Role of Glutathione *S*-Transferases A1-1, M1-1, and P1-1 in the Detoxification of 2-Phenylpropenal, a Reactive Felbamate Metabolite

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Felbamate has proven to be an effective therapy for treating refractory epilepsy. However, felbamate therapy has been limited due to the associated reports of hepatotoxicity and aplastic anemia. Previous research from our laboratory has proposed 2-phenylpropenal as the reactive metabolite in felbamate bioactivation and identified its mercapturates in the urine of rats and patients undergoing felbamate therapy. While the reaction between 2-phenylpropenal and GSH has been shown to occur spontaneously under physiological conditions, the potential catalysis by glutathione transferases (GST) has remained unknown. The work presented here demonstrates a role for GST in the detoxification of 2-phenylpropenal. The kinetic data show that 2-phenylpropenal is a substrate for all three isoforms tested, with a k_{cat}/K_m of 0.275 ± 0.035 μM^{-1} s^{-1} for GSTM1-1, 0.164 ± 0.005 μM^{-1} s^{-1} for GSTP1-1, and 0.042 ± 0.005 μM^{-1} s^{-1} for GSTA1-1. Given that electrophilic substrates such as 2-propenal have been shown to inhibit GSTs, we also examined the inhibition of GSTM1-1, GSTP1-1 and GSTA1-1 by 2-phenylpropenal. The enzyme inhibition studies demonstrate that 2-phenylpropenal inhibits GSTP1-1 and GSTM1-1. The inhibition of GSTP1-1 was completely reversible upon filtration and reconstitution in buffer containing 10 mM GSH. However, 2-phenylpropenal inhibition of GSTM1-1 was irreversible under the same conditions. The irreversible inhibition of GSTM1-1 may be important in understanding the toxicities associated with felbamate. Given that 2-phenylpropenal is both a substrate and irreversible inhibitor for GSTM1-1, GSTM1-1 represents a potential target for 2-phenylpropenal haptenization in vivo, which may in turn mediate the observed idiosyncratic reactions.

Introduction

Previous research has demonstrated the bioactivation of the antiepileptic drug felbamate to the highly reactive α,β-unsaturated aldehyde, 2-phenylpropenal (*1*), which is thought to mediate felbamate-associated toxicities, namely, hepatotoxicity and aplastic anemia. The detoxification of 2-phenylpropenal by GSH has been shown to occur in vivo by identification of the corresponding mercapturates in the urine of both rats and patients being treated with felbamate (*1*). In addition, the conjugation of 2-phenylpropenal with GSH was shown to occur spontaneously under physiological conditions with an extremely short half-life (*2*). However, the potential role for glutathione transferases in mediating this reaction has remained unexplored.

GSTs[1] are a family of enzymes (GST-A, GST-M, GST-P, and GST-T) characterized by their ability to catalyze the reaction between GSH and hydrophobic electrophiles. The active site of GSH has two binding sites, the G-site, which binds GSH, and the H-site, which binds hydrophobic electrophilic species (*3*). The binding of GSH in the G-site lowers the pK_a of the cysteine sulfhydryl from 9 to 6.5 such that bound GSH is thought to exist as the thiolate anion under physiological conditions (*3*). GSTs are expressed ubiquitously with isoform specific tissue localization: GSTA1-1 in the liver, kidney, intestine, and lung, GSTM1-1 in liver, muscle, testis, and brain, and GSTP1-1 in kidney and neoplastic tissue with notable absence in the liver (*3*). Regulation of enzyme expression has been correlated with numerous response factors and shown to be able to be induced by polycyclic aromatic hydrocarbons, phenolic antioxidants, Michael reaction acceptors, reactive oxygen species, organic isothiocyanates, barbiturates, and synthetic glucocorticoids (*3*).

Several α,β-unsaturated aldehydes, including the reactive cylcophosphamide metabolite 2-propenal (acrolein), nucleotide base propenals, and 4-hydroxyalkenals, have been characterized as substrates for GSTs (*4*) and led us to consider the possible role of GSTs in the detoxification of 2-phenylpropenal. Given that the spontaneous reaction between GSH and 2-phenylpropenal occurs rapidly under normal physiological concentrations of GSH, the roles of GSTs may become important in GSH-depleted individuals or in understanding the metabolic dispositions in polymorphic patients. In addition, irreversible inhibition of GST by α,β-unsaturated aldehydes has also been detected (*5*, *6*) and may occur in the case of 2-phenylpro-

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[1] Abbreviations: GST, glutathione *S*-transferase; CDNB, 1-chloro-2,4-dinitrobenzene.

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penal. Demonstrating the irreversible inhibition of GST by 2-phenylpropenal under GSH-depleted conditions would identify a candidate protein for in vivo alkylation. Identification of the hapten that mediates felbamate-associated toxicity remains an important goal in the mechanistic understanding of felbamate idiosyncratic reactions.

Experimental Procedures

Chemicals and Instruments. The chemicals were purchased from either Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI) and were of the highest grade available. The enzymes were purchased from Panvera (Madison, WI). Panvera reported the GST specific activities for the reaction of CDNB (1-chloro-2,4-dinitrobenzene) with 1 mM reduced GSH in 100 mM potassium phosphate buffer (pH 6.5) at room temperature as follows: 95 μmol min^{-1} mg^{-1} for GSTP1-1, 207 μmol min^{-1} mg^{-1} for GSTM1-1, and 57 μmol min^{-1} mg^{-1} for GSTA1-1. The HPLC analysis was conducted on a Waters 2690 Separations Module using a Waters Symmetry C_8 (2.1 mm × 150 mm) column. The separation was achieved isocratically with a 40% MeOH/60% (0.1%) formic acid mixture and a 200 μL/min flow rate. The flow was directed into a Waters 486 tunable absorbance detector equipped with a microbore flow cell and set at $\lambda = 214$ nm. The HPLC/UV quantification was achieved using a Hewlett-Packard 3394A integrator. The absorbance assays were performed on a Perkin-Elmer UV/Vis Lambda 20 spectrometer set at $\lambda = 340$ nm. The mass spectra were obtained using a Finnigan LCQ ion trap electrospray ionization mass spectrometer, and the NMR spectra were obtained on a 300 MHz General Electric QE300 spectrometer

Synthesis of *N*-Acetyl-*S*-(2-phenylpropan-3-ol)-L-cysteine and *S*-(2-Phenylpropan-3-ol)-GSH. *N*-Acetyl-*S*-(2-phenylpropan-3-ol)-L-cysteine was synthesized as previously published (*1*) except that the alcohol was generated in situ before product purification. The GSH adduct, *S*-(2-phenylpropan-3-ol)-GSH, was obtained in an analogous manner by substituting *N*-acetyl-L-cysteine with GSH. LC/ESI-MS for *N*-acetyl-*S*-(2-phenylpropan-3-ol)-L-cysteine: $t_R = 7.5$ min, $[M + 1H]^+$ *m*/*z* 298. LC/ESI-MS for *S*-(2-phenylpropan-3-ol)-GSH: $t_R = 3.3$ min, $[M + 1H]^+$ *m*/*z* 442. Both products were obtained in quantitative yield and determined to be >95% pure by HPLC.

Synthesis of 2-Phenylpropenal. 2-Phenyl-1,3-propanediol monocarbamate was synthesized as previously published (*1*). To a solution of 2-phenyl-1,3-propanediol monocarbamate (0.55 g, 3.1 mmol) in CH_2Cl_2 in a round-bottom flask (protected from light) was added Dess-Martin Periodinane (1.50 g, 3.5 mmol) in one portion. After 2 h, triethylamine (0.471 mL, 3.4 mmol) was added to the reaction mixture and the solution stirred for an additional 1 h, at which time the reaction was complete. The crude material was purified by flash chromatography on silica (1:1 ether/petroleum ether mixture), affording the product as solid white crystals (0.253 g, 62%): thin-layer chromatography $R_f = 0.75$ (1:1 ether/petroleum ether mixture). The compound was characterized by ^{1}H NMR and ^{13}C NMR, and the results are in agreement with the previously published data (*2*). The purity of the compound was determined to be >99% by HPLC ($\lambda = 214$ nm).

Kinetic Analysis of the Reaction between 2-Phenylpropenal and GSH. Given that the uncatalyzed reaction between 2-phenylpropenal and GSH proceeds on the second time scale, the kinetic experiments required a method that could instantaneously quench the reaction. Sodium borohydride ($NaBH_4$) was selected to quench the reactions because it could both inactivate the enzyme and reduce 2-phenylpropenal to the corresponding alcohol. Control experiments demonstrated that when 2-phenylpropenal (to 150 μM) was added to a reaction mixture containing 500 μM GSH, 2.5 μg of the respective enzyme, and a spatula tip of $NaBH_4$ (~1 mg) in 500 μL of 100 mM potassium phosphate buffer (pH 6.5) at 37 °C, product formation was not observed.

The kinetic analysis of the control and enzyme-catalyzed reactions were performed at 37 °C in 500 μL of 100 mM potassium phosphate buffer (pH 6.5) containing 500 μM reduced GSH, *N*-acetyl-*S*-(2-phenylpropan-3-ol)-L-cysteine as an internal standard, and 2.5 μg of the respective enzyme. The reactions were initiated by the addition of 2-phenylpropenal to a final concentration of 25, 50, 75, 100, or 150 μM and stopped with the addition of $NaBH_4$ (~1 mg). The zero time point was obtained by adding $NaBH_4$ to the reaction mixture immediately before the addition of 2-phenylpropenal. The quenched reactions were neutralized by the addition of 50 μL of 20% acetic acid and quantified by HPLC using UV detection and integration as described above. A calibration curve demonstrated a linear absorbance response for *N*-acetyl-*S*-(2-phenylpropan-3-ol)-L-cysteine and *S*-(2-phenylpropan-3-ol)-γ-GSH to 200 μM, the highest concentration that was examined. The linear relationship allowed for the absolute quantification of *S*-(2-phenylpropan-3-ol)-γ-GSH. Each of the time points and concentrations was analyzed in triplicate.

Without the use of stopped-flow kinetics, which was not available to us, the determination of the kinetic parameters (K_m, V_{max}, and k_{cat}/K_m) was difficult given the reaction half-lives (38 s for no enzyme, 18 s for GSTP1-1, 11 s for GSTM1-1, and 26 s for GSTA1-1). Therefore, the initial rates were obtained by plotting the amount of product formed versus time for several time points and fitting the data to a second-degree equation, e.g., $y = a + bt + ct^2$, where b is the initial rate. Lineweaver−Burk plots of each data set were constructed to determine K_m and V_{max}. Comparative analysis utilizing the Eadie−Hofstee plot produced similar values for K_m and V_{max}, validating the use of the Lineweaver−Burk analysis. The k_{cat} was calculated in the typical manner where $k_{cat} = (v_o K_m)/([\text{substrate}][\text{enzyme}])$. The half-life data are reported for reaction mixtures containing 50 μM 2-phenylpropenal, 500 μM GSH, and 2.5 μg of the respective enzyme.

Inhibition of GSTP1-1, GSTM1-1, or GSTA1-1 by 2-Phenylpropenal. The method for monitoring enzyme inhibition has been adapted from similar experiments (*5*, *7*), and the method for testing enzyme activity is described below. Briefly, the enzyme (1 μM) was incubated in 200 mM potassium phosphate buffer (pH 7.4) containing 200 μM EDTA at 37 °C. The enzyme activity at time zero was measured just prior to the addition of 2-phenylpropenal. The inhibition was initiated by the addition of 2-phenylpropenal from a stock solution in CH_3CN to a final concentration of 25 μM. The concentration of CH_3CN was 0.3% (v/v) of the incubation buffer, and the CH_3CN was added to the control. At the appropriate time points, aliquots were removed from the reaction mixture and tested for enzyme activity. The incubations with 2-phenylpropenal and controls were performed in triplicate, and the percent inhibition is reported as the enzyme activity with 2-phenylpropenal relative to the control.

GST Activity with CDNB and GSH. GST activity was determined at 25 °C by measuring the rate of CDNB-GSH product formation at $\lambda = 340$ nm (*5*, *7*). Briefly, 10 μL of the enzyme reaction mixture described above was added to 240 μL of 100 mM potassium phosphate buffer (pH 6.5) containing 200 mM EDTA, 1 mM GSH, and 100 μM CDNB. The reaction was monitored at $\lambda = 340$ nm for 1 min, and the amount of product formed was calculated using the difference extinction coefficient [$\Delta\epsilon = 9.6$ mM^{-1} cm^{-1} (*7*)].

Reversible Inhibition of GSTP1-1 and GSTM1-1 by 2-Phenylpropenal. The enzyme reaction mixture (125 μL), incubated as described above for the inhibition of GSTP1-1 and GSTM1-1 by 2-phenylpropenal, was incubated for 30 min at 37 °C. After the 30 min incubation, the enzyme activity was determined as described above (reported as time zero) and the remaining enzyme reaction mixture was filtered through a Microcon-10 apparatus (Amicon, Beverly, MA) at 10 000 rpm for 15 min. The Microcon-10 apparatus had been prewashed with 200 μL of 200 mM potassium phosphate buffer (pH 7.4). After filtration, the enzyme was reconstituted in 125 μL of 200 mM potassium phosphate buffer (pH 7.4) containing 200 mM

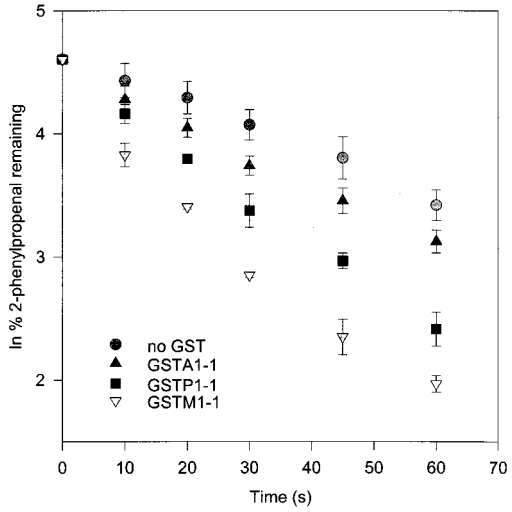


Figure 1. Graph demonstrating a decrease in the half-life of the reaction between 2-phenylpropenal and GSH upon incubation with GSTM1-1, GSTP1-1, or GSTA1-1.

Table 1

	half-life of 2-phenylpropenal (s)
no enzyme	37.8 ± 6.8
GSTM1-1	11.3 ± 1.2
GSTP1-1	17.7 ± 0.4
GSTA1-1	26.4 ± 2.4

EDTA and 10 mM GSH and incubated at 37 °C. At each time point, 10 μL of the enzyme incubation mixture was tested for activity using CDNB as described above. The experiments with 2-phenylpropenal and control experiments were performed in triplicate, and the percent activity is reported as the activity for incubations containing 2-phenylpropenal relative to the controls.

Results

Reaction Order. The order of the non-enzyme-catalyzed reaction between 2-phenylpropenal and GSH was determined by holding the concentration of one reactant constant while varying the concentration of the other reactant and plotting the log of the initial velocity versus the log of the initial concentration. As expected for a bimolecular addition reaction, the reaction was first-order with respect to 2-phenylpropenal, first-order with respect to GSH, and second-order overall.

Half-Life of 2-Phenylpropenal with and without GSTA1-1, GSTP1-1, and GSTM1-1. To determine if GSTA1-1, GSTP1-1, or GSTM1-1 catalyzes the reaction between 2-phenylpropenal and GSH, we measured the half-life of 2-phenylpropenal with and without enzyme. The results are summarized in Figure 1 and Table 1, and show that all three GST isoforms catalyze the reaction. In all cases, a decease in the amount of 2-phenylpropenal corresponded to an increase in the amount of *S*-(2-phenylpropan-3-al)-GSH formed.

Enzyme Kinetic Characterization. To determine which GST isoforms may be important for the in vivo detoxification of 2-phenylpropenal, we determined the K_m, V_{max}, and k_{cat}/K_m values for GSP1-1, GSTM1-1, and GSTA1-1. The kinetic parameters were obtained for each isozyme by varying the concentration of 2-phenypropenal, obtaining the initial rates, and plotting the Lineweaver−Burk plots (Figures 2−4). Comparative analysis using the Eadie−Hofstee plot provided similar values for K_m and V_{max}. The results of triplicate experiments are summarized in Table 2. The reaction between GSH and 2-phenylpropenal is best catalyzed by GSTM1-1 (k_{cat}/K_m = 0.275 ± 0.035 μM^{-1} s^{-1}) followed by GSTP1-1 (k_{cat}/K_m = 0.164 ± 0.005 μM^{-1} s^{-1}) and then GSTA1-1 (k_{cat}/K_m = 0.042 ± 0.005 μM^{-1} s^{-1}). Correcting for the amount of product formed in the non-enzyme-catalyzed reaction did not alter the k_{cat}/K_m. Therefore, the data are reported without modification.

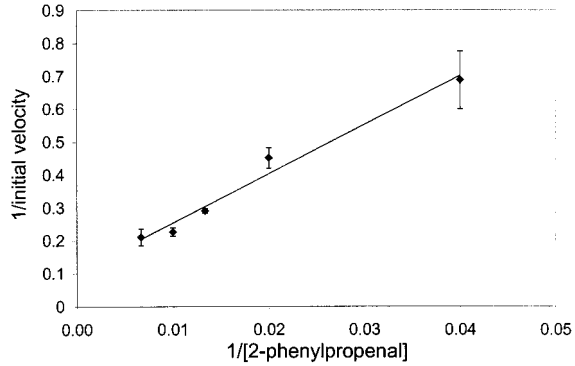


Figure 2. Lineweaver−Burk plot for GSTM1-1.

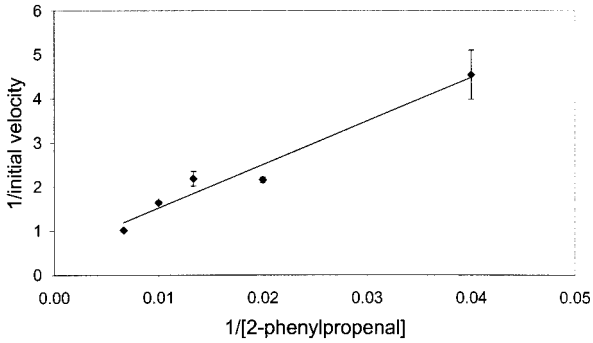


Figure 3. Lineweaver−Burk plot for GSTA1-1.

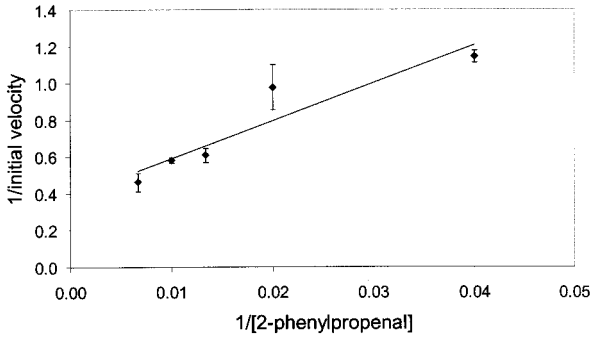


Figure 4. Lineweaver−Burk plot for GSTP1-1.

Table 2

enzyme	K_m (μM)	V_{max} (μM s^{-1})	k_{cat}/K_m (μM^{-1} s^{-1})
GSTM1-1	153 ± 57	10.0 ± 2.2	0.275 ± 0.035
GSTP1-1	55 ± 10	2.6 ± 0.3	0.164 ± 0.005
GSTA1-1	217 ± 107	2.1 ± 0.7	0.042 ± 0.005

Inhibition of GSTM1-1, GSTP1-1, or GSTA1-1 by 2-Phenylpropenal. We sought to determine if 2-phenylpropenal could inhibit GSTM1-1, GSTP1-1, or GSTA1-1 under GSH-depleted conditions. To determine if 2-phenylpropenal could inhibit GSTM1-1, GSTP1-1, or GSTA1-1, we incubated the respective enzyme with 25 μM 2-phenylpropenal at 37 °C and determined the enzyme activity over the course of 2 h. The results for GSTM1-1 and GSTP1-1 are reported as percent activity

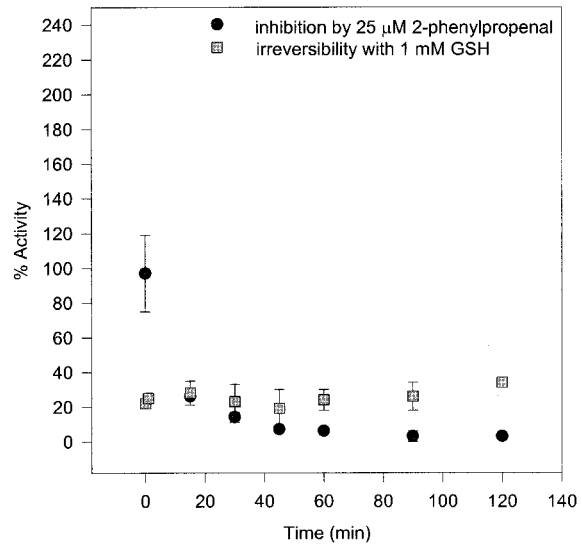


Figure 5. Irreversible inhibition of GSTM1-1 with 25 μM 2-phenylpropenal.

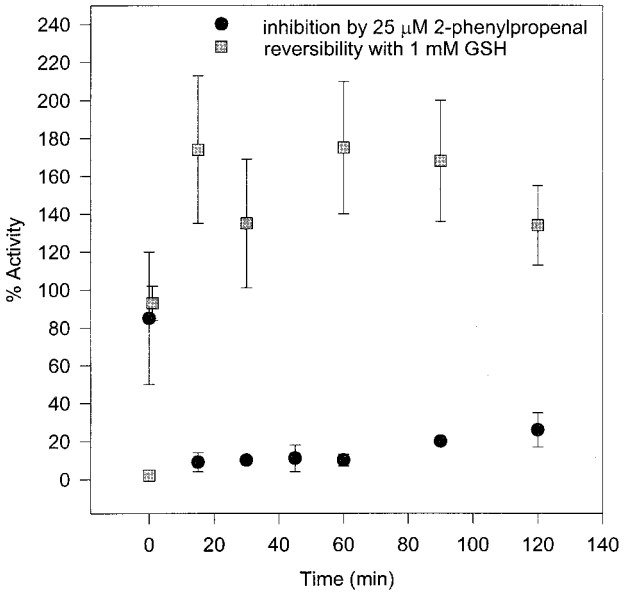


Figure 6. Reversible inhibition of GSTP1-1 with 25 μM 2-phenylpropenal.

as compared to controls without 2-phenylpropenal and are summarized in Figures 5 and 6. The results show that at 25 μM, 2-phenylpropenal is able to inhibit the activity of GSTM1-1 and GSTP1-1; however, at this concentration, 2-phenylpropenal did not inhibit the activity of GSTA1-1. The IC_{50} for the inhibition of both GSTP1-1 and GSTM1-1 by 2-phenylpropenal is ~20 μM. The inactivation of GSTP1-1 occurs rapidly within the first 15 min of incubation. The activity of GSTM1-1 is reduced to 26% of the activity of the control by 15 min and steadily decreases over the course of 2 h.

Reversibility of the Inhibition of GSTM1-1 and GSTP1-1 by 2-Phenylpropenal. Given the inhibition of GSTP1-1 and GSTM1-1 by 2-phenylpropenal, we examined the reversibility of the inhibition by removing unbound 2-phenylpropenal and incubating the inactivated enzyme in 200 mM potassium phosphate buffer containing 10 mM GSH. Aliquots of the enzyme were tested for activity over the course of 2 h, and the results are shown in Figures 5 and 6. The inhibition of GSTP1-1 by 2-phenylpropenal is reversible as soon as the enzyme is incubated with an excess of GSH. However, the inhibition of GSTM1-1 was not reversible when the enzyme was incubated with an excess of GSH, which suggests that 2-phenylpropenal irreversibly inhibits GSTM1-1.

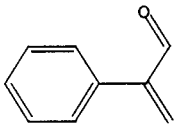
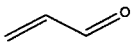
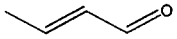
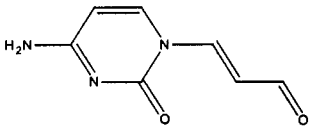
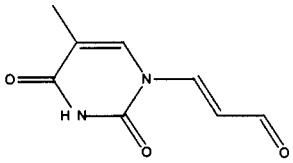
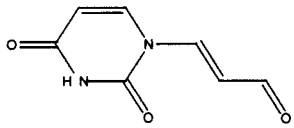
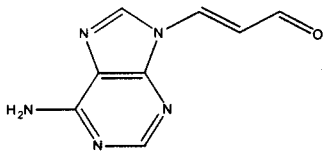
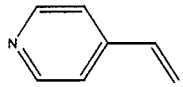
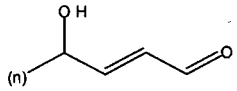
Discussion

The role of glutathione transferases in the detoxification of 2-phenylpropenal may be important, particularly in GSH-depleted patients. Even though the spontaneous reaction between GSH and 2-phenylpropenal occurs rapidly, the reaction exhibits second-order kinetics and should drop precipitously under GSH-depleted conditions. Under our assay conditions, the rate of the reaction is increased 3.1-fold with GSTM1-1, 2.1-fold with GSTP1-1, and 1.4-fold with GSTA1-1. Given that the enzyme concentrations in our reaction conditions are much lower than the physiological levels in the liver (*8*), we suspect that the reaction between 2-phenylpropenal and GSH is catalyzed in vivo. Likewise, it is important to note that the concentration of GSTA1-1 is greater than the concentration of GSTM1-1 in liver (*8, 9*) such that GSTA1-1 may contribute to the in vivo detoxification of 2-phenylpropenal. The differences in reaction rates between the enzyme-catalyzed and non-enzyme-catalyzed reaction suggest that a patient deficient in GST may be more susceptible to felbamate toxicity.

Polymorphisms in GSTM1-1 and GSTP1-1 have been identified (*3*). Polymorphisms in GSTM1-1 have been shown to exist in 50% of the population (*10*). One study was able to demonstrate that human lymphocytes deficient in GSTM1-1 were more prone to epoxide-induced toxicity (*3*). Therefore, patients polymorphic in GSTM1-1 may be more susceptible to felbamate-associated toxicity. Due to its lack of commercial availability, we did not characterize the role of GSTT1-1 in the conjugation between GSH and 2-phenylpropenal. GSTT1-1 catalyzes the conjugation reaction between GSH and compounds such as 4-nitrophenyl bromide, methyl bromide, dichloromethane, and ethylene oxide (*3*). Given typical substrate characteristics, we do not expect GSTT1-1 to catalyze the reaction between GSH and 2-phenylpropenal. However, it is worth mentioning that GSTT1-1 is expressed polymorphically in red cells in ~15% of the population (*11*).

GSTP1-1 is thought to mediate the GSH conjugation of the majority of small molecule α,β-unsaturated aldehydes (*3*). Given the reported literature values for acrolein, we expected GSTP1-1 to be the main isoform for catalyzing the reaction between GSH and 2-phenylpropenal. However, a thorough kinetic analysis has shown that GSTM1-1 has the highest rate of enzyme turnover for the reaction between GSH and 2-phenylpropenal. A comparison of our kinetic data with the reported data for other α,β-unsaturated aldehydes is presented in Table 3. The data in Table 3 show an increase in substrate specificity for GSTM1-1 over GSTP1-1 with an increase in alkyl chain length. A comparison of 2-phenylpropenal to acrolein and the base propenals suggests that the phenyl ring substitution at the C2 position increases selectivity for GSTM1-1 over GSTP1-1. Structural knowledge about GST isoform specificity may be useful in the prediction of other α,β-unsaturated aldehydes and in generating computer-based predictive software.

Table 3[a]

Structure	Compound	k_{cat}/K_m, $mM^{-1}\cdot s^{-1}$ GSTA1-1	GSTM1-1	GSTP1-1
	2-phenylpropenal	42 ± 5	275 ± 35	164 ± 5
	2-propenal	10	60	350
	crotonaldehyde	<0.1	<0.1	29 ± 0.7
	cytosine propenal	<0.1	<0.1	7 ± 0.8
	thymine propenal	<0.1	<0.1	104 ± 10
	uracil propenal	<0.1	<0.1	222 ± 9
	adenine propenal	6 ± 0.6	46 ± 2	769 ± 54
	4-vinylpyridine	<0.1	<0.1	154 ± 5
	4-hydroxyalkenals			
	C-5	0.4 ± 0.9	49 ± 14	34 ± 15
	C-6	6 ± 5	120 ± 6	19 ± 3
	C-7	26 ± 3	401 ± 50	10 ± 3
	C-8	76 ± 11	740 ± 13	26 ± 2
	C-9	111 ± 21	708 ± 58	39 ± 4
	C-10	166 ± 21	839 ± 100	54 ± 7
	C-11	227 ± 15	915 ± 125	93 ± 5
	C-12	258 ± 20	901 ± 61	93 ± 24
	C-14	295 ± 19	786 ± 110	87 ± 8
	C-15	197 ± 19	430 ± 79	48 ± 11

[a] Values for acrolein are from ref *20*; values for crotonaldehyde, base propenals, and 4-vinylpyridine are from ref *4*, and values for 4-hydroxyalkenals are from ref *21*.

Substrates for GST are often found to inhibit the enzyme (*3*, *5*). It has been proposed that GSTs react with electrophilic compounds to protect from intracellular damage (*3*). Not surprisingly, a number of α,β-unsaturated aldehydes inhibit GSTs, particularly GSTP1-1. GSTP1-1 contains a reactive cysteine, Cys-47, which has an estimated pK_a of 4.2 (*12*) and has been shown to become alkylated by a number of electrophiles, including ethacrynic acid (*5*). The reversibility of the inhibition is compound-dependent. We have shown that 2-phenylpropenal reversibly inhibits GSTP1-1. In addition, α,β-unsaturated aldehydes have also been shown to inhibit GSTM1-1 (*13*), although details about the mechanism of inhibition are not as well understood. We have shown that 2-phenylpropenal irreversibly inhibits GSTM1-1 after filtration and reconstitution in 10 mM GSH. The irreversible inhibition of GSTM1-1 represents the identification of a candidate protein for in vivo alkylation, which may contribute to our understanding of felbamate-associated toxicities. Often the proteins implicated in mediating toxic drug-induced reactions are the metabolizing enzymes themselves (*14*). Under GSH-depleted

conditions, 2-phenylpropenal may also alkylate GSTP1-1 and thereby mediate the observed toxicities.

The potential deleterious effects of 2-phenylpropenal inhibiting GSTP1-1 and GSTM1-1 are not limited their inability to detoxify atropaldehyde but extend to implications concerning the general redox status of a cell. The delicate balance between normal cellular metabolism generating reactive oxygen species and lipid oxidation products, such as 4-hydroxynonenal, and detoxification of those molecules remains critical to cell survival. A reduction in GSH concentrations and the level of inhibition of GST may lead to a cumulative overload of reactive oxygen species and its products, resulting in cellular death and toxicity.

In addition to the role of GST, the aldehyde dehydrogenase (ALDH) that oxidizes 3-carbamoyl-2-phenylpropanal, to the corresponding acid, 3-carbamoyl-2-phenylpropionic acid must also be considered and is the focus of future research. Previous studies have demonstrated the formation of 3-carbamoyl-2-phenylpropionic acid from its metabolic precursor, 2-phenyl-1,3-propanediol monocarbamate, upon incubation with human liver S9 fractions (*15*). Additional studies with human liver S9 fractions were able to demonstrate that atropaldehyde inhibits ALDH and conversion of 3-carbamoyl-2-phenypropanal to 3-carbamoyl-2-phenylpropionic acid (*16*). Studies with the structurally similar cyclophosphamide metabolite, aldophosphamide, have demonstrated substrate specificity for ALDH1 and ALDH3 in the generation of the nontoxic metabolite carboxycyclophosphamide (*17*). Like 3-carbamoyl-2-phenypropanal, aldophosphamide undergoes β-elimination affording 2-propenal that is thought to inhibit erythrocyte ALDH1, affecting the overall pharmacokinetics of 4-hyrdoxycyclophosphamide (*18*). Given the similarities between cyclophosphamide metabolism and felbamate metabolism, we propose that ALDH1 may be important in the detoxification of 2-phenylpropenal and may be the enzyme inhibited by 2-phenylpropenal in the human liver S9 fractions. Polymorphisms in several ALDH enzymes, including ALDH1, are well-characterized (*19*) and may contribute to the prediction of patients at risk for felbamate toxicity.

In summary, all of our data at present support the hypothesis that 2-phenylpropenal is the reactive metabolite that mediates felbamate-associated hepatotoxicity and aplastic anemia. We further propose that maintaining normal GSH levels remains the key determinate in preventing felbamate toxicity. Our results suggest that GSTs catalyze the reaction between 2-phenylpropenal and GSH in vivo. Particularly, in patients who become GSH-depleted, the role of GST may become increasingly important and any GST polymorphisms resulting in a loss of activity may further promote felbamate toxicity. In addition, polymorphisms in ALDH activity may result in a relative increase in the amount of atropaldehyde that is formed. An increase in the amount of atropaldehyde would promote GSH depletion.

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