Glutathione S-Transferase Catalyzed Desulfonylation of a Sulfonylfuropyridine

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Glutathione conjugation of xenobiotics is a detoxification pathway that inactivates reactive electrophiles, which may alternatively co-valently bind to endogenous proteins and elicit potential detrimental effects (Evans and Baillie, 2005; Zhou et al., 2005). After glutathione conjugation, the molecule may be directly eliminated via biliary excretion, or the conjugate may proceed through the so-called “mercapturic acid pathway,” where the glycine and glutamic acid residues are eliminated, and the remaining cysteine residue is further conjugated to endogenous proteins and elicit potential detrimental effects. In vitro experiments using rat and monkey liver microsome preparations showed that the formation of M1 was independent of NADPH and molecular oxygen, suggesting that this reaction was not mediated by an oxidative reaction and a glutathione S-transferase (GST) was likely involved in catalyzing this reaction. Furthermore, a rat hepatic GST was capable of catalyzing the conversion of MRL-1 to M1 in the presence of GSH. When a close analog of MRL-1, a p-chlorobenzenesulfonyl furopyridine derivative (MRL-2), was incubated with rat liver microsomes in the presence of GSH, p-chlorobenzenesulfonic acid (M2) was also identified as a product in addition to the expected M1. Based on these data, a mechanism is proposed involving direct nucleophilic addition of GSH to sulfonylfuropyridine, resulting in an unstable adduct that spontaneously decomposes to form M1 and M2.

Materials and Methods

Materials. MRL compounds 1 through 6 (Fig. 1) were synthesized by the Medicinal Chemistry Group, and [3H]MRL-1 was prepared by the Labeled Compound Synthesis Group, both of MRL (Rahway, NJ). The specific activity of [3H] tracer was ~50 Ci/nmol, and the radiochemical purity, as determined by high-performance liquid chromatography (HPLC), was 99.5%. Acetonitrile and methanol (HPLC grade) were purchased from Thermo Fisher Scientific (Waltham, MA). NADPH, GSH, and GST (from rat liver) were obtained from Sigma-Aldrich (St. Louis, MO). Rat, dog, monkey, and human liver microsomes were prepared as outlined in the literature (Lu and Levin, 1972). The

ABBREVIATIONS: GST, glutathione S-transferase; GSH, reduced glutathione; HPLC, high-performance liquid chromatography; LC/MS/MS, liquid chromatography/tandem mass spectrometry; CID, collision-induced dissociation; LC/MS, liquid chromatography/mass spectrometry.
amount of cytochrome P450 enzyme present in rat, dog, and human liver microsomal protein was 0.21, 0.43, and 0.60 nmol/mg, respectively. The amount of cytochrome P450 enzyme present in monkey liver microsomes was not estimated.

Animal Experiments. Experiments were performed according to procedures approved by the Merck Institutional Animal Care and Use Committee. [3H]MRL-1 (2 mg/ml) was formulated in Imwitor 742/Tween 80 (1:1; w/w) and dosed orally to bile duct-cannulated male Sprague-Dawley rats (n = 3) at 2 mg/kg. Bile, urine, and fecal samples were collected at room temperature for up to 72 h postdose and stored at -80°C until analysis.

Analysis of Rat Bile and Isolation of M1. The pH of an aliquot of rat bile (0–72 h) was adjusted to 6 with 50% acetic acid, and an equal volume of acetonitrile was added. The mixture was vortex-mixed, centrifuged at 14,000g for 10 min, and the supernatant was used for metabolite analysis by liquid chromatography/tandem mass spectrometry (LC/MS/MS) with radiometric detection. For isolation of the GSH conjugate (M1), 10 ml of rat bile (8–24 h) was concentrated to approximately 1 ml under a stream of nitrogen; an equal volume of acetonitrile was added, and the mixture was centrifuged as described above. The M1 metabolite was isolated from the supernatant by semipreparative HPLC.

Incubation of [3H]MRL-1 and Its Analogs with Liver Microsomes or Cytosol. The incubation mixtures contained [3H]MRL-1 or its analogs MRL-2, MRL-3, MRL-4, MRL-5, or MRL-6 (10 μM); liver microsomal or cytosolic protein from rat, dog, monkey, or human (1 mg/ml); MgCl₂ (0.1 mM) in 500 μl of phosphate buffer (100 mM, pH 6.5); and NADPH and/or glutathione (1 mM) and were incubated for 60 min at 37°C in a shaking water bath. Control incubations were run in parallel without NADPH and GSH. Additional control experiments also were conducted in the absence of microsomes and with heat-inactivated microsomes. For anaerobic experiments, reaction mixtures were evacuated and purged with argon, separately, before addition to the incubation mixture. The reactions were quenched with an equal volume of acetonitrile and analyzed by LC/MS/MS. In a separate experiment, to study the possibility of (nonsynzymatic) base-catalyzed formation of M1, [3H]MRL-1 (10 μM) was incubated with 0.1 N NaOH in the presence of GSH (1 mM) at 37°C for 60 min and processed as described above.

Incubation of [3H]MRL-1 with GST. [3H]MRL-1 (2 μM) was incubated with a crude preparation of rat hepatic GSTs (100 U) in 200 μl of phosphate buffer at pH 6.5 and at pH 8.5 containing 0.1 mM MgCl₂, either with or without 1 mM glutathione or cysteine. The reaction mixtures were incubated for 60 min at 37°C in a shaking water bath. At the end of the incubation, they were analyzed by LC/MS/MS as described above.

Instrumentation. Conjugates of glutathione were identified by electrospray LC/MS/MS analysis using a Finnegan LTQ mass spectrometer, which was interfaced with a Shimadzu (Kyoto, Japan) HPLC system equipped with two Series LC-10ADVP micropumps and a Series SIL-10ADVP autosampler. The spray voltage was maintained at 4.1 kV, and the capillary temperature was set at 250°C. Full-scan spectra, from m/z 200 to 800, were obtained in the positive ion mode, and product ion spectra were generated by collision-induced dissociation (CID) of MH⁺ ions of interest. The CID of MH⁺ was achieved with helium as the collision gas at the collision energy of 25 eV. Separation of bile metabolites was achieved on a 5-μm ZORBAX SB-C3 column (4.6 × 250 mm; Agilent Technologies, Santa Clara, CA) at a flow rate of 1 ml/min. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile (B). The column was eluted with a linear gradient from 30% to 90% B over 40 min. One fourth of the column eluate was directed into the mass spectrometer and the remaining into a β-RAM radiometric detector (IN/US Systems, Brandon, FL) for online radioprofiling. These HPLC conditions also were used for the purification of the glutathione conjugate.

For identification of p-chlorobenzenesulfonic acid, the mass spectrometer was operated in the negative ion mode from m/z 120 to 700, with a spray voltage of 4.0 kV, capillary temperature of 350°C, and the collision energy of 30 eV. The chromatography was performed on a 5-μm ZORBAX RX-C8 column (Agilent Technologies) and eluted at a linear rate of 1 ml/min. The mobile phase consisted of water containing 0.1% formic acid (A) and acetonitrile/methanol (1:1; B). The column was eluted at a linear gradient from 10% to 60% B over 10 min.

High-resolution mass spectrometry data were acquired using an LTQ Orbitrap (Thermo Fisher Scientific) operated in positive electrospray ionization mode. Spray voltage was set at 5.0 kV, with a capillary temperature of 300°C. Sheath and auxiliary gas flow rates were set at 37 and 0, respectively. Capillary and tube lens voltages were set at 5 and 90, respectively. Resolution was set at 60,000 for full MS (m/z 300–800) and at 15,000 for MS² and MS³ scan events. Differences between the measured mass and the calculated mass are expressed in parts per million.
[1H]NMR Analysis. NMR spectra of MRL-1 and the purified metabolite were obtained in methanol-d₄ at room temperature using a Varian Inova 600-MHz NMR spectrometer (Palo Alto, CA) equipped with a 3-mm probe. The chemical shifts are expressed as parts per million relative to tetramethylsilane.

Results

Identification of GSH Conjugate of MRL-1 (M1) in Rat Bile. A representative HPLC/radiochromatogram of the bile from rats dosed orally with [3H]MRL-1 is depicted in Fig. 2. A major polar peak eluting at ~13 min constituted approximately ~40% of the radioactivity excreted in the bile. Liquid chromatography/mass spectrometry (LC/MS) analysis of this metabolite indicated the presence of an MH⁺ ion at m/z 645, which is 185 Da higher than that of parent compound (MRL-1, MH⁺ 460 Da). The CID spectrum of the MH⁺ ion m/z 645 yielded fragment ions m/z 516 corresponding to the loss of 129 Da, m/z 570 corresponding to the loss of 75 Da, and m/z 627 corresponding to the loss of 18 Da (Fig. 3). These neutral losses are characteristic of glutathione conjugates: 129 Da resulting from pyroglutamate and 75 Da resulting from glycine (Baillie and Davis, 1993). The mass of the metabolite corresponded well with a structure in which the t-butylsulfonyl group had been replaced by a glutathionyl group. Furthermore, high-resolution mass spectrometry analysis of the metabolite showed MH⁺ ion at m/z 645.0980, which matched well with a molecular formula of C₂₉H₂₅O₅N₄Cl₂S (ΔM 1.3 ppm). Based on the above data, a tentative structure for the metabolite (M1) was assigned as shown in Figs. 1 and 3.

The structure of this conjugate (M1) was confirmed by ¹H NMR analysis (Table 1). The ¹H NMR spectrum of M1 exhibited the signals characteristic of GSH and lacked the signals resulting from the t-butylsulfonyl group (Fig. 4). The regiochemistry of the glutathionyl group was deduced from the one-dimensional ¹H-¹H
nuclear Overhauser effect spectrum (Fig. 5), wherein the lone pyridine proton (8.03 ppm, H-\textit{m}) was selectively inverted and an enhancement of the lone furan proton (7.09 ppm, H-\textit{p}) was observed. Thus, these data suggest the glutathionyl group is located on the furan carbon that was previously occupied by the \textit{t}-butylsulfonyl group at C2.

In Vitro Metabolism of \([3\text{H}]\text{MRL-1}\) in NADPH/GSH-Fortified Liver Microsomes or Cytosol. Incubation of \([3\text{H}]\text{MRL-1}\) with liver microsomes from rats, dogs, monkeys, and humans in the presence of NADPH yielded small amounts of oxidative metabolites (addition of 16 Da; data not shown). When \([3\text{H}]\text{MRL-1}\) was incubated with liver microsomes from these species in the presence of GSH, formation of a new peak was observed in rat and monkey liver microsomal incubations. A representative radiochromatogram depicting metabolite of \([3\text{H}]\text{MRL-1}\) formed on incubation with rat liver microsomes is shown in Fig. 6. The metabolite was confirmed as M1 (see above) based on the LC/MS analysis (MH\textsuperscript{+} at \textit{m}/\textit{z} 1100) and CID spectra of which corresponded well with those of M1 formed from MRL-1. The LC/MS analysis of the reaction mixture under different analytical conditions (Fig. 7B; see under Materials and Methods) indicated the presence of MH\textsuperscript{+} ion at \textit{m}/\textit{z} 175, the CID of which yielded a fragment ion at \textit{m}/\textit{z} 111 resulting from the loss of 64 Da attributed to SO\textsubscript{2} (Fig. 7C). Based on these data and by comparison with LC/MS data of an authentic standard, the metabolite M2 was identified as \textit{p}-chlorobenzensulfinic acid.

Metabolism of \([3\text{H}]\text{MRL-2}\) Analogs in GSH-Fortified Rat Liver Microsomes. Figure 7, A and B, depicts extracted ion chromatograms of metabolites formed from MRL-2 on incubation with rat liver microsomes and GSH. The mass and CID spectra of the peak at \textasciitilde 25 min corresponded well with those of M1 formed from MRL-1. The LC/MS analysis of the reaction mixture under different analytical conditions (Fig. 7B; see under Materials and Methods) indicated the presence of MH\textsuperscript{+} ion at \textit{m}/\textit{z} 175, the CID of which yielded a fragment ion at \textit{m}/\textit{z} 111 resulting from the loss of 64 Da attributed to SO\textsubscript{2} (Fig. 7C). Based on these data and by comparison with LC/MS data of an authentic standard, the metabolite M2 was identified as \textit{p}-chlorobenzensulfinic acid.

Discussion
This study describes a novel desulfonylation of a sulfonyl furopyridine that is catalyzed by a GST. The identification of the resulting GSH conjugate and a mechanism of its formation are also discussed. GST-catalyzed displacement of several hetero groups and activated sulfonamides by GSH have been reported (Livesey et al., 1982; Conroy et al., 1984; Dekant et al., 1988; Zhao et al., 1999; Parkinson, 2001). Common characteristics of these displacement reactions are
that they occur with good leaving groups and on ring systems activated by electron-withdrawing groups. In the case of sulfonamides, a series of \(N\)-substituted aryl sulfonamide derivatives were evaluated for their ability to undergo GST-dependent sulfonamide cleavage (Zhao et al., 1999). The sulfonamide cleavage resulted in the formation of the corresponding aryl-glutathionyl (SG) conjugate, an amine and sulfur dioxide (Koeplinger et al., 1999; Zhao et al., 1999). Based on the metabolites identified, a mechanism was proposed for the cleavage of sulfonamide that involves direct attack of the thiolate anion of GSH on the aryl carbon attached to the sulfonamide, with the subsequent C-S cleavage proceeding via a concerted or a nonconcerted fashion (Zhao et al., 1999). Structure activity relationship of nonenzymatic cleavage of sulfonamides, attached to a series of heterocycles, has also been evaluated (Conroy et al., 1984). The results from these studies showed that sulfonamides with \(pK_a\) values greater than 9 were not cleaved by GSH or L-cysteine.

The structural features of the M1, detected in rat bile after MRL-1 dosing or formed in vitro after incubation with microsomes and GSH, were evident from its NMR and LC/MS analyses, which indicated the absence of the \(t\)-butyl group and the presence of the glutathionyl group. The data obtained by NMR spectroscopy also suggested that the glutathionyl group in M1 was present on the same carbon (C2) of the furan ring on which the \(t\)-butylsulfonyl group was present in MRL-1. The latter finding was rather unexpected because it was reasonable to hypothesize that nucleophilic attack of the thiolate anion would have been blocked at C2 as a result of steric hindrance by the \(t\)-butylsulfonyl group. This hypothesis was further fueled by observations with MRL-6, which differed from MRL-1 by only an additional methyl group at the C3 position and did not form the analogous glutathionyl conjugate; initially, this was presumed to be because of the blockade of the C3 position to nucleophilic attack by the thiolate anion.

To study the mechanism of this reaction further, the formation of M1 was investigated in rat, dog, monkey, and human liver microsomes in vitro. The data showed that the M1 metabolite was formed only in rat and monkey liver microsomes but not in dog and human liver microsomes. In addition, formation of M1 was independent of NADPH and molecular oxygen, and GSH could not be replaced by...
L-cysteine as a cosubstrate (a cysteine conjugate similar to M1 was not formed; data not shown). These data and the experiments conducted with partially purified GST (from rat liver) confirmed that the formation of M1 is catalyzed by one or more GST enzymes. There are three distinct families of glutathione transferases that are localized in mitochondria, cytosol, and/or microsomes with varying substrate specificity (DeJong et al., 1988; Pickett and Lu, 1989; Jakobsson et al., 1999; Hayes et al., 2005). In the present study, the glutathione conjugation of MRL-1 appeared to be catalyzed by a microsomal GST, as formation of M1 was not observed in liver cytosolic fractions obtained from rat, dog, monkey, and human (data not shown).

In many instances, the nucleophilic attack on electrophilic centers by GS proceeds at a slow rate even in the absence of GST. In the present investigation, M1 was not detectable in incubations of MRL-1 with GSH and heat-inactivated rat liver microsomes. However, M1 was detectable in trace amounts when MRL-1 was incubated with GSH and sodium hydroxide, suggesting that C-S bond in MRL-1 is strong and harsh reaction conditions are required to break the bond.

Based on the above data, a mechanism was proposed for the formation of M1 that involves direct attack of the enzymatically stabilized (by GST) thiolate anion on the electron-deficient C2, as shown in Fig. 9. The resulting adduct spontaneously loses t-butylsulfenic acid to produce M1. Because of its small mass, it was difficult to directly identify t-butylsulfenic acid by LC/MS analysis in support of this proposed mechanism. Therefore, MRL-2, a p-chlorobenzene-sulfonic acid analog of MRL-1 (Fig. 1), was used to study the nature of coproduct formed in this reaction. In GSH-supplemented rat and monkey liver microsomal incubations, MRL-2 underwent metabolism similar to MRL-1 to produce M1. More importantly, p-chlorobenzensulfenic acid (M2) was identified as a coproduct in these incubations. Thus, formation of M2 from MRL-2 supports the proposed mechanism as shown in Fig. 9. This mechanism of nucleophilic displacement of the sulfonyl group by a thiolate anion, which occurs like an S$_{2}$,2 addition-elimination process, is similar to that proposed for the nonenzymatic displacement of sulfonamide by GSH or L-cysteine in 1,3,4-thiadiazole-2-sulfonamides (Conroy et al., 1984). However, as mentioned above, the formation of M1 from MRL-1 (or M2 from MRL-2) does not proceed in the absence of GST enzyme in the present study.

It is interesting to note that MRL-4 and MRL-5 were not substrates for GST and did not produce M1; both are analogs of MRL-1 differing only in the moiety at the C2 position on the furan ring. This can be explained by the fact that the carbonyl moieties at C2 would make poor leaving groups. However, of interest was the fact that MRL-6 (3-methylated analog of MRL-1) did not produce the corresponding GS conjugate. This result stands in stark contrast to the nonenzymatic GSH additions to related benzofuran systems substituted with 2-sulfonylamides (Graham et al., 1990). In these benzofuran systems, 3-methylated analogs were appreciably more reactive to GSH than the desmethyl compounds. It is unclear why in our investigations with this furopyridine chemical series MRL-6 is not reactive toward GSH conjugation. One could speculate that the C3 methyl group blocks a key enzymatic transition state or destabilizes the C3 carbanion as a result of its positive inductive effect, thus making the nucleophilic attack of thiolate anion at C2 less favorable (Fig. 9). It might be intriguing to investigate whether electron-withdrawing groups at C3 will allow desulfonylation in related structures. The electrophilicity of sulfonamides, attached to various heterocycles, has been assessed by...
measuring their rate of reaction with GSH (Graham et al., 1989, 1990; Woltersdorf et al., 1989; Hartman et al., 1992). Sulfonamides attached to benzothiadiazole were more reactive with GSH compared with those attached to benzofuran, benzoxazole, or indole. Contrary to these sulfonamides, benzo[1,2-c:4,5-c]dithiophene sulfonamides were practically inert toward glutathione. The GSH reactivity of these sulfonamides was found to be well correlated with dermal sensitization reaction in guinea pigs. On the other hand, benzo[1,2-c:4,5-c]dithiophene sulfonamides, which were inert toward glutathione, were devoid of dermal sensitization potential assessed in guinea pigs. In the present study, no dermal sensitization was observed in rodents after administration of MRL-1 or related compounds. Clinical significance of glutathione conjugation with MRL-1 is not known. As discussed above, only rat and monkey liver microsomes were active in GST-mediated conjugation reaction described in this study. It is possible that there is a species difference in GST activities, and MRL-1 may be cleared in humans via non-GST-mediated metabolism routes.

In conclusion, we have identified a novel GST-catalyzed desulfonation of a 2-sulfon furylpyridine series that appears to proceed via addition-elimination reaction. Initial nucleophilic attack of glutathione sulfeny1 sulfur on the C-2 position of the sulfon furylpyridine produces an unstable adduct that leads to the formation of rearromatized 2-glutathionylpyridine conjugate with the elimination of corresponding sulfenic acid derivative. 

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References
Dekant W, Vamvakas S, Henschler D, and Anders MW (1988) Enzymatic conjugation of hexachloro-1,3-butadiene with glutathione. Formation of 1-(glutathionyl-S-yl)-1,2,3,4,4-pentachlorobuta-1,3-diene and 1,4-bis(glutathionyl-S-yl)-1,2,3,4-tetrachlorobuta-1,3-diene. Drug Metab Dispos 16:701–706.

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