST isoforms. In addition, the distances between the active sites on each subunit vary. These differences have not been fully exploited in the design of isoform-specific GST inhibitors. Therefore, we have explored the concept of 'multivalency' to design compounds that would interact with the active sites on each GST monomer simultaneously, and thereby increase the overall binding affinity and selectivity (fig. 7). Several bivalent compounds (compounds **14, 15**) with varying binding elements and linkers were synthesized [79]. The compounds have higher affinity as compared to their monovalent counterparts, and they also have higher isoform selectivity. For example, **14** is a bivalent analog of the product resulting from conjugation of GSH with CDNB, and it has a nearly 10-fold lower IC_{50} for GSTA1-1 than for GSTP1-1. Compound **15**, which consists of two cibacron blue moieties attached to alkyl hydroxyl linker, is a highly potent and selective inhibitor of GSTP1-1 [79]. Binding stoichiometry indicated that the compounds bind in bivalent manner, which further validates the strategy. A striking observation based on calorimetry with a subset of these compounds is that the 'entropic' advantage of the bivalent binding provides only a portion of the differential affinity between monovalent and bivalent compounds. Enthalpic interactions appear to contribute significantly to the 'bivalent advantage'. This suggests that significant linker-protein interactions contribute to binding. Obviously, the bivalent compounds made so far are not good drug candidates, but they demonstrate the potential utility of bivalency. Further work is in progress to explore more of cleft region as a source of selectivity for bivalent compounds. Currently, this strategy is being explored by Syntrix Biosystems (www.syntrixbio.com) for GST inhibitor design.

Ligandin-type inhibitors

Historically, inhibitors of GST catalysis that demonstrate non-competitive steady-state kinetic behavior, with respect to H-site substrates such as CDNB, have been referred to as 'ligandin' inhibitors or even 'ligandin substrates'. Many of the early ligandin inhibitors were hydrophobic planar aromatic compounds with anionic functional groups or steroids, as shown in figure 3. Based on apparent stoichiometries of binding of 1 inhibitor/ GST dimer, and on the non-competitive nature of the inhibition, it is widely speculated that ligandin molecules bind in the intersubunit cleft and some may 'partially' occupy the catalytic H-site from within the cleft. Several lines of evidence support this simple model.

The X-ray crystal structures of several GTP1-1/inhibitor complexes demonstrate a binding site at the edge of the subunit directly facing the cleft, and partially overlapping the H-site in the same subunit. In these structures, the electron density suggests partial occupancy or 'half-of-sites' binding in some cases [59]. An additional X-ray structure, of sigma-class, squid, GST indicates a binding site for a GSH-conjugate within the intersubunit cleft, near the 'mouth' and distinct from the active sites [60]. Similarly, *S. japonica* GST exhibits an intersubunit binding site for the anti-schistosomal drug praziquantel [54]. Thus, it is clear that some drugs or inhibitors could bind within this cleft, but it remains uncertain whether these binding sites observed in crystallographic structures correspond to the inhibitory binding sites, because as described below, there is also evidence for differential inhibitory activity of different binding sites when multiple sites exist for a single compound. In essence, the degeneracy described above for the H-site appears to also occur with the ligandin site, which may consist of numerous subsites within an expansive binding surface.

Calorimetric and spectroscopic studies have suggested that bromosulfophthalein (BSP) binds within the intersubunit cleft of GSTA1-1 and GSTP1-1 [80, 81], but also partially overlaps with the H-site. Interestingly, this inhibitor has also been shown to bind with higher affinity to a separate site on GSTA1-1, near the domain-domain interface within the subunit. This high-affinity site, occupied at lower BSP concentrations, may not be function-

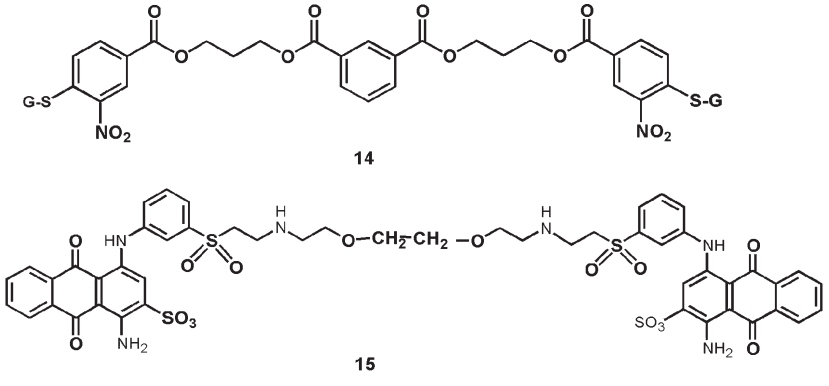

Figure 7. Bivalent inhibitors based on the GS-CDNB conjugate (14) and on the ligandin-type inhibitor cibacron blue (15).

ally important, and inhibition only occurs at concentrations high enough to populate the lower affinity ligandin site. Chemical modification of GST with steroid analogs has also led to the conclusion that these inhibitors bind within, and partially span, the intersubunit cleft [82]. Peptide mapping and mass spectral identification of peptides clearly indicates that Cys-17 or Cys-111 in the cleft GSTA1-1 (rat) may be adducted, but in a mutually exclusive manner. This further demonstrates the possibility of multiple binding modes for a single ligandin-type inhibitor. Furthermore, a combination of steady-state inhibition studies and pre-steady-state binding of the GSH conjugate of aflatoxin suggested that the aflatoxin moiety attached to the inhibitor in the catalytic site of one subunit could 'spill' into the cleft and prevent binding to the catalytic site of the other subunit [68]. Together, these data supported the noncompetitive and degenerate nature of ligandin-type inhibitors, which bind at a site that includes portions of the cleft and one of the H-sites.

However, there is still uncertainty about the location of the ligandin site. For example, a separate study examined the nature of inhibition and affinity of a steroid, hematoporphyrin and lithocholic acid, a classic ligandin inhibitor [83]. In this study, the Cys-112 within the intersubunit cleft of human GSTA1-1 was modified with GSH, or crosslinking agents that provided an intersubunit tether within the cleft. Based on the widely accepted hypothesis that the ligandin site included the intersubunit cleft, this modification was expected to cause changes in K_Is for ligandin inhibitors. However, this modified enzyme behaved identically to the unmodified protein, thus raising some doubt about the location of this binding site. Moreover, the observed kinetic patterns for inhibition by these compounds were competitive rather than noncompetitive, suggesting that they fully occupy the H site. Of course, it is possible that these chemical modifications block only one of several binding sites for these ligands, thus forcing them to behave as competitive H-site ligands. In addition, mutations in the C-terminal helix of GSTA1-1 alter the ligandin behavior towards some inhibitors, such as ANS [84]. To the extent that the C-terminal helix does not contribute significantly to the intersubuit cleft, this suggests that this cleft is not the only site for ligandin-type inhibitors. Taken together the results suggest that there may be a large contiguous hydrophobic site that spans the intersubunit cleft and the H-site. Such a large site may include discrete subsites that preferentially accommodate various hydrophobic ligands in partially overlapping regions [85]. In general the ligandin site is a well-appreciated aspect of GSTs, but its structural characterization is minimal, and this brief summary emphasizes the potential existence of degenerate binding sites that contribute to an expansive ligandin site. Therefore, there are few, if any, examples of rational inhibitor design aimed at any well-defined ligandin site(s).

Natural plant polyphenols and tocopherols

A further set of inhibitors that share some of the properties of classic ligandin-type inhibitors are naturally occurring polyphenols, such as quercetin, or tocopherols, found in plants an even food constituents. These compounds, which are hydrophobic aromatic compounds with potential anionic character, are particularly prevalent in grapes and red wine, some vegetables and some teas. These and other dietary constituents have received great attention, mainly due to their ability to induce various GSTs and their potential anti-carcinogenic properties. However, beyond these genetic responses, there are also reports of the ability of these compounds to directly inhibit some GSTs, mainly GSTP1-1 [86–90]. At least in some cases, these inhibitors appear to lie outside the paradigm of the ligandin-type inhibitors because they are competitive with respect to hydrophobic substrate or they lead to time-dependent covalent modification. Although these compounds represent a potential source of interesting leads for rational inhibitor design, their mechanism of inhibition and their degree of isoform selectivity remain to be fully determined.

Pro-drugs

It has been known for several years that GSTs posses the ability to hydrolyze or cleave GSH-conjugates, albeit with slow turnover rates [91, 92]. GSH thioesters and isothiocyanates are among the known substrates for 'reverse' GST catalysis. Due to the over-expression of GSTs in tumors, and possibly in infectious agents, several groups have considered the utility of these reverse reactions for GST-activated pro-drugs that release electrophilic toxins or other chemical species. The proof-of-principle for this strategy is best exemplified by TLK286, a GSH analog (γ-glutamyl-cys-phenylglycine) containing a phosphoramidate sulfonyl moiety that undergoes enzyme-dependent release of a DNA alkylating agent and a GS-vinyl sulfone [93–95]. It is speculated that the active site Tyr of GSTP1-1 acts as a general base to promote the β-elimination that yields the alkylating agent (fig. 8, top). However, TLK286 also has been shown to inhibit DNA-dependent protein kinases [95], which may contribute to its cytotoxicity. Interestingly, the DNA-dependent kinases appear to interact with the parent compound (TLK286) rather than the activated products. Regardless of the potential contribution to the therapeutic effects by a non-GST target, TLK286 is currently being evaluated in several clinical trials for several types of cancer [96, 97]. It is also interesting that the GS-vinyl sulfone that is generated is likely to be a reactive electrophile. However, its fate has not been considered in studies of TLK286. The possible importance of this is described below.

A further class of GST activated pro-drugs includes diazeniumdiolate nitric oxide donors, which are activated

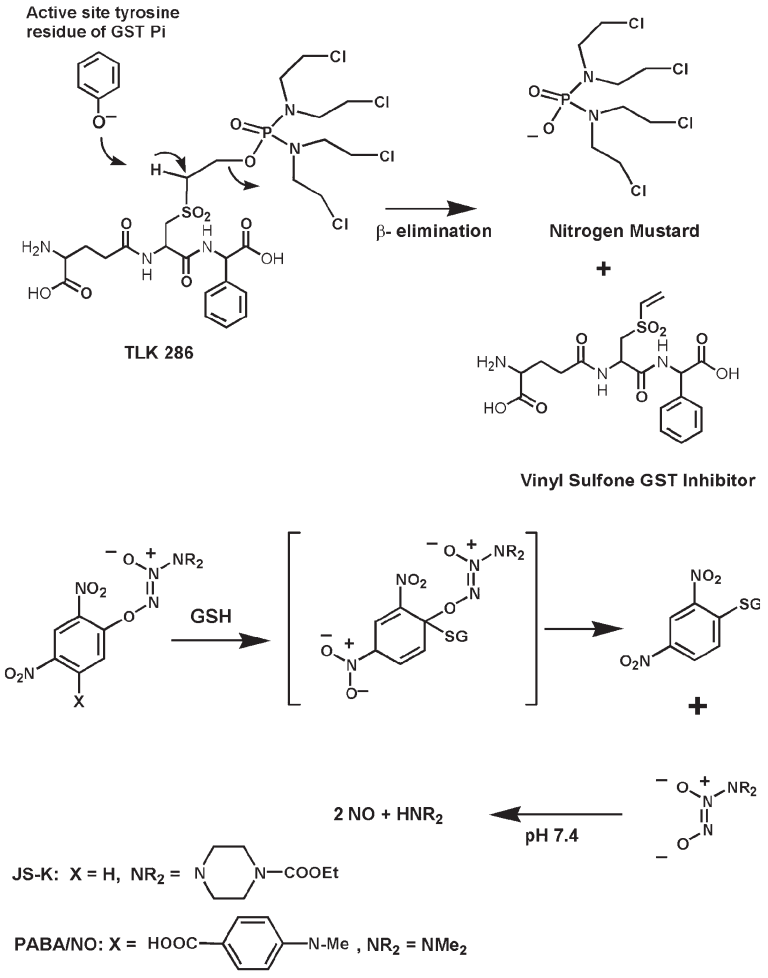

Figure 8. Pro-drugs targeted to GSTP1-1. (Top) TLK286 releases an electrophilic nitrogen mustard and a vinyl sulfone. (Bottom) The diazeniumdiolates release NO•.

upon GST-dependent reaction with GSH. Two such analogs have been examined [98, 99]. The first was JS-K (2-(2,4-dintrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate), which has been studied with HL-60 cells, as a model for human myeloid leukemia, which has been known to be sensitive to nitric oxide (NO•). The elegant strategy used here was to exploit the well-documented GST reaction wherein CDNB forms a GSH-Meisenheimer complex before eliminating Cl^- anion. With JS-K, the diazeniumdiolate replaces the Cl^-, and is released subsequent to formation of the analogous Meisenheimer complex (fig. 8, bottom). Notably, the reaction occurs nonenzymatically in physiological conditions, as with CDNB, but GSTM1-1 and GSTA1-1 catalyze the reaction significantly. Computational docking experiments with JS-K and the available crystal structures for GSTs A1-1, P1-1 and M1-1 suggested that the steric bulk of the diazeniumdiolate would be well tolerated by GSTM1-1 and GSTA1-1, but not GSTP1-1. In subsequent work [99], the steric bulk at this position was reduced, and steric bulk on the opposite side of the aromatic ring was increased. The increased steric bulk here was anticipated to provide specificity for GSTP1-1, based on the docked models. Indeed, this compound (PABA/NO/fig. 8) appears to be P1-1 specific, and its relative toxicity to fibroblast cells is proportional to their GSTP1-1 expression level.

An interesting, and relatively unappreciated, aspect of the design of pro-drugs targeted to GSTs is that the promiscuous nature of the GST active site and ligandin site(s) makes the activating GST a potential target for reaction with, or inhibition by, the electrophilic drug that is generated. In the two examples described here, the alkylating agent released from TLK286 or the NO• released from PABA/NO could react with the GST, thus inhibiting it and preventing further activation of the pro-drug. In fact, nitration of Tyr residues is one of the common modifications of proteins exposed to NO•, and it is widely speculated that the active site Tyr-7 of GSTP1-1 is responsible for pro-drug activation. Ideally, these potential complexities should be considered when designing GST-activated pro-drugs [100]. The reactivity of any species released by

a GST-catalayzed process may be self-limiting if it attacks the GST from which it is generated.

Conclusions and prospective

Due to the multiple biological roles of GSTs, these enzymes are potential targets in several unrelated therapeutic areas. The diverse functions, including catalytic GSH conjugation, passive ligandin-type binding and modulation of signal transduction, may be selectively targeted by different inhibitors with sufficient understanding of the relevant binding epitopes. In addition, the over-expression of GSTs in tumors and infectious organisms may be exploited for pro-drug activation. Rational design of drugs affecting the individual functions of GSTs is difficult because of the apparent degeneracy of the H-site recognized by substrates, uncertainty about the location and nature of the ligandin site, and of the JunK binding site. Regardless of the specific therapeutic goal of any GST inhibitor or pro-drug, new design principles and strategies are needed to achieve isoform specificity.

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