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Cancer Drugs, Genetic Variation and the Glutathione-*S*-Transferase Gene Family

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Abstract

The glutathione-*S*-transferase (GST) super family comprises multiple isozymes (Alpha, Mu, Pi, Omega, Theta, and Zeta) with compelling evidence of functional polymorphic variation. Over the last two decades, a significant body of data has accumulated linking aberrant expression of GST isozymes with the development and expression of resistance to cancer drugs. Clinical correlation studies show that genetic differences within the human GST isozymes may play a role in cancer susceptibility and treatment.

The initial confusion was presented by the fact that not all drugs used to select for resistance were substrates for thioether bond catalysis by GSTs. However, recent evidence that certain GST isozymes possess the capacity to regulate mitogen activated protein kinases presents an alternative explanation. This dual functionality has contributed to the recent efforts to target GSTs with novel small molecule therapeutics.

While the ultimate success of these attempts remains to be shown, at least one drug is in late-stage clinical testing. In addition, the concept of designing new drugs that might interfere with protein:protein interactions between GSTs and regulatory kinases provides a novel approach to identify new targets in the search for cancer therapeutics.

1. Glutathione-*S*-Transferase (GSTs) and Anticancer Drug Response

In cancer chemotherapy, development of drug resistance is a key element in the eventual failure of effective therapeutic treatments. In both preclinical models and in patients, exposure to anticancer agents can provide the selective pressure, which leads to induced expression of protective gene products. Although the drug-resistant phenotype is frequently characterized by multiple and pleiotropic changes, one frequent adaptation is altered expression of glutathione-*S*-transferases (GSTs).[1,2] GSTs are a family of phase II detoxification enzymes that have as a primary function the protection of cellular macromolecules through catalyzing the conjugation of glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds. While GSTs play a quintessential role in protecting cells from environmental and oxidative stress, they can provide an obstacle to the successful treatment of patients with cancer. For example, a survey of the National

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Cancer Institute cancer drug screening panel of cell lines showed an inverse correlation between GST expression and sensitivity toward alkylating agents.[3]

Table I provides a list of anti-cancer agents for which resistance has been associated with elevated levels of GSTs. Some of these drugs are substrates of GSTs and can be directly inactivated through catalytic thioether bond conjugation to GSH. However, an early conundrum provided by the GST literature was that many tumor cells over-expressed GST isozymes even if resistance was not to a drug that could act as a substrate. Such apparently conflicting information has been explained by recent studies that have identified new functions for old enzymes. This review will summarize those issues most pertinent to understanding the genetic differences in human GST expression and their relevance to cancer susceptibility and treatment.

2. GSTs and Kinase Regulation

Arguably, one of the more interesting developments in understanding the role of phase II metabolism enzymes in maintaining cellular homeostasis is the recent publication of evidence linking GSTs with kinase-mediated signaling cascades. At this stage, it is premature to offer conjecture on the generality of this regulatory pathway, however, at least two examples of non-catalytic functions for GST isozymes now exist.

Mechanistically, GSTs play a regulatory role in kinase signaling by forming ligand-binding interactions with critical cellular kinases involved in the regulation of proliferation and apoptosis. Through protein : protein interactions, GSTs function to sequester signaling kinases and act as negative regulators. It is ironic to reflect that the recently reported protein ligand binding of the GSTP1–1 isozyme with c-Jun N-terminal kinase (JNK) and of the GSTM1 isozyme with apoptosis signal-regulating kinase (ASK1) present properties reminiscent of the ‘ligandin’ functionality ascribed to the capacity of liver GST to bind reversibly to heme and bilirubin.[5] Thus far, two distinct interactions have been characterized. For example, GSTM1 plays a regulatory role in the heat shock-sensing pathway by binding to, and inhibiting, the activity of ASK1.[6,7] ASK1 is a mitogen-activated protein (MAP) kinase that activates the JNK and p38 pathways leading to cytokine- and stress-induced apoptosis.[8] The activity of ASK1 is low in non-stressed cells due to its sequestration via protein:protein interactions with GSTM1 to form a GSTM1:ASK1 complex. Oxidative stress and heat shock lead to the dissociation of the GSTM1:ASK1 complex resulting in liberation and activation of ASK1.[7,9] Forced expression of GSTM1 blocked ASK1 oligomerization and repressed ASK1-dependent apoptotic cell death.[6] GSTM1 expression is altered in a variety of tumor types and is associated with impaired clinical response to therapy. Thus, in addition to any role that GSTM1 may play in catalyzing GSH conjugation to anti-cancer agents (and this is likely to be minor), it may also influence the apoptotic response through kinase regulation.

The other published example of kinase regulation is provided by the GSTP1 family that plays an integral role in controlling stress response, apoptosis and cellular proliferation through interacting with JNK. JNK has been implicated in pro-apoptotic signaling and may be required for the induced cytotoxicity of a variety of chemotherapy agents,

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including several of those listed in table I.[4,10] JNK activity is propagated through phosphorylation of c-jun and subsequent downstream effectors. In non-stressed cells, low JNK1 catalytic activity is maintained as a consequence of the sequestration of the protein in a GSTP1:JNK complex.[11,12] However, under conditions of oxidative stress (e.g. ultraviolet [UV] irradiation or hydrogen peroxide treatment) a dissociation of the GSTP1:JNK complex occurs, producing oligomerization of GSTP1 and subsequent induction of apoptosis.[11]

Additional support for this model of GST regulation is provided by the observations that either immunodepletion of GSTP1, or its inhibition by a rationally designed GSH-based peptidomimetic inhibitor, γ-glutamyl-*S*-(benzyl) cysteinyl-R(–)-phenyl glycine diethyl ester (TLK199), also results in the activation of JNK.[11,12] Collectively, these data provide a plausible explanation of why GST over-expression is a mechanism of drug resistance when the selecting drug is not a substrate for GSH conjugation.

The rational design and synthesis of TLK199 was based on the principle that modulation of drug resistance could be utilized as a viable clinical approach to cancer treatment. While the protein-protein interactions of GSTP1 and JNK have been shown to have a binding constant of approximately 200 nmol/L, the exact site(s) of interaction has not been characterized, other than to implicate the C-terminal end of JNK.[13] It is possible that polymorphisms within each isozyme class may produce distinct binding constants and thereby alter kinase signaling kinetics. It is also apparent that the GSTM1 null phenotype would imply that the mechanism for negative regulation is not universal. However, it is possible for other members of the GST family to substitute for Mu deficiency, illustrating, once again, the potential importance of functional redundancy in GSTs. By implication, the non-enzymatic roles for GSTs in regulating drug response might be a significant consideration when analyzing what impact GST polymorphisms may have in determining cellular response to drug exposure.

3. The GST Gene Super Family: Classification

Human GSTs are divided into two distinct super family members: membrane bound microsomal and cytosolic. Microsomal GSTs contain three isoforms designated mGST 1, 2, and 3 that are encoded by a single gene located on chromosome 12 (*MGST1*).[14,15] Like cytosolic GSTs, the microsomal isozymes catalyze the conjugation of GSH to electrophilic compounds. Microsomal GSTs play a key role in the endogenous metabolism of leukotrienes and prostaglandins.[14]

The cytosolic GSTs are subject to significant genetic polymorphism in human populations. They are divided into 6 classes that share ~30% sequence identity: Alpha, Mu, Omega, Pi, Theta, and Zeta (see table II). Multiple alleles exist within each class and these share >50% sequence identity (table III).[16] The 5′ promoter region varies between classes and can contain one or more of the following response elements: the antioxidant-response element, the xenobiotic response element, the GSTP enhancer 1, the glucocorticoid-response element, and the Barbie box element.[17–19] Also, the promoter region contains putative binding sites for transcription factors including, AP-1, MAF, Nrfl, Jun, Fos, and NF-kappa B.[18] It is important to note that the occurrence and/or prevalence of these elements are quite

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species specific and there are particular differences between rodents and humans. As such, while these features provide an adaptive response mechanism to up-regulate GST expression following cellular stress and exposure to toxic xenobiotics, the data do not always lend themselves to extrapolation from mouse to man.

Cytosolic GSTs function as homo- and hetero-dimeric proteins, allowing the formation of a larger number of enzymes from a limited number of genes, however, dimerization is limited to subunits within the same class.[20] The subunits range in size from 24 to 29 kDa.[21] Each subunit contains an active site with two subsites: a highly conserved G site for GSH binding and an H site for hydrophobic substrates.

Less than 10% of the protein is strictly conserved, and yet all GST isozymes have two domains and a similar topology. The N-terminal domain (residues 1–80) comprises one-third of the protein and forms the G site. It is composed of four β sheets with three flanking α helices, a structural motif common to thioredoxin and other proteins evolved to bind GSH or cysteine.[22] This region contains a catalytically essential tyrosine, serine or cysteine residue that interacts directly with the thiol group of GSH.[22] The C-terminal domain (residues 87–210) is α helical and together with a loop from the N-terminal domain forms the H site. Amino acid variation in the H site accounts for substrate specificity. In addition, structural variations within the C-terminus exist in the Alpha, Theta, and Mu classes. Specifically, an additional C-terminal α helix is present in the Alpha and Theta classes while the Mu class has an extra loop.[22] Both differences are located proximal to the H site, creating a more constricted active site.

Although GSTs are ubiquitously expressed their tissue distribution in mammals is complex. Fetal tissues contain a GST expression profile that is distinct from adults and within some organs, such as the kidney, there are different isoforms expressed even between cell types.[23,24] Adding to the complexity, GSTs have been shown in rodent models to be induced by structurally unrelated compounds known to result in chemical stress and carcinogenesis including: phenobarbital, planar aromatic compounds, ethoxyquin, butylated hydroxyanisol (BHA), and trans-stilbene oxide.[25] Some of the compounds known to induce GSTs are themselves substrates for the enzyme, suggesting that induction may be an adaptive response mechanism.

4. Genomic Considerations: Human Polymorphisms

4.1 Alpha Class

The Alpha class of GSTs is the major isoform expressed in the liver. In fact, an increase in GSTA1*A concentration in blood is a specific marker of hepatocellular impairment.[26] Five genes have been identified in a cluster on chromosome 6 that encode proteins belonging to the Alpha class (GSTA1, A2, A3, A4 and A5).[27–30] GSTA1, GSTA2 and GSTA4 are widely expressed in human tissues, whereas GSTA3 is rare and GSTA5 was not detected in any tissues examined.[27] The *GSTA1* and *GSTA2* genes span a region of ~12kb and contain 7 exons.[31,32] *GSTA1* contains 8 single nucleotide polymorphisms. A silent mutation in exon 5 (A375G) and seven other single nucleotide changes in the promoter region, none of which appear to alter function, have been identified.[27,33] However, the promoter region

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of *GSTA1**A and *GSTA1**B differ by 3 base substitutions at positions −567, −69, and −53 which alter expression.[27] Specifically, a G→A change at position −52 alters binding of Sp1, rendering the *GSTA1**A promotor more active and therefore more highly expressed.[27] GSTA1 was originally described as ligandin based on its ability to bind to a number of electrophilic compounds.[5] Polymorphisms within this class that lead to differences in expression may alter an individual's capacity to metabolize drugs and xenobiotics as well as to sequester molecules that may alter kinase signaling. Supporting these conclusions, *GSTA1*B* gene shows a decreased hepatic expression and appears to confer susceptibility to colon cancer.[34]

The gene encoding GSTA2 was cloned in 1987, and the protein was shown to contain 221 amino acids with a molecular mass of 25 kDa.[28] GSTA2 has two polymorphisms: Thr112/Glu210 and Ser112/Ala210.[35,36] The structure and function of these two isozymes appear identical.[37] As part of the conditioning process, patients undergoing hematopoietic stem cell or bone marrow transplantation are pretreated with the myelosuppressive drug busulfan. While busulfan conjugation to GSH occurs primarily through GSTA2, polymorphisms at this locus appear to have no impact on its biotransformation.[33]

GSTA3 and *GSTA4* were identified using the Expressed Sequence Tag database and shown to share approximately 93% and 52% nucleotide sequence identity with *GSTA1*, respectively.[38] Full-length *GSTA3**C was recently cloned and shown to catalyze the double bond isomerization in the biosynthetic pathway of steroid hormones.[39] The isomerase activity in GSTA3 is distinct from other GSTs. Board[38] reported that *GSTA3* was a rare transcript. More recent studies show that GSTA3*C is expressed only in tissues characterized by active steroid hormone biosynthesis, including testis, ovary, adrenal gland, and placenta.[39] Polymorphism within GSTA3 and the contribution this subclass may have in the pathology of hormone producing tissues has not been investigated.

GSTA4 was isolated and cloned from a human adult brain cDNA library and shown to have a high activity with reactive carbonyl compounds, including alkenals.[38] The expression has been examined in normal and pathological human tissues and shown to be widely expressed throughout many organs, including the liver, kidney, colon, heart, brain, and skin.[19] In rat neuronal tissue, GSTA4 activity increases with age; however these studies have not been extended to the human isoform.[40] Increased expression of GSTA4 was observed in tissues damaged from reactive oxygen species, including the liver, UV-irradiated skin and the heart. However, expression was decreased in hepatocellular carcinoma.[19] In a separate study using a mouse model, these authors showed that GSTA4 expression is induced in the liver and kidney following iron overload.[41] Based on these two studies, these authors conclude that GSTA4 expression may increase with the formation of free radicals.

The role of GSTA4 and GSTA5 in response to chemotherapeutic agents has been investigated with mouse and rat homologs.[42,43] GSTA4 has been shown to confer resistance to doxorubicin in Chinese hamster ovary cells.[43] The mechanism of resistance was attributed to the inactivation of lipid peroxidation products via GSTA4. Resistance to doxorubicin and other alkylating agents was observed in a hamster fibroblast cell line transfected with the *GSTA5* gene.[42] Induction of GSTA5 in rat hepatocytes was observed

following exposure of chemotherapy agents.[44] Computer modeling of the promotor region in rat *GSTA5* identified a putative antioxidant response element that may be responsible for the induction of this isozyme by chemotherapeutic agents.[44,45] The information on GSTA4 and A5 is limited in human studies, however, rodent models suggest that GSTA may play a role in clinical response to therapeutic agents.

4.2 Mu Class

Five genes have been identified that encode proteins belonging to the Mu class (*GSTM1-5*).[46] GSTM proteins are encoded by a gene cluster located on chromosome 1.[46,47] Four of the *GSTM* genes are spaced ~20kb apart in the following orientation; 5′ *GSTM4—GSTM2—GSTM1—GSTM5* 3′.[48] The *GSTM1* gene contains four alleles and has been the most widely studied member of the class (table IV). GSTM1*A and *B differ by one amino acid change (table III) and are enzymatically identical.[49] GSTM1*A has been associated with a decreased risk of bladder cancer and has an allele frequency of 20%.[50] Some Saudi Arabian individuals have demonstrated an enhanced GSTM1 enzyme activity that has been characterized as a gene duplication at this locus.[51] The frequency of this genotype and corresponding risk assessment has not yet been defined.

GSTM1 activity is absent in a large number of individuals due to a gene deletion. *GSTM1* and *M2* genes are in close physical proximity and share 99% nucleotide sequence identity. It is proposed that an unequal crossing over of these two genes resulted in a 15kb gene deletion (the *GSTM1**0 allele).[46] *GSTM1**0 is surprisingly common, with an average frequency of 50% in human populations with a range of 22% in Nigerians to 67% in Australians.[50]

The GSTM1 null phenotype has been extensively studied as a risk factor for a variety of cancers as these individuals may be subject to an increased sensitivity to carcinogens.[52,53] The null phenotype (homozygous *GSTM1**0) is associated with an increased risk of lung, colon, head and neck, and bladder cancer, and aplastic anemia, and is a risk factor for pulmonary asbestosis.[54,55]

The literature defining the role that GSTM1*0 plays in response to chemotherapy agents is contradictory. Patients with breast cancer (homozygous *GSTM1**0) treated with cyclophosphamide and adriamycin had a reduced risk of recurrence compared with patients with a wild-type phenotype.[56] Yet, patients with ovarian cancer with a null phenotype treated with alkylating agents showed a poorer prognosis.[57] Defining a causal relationship between the GSTM1*0 phenotype and risk assessment in colorectal cancer is also contradictory.[58,59] However, more recent studies show that individual susceptibility to colorectal cancer was increased when patients expressed both GSTM3*B and GSTM1*0.[60] These data support the general conclusion that the individual phenotype for the GST family members as a whole, rather than a single isoform, should be considered when viewing risks and outcome.

Neurodegenerative diseases such as Parkinson's disease and schizophrenia are characterized by the degeneration of dopaminergic neurons. Cytosolic prostaglandin E synthase was identified in human brain and characterized as GSTM2.[61] GSTM2*B has been shown to catalyze the conjugation of GSH to aminochrome, a reactive oxygen species generated in

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the redox cycling of orthoquinones within dopaminergic neurons.[62] Hence, GSTM2*B has been proposed to play a protective role against neurodegenerative diseases.

GSTM3 was isolated in brain extracts and later shown to be expressed in brain and testis.[63,64] The *GSTM3* locus contains 2 alleles, *A and *B. The *GSTM3**B allele has a three base pair deletion in intron 6 that introduces a recognition sequence for the multifunctional transcription factor YY1.[65] YY1 has been shown to activate and repress transcription thereby altering many cellular responses.[66] The *GSTM3**AA genotype was shown to occur more frequently in patients with multiple cutaneous basal cell carcinoma than *GSTM3**BB.[67] *GSTM3**AA is also associated with an increased risk for laryngeal squamous cell carcinoma, while *GSTM3**BB was putatively protective.[68] In contrast, *GSTM3**A and *B are expressed in brain, however, there appears to be no direct relationship between GSTM3 and the incidence of astrocytomas.[69]

GSTM4 was cloned and shown to be 87% identical with *GSTM1*.[70,71] The *GSTM4* locus also contains two alleles, *A and *B.[72] The *GSTM4**B allele has been recently implicated as a risk factor in the development of lung cancer.[73] The Mu class of GSTs was analyzed in leukemic blasts from 21 children with acute lymphoblastic leukaemia (ALL).[73] GSTM3 and GSTM4 were expressed in 62% and 24% of patients, respectively.[73] These studies showed that GSTM3 was positively related to good prognosis and further information on this class might provide more information for treatment of children with ALL.

4.3 Omega Class

The Omega class of GSTs has recently been described and contains two members, GSTO1 and GSTO2. While this class shares sequence similarity with other GSTs, it is important to recognize that they are structurally and functionally distinct. The GSTO1*A protein is encoded by a single gene on chromosome 10 and is expressed abundantly in liver, macrophages, glial and endocrine cell.[74] GSTO1*A has been shown to be up-regulated in estrogen receptor-negative human breast cancer cell lines.[75] GSTO1*A has 2 unique features that may define this family, separating it from other eukaryotic GSTs. First, using X-ray crystallography, a 19 residue N-terminal extension has been identified that forms a novel structural unit, the function of which remains unclear.[74] Secondly, known substrates of other GSTs are not turned over by GSTO1*A. However, GSTO1*A demonstrated a GSH-dependent reduction of dehydroascorbate, a function characteristic of glutaredoxins rather than GSTs.[74,76] These issues have made the identity and characterization of GSTO1*A an anomaly. Adding to the complexity, a protein previously described as nuclear chloride channel, NCC27, was shown to share sequence homology to the GSTO1 family.[77] Like GSTO1*A, NCC27 has the N-terminal extension and lacks typical transferase activity. NCC27 is co-localized in cardiac and skeletal muscles with ryanodine receptors (RyRs), which are calcium-releasing channels. The addition of GSTO1*A to a cytoplasmic solution inhibited the activity of RyR2 by 50%.[77] These data along with the ubiquitous expression suggests that GSTO1 may have a fundamental, yet uncharacterized, role in cellular calcium homeostasis.

GSTO1*A was originally identified as the human monomethylarsonic acid reductase, (MMA[V]), and described as being the rate-limiting enzyme of inorganic arsenic

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metabolism.[78] More recently, variations within GSTO1 have been identified that might contribute to an individual's ability to metabolize arsenic.[79] Specifically, thioltransferase activity is decreased 75% in the Ala140Asp, and 40% in the Thr217Asn GSTO1 variant, compared with the wild-type GSTO1*A.[79]

Three alleles have been identified in the GSTO1 class; *GSTO1**A, *GSTO1**B and *GSTO1**C.[80] Among the Australian, African, and Chinese populations, *GSTO1**A was the most prevalent haplotype with a frequency ranging from 0.6–0.9; while *GSTO1**B*A was the least common, with a frequency ranging from 0.01–0.05.[80] The impact of heterogeneity within this class has yet to be defined. However, we do know that the thioltransferase and GSH-conjugation activity among the haplotypes are equivalent for GSTO1*A and *C, however, GSTO1*B had significantly higher activity despite the deletion of E155 (Glu155).[80]

GSTO2 has been recently identified and been shown to share 64% amino acid identity with GSTO1.[80] *GSTO2* is separated from *GSTO1* by 7.5kb on chromosome 10.[80] GSTO2 expression was most abundant in testis, however, it was observed in a variety of tissues including liver, kidney, skeletal muscle, and prostate.[80] The function of GSTO2 has not been identified. A third omega class member was identified and shown via *in situ* hybridization to exist on chromosome 3 (*GSTO3p*).[80] The lack of introns and representation in the express sequence tag database led researchers to believe that *GSTO3p* is a pseudogene.[80] Further investigation of *GSTO3p* is merited by the fact that the site of the pseudogene corresponds to a region that was previously believed to contain a gene that may influence the age of onset for Alzheimer's and Parkinson's disease.[80]

4.4 Pi Class

A single gene located on chromosome 11 encodes for proteins designated in the Pi class (GSTP1). The *GSTP1* gene spans ~3kb, encodes 210 amino acids in seven exons.[81] Expression of GSTP1 has been identified in all tissues and cells, except red blood cells.[82] GSTP1 has been of particular interest because it is overexpressed in a wide variety of tumors.[83,84] The allele frequencies for *GSTP1* *A, *B, and *C in Caucasian populations are 0.685, 0.262, and 0.068, respectively.[85] The promoter region contains a TATA box, two SP1 sites, an insulin response element and an antioxidant response element within an AP1 site.[86]

Numerous studies have been published showing the expression of GSTP1 is associated with clinical outcome in cancer (table V).[52,87,88] Polymorphisms at the *GSTP1* locus result in four alleles, *GSTP1**A-D, that differ structurally and functionally.[86,89] Normal lung tissue from 34 patients was genotyped and analyzed for GST enzyme activity as measured by 1-chloro-2,4-dinitrobenzene conjugation.[90] In these studies, enzyme activity was reduced in individuals expressing one of the *GSTP1* Val105 alleles (*B and *C) compared with individuals containing the Ile105 alleles, (*A and *D) [see table III].[90] In separate studies, the Val105 and Ile105 alleles were analyzed to determine if the genotype was a risk factor for a subgroup of basal cell carcinoma patients who develop multiple tumors.[91] *GSTP1* Val105/Val105 genotype (BB, BC, or CC) was associated with an increased number of tumors compared with the Ile105/Ile105 genotype (AA, AD, or DD).[91] Perhaps this

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reflects some degree of susceptibility differences to carcinogen exposure as a consequence of different detoxification profiles for these isozymes.

The *GSTP1* genotype has been associated with differences in cancer and respiratory disorder susceptibilities and response to chemotherapeutic agents. For example, *GSTP1**A has been reported to play a role in the acquisition of resistance to cisplatin via formation of platinum-glutathione conjugates.[92] *GSTP1**B is an allele in which a single nucleotide (A→G) substitution at position 313 results in the Ile→Val substitution that substantially reduces catalytic activity.[90] Individuals expressing the Val313 allele have a diminished detoxification capacity.[97] Homozygosity for *GSTP1**B is favorable in the treatment of patients with cancer because such patients have a diminished capacity to detoxify platinum-based anticancer agents.[93] However, this phenotype is also associated with an increased susceptibility to lung, bladder, and testicular cancers.[94,95] *GSTP1**C, an allelic variant that is predominant in malignant glioma cells, differs from other *GSTP1* variants by two transitions resulting in Ile105Val and Ala113Val.[86] The *GSTP1**C was shown to be protective against breast cancer.[96] However, the precise relevance of this variant to disease occurrence or progression is not yet clear.

4.5 Theta Class

Two genes separated by 50kb on chromosome 22 encode for proteins designated in the Theta class of GSTs, GSTT1 and GSTT2.[98–100] Polymorphisms exist within both genes. *GSTT1**A and *GSTT1**B differ by a single nucleotide substitution that alters the amino acid residue 104 from threonine (*GSTT1**A) to proline (*GSTT1**B) [see table III].[101] Introduction of a proline in this region containing an alpha helix results in a conformational change that significantly decreases the activity and mimics the null phenotype. In Swedes, the allele frequency for *GSTT1**A is 0.65 versus 0.35 for the non-functional *GSTT1**B allele.[101] A deletion in the *GSTT1* locus (*GSTT1**0) results in a null phenotype in which individuals do not express catalytically active protein. Occurrence of the null phenotype varies between ethnic groups and is found to be highest in Chinese (64.4%) and lowest in Mexican Americans (9%).[102] The null phenotype is also associated with an increased risk for tumors of the head and neck, oral cavity, pharynx, and larynx.[59,88] Countless studies have been published addressing the role of *GSTT1* polymorphisms and clinical outcome.[52,53,103]

A new allele has recently been identified in *GSTT2* that has a rare amino acid substitution (Met139 Ile).[98] While the frequency of this allele is more prominent in Australian and European populations, any possible phenotype has yet to be identified.

4.6 Zeta Class

A single 10.9kb gene located on chromosome 14 encodes the protein designated inthe Zeta class (GSTZ1).[104,105] GSTZ1 catalyzes the GSH-dependent transformation of a variety of alpha-halogenated acids. GSTZ1 was independently characterized and described as maleylacetoacetate isomerase (MAAI) because it plays a putative role as an isomerase in the catabolic pathway of phenylalanine and tyrosine.[106] GSTZ1, a 29 kDa protein, is expressed in hepatocytes and proximal convoluted tubules. Polymorphisms within the *GSTZ1* gene

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have been identified, and are designated *GSTZ1**A–D.[105,107,108] The GSTZ1*A isozyme has been shown to have the highest catalytic activity toward dichloroacetic acid and is predicted to play a key role in the treatment of lactic acidosis where dichloroacetic acid is prescribed.[109] Indeed, repeated treatments with dichloroacetic acid increases its plasma elimination half-life, suggesting that it may induce GSTZ activity.[110] In contrast, GSTZ1*D, characterized by a Thr82Met substitution, has a lower catalytic activity with dichloroacetic acid.[107] This isozyme has been associated with tyrosine metabolism. However, while inborn errors in tyrosine metabolism have been attributed to mutations in other enzymes, none have so far been associated with GSTZ1.[106] Nonetheless, deficiency of GSTZ1 expression has been identified in four families and is associated with mortality within the first year of life.[106] While the precise role (if any) of GSTZ1*D in contributing to premature death remains unclear, it remains plausible that a perturbation in tyrosine metabolism may be contributory.

4.7 The GST Null Phenotype

The Theta and Mu GST classes contain gene deletions that result in a 'null' genotype and the absence of enzyme expression and activity. However, a 'null' phenotype is observed in individuals with alleles that have a decreased rate of detoxification. GSTM3, GSTP1, and GSTZ1 provide examples of polymorphisms with decreased enzymatic activity. These individuals (homozygous *GSTM3**B, *GSTP1**B and *A, and *GSTZ1**C and *D) are thought to be at risk of a higher level of carcinogen induced damage and, therefore, at higher risk of developing cancers.

Several studies have investigated the expression of GSTs as a unpredictive factor for treatment outcome and survival in patients with cancer.[52,53,103] The results have been varied based on tumor type and progression. In 81 women with invasive ovarian cancer, patients with a null phenotype for GSTM1 or GSTT1 had a better survival after chemotherapy than other patients.[111] In contrast, 148 women with epithelial ovarian cancer who had a null genotype for GSTM1 or GSTT1 showed a poorer prognosis and decrease in disease free interval as compared with women who had GSTM1 or GSTT1 activity.[57]

GSTM1 and *GSTT1* genotypes were examined in children with acute myeloid leukemia (AML). These studies showed that individuals who lacked GSTT1 expression (GSTT1*0) displayed a greater toxicity and reduced survival following chemotherapy.[112] In contrast, the null genotype for *GSTM1* and *GSTT1* conferred a two-fold reduced risk of relapse in children with ALL (see section 4.2).[113] Collectively these studies suggest that the mechanisms that determine survival and disease free interval remain unclear, however, GSTs appear to have a contributory role that varies contingent upon tumor type.

A null genotype for *GSTP1* has not been reported. However, hypermethylation of the *GSTP1**A regulatory region is the most common somatic alteration identified in human prostate cancer.[114] This alteration results in the loss of GSTP1 expression and is proposed to occur during pathogenesis of the disease.[115] The impact of this phenotype has been extensively studied.[116] Recently, a methyl-CpG binding domain (MBD) protein has been identified that mediates hypermethylation of the *GSTP1**A regulatory region.[117] These findings provide a possible target for restoration of GSTP1*A activity. GST expression

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(and/or activity) of specific isoforms is lost in some individuals with allelic variation. Although it has been speculated that reduced detoxification of possible carcinogens may be causal to malignant transformation and disease progression, a more plausible link may be through an altered capacity to regulate kinase-dependent proliferation pathways.

5. Proteomic Considerations

Evidence for post-translational modifications of GSTs does exist. However, the interpretation of their biological relevance is hampered by the consideration that the experimental evidence is *in vitro* in nature. For example, GSTP1*A may be subject to phosphorylation at Thr109, Ser28, Ser154 and Ser184; *O*-glycosylation at Thr5; methylation at unknown sites; *N*-glycosylation at unknown sites. While the phosphorylation and *O*-glycosylation are based on predictive sequence modeling, there is direct experimental evidence (albeit with purified proteins) for the methylation[118,119] and *N*-glycosylation.[120] Whether or not these modifications are species-specific or exist in a cellular milieu, and whether they influence GST function, remains to be established.

6. Pharmacogenetics of GSTs

As is apparent from the foregoing discussion, a number of interrelated factors contribute to making GSTs a viable target for cancer drug design. In tumors where GST over-expression imparts a decreased therapeutic response, distinct strategies have been adopted to target GSTs. The first involves the design of GST inhibitors to sensitize tumors where conventional anticancer agents are subject to catalytic detoxification by GSTs. Somewhat novel in concept, progress has been made in the design of compounds to disrupt the protein:protein interactions of GST with interacting proteins, exemplified by stress kinases. This approach, in particular, takes advantage of an emerging principle in drug discovery. While the historical method of discovering new agents has relied upon the targeting of a specific protein to alter its function, it is now possible to target interacting proteins causing interference, with the expectation of a resultant pharmacological effect (see figure 1). Perhaps the most logical of the design strategies is to exploit the elevated expression of GST in tumors, particularly GSTP1*A, through design of GST-activated prodrugs.

6.1 Modulating Drug Resistance in Tumors by GST Inhibition

6.1.1 GST Inhibition by Ethacryinic Acid—A variety of GST inhibitors have been shown to modulate drug resistance by sensitizing tumor cells to anticancer drugs.[121–123] The first clinical studies tested an FDA-approved drug, ethacrynic acid (EA). EA acts non-specifically to inhibit GST Alpha, Mu, and Pi class isozymes by binding directly to GSTs, as well as to deplete its cofactor, GSH, via EA-GSH conjugation, where the thioether conjugate is also an inhibitor of the enzyme.[124,125] EA has been reported to potentiate the cytotoxic effects of alkylating agents, including chlorambucil in human colon carcinoma cell lines and melphalan in human colon tumor xenografts in SCID mice.[121,126]

As a chemosensitizer, the therapeutic value of EA has been demonstrated in patients. A phase I clinical trial showed that EA could suppress GST activity by approximately 50% in white blood cells. This could be correlated with preclinical data showing a corresponding

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two- to three-fold increase in sensitivity to alkylating agents.[121] However, the efficacy of EA in the clinical management of patients with cancer was limited by a lack of isozyme specificity and its dose-limiting diuretic properties.[127] Clinical correlates to laboratory-based observations are not easily demonstrated. However, in a population of patients with chronic lymphocytic leukemia (CLL), an elevated level of GST activity was found in patients who had received significant treatment with chlorambucil/corticosteroid combinations.[128] A small scale clinical study showed that these same patients, who had developed resistance to therapy, were able to achieve further remission when chlorambucil was administered in combination with EA.[129]

6.1.2 GST Inhibition by TLK199—

Efforts to develop inhibitors continued, with improved isozyme specificity and superior clinical application. One such lead compound, TLK199, is a glutathione analog that is a selective inhibitor of GSTP1*A.[130,131] TLK199 acts as a chemosensitizer and was shown to potentiate the toxicity of numerous anticancer agents in different tumor cell lines. In the same study, sensitivity to melphalan was enhanced in xenograft models with elevated GST levels.[132] TLK199 has also been shown to be an effective micromolar inhibitor of the multidrug resistance-associated protein1 (MRP-1), achieving a reversal in the resistance of a variety of agents in NIH3T3 cells transfected with MRP-1.[133]

A serendipitous outcome to the preclinical studies with TLK199 was the observation that TLK199 behaves as a small molecule myeloproliferative agent in rodents.[134] A plausible mechanism for the myelostimulatory effects of TLK199 may be the previously discussed capacity of the drug to disrupt protein: protein interactions in the GSTP:JNK complex. As a consequence of this effect, JNK activity is enhanced and this could be causally associated with the mitogenic response in bone marrow progenitor cells. Elevated levels of JNK have been identified in HL60 cells chronically exposed to, and grown in the presence of, TLK199.[134] This was distinct from the parental (wild-type) cell line where drug exposure induced apoptosis. *In vivo* studies in myelosuppressed rodents showed a dose-dependent increase in peripheral platelet and neutrophil counts within 24 hours of treatment with a physiological concentration of TLK199.[132,135]

6.2 Modulating Antimicrobial Drug Response by GST Inhibition

GSTs may also be viable drug targets in disease states unrelated to cancer. For example, GSTs distantly related to the mammalian counterparts are present in parasitic organisms and can provide potential targets for therapeutic intervention. Because of the restricted homology, there is always the possibility of an enhanced therapeutic index, since targeting the parasitic protein might have the potential advantage of not compromising the human host. Many anti-parasitic drugs form free radicals that may be inactivated by GSTs from the parasite.[136] In particular, chloroquinone, an antimalarial agent is inactivated by GSH conjugation.[137] The GSH depletion and GST inhibition can result in an enhanced efficacy of chloroquinone against the malarial parasite. Hence, inhibition of parasitic GSTs or destabilization of intraparasitic pools of GSH give a duality of function to therapy with chloroquinone.[137,138] To discover the next generation of anti-malarial drugs, structure-

based drug design investigations are underway utilizing crystal structures of the malarial parasites' GST.[138]

Schistosomiasis, a debilitating tropical disease caused by the parasite *Schistosoma japonicum*, affects over 200 million people world-wide and results in about 500 000 deaths annually.[138,139] Present therapy for the disease uses oltipraz, a drug which binds directly to the schistosome GST in the integument of the trematode.[140] Development of an effective vaccine is a viable goal for long-term prevention. In fact, a variety of schistosome target antigens are capable of protecting experimental animals from challenge. One of these is a potential vaccine, the 28 kDa *S. mansoni* GST (Sm28GST) that confers protective immunity in transgenic mice expressing Sm28GST.[48,140] Vaccination with Sm28GST was shown to decrease parasite fecundity and effect egg maturation, thereby decreasing disease pathology in host rats, mice and baboons. Following vaccination in human populations, an inverse correlation was found between IgA antibody production to Sm28GST and a decrease in parasitic egg production.[139] An alternative vaccine directed against the *S. haematobium* GST (Sh28GST) was shown to be well tolerated in phase I and II clinical trials and demonstrated the capacity to block parasite transmission.[141]

6.3 GSTP1-Activated Prodrugs

Traditional cancer drugs are cytotoxins that target rapidly dividing cells. In most cases, the therapeutic index is compromised because normal tissues, such as bone marrow, gut mucosa, and hair follicles receive exposure equivalent to the tumor. In an effort to improve drug efficacy, greater tumor targeting is a desirable endpoint. Prodrugs are rationally designed inactive agents that are converted to active cytotoxic agents that can target tumor tissues with high expression of activating enzymes. This strategy allows for an increased delivery of active agent to the tumor tissue while minimizing the toxicity towards normal tissues. GSTs provide a promising target because expression is enhanced in many tumors and high levels are sometimes correlated with poor prognosis. In addition, GSTP1*A is frequently elevated in drug resistant tumors. Thus, a two-pronged attack may be afforded by such an activation strategy.

One such approach has been to attempt to exploit the ability of GSTs to catalyze GSH conjugation. For example, cis-3-(9H-purin-6-ylthio)acrylic acid (PTA) is a prodrug of the antitumor and immunosuppressive antimetabolite 6-mercaptopurine that requires GSH conjugation and subsequent metabolism for activation.[142] Renal and hepatic GSTs enhance activation nearly two-fold both *in vivo* and *in vitro* as compared with spontaneous GSH conjugation.[142] At this time, this drug is in early preclinical development. However, future success may be restricted by the somewhat narrow spectrum and limited efficacy of the parent drug 6-mercaptopurine.

A second approach in drug development has been to design prodrugs that are selectively activated by GSTs, which are known to be over-expressed in a wide variety of tumors. This design exploits GSTs ability to mediate cleavage of sulfonamides by promoting a β-elimination reaction. Synthesis of drugs as inactive compounds via GSH conjugation through a sulfone linkage was a logical extension of this concept. One such drug is a

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GSH analog of cyclophosphamide that was shown to selectively enhance toxicity via GST activation in cell and animal models.[143]

TLK286, γ-glutamyl-α-amino-β-(2-ethyl-N,N,N′,N′-tetrakis [2-chloroethyl]phosphorodiamidate)-sulfonyl-propionyl-(R)-(−) phenylglycine, is the lead candidate from a novel class of prodrugs activated in cancer cells by GSTP1*A.[131] GSTP1*A promotes a β-elimination reaction that cleaves TLK286 into a GSH analog and a nitrogen mustard that can alkylate cellular nucleophiles.[144] Sensitivity to the drug is correlated with GSTP1*A expression both in cell culture and in animal models.[144,145] In contrast, lower expression of GSTP1*A at both the protein and transcript level was reported as an adaptive survival trait following chronic exposure to TLK286.[145] These results support the concept that tumors expressing high levels of GSTP1*A will be more sensitive to the cytotoxic effects of TLK286.

The efficacy of TLK286 was examined both *in vitro* and *in vivo*. Clonogenic assays showed TLK286 had significant activity against 15 of 21 lung tumors and 11 of 20 breast tumors.[146] Reports on phase I clinical trials showed minor drug related adverse effects that included hematuria and myelosuppression, combined with anti-tumor activity and/or disease stabilization in patients with various advanced malignancies.[147,148] None of the toxicities were greater than grade III, with no indications to limit further clinical development. Clinical benefit has now been reported in phase II trials underway in patients with advanced non-small cell lung cancer (NSCLC) and platinum-resistant ovarian cancer.[149] Such results have encouraged the formulation of phase III trials, which will initially focus on combinations of TLK286 and docetaxel (taxotere) in the treatment of NSCLC.

A general conclusion from the initial foray into targeting GSTs is that the approach is viable. Whether the drugs presently traversing the preclinical and clinical testing protocols will reach eventual widespread utility remains to be determined. However, at this point in development the results are encouraging.

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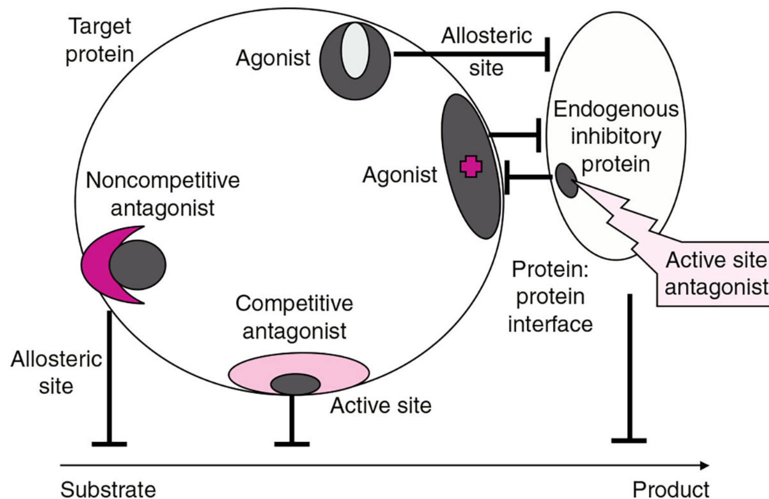

Fig. 1.

Drug discovery has frequently involved the targeting of a specific protein with small molecules that act as antagonists or agonists either at active or allosteric sites. Whether resultant drugs act competitively or non-competitively depends upon the actual site and avidity of binding. The impact on a target enzyme is usually interference with substrate-to-product conversion. More recently, the principle of interference with protein:protein interaction as a viable drug discovery approach has gained credibility. Shown in the figure is the concept of targeting an endogenous inhibitory protein (in context of this review, for example GSTP1*A). The outcome is to disassociate this protein from its partner (e.g. c-Jun N-terminal kinase) producing the agonist effect and concomitant activation of the kinase.

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Table I.

Anti-cancer agents associated with increased levels of glutathione-*S*-transferase (GST) and resistance (adapted from Tewi[2] and Fan and Chambers[4])

	Agent
Substrates of GST and thereby inactivated via GSH-conjugation	Chlorambucil
	Acrolein
	Hydroxyalkenals
	Carmustine
	Nitrogen mustard
	Melphalan
	Ethacrynic acid
	Corticosteroids
	Phosphoramide mustard
Non-substrates	Bleomycin
	Hepsulfam
	Carboplatin
Non-substrates but require JNK activation to elicit cytotoxicity	Antimetabolites
	Antimicrotubule drugs
	Topoisomerase I and II inhibitors
	Mitomycin C
	Adriamycin
	Cisplatin

GSH = glutathione; **JNK** = c-Jun N-terminal kinase.

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Table II.

Cytosolic glutathione-*S*-transferases (GSTs)

Class	Gene(s)	Protein (MW)	Chromosome location
Alpha (α)	GSTA1–5	25 900	6
Mu (μ)	GSTM1–5	26 000–26 700	1
Omega (ω)	GSTO1–5	27 566	10
Pi (π)	GSTA1	24 700	11
Theta (θ)	GSTA1–2	25 100	22
Zeta (ζ)	GSTZ1	25 000	14

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Table III.

Genetic variation in selected glutathione-*S*-transferases (GSTs)

Gene	Allele[a]	Amino acid change	Enzyme activity	References
GSTA1	*A	Reference	Reference	27
	*B	Pt mutation, promotor	Decreased expression	27
GSTA2	*A	Thr112;Glu210	Reference	35
	*B	Ser112;Ala210	No change	36
GSTM1	*A	Lys173	Reference	49
	*B	Asp173	No change	49
	*O	Deletion	None	50
	*Ax2	Duplication	Hyper	51
GSTM3	*A	Reference	Reference	63,64
	*B	3 bp deletion	Altered transcription factor recognition	65
GSTM4	*A	Tyr2517	Reference	72
	*B	Cyt2517	No change	72
GSTO1	*A	Ala140;Glu155	Reference	80
	*B	Ala140;155 deleted	Increased	80
	*C	Asp140;Glu155	No change	80
GSTP1	*A	Ile105;Ala114	Reference	87,89
	*B	Val105;Ala114	Decreased	87,89
	*C	VaM05;VaM14	Decreased	87,89
	*D	Ile105;Val114	No change	88,89
GSTT1	*A	Thr104	Reference	98,101
	*B	Pro104	Deceased	98,101
	*O	Deletion	None	98
GSTT2	*A	Met139	Reference	98
	*B	Ile139	Not characterized	98
GSTZ1	*A	Lys32;Arg42;Thr82	Reference	107,108
	*B	Lys32;Gly42;Thr82	Decreased	108,109
	*C	Glu32;Gly42;Thr82	Decreased	108,109
	*D	Glu32;Gly42;Met82	Decreased	107

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[a]Enzyme activity is measured within each gene using *A, the first allele identified, as a reference.

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Table IV.

Polymorphisms in the *GSTM1* gene and associated cancer risks[49,50,52–54]

Allele	Frequency in Caucasian populations	Frequency in colorectal cancer patients	Cancer risks
*M1**A	0.2	0.19	Decreased risk of bladder and breast cancer
*M1**B	0.2	0.14	Decreased risk of pituitary adenomas
*M1**O	0.59	0.65	Increased risk of lung, colon, bladder, and post-menopausal breast cancer

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Table V.

Polymorphisms in the *GSTP1* gene and associated cancer risks[52,83–85,87,92–96]

Allele	Frequency in Caucasian populations	Cancer risks
*P1**A	0.65	Cisplatin resistance
*P1**B	0.262	Favorable response to cisplatin. Increased susceptibility to lung, bladder and testicular tumors
*P1**C	0.068	Predominant genotype in malignant glioma. Protective for breast cancer