MECHANISM, STRUCTURE-ACTIVITY STUDIES, AND POTENTIAL APPLICATIONS OF GLUTATHIONE-S-TRANSFERASE-CATALYZED CLEAVAGE OF SULFONAMIDES

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ABSTRACT:
The mechanism of sulfonamide cleavage of PNU-109112, a potent HIV-1 protease inhibitor, by glutathione-S-transferase (GST) was investigated in the presence of reduced GSH. GST-catalyzed sulfonamide cleavage takes place via the nucleophilic attack of GSH on the pyridine moiety of the substrate with formation of the GS-para-CN-pyridinyl conjugate, the corresponding amine, and sulfur dioxide. Structure activity studies with a variety of sulfonamides indicate that an electrophilic center α to the sulfonyl group is required for cleavage. Substituents that withdraw electron density from the carbon atom α to the sulfonyl group facilitate nucleophilic attack by the GS thiolate bound to GST. The rate of sulfonamide cleavage is markedly affected by the nature of the electrophilic group; replacement of para-CN by para-CF3 on the pyridine ring of PNU-109112 confers stability against sulfonamide cleavage. On the other hand, stability of sulfonamides is less dependent on the nature of the amine moiety. These principles can be applied to the synthesis of sulfonamides, labile toward cellular GST, that may serve as prodrugs for release of bioactive amines. Tumors are particularly attractive targets for these sulfonamide prodrugs as GST expression is significantly up-regulated in many cancer cells. Another potential application could be in organic synthesis, where protection of amines as the corresponding activated sulfonamides can be reversed by GST/GSH under mild conditions.

In the companion paper (Koeplinger et al., 1999), glutathione-S-transferases (GST) are identified as the enzymes that, in the presence of the cosubstrate-reduced glutathione, mediate cleavage of the sulfonamide bond in PNU-109112, a potent inhibitor of the HIV-1 protease. The GST family comprises a group of isoenzymes (designated class α, μ, π, σ, and θ GST) ubiquitously present in living organisms. GSH and GST constitute the major intracellular detoxification system for both endogenous and exogenous peroxides, reactive electrophiles, and alkylating agents (Tew, 1994; Hayes and Pulford, 1995; Vermulen et al., 1996). Furthermore, the presence of GST-like domains in a variety of proteins suggests that they may also play other roles, such as in protein assembly and folding (Blocki et al., 1992; Koonin et al., 1994).

A significant obstacle contributing to the lack of effective chemotherapy for some cancers is the multidrug resistance (MDR) that develops during chemotherapy. Consistent with its detoxification function, GST is often overexpressed in cell lines selected in vitro for resistance to anticancer drugs and is thought to contribute significantly to many types of MDR. Heightened interest in GSH and GST has resulted from recent discoveries of the key role that intracellular GSH homeostasis plays in both apoptosis resistance (Kearns and Hall, 1998) and regulation of the 190-kDa MDR-associated glycoprotein (Loo et al., 1996). In cancer chemotherapy there is a need to develop more selective cytotoxic agents. Due to differential expression of enzymes in cancer versus normal cells, an active area of investigation has been the design of masked cytotoxic prodrugs that are activated preferentially in cancerous cells.

In this work we have investigated the molecular mechanism of GST-catalyzed sulfonamide cleavage and have conducted studies on a variety of sulfonamides to evaluate substituent effects on the rate of sulfonamide cleavage. We observe enhanced rates of intracellular sulfonamide cleavage in a human colon adenocarcinoma cell line, Caco-2, relative to cells or cell lines derived from noncancerous tissue. Potential applications of our findings to prodrg design and amino group protection/deprotection in chemical synthesis are discussed.

Experimental Procedures

Materials. PNU-109112, its metabolite PNU-143070, and other sulfonamides reported were prepared by the Medicinal Chemistry Research or Fine Chemical Research Preparation units of Pharmacia and Upjohn, Inc. (Kalamazoo, MI). Ethacrynic acid (EA), GSH, GSSG, 1-chloro-2,4-dinitrobenzene (chlorodinitrobenzene, CDNB), and paraarsonil hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). Mercuric chloride (Hg(II) chloride), formaldehyde (40%), and sodium sulfite were obtained from Merck. All other reagents were of analytical grade.

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2 Abbreviations used are: GST, glutathione-S-transferase; MDR, multidrug resistance; EA, ethacrynic acid; CDNB, chlorodinitrobenzene (1-chloro-2,4-dinitrobenzene); Hg(I) chloride, mercuric chloride; ESI, electrospray ionization; MDCK, Madin-Darby canine kidney.
[\textsuperscript{14}C]Mannitol was obtained from DuPont-New England Nuclear. Culture media and PBS buffer were obtained from Life Technologies, Inc. d-Glucose was obtained from Mallinckrodt and HEPES was obtained from Aldrich. Cluster well plates and Transwell filters were obtained from Costar Corporation (Cambridge, MA). Caco-2 cells were obtained from American Type Culture Collection and used at passage 36 after 16 days in culture. Madine-Darby canine kidney (MDCK) cells were obtained from American Type Culture Collection and used at passage 50 after 5 days in culture. Rat, dog, and human primary hepatocytes were isolated as described previously (Koeplinger et al., 1999).

**HPLC Assay of the GS Conjugate Derived from PNU-109112.** The GS-cyanopyridine conjugate formed as a metabolite coproduct of sulfonamide cleavage of PNU-109112 (Koeplinger et al., 1999) was very polar and not retained by any reversed-phase HPLC conditions attempted. Because of its net negative charge, the conjugate was retained on a weak anion exchange amine-silica column and resolved from GSH and GSSG using 0.1 M ammonium acetate, pH 6.6 as the mobile phase. A sample (100 \( \mu \text{L} \)) was injected into a Kromasil 100–5NH\textsubscript{2} column (25 mm x 0.46 cm; Eke Nobel AB- Bohus, Sweden) and eluted with 0.1 M ammonium acetate, pH 6.6 at a flow rate of 1.0 ml/min. The effluent was monitored at 260 nm.

**Preparation of N-benzyloxy carbonyl Bis Methyl Ester Derivative of the GS-Cyanopyridine Conjugate.** The unknown GS conjugate produced as a coproduct of the reaction of GSH and PNU-109112 was isolated as described above and 0.1 g was dissolved in 1 ml of 0.1 M sodium carbonate buffer, pH 9.0. Benzyl chloroformate (50 \( \mu \text{L} \)) was added and the two-phase suspension was stirred at room temperature under argon for 30 min. The pH was adjusted again to 9.0 with the addition of sodium hydroxide and an additional 50 \( \mu \text{L} \) of benzyl chloroformate was added. The reaction mixture was stirred for 30 min and excess benzyl chloroformate was removed by extraction three times with 1 ml portions of methylene chloride. Water was then removed by azetrope evaporation with methanol, yielding a solid.

Methanolic HCl (4 M) was prepared by the dropwise addition (on ice) of 2.8 ml of acetyl chloride (Alltech Instant Methanolic HCl GC Derivatization Reagent) to 5 ml of methanol. The methanolic HCl was then added to the flask containing the solid product formed from the benzyl chloroformate derivatization step. The solution was stirred for 15 min and then solvent and HCl were removed by rotary evaporation. The derivatized product was dissolved in methanol/water (50:50, v/v), purified by reversed-phase chromatography on a Zorbax SB phenyl column and analyzed by liquid chromatography/mass spectrometry [electrospray ionization (ESI)].

**Colorimetric Assay for Sulfite.** A colorimetric pararosaniline/formaldehyde assay described previously for measurement of sulfur dioxide and metabolic formation of sulfite by bacteria (Leinweber and Monty, 1987) was modified to measure sulfite formation in in vitro incubations of GST and PNU-109112. A volume of 93.6 ml of Milli-Q purified water, 1.6 ml of (0.3035 g pararosaniline HCl dye/10 ml of absolute ethanol), and 4.4 ml of sulfur dioxide color reagent was added to the two-phase suspension. After mixing, 0.4 ml of 40% aqueous formaldehyde was added. This solution was prepared fresh before use as the color darkened significantly upon standing.

Incubations of 100 \( \mu \text{M} \) PNU-109112 and other sulfonamides with rat liver GST (0.05–0.5 mg/ml) and 500 \( \mu \text{M} \) GSH were carried out at 37°C in 10 mM potassium phosphate buffer pH 7.4. Stock solutions (10 mM) of all drugs were prepared in methanol. Control incubations were carried out in the absence of GST or drug with corresponding solvent controls. At specified time points, 100–\( \mu \text{L} \) samples of the incubations were added to 40 \( \mu \text{L} \) of saturated aqueous HgI\textsubscript{2} chloride to precipitate GSH, which interfered strongly with the colorimetric assay. The samples were vortex-mixed and then centrifuged to sediment insoluble solids. Fifty microliters of each sample supernatant was then added (in duplicate) to separate wells on a 96-well microtiter plate.

Sodium sulfite standards of concentration 0, 10, 20, 50, 100, and 500 \( \mu \text{M} \) were prepared in 10 mM potassium phosphate buffer pH 7.4. Each sulfite standard was also prepared to contain 500 \( \mu \text{M} \) GSH to match the concentration of GSH contained in samples. One hundred microliters of each standard was added to 40 \( \mu \text{L} \) of saturated aqueous HgI\textsubscript{2} chloride. The standards were then vortex-mixed and insoluble solids were sedimented by centrifugation. Fifty microliters of each standard solution supernatant was then added to corresponding standard wells on the 96-well plate in duplicate.

Using a repeat-pipetor, 200 \( \mu \text{L} \) of sulfur dioxide color reagent was added to each well and the plate was allowed to stand at room temperature for 15 to 30 min to allow color formation. The plate was then read at 570 nm using a Molecular Devices (Menlo Park, CA) Thermomax computer-controlled microtiter plate reader with Softmax version 2.34 software operating under Microsoft Windows 3.1. Sulfite standard curves were linear (correlation coefficient = 1.00). Calibration curves were directly applied to unknowns and sample sulfite concentrations were reported in micromolar units.

**Caco-2 Cell Transport Studies.** The preparation of confluent Caco-2 cell monolayers on polycarbonate Transwell filters has been described in detail previously (Hilgers et al., 1990). Transport experiments were performed as described previously with minor modifications (Hilgers et al., 1990). PBS buffer supplemented with 15 mM HEPES and 0.1% glucose was used in place of Hanks’ balanced salt solution and for inhibition studies. PBS was also supplemented with 20 or 200 \( \mu \text{M} \) EA. Briefly, the monolayers were washed three times with the appropriate buffer and allowed to sit 15 min before adding a 25 \( \mu \text{M} \) donor solution of PNU-109112 in appropriate buffer to the apical compartment of the Transwell cup. At 15-min intervals the cup containing monolayer and donor solution was transferred to a fresh receiver compartment. Donor and receiver compartments were analyzed by HPLC and liquid scintillation counting and permeability coefficients were calculated as described previously (Conradi et al., 1991). Two monolayers were used at each concentration of EA. Transport of PNU-109112 across Caco-2 cell monolayers was studied in the presence of zero, 20, or 200 \( \mu \text{M} \) EA, a nonspecific inhibitor of GST. To detect potential damage to cells by EA, leakage of [\textsuperscript{14}C]mannitol across the monolayers was also monitored by liquid scintillation counting.

The concentrations of PNU-109112 and its metabolite were determined by HPLC on a Keystone Scientific C-18 Hypersil BDS column (150 x 4.7 mm) from Resolution Systems (Holland, MI). The mobile phase consisted of 75% methanol, 26.08% water, 0.01% trifluoroacetic acid, and 0.01% dimethylsulfoxide. Flow rate was 1.5 ml/min and the temperature was 30°C. Peaks were detected by absorption at 254 nm. Injection volume for receiver solutions was 400 \( \mu \text{L} \) and for donor solutions, 40 \( \mu \text{L} \). These solutions were acidified with acetic acid before injection.

**Results**

Two mechanisms are possible for GST-catalyzed sulfonamide cleavage of PNU-109112, the products of which include PNU-143070 (cf. Fig. 5 and Koeplinger et al., 1999) and a GSH conjugate exemplified by one or the other of structures A or B shown in Fig. 1. Reaction could involve initial GS\textsubscript{2} attack on either the sulfonyl S atom to give A or the adjacent \( \alpha \) carbon atom in the cyanopyridine ring to give structure B. The net result is either direct (a) or indirect (b) sulfonamide N-S bond cleavage.

The extremely polar and nonvolatile nature of the unknown GS\textsubscript{2} conjugate product precluded direct mass spectral characterization by either ESI, atmospheric pressure chemical ionization, or fast atom bombardment techniques due to its poor ionization characteristics. Poor ionization for underivatized GS\textsubscript{2} conjugates is commonly observed and can be overcome by formation of the benzyloxycarbonyl bis methyl ester derivatives (Pearson et al., 1990). Analysis of the mass spectrum (ESI) obtained from the benzyloxycarbonyl bis methyl ester derivative of the unknown GS\textsubscript{2} conjugate indicated a molecular ion \( m/z \text{ 572 (MH}^{+}) \) (Fig. 2A), consistent with the expected molecular mass of the postulated GS\textsubscript{2} conjugate of structure B (Fig. 1). Another ion representing the corresponding sodium adduct ion at \( m/z \text{ 594 (Na}^{+}) \) was also observed. Collision activation/fragmentation of ion \( m/z \text{ 572} \) resulted in the mass spectrum shown in Fig. 2B. Fragmentation of the peptide backbone to yield an ion of \( m/z \text{ 178} \) is consistent with the assigned structure.

A loss of 64 (sulfur dioxide) was inferred by identification of the PNU-109112 sulfonamide cleavage products as PNU-143070 and the GS-cyanopyridine conjugate. In aqueous media at pH 7.4, sulfur dioxide exists in hydrated form as a mixture of bisulfite (HSO\textsubscript{3}\textsuperscript{–}) and sulfite (SO\textsubscript{2}\textsuperscript{2–}). Because of this hydration, sulfur dioxide is very soluble in water and would not be expected to be volatilized out of..
incubation mixtures. The colorimetric pararosaniline/formaldehyde assay described in Experimental Procedures was used to confirm that sulfite was formed as a metabolic coproduct in in vitro incubations with GST and PNU-109112.

Identification and Kinetic Characterization of Sulfonamide Substrates. Mechanistic studies revealed that all GST-cleaved sulfonamides share sulfur dioxide/sulfite as a common reaction product. The variable structure of the amine and GSH conjugate coproducts of structurally diverse sulfonamides would require development of specific assays if either of these products were used to monitor reaction. The colorimetric sulfite assay described in Experimental Procedures was thus developed into a rapid general screening method for identification of sulfonamides cleavable by GSH and/or GSH/GST. Through screening, a variety of sulfonamides in addition to PNU-109112 were found to be substrates for GST. Structures and catalytic specificity ($k_{cat} / K_m$) for selected sulfonamides found to be substrates for GST are provided in Table 1. In Fig. 3 are shown the structures of selected sulfonamides that were found not to be cleavable by GST. As shown in Table 1, compounds containing the para-cyano- (PNU-109112, PNU-109092, I, PNU-144388, II, and PNU-107045) or paranitro- (PNU-108366) pyridinyl moiety were cleaved by GST. However, when the para-cyano or -nitro is replaced by para-CF3 (PNU-140690), -NH2 (109991), -NHOH (PNU-144378), -COOH (PNU-144310), or -H (PNU-107045 versus PNU-105241), cleavage of the sulfonamide bond was not observed (Fig. 3). If the ortho or para-CN substituent was present on a phenyl rather than a pyridinyl ring no cleavage occurred (Table 1: PNU-109092 versus Fig. 3: PNU-109115). Analogs with an unsubstituted phenyl group were not cleavable. However, phenyls bearing strong electron-withdrawing groups such as nitro (Table 1: PNU-20461, PNU-15189, and PNU-125098) or nitro in combination with -Cl (Table 1: PNU-11958), or -CF3 (Table 1: PNU-136710, PNU-125042, PNU-138600, and PNU-125046) were sufficiently activated toward enzymatic cleavage. Benzothiazole sulfonamides (e.g., Table 1: III and PNU-4191) were also good substrates for GST.

Stereoselectivity of GST-Mediated Sulfonamide Cleavage. PNU-109112 is a pure stereoisomer of absolute stereochemistry $3^R,6^R$ (structure shown in Table 1). The molecule has two asymmetric centers; four stereoisomers exist (enantiomers: PNU-109111, $3^S,6^R$ and PNU-109114 $3^S, 6^S$; and enantiomers: PNU-109112 $3^R, 6^R$ and PNU-109113, $3^S, 6^S$). GST-mediated sulfonamide cleavage of the stereoisomers exhibited a modest degree of stereoselectivity. Sulfonamide cleavage of PNU-109112 was more than twice as fast as PNU-109111, which was cleaved at the slowest rate. The relative rate of GST catalyzed cleavage of the different stereoisomers is shown in Table 2.

Cellular Metabolism of Sulfonamides. A facile rate of intracellular cleavage of PNU-109112 and several other activated sulfonamides was observed in Caco-2 but not MDCK cells during cell transport studies. The rapid rate of metabolism in Caco-2 cells was effectively inhibited by EA, a known GST inhibitor (Table 3, Fig. 4).
### TABLE 1
Sulfonamide substrates for GST-mediated cleavage

<table>
<thead>
<tr>
<th>Structure</th>
<th>$k_{cat}/K_m$ min·mM$^{-1}$</th>
<th>Structure</th>
<th>$k_{cat}/K_m$ min·mM$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>PNU-11958</td>
<td>1.67</td>
<td>PNU-15189</td>
<td>0.38</td>
</tr>
<tr>
<td>PNU-109112 (3R 6R)</td>
<td>1.54</td>
<td>PNU-12568</td>
<td>0.016</td>
</tr>
<tr>
<td>PNU-158600</td>
<td>1.30</td>
<td>PNU-20461</td>
<td>0.08</td>
</tr>
<tr>
<td>PNU-109092</td>
<td>1.29</td>
<td>PNU-108366</td>
<td>0.08</td>
</tr>
<tr>
<td>PNU-136710</td>
<td>0.94</td>
<td>PNU-144388</td>
<td>ND</td>
</tr>
<tr>
<td>PNU-125046</td>
<td>0.78</td>
<td>I</td>
<td>ND</td>
</tr>
<tr>
<td>PNU-125042</td>
<td>0.83</td>
<td>PNU-107045</td>
<td>ND</td>
</tr>
<tr>
<td>PNU-4191</td>
<td>0.41</td>
<td>PNU-35299</td>
<td>ND</td>
</tr>
<tr>
<td>III</td>
<td>0.40</td>
<td>II</td>
<td>ND</td>
</tr>
</tbody>
</table>

N.D. = sulfonamide was cleaved and served as a GST substrate. However, kinetic characterization was not carried out.
EA at 20 \( \mu M \) reduced metabolite formation by 70%, whereas 200 \( \mu M \) completely blocked cleavage of PNU-109112. When metabolism was blocked by EA, the apparent permeability of PNU-109112 increased over 2-fold, based on appearance of PNU-109112 in the receiver. The high rate of metabolism of PNU-109112 in Caco-2 cells was shown by the fact that 60% of the compound underwent cleavage during dynamic cell transport studies, even with inherently short cell contact times. In contrast, even after static 2-h incubations of 25 \( \mu M \) PNU-109112 with MDCK cells, no detectable level of metabolic cleavage occurred. In rat, dog, and human primary hepatocyte incubations, relatively lower rates of cleavage PNU-109112 and other sulfonamides were observed as compared to Caco-2 cells. These results underscore the potential selectivity for metabolic release of a bioactive component from a sulfonamide prodrug by cells overexpressing GST.

**Discussion**

**Sulfonamide Cleavage Mechanism.** PNU-109112, a sulfonamide inhibitor of the HIV-1 protease, is metabolically inactivated by GST (Koeplinger et al., 1999). Our studies of GST-mediated cleavage of PNU-109112 and a wide variety of other sulfonamides (selected examples shown in Table 1) reveal two striking structural features common to sulfonamide substrates. Groups capable of withdrawing sufficient electron density from the carbon atom \( \alpha \) to the sulfonyl group are an absolute requirement. Such groups positioned ortho and/or para on aromatic (e.g., para-nitro, para-nitro with ortho-trifluoromethyl) or heteroaromatic rings (e.g., para-cyanopyridyl, benzothiazoles) were shown to activate the corresponding sulfonamides. The electrophilic substructure of the sulfonyl group is solely responsible for activation of the sulfonamide bond toward cleavage. On the other hand, the amine portion has little or no impact on the cleavability of sulfonamide substrates but does influence the catalytic rate. Widely variable structures are apparently accommodated by the H-site of GST, as sulfonamide derivatives of simple primary or secondary amines, as well as much larger complex amines (Table 1) may serve as GST substrates. The latter feature is very significant because it suggests that complex drugs having free amino groups can be made as sulfonamides that are activated for cleavage by GST to release the bioactive amines at appropriate sites.
Findings presented herein suggest that GST-mediated sulfonamide cleavage occurs by the mechanism shown in Fig. 5. Binding of GSH at the G site of GST results in formation of the thiolate anion $\text{GS}^-$.

This nucleophile then attacks the carbon atom $\alpha$ to the sulfonyl moiety of the substrate, which is bound at the H site of GST. Sulfonamide cleavage results in the formation of the GS-conjugate, the corresponding amine, and sulfur dioxide. Release of sulfur dioxide and the amine is either concerted or results from subsequent hydrolysis of an initially formed sulfur dioxide-amine complex. Amines are known to form analogous complexes of sulfur dioxide under anhydrous conditions. Such complexes are unstable in water and are completely hydrolyzed to yield the corresponding amines and sulfite/bisulfite (Testa, 1995).

Studies on the nonenzymatic displacement of the sulfonyl group from different classes of aromatic compounds by GSH and cysteine (Conroy et al., 1984) led to the conclusion that 1,2,3-thiadiazole-2-sulfonamides underwent nucleophilic displacement of the sulfonyl moiety at C-2 of the thiazole ring by GSH. These investigators found that electron-withdrawing substituents in the 1,3,4-thiadiazole-2-sulfonamides serve to increase the electrophilicity of the C-2 carbon, which stabilizes the transition state and favors reaction with GSH. Likewise, we have found that certain activated sulfonyl groups (e.g., ortho-, para-dinitrophenyl analogs) are chemically unstable and are cleaved by rapid spontaneous reaction with GSH even in the absence of GST. However, sulfonamides incorporating many of the activating groups we have identified (e.g., Table 1: para-cyanopyridinyl, para-nitrophenyl, benzothiazole) are sufficiently stable such that the rate of chemical degradation with GSH is more than an order of magnitude slower than the enzymatic process. All of our kinetic data have been corrected for low background rates of spontaneous chemical cleavage.

**Cellular Metabolism of Sulfonamides.**

Caco-2 and MDCK cell monolayers are frequently used to model polarized epithelial cellular absorption and transport (Audus et al., 1990). Transport studies of PNU-109112 revealed a facile rate of cellular metabolism in Caco-2 but not MDCK cells. The Caco-2 cell line derives from a human colon adenocarcinoma whereas the MDCK is a cell line of nonmalignant origin. Other investigators have reported that GST is substantially elevated in Caco-2 cells (Prueksaritanont et al., 1996). Using CDNB, Caco-2 cell cytosol was shown to have conjugating activity of 0.82 ± 0.06 nmol/min/mg compared with 0.28 ± 0.13 for control human intestinal tissue (Prueksaritanont et al., 1996). Caco-2 cells express both $\mu$ and class $\alpha$ GST, the former appearing first and the latter predominating after a longer time in culture (Peters and Roelofs, 1989). Overexpression of GST has been suggested to correlate significantly with MDR in Caco-2 cells (Peters and Roelofs, 1992), CHO cells (Hall et al., 1989), and other cells derived from cancerous tissue resistant to cytotoxic drug therapies (Hayes and Pulford, 1995). Expression of functional GST in the Caco-2 cell line has been demonstrated for a variety of other electrophiles in addition to CDNB (Oude-Elferink et al., 1993).

**Potential Applications.**

GST overexpression is common in cancer and has been correlated with the development of MDR (Tew, 1994; Hayes and Pulford, 1995; Vermulen et al., 1996). Our observations of intracellular sulfonamide cleavage mediated by GST suggest that prodrugs activated by this enzyme, particularly in tumor cells, might provide a strategy for improved cancer chemotherapy. The concept is based, first, upon the ability to design sulfonamide prodrug derivatives maximizing their selective breakdown by intracellular GST. Second, selective targeting of these drugs to cancer tissue might be made possible by engineering in a chemical moiety that serves as a ligand for some cancer tissue-specific receptor. Because GST-mediated sulfonamide cleavage is relatively independent of the nature of the amine derivatized, any amine, in principle, can be derivatized by linkage with sulfonyl moieties, which confer a high or low degree of intracellular lability. Recently the use of unrelated GST-activated cytotoxins as antitumor therapeutics has been described (Kauvar, 1996). A latent cytotoxic prodrug was prepared by coupling cyclophosphamide to the sulfhydryl group of GSH via a sulfone linkage. The prodrug is cleaved by GST to release cyclophosphamide, the active toxin. Encouraging results in both cell and animal models of cancer have suggested that this cyclophosphamide prodrug exhibits improved cytotoxic selectivity toward cancer cells, most likely due to the high levels of GST in these cells. This approach exemplifies that described above, but lacking the second principle, namely, a structural element that would target the drug to tumor cells selectively.

Working on the two basic principles, high GST levels and unique cell surface receptors in tumor cells, we postulate a chimeric activated sulfonamide that might deliver a warhead to a tumor. For example, the folate receptor is up-regulated in many cancers (Campbell et al., 1991; Weitman et al., 1992; Anderson et al., 1992). Folate-mediated targeting of antineoplastic drugs, toxins, and nucleic acids to tumor cells has

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**Fig. 5.** Proposed mechanism of GST-catalyzed PNU-109112 sulfonamide cleavage.
been reported (Leamon et al., 1992; Low et al., 1997). Folic acid, coupled to a sulfonamide linker and doxorubicin (Fig. 6), a drug efficacious in a variety of malignancies (Barbieri et al., 1987), provides a potential vehicle for selective delivery to cancer cells and intracellular GST-mediated release of the cytotoxic agent. Other possibilities may be envisioned. For example, peptides selectively recognized by as yet unknown tumor cell surface receptors have been discovered recently using a novel plasmid library approach (Arap et al., 1998) and could serve as the targeting element. No doubt, a number of tumor-selective receptors, which could be potentially exploited in a similar fashion for selective drug delivery, remain to be discovered.

Currently the use of sulfonamides as protecting groups for amines in organic synthesis is limited by the often harsh conditions required for deprotection. A recent report (Fukuyama et al., 1995) suggests general synthetic utility in the protection of secondary amines as 2- and 4-nitrobenzene sulfonamides. The latter sulfonamides were found to be sufficiently activated as to be cleaved chemically by thiols (e.g., propane thiol) in the presence of strong base. Because of the similar pKa of the thiol S-H and the sulfonamide N-H, the chemical deprotection step apparently is useful only for secondary amines, which do not have an acidic proton in the protected sulfonamide derivative, and is not applicable to primary amines. However, GST catalysis affects the desired deprotection for either primary or secondary amines by selectively lowering the pKa of GSH at neutral pH at which the sulfonamide N-H (derived from primary amines) remains protonated and thus the sulfonamide is cleavable. Enzymatic rather than base catalyzed cleavage of the sulfonamide bond releases either primary (Table 1: PNU-15189, PNU-144388) or secondary amines (Table 1: I, PNU-109092) under mild experimental conditions. GST is broadly specific, suggesting general applicability. The stereoselectivity of GST mediated cleavage, although modest (e.g., Table 2), may be useful in the kinetic resolution of stereoisomers during the deprotection step.

In conclusion, we have shown that activated sulfonamides are cleaved by GST in the presence of GSH both in vitro and in vivo. Unlike hydrolytic cleavage of carboxamides by amidases, GST-mediated sulfonamide cleavage does not involve direct hydrolysis of the amide S-N bond but occurs via nucleophilic attack by the enzyme-bound base G:\S on the alpha to the sulfonyl. This results in formation of the GS-conjugate, the corresponding amine, and sulfur dioxide.

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