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Commentary

Redox in redux: Emergent roles for glutathione S-transferase P (GSTP) in regulation of cell signaling and S-glutathionylation

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ABSTRACT

Glutathione (GSH) provides a major source of thiol homeostasis critical to the maintenance of a reduced cellular environment that is conducive to cell survival. Mammals have accumulated a significant cadre of sulfur containing proteins, the interactive significance of which has become clear in recent times. Glutathione transferases (GST) are prevalent in eukaryotes and have been ascribed catalytic functions that involve detoxification of electrophiles through thioether bond formation with the cysteine thiol of GSH. The neutralizing impact of these reactions on products of reactive oxygen has contributed to the significant evolutionary conservation and adaptive functional redundancy of the multifaceted GSH system. Amongst the GSTs, GSTP has been implicated in tumorigenesis and in anticancer drug resistance. Emerging studies indicate that GSTP has ligand binding properties and contributes in the regulation of signaling kinases through direct protein:protein interactions. Furthermore, S-glutathionylation is a post-translational modification of low pK_a cysteine residues in target proteins. The forward rate of the S-glutathionylation reaction can be influenced by GSTP, whereas the reverse rate is affected by a number of redox sensitive proteins including glutaredoxin, thioredoxin and sulfiredoxin. The functional importance of these reactions in governing how cells respond to oxidative or nitrosative stress exemplifies the broad importance of GSH/GST homeostasis in conditions such as cancer, ageing and neurodegenerative diseases. GSTP has also provided a platform for therapeutic drug development where some agents have completed preclinical testing and are in clinical trial for the management of cancer.

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1. Glutathione and glutathione transferases

The biological importance of glutathione has been appreciated since it was first described as ''philothion,'' literally, love of sulfur [1]. The glu–gly–cys tripeptide was subsequently crystallized by a number of investigators, one of whom determined that it contained sulfur and renamed it glutathione [2]. Glutathione (GSH) is present in virtually all prokaryotes, eukaryotes and mammals; even red blood cells that lack protein synthetic capacity can synthesize GSH. In humans, the usual range of plasma GSH is 10–30 μM and with a flow rate to the kidney of 1 l/min, ~60 μmol, GSH is delivered to the lumen of the proximal tubule [3]. Within the proximal tubule concentrations of GSH approach 3 mM with a turnover half-life of ~20 min

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[4]. Turnover of renal GSH suggests that a further 2.4 μmol/h is delivered to the tubule lumen. Since the concentration in urine is 1–3 μM, this is an excretion rate of <1 nmol/h, implying that 99% of the GSH is reabsorbed. This salvage mechanism helps to supplement the *de novo* synthesis. Two enzymes, γ-glutamylcysteine synthetase (γ-GCS) and glutathione synthetase function sequentially to achieve *de novo* biosynthesis of GSH. The γ-GCS reaction represents the committed step in the biosynthesis and is subject to feedback inhibition by GSH. Availability of cysteine is a limiting factor in the synthetic scheme. Reactions of GSH can be sub-divided into those involving the γ-glutamyl and those of the sulfydryl of cysteine, the latter being the focus of this commentary article. The amino terminal peptide bond is formed through the γ-carboxyl of the glutamate residue. This bond is resistant to degradation by serum aminopeptidases or proteolytic enzymes and this permits inter-organ transport of GSH. Nucleophilicity is provided by the thiol group of cysteine and with a pK_a of 9.65, is basic enough to imbue important chemical properties.

As part of the redox environment, glutathione S-transferases cooperate with GSH to maintain thiol homeostasis. A variety of publications have discussed the merits of nomenclature for GST. For the most part, avoidance of the Greek letter symbols has been recommended. It is nevertheless fairly common to find families described by the major six cytosolic classes that share ~30% sequence identity designated by α, μ, ω, π, θ, and ζ. Multiple isozymes exist within each class and these share >50% sequence identity [5]. In humans, a single gene located on chromosome 11q13 encodes for proteins designated in the human π class (GSTP1). The GSTP1 gene spans ~3 kb and encodes 210 amino acids in seven exons [6]. Polymorphisms at the GSTP1 locus result in three alleles GSTP1*A–D that differ structurally and functionally [7,8]. The allele frequencies for *A–C in Caucasian populations are 0.685, 0.262, and 0.068, respectively [9]. The promoter region contains a TATA box, two SP1 sites, an insulin response element and an anti-oxidant response element within an AP1 site [7]. In mice, there are two GSTP genes designated mGSTP1 and mGSTP2 that contain seven exons and are ~3 kb in length. These genes lie adjacent to one another on chromosome 19 [10] a fact that was relevant to the targeting strategy in creating knockout animals [11].

2. What happens in the absence of GSTP?

Mice null for GSTP1-1 and GSTP2-2 are viable, fertile, with life spans essentially similar. General physiology and development do not appear to be grossly aberrant [12]. Early characterization of these mice suggested that there was an enhanced susceptibility to carcinogen induced skin papillomas when mice were exposed to polycyclic aromatic hydrocarbons [11]. In mouse embryo fibroblast cells isolated from wild type or GSTP null animals, a number of characteristics related to signaling and growth were different [13]. For example, the doubling time for wild type cells was 33.6 h compared to 26.2 h for GSTP null. Both early passage and immortalized mouse embryo fibroblast (MEF) cells from GSTP null animals expressed significantly elevated activities of extracellular regulated kinases (ERK1/ERK2). More recent results [14] showed that null animals had constitutively elevated c-jun NH_2-terminal kinase (JNK) activities compared to wild type and that this correlated with altered regulation of genes downstream of JNK control. As a whole, the genetic absence of GSTP influences the capacity of stress kinases to regulate gene expression and this can have an impact on cell proliferation pathways. The non-lethality of the deletion points to possible functional redundancy and implies that other GST (or other redox proteins) may substitute for the absence of GSTP. This conclusion would seem to be supported by the data suggesting general redundancy of function amongst and within this protein cluster.

Aberrant cellular signaling is also a hallmark of the malignant phenotype and as we shall see, high levels of GSTP in many tumors may be either a cause or effect of the transformation process. Prostate cancer does not follow this expression pattern. Hypermethylation of the GSTP regulatory region is a common somatic alteration identified in human prostate cancer [15]. This alteration results in the loss of GSTP expression and is proposed to occur during pathogenesis of the disease [16]. Recently, a methyl-CpG binding domain (MBD) protein has been identified that mediates hypermethylation of the GSTP regulatory region [17]. These findings led to speculation that there could be some therapeutic value in restoring GSTP activity in this disease, although this contingency has yet to be tested. In addition, GST expression (and/or activity) of specific isoforms is lost in some individuals with allelic variation. It has been speculated that reduced GSTP may alter the capacity to detoxify possible carcinogens and thus may be causal to malignant transformation and disease progression in the prostate. However, perhaps a more plausible (and as yet untested) connection may be through an altered capacity to regulate kinase dependent proliferation pathways or to control protein S-glutathionylation. These issues are now discussed in the context of whether a cell can have too much GSTP. Pharmacological suppression of GSTP, while obviously not as efficient as the genetic deletion approach, also causes changes in these same pathways. As will be discussed later, inhibitors of GSTP can influence cell proliferation (particularly in the bone marrow) in a manner that is pharmacologically useful.

3. Can a cell have too much GSTP?

High levels of GSTP are found in many tumors (in particular ovarian, non-small cell lung, breast, colon, pancreas and lymphomas) and in a wide range of drug resistant cell lines and tumors [18]. In perspective, these enhanced expression ratios (when compared to normal tissues or wild type cell lines) have not always been easily explained. For example, in two of the earliest reports of increased GST expression in drug resistant cell lines, one was in response to chlorambucil [19], where evidence of catalytic formation of the thioether conjugate of this alkylating agent was subsequently documented [20] and could in principle explain a cause:effect relationship for selection of GST over-expression. However, for a later example, an MCF7 human breast carcinoma cell line resistant to adriamycin was found to have approximately 50-fold more GSTP than the corresponding wild-type line which

expressed extremely low levels [21]. This connection was less readily explained by any GST catalytic properties, since GSH conjugates of adriamycin do not occur under physiological conditions. In the years since these reports, tacit (and frequently unjustified) assumptions have linked GST mediated detoxification processes with such acquired resistant phenotypes. The advent of the importance of GST in kinase regulation and proliferation and more recently, the link of GSTP to the forward reaction of S-glutathionylation has created possible new ways to view GSTP expression patterns. For example, could it be that some tumors and drug resistant cells have developed an acquired dependence on the protein? Because of the proliferative nature of tumor cells, kinase pathways are frequently dysregulated, and as a consequence, tumor cells could conceivably attempt to compensate by enhancing expression of GSTP to counterbalance increased kinase activity. Such ''addiction'' to over-expressed proteins has been promulgated as a characteristic of the transformed phenotype. Moreover, every month new reports emerge of the potential importance of S-glutathionylation in regulating protein functions. Not inconsequentially, phosphatases such as PTP1B [22] and cdc25 [23] are subject to regulation by S-glutathionylation of critical cysteine residues. The kinase/phosphatase cycle impacts many pathways critical to uncontrolled cell growth. With these factors in mind, it is tempting to speculate that the relative abundance of GSTP in certain cancer tissues may reflect a role(s) that is unrelated to straightforward detoxification. Viewed in context, in the absence of electrophilic stress, it has always seemed odd that GSTP can be such a prevalent protein (indeed in some cases the most prevalent non-structural protein) in many tumor cells. Selective pressures or conditions of convergent evolution could have favored the emergence of the non-enzymatic, non-catalytic properties of GSTP and could have imbued them with biological relevance.

4. GSTP in drug resistance and small molecule detoxification

The GSTP1 genotype has been associated with both differences in chemotherapeutic response and in cancer susceptibility. In fact, the literature is replete with examples of correlative studies where elevated GST levels are present in selected drug resistant cell lines. Some examples of the type of drugs are shown in Table 1. For the examples shown, drug resistant cell lines have generally been established by selection through chronic exposure to the selecting agent. While few studies have detailed increased capacity to form thioether conjugates of the selecting drug, the left column shows some specific links. For those agents in the right column, there is no indication that GSTP participates in any catalytic detoxification process that involves the parent drug. Prior to the detailed analysis of GSTP allele variation, it was common for authors to use general GST substrates or immunoblots with GST antibodies to detail the expression differences. More detailed examples have been published recently. For example, GSTP1*A can play a role in the acquisition of *cis*-platinum resistance reportedly through enhancing the formation of platinum–glutathione conjugates [24]. GSTP1*B is the allele in which a single nucleotide (A → G) substitution at position 313 produces an isoleucine to valine conversion that substantially reduces catalytic activity [25], such that individuals expressing the valine allele have a diminished potential to detoxify the drug. Additionally, homozygocity for GSTP1*B is favorable in the treatment of cancer patients because they have a diminished capacity to detoxify a number of platinum based anticancer agents [26]. This phenotype is also associated with an increased susceptibility to lung, bladder, and testicular cancers [27]. GSTP1*C is an allelic variant predominant in malignant glioma cells and differs from other GSTP1 variants by two transitions resulting in Ile104Val and Ala113Val [7]. Without a clear understanding of why, GSTP1*C has been correlated with lower incidence of breast cancer [28].

Table 1 – Drug resistance associated with selected increased GSTP expression

Drugs where evidence for substrate catalysis with GSTP exists	Non-substrates
Carboplatin	Adriamycin
Chlorambucil	Etoposide
Cis-platin	Mitomycin C
Ethacrynic acid	Vinca alkaloids (MDR)
Melphalan	
Nitrogen mustard	
Phosphoramide mustard	
Select steroids	

These few specific examples under-represent the voluminous literature of correlative associations between GST expression patterns and sensitivity to drugs or to cancer incidence. So many reviews have been written that there would presently be limited value in reassessing the epidemiological or the drug response data in detail. For further information the reader is referred to Ref. [29]. What has become clear in recent times is that the GST family, and in particular GSTP, has a functional pleiotropy that extends well beyond enzymology.

5. GSTP as a component of the S-glutathionylation cycle

Published evidence for various post-translational modifications of GSTP does exist. However, analysis of whether these alterations imbue *in vivo* biological importance is complicated by the consideration that the majority of the experimental evidence is *in vitro* in nature. For example, GSTP is subject to phosphorylation at Thr109, Ser28, Ser154 and Ser184; *O*-glycosylation at Thr5; methylation at unknown sites; *N*-glycosylation at unknown sites. The phosphorylation and *O*-glycosylation conclusions are premised on predictive sequence modeling, but there is direct experimental evidence (albeit with purified proteins) for the methylation [30] and *N*-glycosylation [31]. Whether or not these modifications occur in real life and whether they influence GSTP function remains to be firmly established. However, one post-translational modification where there is evidence for GSTP association and functional significance is S-glutathionylation.

Cysteine residues in proteins provide a nucleophilic site for a number of disparate post-translational modifications. For example, disulfide bonds can occur between vicinal thiols with concomitant major implications for three dimensional protein structures. In addition, contingent upon the steric properties and local environment of the cysteine, lipidation of these residues may occur through S-isoprenylation, S-farnesylation, S-geranylgeranylation or S-palmitoylation. These add considerable peripheral bulk with resultant impact upon protein structure, function and sub-cellular localization. Perhaps one of the more studied examples of a cancer specific target is the ras oncogene where these post-translational events have an impact upon cellular transformation. More recently, another cysteine modification has received attention. The direct addition of GSH to target cysteines creates an S-glutathionylated residue, resulting in an increase in molecular weight of 605 and a net increase in negative charge (from the glutamic acid residue of GSH). The scheme in Fig. 1 summarizes some of the possible intermediates from the interaction of nitric oxide and GSH that may lead to S-glutathionylation of cysteine residues in target proteins. These pathways are complex and a complete understanding of them is still evolving. Nevertheless, there is value in considering the implications of NO interactions with thiol molecules. Briefly, the example illustrates how NO (in this case from activation of PABA/NO) creates nitrosative stress and in one exigency through intermediary GSNO, a glutathionyl radical $GS^{\bullet}$ and nitroxyl (HNO; step 1). Nitroxyl has recently been considered as a biologically important molecule that has pharmacological properties that may appose NO [32]. The degree of electrophilic selectivity of HNO for thiols is higher than for other nuceophiles (thiol > amine > oxygen nucleophiles; [33]) and can react with GSH (step 2) to give *N*-hydroxysulfenamide (GS–NH–OH); this can rearrange to generate a sulfinamide ($GS(O)NH_2$; step 3). Reaction of $GS(O)NH_2$ with GSH (step 4) can produce glutathione disulfide-S-oxide (GS(O)SG), a product that has the potential to act as the proximal donor in S-glutathionylation reactions (step 5). A sulfinic acid (GS(O)OH)

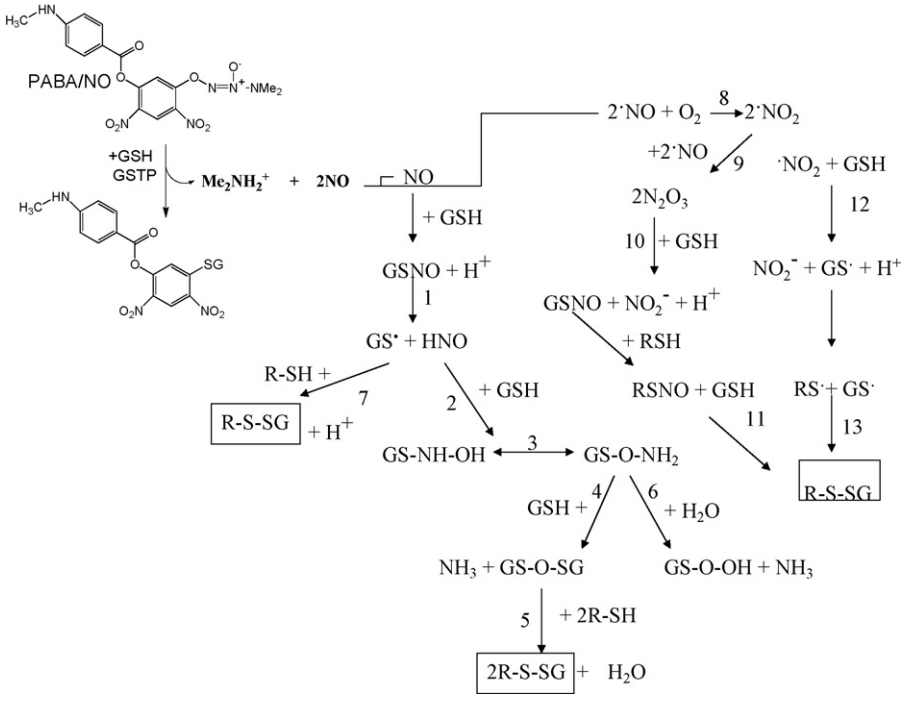

Fig. 1 – Scheme by which GST activated PABA/NO can produce intermediates important to S-glutathionylation reactions. PABA/NO releases NO that can react with GSH (1) to form a glutathionyl radical $GS^{\bullet}$ and nitroxyl (HNO; [39]). Nitroxyl reacts with GSH (2) to give *N*-hydroxysulfenamide (GS–NH–OH), that can rearrange (3) to form a sulfinamide ($GS(O)NH_2$). Reaction of $GS(O)NH_2$ with GSH (4) forms glutathione disulfide-S-oxide (GS(O)SG) and NH_3 and with H_2O (6) to form a sulfinic acid (GS(O)OH) and NH_3. Key intermediates leading to the synthesis of GS(O)SG are the sulfinamides ($GS(O)NH_2$ and GS(O)–NH–SG. The reaction of GS(O)SG (5) or $GS^{\bullet}$ (7) with a reduced protein thiol (R-SH) leads to the formation of mixed disulfide. Arguments in favor of glutathione disulfide S-oxide (GS–O–SG) acting as the proximal donor for S-glutathionylation reactions are presented in Refs. [34,35]. A further route of NO metabolism is also shown on the right side of the schema. Reactive nitrogen oxide species formed with oxygen (8) can nitrosate protein thiols. Evidence suggests that this may be mediated through the formation of dinitrogen trioxide (9). Furthermore, dinitrogen trioxide (N_2O_3) can rapidly react with GSH to produce GSNO (step 10). The later can then react with cysteine residues through *trans*-nitrosylation followed by thiol-disulfide exchange resulting in S-glutathionylation (step 11). The levels of NO together with the hydrophobicity of the local cellular environment could favor the principle that N_2O_3 may be an important species in nitrosation and S-glutathionylation reactions. It is also possible for reactive nitrogen dioxide to produce thiyl radicals that may lead to eventual S-glutathionylation (steps 12 and 13). An in-depth assessment of the biochemistry surrounding these latter pathways is presented in Ref. [38].

and NH_3 are possible hydration products from sulfinamide (step 6). Key intermediates leading to the synthesis of GS(O)SG are the sulfinamides ($GS(O)NH_2$ and GS(O)–NH–SG). The reaction of GS(O)SG (step 5) or $GS^{\bullet}$ (step 7) with a cysteine residue (R-SH) can lead to the formation of the S-glutathionylated mixed disulfide [34,35]. While it is apparent that GSH itself is not a proximal donor in S-glutathionylation, there are a few candidates that may be involved and these include GSSG, GS–O–SG, GS–O–O–SG and/or the products shown on the right side of Fig. 1 (steps 8–12) [34–39]. NO can produce oxidized intermediates (steps 8 and 9) that can lead to the highly nitrosative species N_2O_3 which rapidly react with GSH to produce GSNO (step 10). The later can then react with cysteine residues through *trans*-nitrosylation followed by thiol-disulfide exchange resulting in S-glutathionylation (step 11). It is also possible that nitrosative species such as nitrogen dioxide interact directly with GSH to lead to S-glutathionylated cysteine residues (steps 12 and 13). Some, or all, of these reactions may contribute to the modification and ongoing work will help to establish which are the most important. In addition to S-glutathionylation, nitrosation of proteins results from the interaction of oxidation products of NO to NO_2 and N_2O_3 with susceptible protein nucleophiles. In the case of PABA/NO there are only low levels of this modification, while S-glutathionylation predominates [40,41].

GSTP has been shown to play a necessary role in the S-glutathionylation of 1-cysperoxiredoxin (1-cysPrx). Oxidation of the catalytic cysteine of 1-cysPrx has been associated with its loss of peroxidase activity [42]. Recently, it was shown that heterodimerization of 1-cysPrx with GSTP mediates the S-glutathionylation of the previously oxidized cysteine thus restoring its peroxidase activity [42]. From this study, it was concluded that the S-glutathionylation and subsequent GSH-mediated reduction of 1-cysPrx requires heterodimerization with GSTP [42]. This provides the first example in which GSTP functions in the S-glutathionylation of oxidized cysteine residues. In addition, mouse embryo fibroblast cells deficient in GSTP1-1 and/or GSTP2-2 have a reduced capacity to respond to oxidative or nitrosative stress by enacting S-glutathionylation of a select group of target proteins [40]. These findings imply that GSTP may play a direct role in control of post-translational S-glutathionylation reactions. As such it is possible to construct a scheme for the forward and reverse steps that contribute to the glutathionylation cycle (Fig. 2).

S-glutathionylation can serve to regulate directly the structure/function of a quite diverse range of proteins and also serves to prevent the sequential oxidation of thiol groups to sulfenic, sulfinic and sulfonic acids; the latter product is generally resistant to any type of ''repair'' and leads to the proteosomal degradation of the protein (Fig. 3). Reversibility of S-glutathionylation provides a switch in controlling response to changes in redox conditions and cells have evolved a degree of functional redundancy in regulation of this reversible reaction. Proteins involved in the reduction of oxidized cysteine residues include thioredoxin (Trx), glutaredoxin (Grx) and sulfiredoxin (Srx). Trx can reduce protein disulfides and protein sulfenic acid intermediates [43]. Grx enzymatically deglutathionylates certain proteins [44], but has also been shown to catalyze the oxidative modification of several proteins in the presence of a GS-radical generating system [45]. Hence, Grx is capable of catalyzing both S-glutathionylation and deglutathionylation of proteins via distinct mechanisms [46]. Grx contains a conserved two cysteine residue motif (CXXC) where both cysteines are required for its reductive

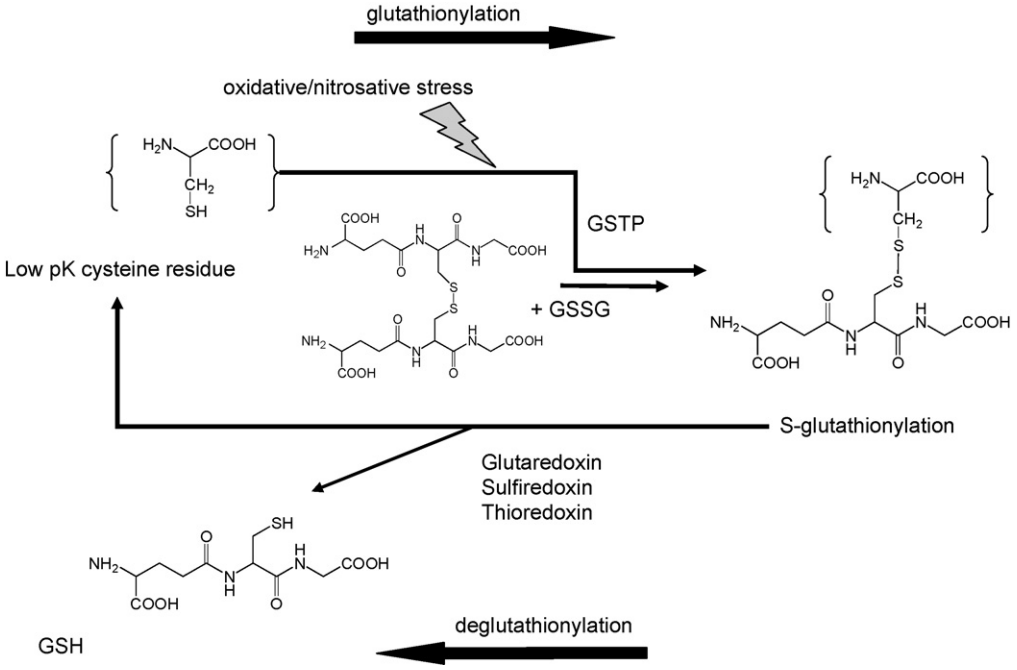

Fig. 2 – Forward and reverse reaction of the S-glutathionylation cycle. In the example shown, a cysteine residue in a target protein under oxidative or nitrosative stress can form an intemediate that can be S-glutathionylated. The proximal donor in this reaction is GSSG and GSTP can act as a catalyst in the reaction. The modified cysteine can be deglutathionylated by one of three known redox active proteins, glutaredoxin, sulfiredoxin or thioredoxin.

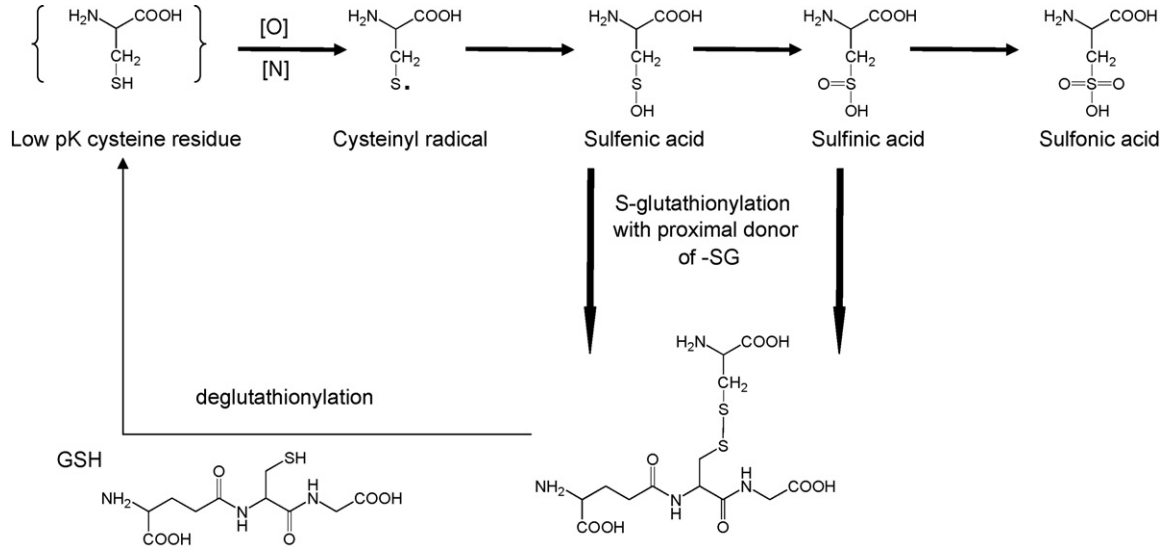

Fig. 3 – Oxidation states of sulfur following exposure to electrophilic oxygen or nitrogen species determine the intermediates of cysteine modifications in proteins. Sulfenic and sulfinic acid residues can generally be salvaged through S-glutathionylation. Sulfonic acids generally consign the protein to proteosomal degradation.

deglutathionylation function via a dithiol mechanism of disulfide exchange. However, studies with mutant Grx containing only the N-terminal cysteine residue within the conserved motif (CXXS) showed that Grx can also function as an oxidase through a monothiol mechanism. Grx can form a mixed disulfide (Grx-SG) as a consequence of both its oxidative and reductive mechanisms. Human Srx1 contains only one cysteine residue within its sequence and it is involved specifically in the reductive deglutathionylation of proteins. Although the conserved cysteine residue in Srx1 (Cys 99) is essential for the deglutathionylation reaction, it is not a direct acceptor for the GSH moiety, as Srx1 does not form a mixed disulfide with GSH during the deglutathionylation reaction [47]. In yeast, five different Grxs have been described. Grxs 1 and 2 are classic dithiolic Grxs containing both conserved cysteine residues [48]. Interestingly, Grxs 3–5 contain only one conserved N-terminal cysteine residue and function through a monothiol mechanism [49]. However, Grx5 contains an additional non-conserved C-terminal cysteine residue that is involved in deglutathionylation of mitochondrial proteins, through a monothiol mechanism [50]. This 'non-conserved' C-terminal cysteine residue aligns with the Cys99 residue in human Srx1. The similarity of human Srx1 to Grx5 in yeast suggests a potential monothiol mechanism of disulfide exchange reaction, even though a S-glutathionylated Srx1 intermediate does not form. The crystal structure of human Srx1 [51] identifies the active site motif as FGGCHR, along with detail of H-bonding with residues surrounding the active site cysteine. However, the importance of this residue to the deglutathionylation reaction has yet to be established.

Srx1 also appears to have ligand binding capacity with the consequence of interference with S-glutathionylation, presumably through steric hindrance. The ligand binding properties of Srx1 are apparently independent of its conserved cysteine residue, since both inhibition of S-glutathionylation and co-immunoprecipitation of the protein with actin are observed with the cysteine mutant Srx1 [47].

6. GSTP as a ligand binding protein-cellular kinases

GSTs were initially determined to have significant ligand binding potential for molecules such as heme and bilirubin, a determinant factor in the applied early nomenclature of ligandin Ref. [52]. In the years between the early 1970s and the present day, the biological importance of the catalytic properties of GSTs has been the focus of much of the published work. Somewhat ironically, recent publications have led us full circle, with a renewed reconsideration of the protein:protein interaction properties of, in particular, GSTP.

It has been speculated that the absence or decreased expression of GSTP results in a reduced detoxification of possible carcinogens that may be causal to malignant transformation and disease progression. In addition, the GST-mediated conjugation of GSH to a number of anticancer drug substrates has long been linked to anticancer drug resistance in a variety of tumors. The paradox is that GSTP has a weak affinity for the majority of anticancer drugs, even though its increased expression can in some cases be correlated with multidrug resistance. From this it can be inferred that the capacity of GSTs to regulate kinase dependent proliferation pathways, especially in the case of GSTP, may be of more consequence than its catalytic properties alone.

GSTs play a regulatory role in cellular signaling by forming protein:protein interactions with critical kinases involved in controlling stress response, apoptosis, and proliferation. The ligand-binding capacity of GST results in the negative regulation of signaling pathways through sequestration of

signaling kinases. The first example of GST-mediated kinase regulation is the characterization of GSTP as a c-NH_2 terminal jun kinase (JNK) inhibitor [53]. JNK has been implicated in pro-apoptotic signaling and may be required for the induced cytotoxicity of a variety of chemotherapy agents [54]. Phosphorylation of JNK activates c-jun resulting in subsequent activation of downstream effectors. In non-stressed cells, low JNK1 catalytic activity is orchestrated and maintained through its sequestration within the protein complex that includes at minimum GSTP, JNK and c-jun. However, under conditions of oxidative or chemical stress, a dissociation of the GSTP: JNK complex occurs releasing GSTP that subsequently undergoes oligomerization, and activation of released JNK allows for the subsequent induction of downstream events (Fig. 4) [55]. Other GST isozymes seem capable of mediating this interaction. For example, GSTA1 has been shown to suppress activation of JNK signaling caused by either inflammatory cytokines or oxidative stress [56]. The authors invoke a mechanism similar to that shown for the GSTP:JNK interaction with the underlying premise that within the family of GST isozymes, ligand binding may be promiscuous.

Recent research has shown that peroxiredoxin1 (Prx1) can directly associate with the GSTP/JNK complex. For example, Ralat et al. have shown that GSTP interacts directly with Prx1 through heterodimer formation that involves the Cys47 and Tyr7 of GSTP and that GSH must be bound to GSTP [57]. Reactivation of oxidized Prx1 occurs through S-glutathionylation of Cys 47 and subsequent formation of an inter-subunit disulfide. Finally GSH mediated reduction of the disulfide regenerates the reduced active site sulfhydryl of Prx1. Overexpression of Prx1 has been associated with cellular resistance to irradiation and a suppression of ionizing radiation induced JNK activation and apoptosis [58]. Mutating the Cys52 residue to Ser52 attenuated the peroxidase activity of Prx1and reduced the JNK activation; nevertheless, both proteins could be immunoprecipitated with the GSTP/JNK complex. These authors also concluded that Prx1 was able to suppress apoptosis through inhibition of JNK activation.

A further ligand binding function for GSTP has defined a regulatory role in tumor necrosis factor-alpha (TNF-alpha)-triggered signaling. Wu et al. [59] showed that GSTP physically associated with tumor necrosis factor receptor-associated factor 2 (TRAF2). Increased expression of GSTP inhibited TRAF2-induced activation of both JNK and p38 but not of NF-kB. GSTP also attenuated TRAF2-enhanced apoptosis signal-regulating kinase 1 (ASK1) autophosphorylation and inhibited

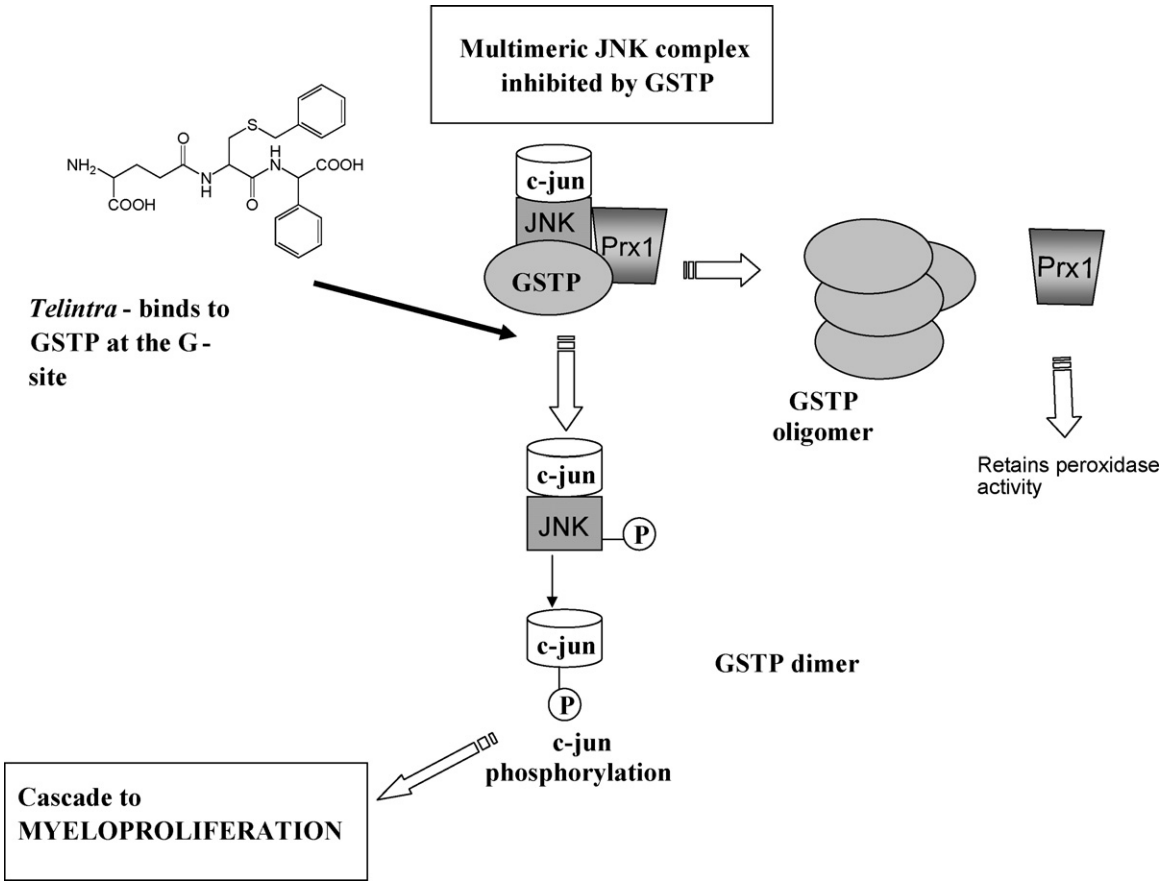

Fig. 4 – Representative cartoon model of multimeric protein complex involving GSTP, JNK, Prx1 and c-jun. While there are presently very few details of the determinants of the GSTP:JNK binding, the C-terminal of JNK is critical to the interaction. The apparent binding constant for a C-terminal fragment (residues 200-424) of JNK with GSTP is 217 nM [71]. In the example shown, the GSTP inhibitor Telintra binds to the protein causing disassociation of the constituents. GSTP can oligomerize and Prx1 released activating JNK. Downstream events can eventually lead to a myeloproliferative response in normal bone marrow. For more in depth discussion see text.

TRAF2-ASK1-induced apoptosis by suppressing the interaction of TRAF2 and ASK1. Conversely, reduced GSTP expression increased TNF-alpha-dependent TRAF2-ASK1 association followed by hyper-activation of ASK1 and JNK. GSTP lacking the TRAF domain-binding motif had a reduced capacity to bind TRAF2 and block TRAF2-ASK1 signaling compared with the wild type. These effects were independent of the catalytic activity of GSTP. Thus the ligand-binding regulatory role(s) of GSTP extend into TNF-alpha-induced signaling by forming interactions with TRAF2, adding further credence to the importance of GSTP to signaling events in tumor cells.

A further example of GST-mediated kinase regulation is evidence that GSTM1 binds to and inhibits the activity of ASK1 [60]. Redundancy of this activity is evidenced by the fact that thioredoxin can mediate the same suppression [61]. ASK1 is a MAP kinase kinase kinase that activates the JNK and p38 pathways leading to cytokine- and stress-induced apoptosis [62]. Under normal conditions, ASK1 exhibits low activity due to its sequestration via GSTM1. This protein:protein interaction forms a GSTM1:ASK1 complex which is dissociated under stressful conditions leading to the release and activation of ASK1 [63]. This mechanism is similar to the one proposed for GSTP:JNK. Under conditions such as oxidative stress or heat shock, GSTM1 oligomerizes allowing for the release of ASK1 and subsequent induction of apoptosis. Impaired clinical response to therapy in a variety of tumor types has been associated with an altered expression of GSTM1. Thus, any enzymatic influence GSTM1 plays in anticancer drug resistance is (similar to GSTP) further augmented by its role in kinase regulation.

7. Drugs that target GSTP

Epidemiological data show the existence of significant variations in individual patient response to cancer chemotherapy. Genetic polymorphisms could alter drug metabolism and the pharmacogenetic opportunities provided by GSTP represent a viable platform for rational drug design and serve to make GSTP an applicable target for anticancer therapy [64]. For example, in tumors where GSTP over-expression has an adverse effect on therapeutic response, distinct strategies have been adopted to target GSTs. One is the design of GSTP inhibitors to increase the efficacy of chemotherapeutics that would otherwise be detoxified by these proteins. This comes with the previously discussed caveat that the k_{cat} of GSTP for many anticancer drugs is not impressive. Nevertheless, in the early 1990s drug discovery efforts were initiated. Broadly, GST inhibitors can be classified as competitive or noncompetitive. At present, two of the best-characterized inhibitors are ethacrynic acid (EA) and the peptidomimetic glutathione analog, TLK199 [γ-glutamyl-S-(benzyl)cysteinyl-R-phenyl glycine diethyl ester]. EA acts as a non-competitive inhibitor of GSH for GST binding as well as depleting GSH by forming an EA-GSH conjugate [65]. The binding to both GST and GSH serves to inhibit enzyme activity. Earlier studies showed this to be effective in sensitizing tumors that otherwise exhibited chemotherapy resistance [66]. Clinical trials with ethacrynic acid were instigated, but dose limiting toxicities caused by the diuretic properties of the drug were restrictive [67]. TLK199 is a selective inhibitor of GSTP and can also act as a chemosensitizer, potentiating the toxicity of a number of anticancer agents in different tumor cell lines. Sensitivity to a range of anticancer drugs, including nitrogen mustards, was enhanced in mouse xenograft models with elevated GST levels [68]. Because of the structural similarities to GSH, TLK199 has also been shown to effectively inhibit the multidrug resistance-associated protein 1 (MRP-1) transporter, reversing the resistance of a variety of agents in NIH3T3 cells transfected with MRP-1 [69].

The early plans for pharmaceutical development of TLK199 did not follow the logic for its synthesis. A number of reasons contributed to this. Initially, chemosensitization through combination regimens fell out of favor as the decade advanced. Clinical trials for such combination approaches are difficult to design and implement. Fortunately, during the course of preclinical characterization, TLK199 was found to exhibit unanticipated myeloproliferative effects in mice [13]. Furthermore, *in vitro* hematopoiesis experiments showed that mice deficient in GSTP1 exhibit an increase in myeloid cell differentiation and proliferation, where the molecular basis for this effect is linked with an increase in bone marrow progenitor cells that populate circulating mature blood cells [70]. In preparing for clinical testing and FDA review, it is generally beneficial to have a mechanism to explain the pharmacological behavior of a new drug. As such, one cause for these myeloproliferative effects would be the ability of TLK199 to disrupt the protein:protein interaction of the JNK:GSTP complex [71]. As a consequence of this effect, JNK activity is enhanced and, in the case of the bone marrow compartment, this has been causally associated with the increase in cellular proliferation [70,72]. Company sponsored (Telik Inc., Palo Alto, CA) clinical trials of TLK199 (now named *Telintra*) in patients with myelodysplastic syndrome (MDS) have been instigated. An ongoing multi-center Phase II trial has produced interim results as of the last quarter of 2004. For 34 MDS patients, clinically significant improvement in one or more blood cell lineages was observed in 61.5% of patients from all major FAB subtypes [73]. Responses were associated with decreased requirements for red blood cell, platelet and growth factor support, in some cases leading to transfusion independence. While these results are preliminary in nature, they do provide encouragement for the translational relevance of targeting GSTP and for the mechanism of action data for Telintra (Fig. 4).

A new class of 7-nitro-2,1,3,-benzoxadiazole derivatives have been designed as suicide inhibitors of GSTs. These NBDs thioethers have micromolar to nanomolar affinities for binding to the H-site of GSTA1-1, GSTP1-1, and GSTM2-2 [74]. NDBs have been shown to induce tumor cell death by dissociating the JNK–GSTP1-1 complex thus initiating apoptosis [75]. When compared to the earlier example of Telintra in the bone marrow compartment, this serves to illustrate a different role for JNK in transformed cells. A representative model of these NBD derivatives is 6-(7-nitro-2,1,3,-benzoxadiazole-4-ylthio)hexanol or NBDHEX. The utility of this class of agents as possible anticancer drugs has yet to be established; however, mechanistically their impact upon kinase pathways does conform to literature predictions.

Another GST inhibitor is the GSH conjugate L-γ-glutamyl-(S-9-fluorenylmethyl)-L-cysteinyl-glycine [76]. This inhibitor possesses a fluorenylmethyl group as a cysteine S-derivatized moiety. It was predicted that the H-site of GSTA1-1 would favor the binding of this isoform with a greater affinity while the fluorescent properties would allow for monitoring during biological testing. L-γ-Glutamyl-(S-9-fluorenylmethyl)-L-cysteinyl-glycine was shown to be effective in inhibiting GSTA1-1, GSTP1-1 and GSTM2-2 with the greatest inhibition shown against the alpha isoform. Any practical utility as an anticancer drug has yet to be established.

Extending this drug platform, an alternative approach was adopted to take advantage of the high levels of GSTP expression in tumor cells. Prodrugs have been designed as inactive agents that are converted to active cytotoxics upon exposure to tumor tissues exhibiting high expression of activating enzymes. This targeting strategy is designed to minimize collateral toxicity towards normal tissues while enhancing delivery of active agent to the tumor tissue. This makes GSTP a promising target for prodrug therapy, with one approach exploiting the ability of GSTP to mediate cleavage of sulfonamides by promoting a β-elimination reaction. Extension of this concept led to the synthesis of drugs as inactive compounds via GSH conjugation through a sulfone linkage. 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 latent drugs activated in cancer cells by GSTP [77]. TLK286 is cleaved by a GSTP1*A-promoted β-elimination reaction into a nitrogen mustard and GSH analog that can then in principle alkylate cellular nucleophiles. GSTP expression was correlated to drug sensitivity both in cell culture and in animal models [78]. In addition, GSTP was adaptively down-regulated at both the protein and transcript level following selection of resistance through chronic exposure to TLK286 [79]. Collectively, these findings supported the concept that TKL286 selectively targets tumors expressing high levels of GSTP, thus preferentially exerting its cytotoxic effect on these tissues.

Approximately 8 years encompassed the design, synthesis and pre-testing of TLK286. As such, many of the preclinical studies were instrumental in leading to clinical trial design. Telik have also instigated clinical trials with TLK286, now called *Telcyta*. Completion of phase I studies [80] led to implementation of efficacy trials. To date, four positive clinical phase II studies in women with platinum refractory or resistant ovarian cancer have been reported, including two studies which demonstrated that TLK286 is active and well-tolerated as a single agent in platinum refractory or resistant ovarian cancer, and two studies which demonstrated synergy of TLK286 in combination with either carboplatin or liposomal doxorubicin in platinum refractory or resistant disease [81]. Two randomized phase III studies of TLK286 in platinum refractory or resistant ovarian cancer are currently underway. In the ASSIST-1 (ASsessment of Survival In Solid Tumors) trial, TLK286 is being evaluated as monotherapy in the third-line treatment setting. In the ASSIST-3 trial, the combination of TLK286 and carboplatin are being evaluated in the second-line treatment setting. This drug is close to NDA review by the FDA. The result of this process will have a significant impact upon proof of principle for the overall drug design approach.

A series of sulfonylhydrazine prodrugs have been synthesized that utilize the reductive environment of hypoxic tumors to generate cytotoxic species [82]. 1,2-Bis(methylsufonyl)-1-(2-chloroethyl)2-[[1-(4-nitrophenyl)ethoxy]carbonyl]hydrazine (KS119) and 1,2-bis(methylsufonyl)-1-(2-chloroethyl)2-[[1-(4-nitrobenzyloxyl)ethoxy]carbonyl]hydrazine (PNBC) are activated under hypoxic conditions through the reduction of their nitroaromatic groups. Upon reductive activation, these drugs release the cytotoxic alkylating species 1,2-bis(methylsulfonyl)-1-(2-chloroethyl)hydrazine. KS119 requires reduction of its nitro group to activate the release of the alkylating species, yet PNBC can also be activated through nucleophilic attack by thiols such as GSH and GST. *In vitro* studies demonstrated the ability of KS199 and PNBC to kill cells from solid tumors while maintaining minimal toxicity to normal cells under ordinary aerobic conditions. This enhances hypoxia-selective activity against tumor tissue while remaining relatively non-toxic to normal tissue.

Nitric oxide (NO) an endogenous, diffusible, *trans*-cellular messenger has been shown to impact regulatory and signaling pathways and cell survival. Exposure to NO can impart direct post-translational modifications on target proteins such as nitration and/or nitrosylation. Alternatively, after interaction with oxygen, superoxide, glutathione or certain metals, NO can lead to S-glutathionylation and influence signaling pathways (Fig. 1). A group of nitric oxide releasing prodrugs has been designed to target GST-associated drug resistance. Synthesis [83] of two NO-releasing diazeniumdiolates [O2-(2,4-dinitrophenyl)-1-[(4-ethoxycarbonyl)piperazin-1-yl]diazen-1-ium-1,2-diolate] (JS-K) and [O2-(2,4-dinitrophenyl)-1-[4-(*N*,*N*-diethylcarboxamido)piperazin-1-yl]diazen-1-ium-1,2-diolate] (CB-3-100), have been shown to effectively inhibit GSTP while increasing arsenic and *cis*-platin toxicity in previously resistant tumor cells. GSTP1 inhibition by JS-K and CB-3-100 also led to an enhanced activation of JNK and ERK, an observation in agreement with the role of GSTP in MAPK binding [84,85]. It was also shown that *n*-ethyl maleimide and certain NO donors caused an inhibition of GSTP activity [86] with the authors suggesting the involvement of specific cysteine residues subject to either nitrosylation or nitration.

While these prodrugs are potential candidates for cancer therapy, a different nitric oxide releasing drug O(2)-[2,4-dinitro-5-(*N*-methyl-*N*-4-carboxyphenylamino) phenyl] 1-*N*,*N*-dimethylamino)diazen-1-ium-1,2-diolate (PABA/NO; Fig. 1) has some advantageous pharmacological properties. GSTP efficiently metabolizes PABA/NO to release nitric oxide. Structure-based optimization has produced three analogues with improved solubility and stability in aqueous solution [87]. Nevertheless, PABA/NO maintains unique physicochemical properties and the aminobenzoic acid precursors differ structurally only in the presence or absence of the *N*-methyl group and/or the position of the carboxyl moiety (*meta* or *para*). X-ray crystallography data showed that the *N*-methyl-*para*-aminobenzoic acid substituent of PABA/NO, in contrast to the bonding pattern in its three analogues, was bound to the dinitrobenzene ring via its carboxyl oxygen (whereas the other three are linked through the aniline nitrogen). Rate constants for hydrolysis as well as for reactivity with GSH showed that PABA/NO was the most reactive, with values of $5 \times 10^{-4}\,s^{-1}$ and $0.30\,M^{-1}\,s^{-1}$,

respectively, at room temperature and pH 7.4. GSH reacted with the three PABA/NO analogues where the 5-substituents contained the diarylamine structural unit to give stable ArSG derivatives, while PABA/NOs ArSG adduct also reacted to cleave the ester grouping. Under these conditions, all four analogues produced nitric oxide (NO) by spontaneous hydrolysis of the nucleophilically displaced $Me_2NN(O) = NO^-$ ion [87].

By releasing NO, PABA/NO activates p38, JNK, and ERK [41]. Whether kinase activation is caused by direct interaction with NO or interaction with the drug and/or its metabolites is not yet clear; however, this ability to activate stress-related kinases is significant due to the observed function of GSTP as a negative regulatory switch for these pathways. Nitrosative stress resulting from low micromolar concentrations of PABA/NO caused undetectable nitrosylation, limited nitration and high levels of S-glutathionylation of a number of target proteins in tumor cells grown in culture [40]. S-glutathionylation occurred in <5 min and was sustained for ~7 h, implying a half-life for the deglutathionylation process (Fig. 2) of approximately 3 h. S-glutathionylated proteins included: β-lactate dehydrogenase, Rho GDP dissociation inhibitor β, ATP synthase, elongation factor 2, protein disulfide isomerase, nucleophosmin-1, chaperonin, actin, PTP1B and glucosidase II. Sustained S-glutathionylation was temporally concurrent with drug-induced activation of stress kinases linked to cell death pathways. This is consistent with the fact that PABA/NO induced S-glutathionylation and inactivation of PTP1B [22], a phosphatase that can, in principle, counteract the kinase effects.

MRP1 over-expressing cells were less sensitive to the drug and had reduced levels of S-glutathionylated proteins confirming that the cytotoxic activity of PABA/NO is dependent upon the presence of intracellular PABA/NO or its metabolites [41]. Since MRP1 effluxes glutathione conjugates of a wide variety of electrophiles, it is possible that GSNO is a substrate for MRP1.

Preclinical testing of PABA/NO as an anticancer drug is in progress. The *in vivo* efficacy of PABA/NO has been shown in tumor bearing animals [84,41], where significant growth delay in a human ovarian cancer model in SCID mice yielded results comparable to those seen with *cis*-platin (the standard of care for management of ovarian cancer). Continued preclinical development is required to test the possibilities that PABA/NO (or related analogues) may possess clinical activity. As such, while NO release is certainly a factor in drug efficacy, formulation and solubility issues may drive the development of the next generation of NONOates as possible cancer drugs [87,88].

One new drug that is undergoing both preclinical and clinical testing is NOV-002. The molecule is essentially the sodium salt of oxidized glutathione (GSSG; see Fig. 2) stabilized with *cis*-platin at a ratio of 1000:1. The GSSG is the active component of NOV-002 and administration of NOV-002 *in vivo* delivers a stabilized form of GSSG as reflected by sustained elevation of serum and tissue levels of GSSG to a degree that is not achieved by commercial GSSG. GSSG can act as a proximal donor in S-glutathionylation reactions (Fig. 2) Although generally believed to be impermeable to cells, exogenous GSSG can cause an alteration of intracellular GSH levels by shifting the equilibrium towards the formation of mixed disulfides [89]. Exogenous triggers could activate transmembrane proteins through S-glutathionylation of cysteine residues, and transduce the signal through a cascade of events. Addition of NOV-002 results in increased intracellular levels of GSSG, rapid S-glutathionylation of cellular proteins, and alterations in phosphorylation of ERK and p38 [90]. Similar induction of ERK and p38 phosphorylation is observed with GSSG indicating that the active component of NOV-002 is GSSG. More recently, NOV-002 has been shown to increase phosphorylation of EGFR [91].

In a phase I/II trial conducted in the US in 41 patients with stage IIIB/IV NSCLC, the addition of NOV-002 to standard carboplatin/paclitaxel chemotherapy significantly increased objective tumor response rates over that seen with chemotherapy alone (65% versus 45%) [92]. A randomized phase III trial in patients with newly diagnosed stage IIIB/IV NSCLC with carboplatin/paclitaxel ± NOV-002 is presently ongoing.

8. Conclusions

For anticancer drugs, the catalytic value of GSTP to form thioether conjugates is not substantial. Yet, high levels of GSTP expression are common in cancers and in drug resistant cell lines. Convergent evolution could favor the efficient use of GSTP and related redox proteins for multiple cellular functions. As such, GSTP has developed additional roles in the regulation of kinase activity and post-translational S-glutathionylation reactions. The biological importance of these reactions may have provided strong selection pressures and evolutionary efficiency. Drug discovery and development with GSH and GSTP as a platform has produced some interesting candidates that are in varying stages of preclinical and clinical development. Telcyta would seem to be the lead agent for clinical FDA approval; however, the pipeline for other possible drugs is in place and continued development of these agents will provide proof of principle for GSTP as a viable drug target.

Conflict of interest

KDT was a member of the scientific advisory board of Telik from 1991 to 2003; he is presently on the scientific advisory board of Novelos Inc. Waltham, Mass.

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