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Variants of glutathione s-transferase pi 1 exhibit differential enzymatic activity and inhibition by heavy metals

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Abstract

Nonsynonymous single nucleotide polymorphisms in glutathione s-transferase pi 1 (*GSTP1*; Ile/Val 105, Ala/Val 114) have been associated with altered toxicant metabolism in epidemiological cohorts. We explored the impact of *GSTP1* genotype on enzyme kinetics and heavy metal inhibition *in vitro*. Four *GSTP1* allozymes (105/114: Ile/Ala, Val/Ala, Ile/Val, Val/Val) were expressed in and purified from *E. coli*. Enzyme activity assays quantifying the rate of glutathione conjugation with 1-chloro-2,4-dinitrobenzene (CDNB) revealed significant differences in kinetic parameters depending on genotype ($p < 0.01$). Allozymes with Ile105 had better catalytic efficiency and greater affinity for CDNB (mean $\pm$ SEM: Ile105 Ala114 $K_m = 0.33 \pm 0.07$ mM vs. Val105 Ala114 $K_m = 1.15 \pm 0.07$ mM). Inhibition of *GSTP1* activity by heavy metals was assessed following treatment with mercury (inorganic- $HgCl_2$, methylmercury- MeHg), selenium, cadmium, lead, arsenic, and manganese. All allozymes were inhibited by $HgCl_2$ (IC_{50} range: 24.1–172 μ M), MeHg (93.9–480 μ M), and selenium (43.7–62.8 μ M). Genotype significantly influenced the potency of mercury with *GSTP1* Ile105 Val114 the least sensitive and Val105 Ala114 the most sensitive to inhibition by $HgCl_2$ and MeHg. Overall, genotype of two nonsynonymous polymorphisms in *GSTP1* influenced enzyme kinetics pertaining to an electrophilic substrate and inhibition by two mercury species.

Keywords

inorganic mercury; methylmercury; glutathione s-transferase; polymorphism; enzyme activity

INTRODUCTION¹

The influences of gene-environment interactions on the toxicokinetics of chemicals and susceptibility to toxicity have recently been considered important aspects of risk assessment (Omenn, 2001). Pharmacological studies have established that genetic factors may underlie the immense variability in an individual's ability to absorb, distribute, metabolize, and

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

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¹Abbreviations used: CDNB (1-chloro-2,4-dinitrobenzene); GSH (glutathione); GST (glutathione s-transferase); *GSTP1* (GST pi 1); *GSTP1* 1A, 1A, 1V, 1V (105/114: Ile/Ala, Val/Ala, Ile/Val, Val/Val); Hg (mercury); $HgCl_2$ (mercury (II) chloride); K_m (Michaelis-Menten constant); IC_{50} (concentration inhibiting 50% activity); MeHg (methylmercury); SNP (single nucleotide polymorphism); V_{max} (maximum velocity)

eliminate drugs (Ingelman-Sundberg, 2004). Similarly in toxicology, inter-individual differences in the metabolism and toxicodynamics of hazardous chemicals may be influenced by genetic variation. For example, in human epidemiological studies focused on heavy metals, polymorphisms in key metabolic and antioxidant genes have been associated with altered metabolism and elimination of methylmercury (MeHg; Custodio et al., 2004; Engström et al., 2008; Goodrich et al., 2011; Gundacker et al., 2009), elemental mercury (Custodio et al., 2005; Goodrich et al., 2011), lead (Gundacker et al., 2009), and arsenic (Engström et al., 2007; Marcos et al., 2006) using common biomarkers of exposure.

Many of the polymorphisms associated with metal biomarker levels are in the glutathione s-transferase (GST) family. GSTs are implicated in phase II detoxification of various endogenous metabolic byproducts and environmental chemicals including heavy metals (e.g. inorganic arsenic, inorganic mercury-HgCl₂, MeHg) (Adamis et al., 2004; Ballatori and Clarkson, 1985; Hayes et al., 2005; Wang and Lee, 1993). GSTs catalyze the conjugation of glutathione (GSH) to electrophilic substrates, resulting in less reactive products ready for elimination (Hayes et al., 2005). Conjugation of GSH to HgCl₂ and MeHg promotes elimination via the biliary system. While GSH is able to conjugate spontaneously with Hg, GSTs have also been implicated in the process. The exact role of GSTs in Hg conjugation is debated and may involve reaction catalysis, transport of the conjugated product, or both (Ballatori and Clarkson, 1985). *In vitro* results suggest that GSH conjugation of arsenic and cadmium followed by cellular efflux are GST-dependent (Adamis et al., 2004; Wang and Lee, 1993). The aforementioned studies highlight the important role that GSTs play in the detoxification of heavy metals. However, the interactions between GSTs and heavy metals are further complicated by the ability of some metals (e.g., HgCl₂, MeHg) to bind and inhibit GSTs (Almar and Dierickx, 1990; Dierickx, 1982; Poon and Chu, 2000; Reddy et al., 1981).

The catalytic efficiency of GSTs varies tremendously among individuals. Seven classes of cytosolic GSTs exist in humans, and polymorphisms are ubiquitous among the genes encoding these enzymes (Strange et al., 2000). The NCBI database reveals 155 single nucleotide polymorphisms (SNPs) in GSTP1 pi 1 (*GSTP1*) with minor allele frequencies as high as 50% in some populations. Polymorphisms in GSTs resulting in altered catalytic activity or expression have been linked to increased risk for cancer (Cote et al., 2005; Strange et al., 2000) and differential metabolism of many toxicants (Ishimoto and Ali-Osman, 2002; Srivastava et al., 1999) including heavy metals (Custodio et al., 2004; Engström et al., 2007, 2008; Goodrich et al., 2011; Gundacker et al., 2009; Marcos et al., 2006). GSTP1 is an enzyme of particular interest as it is the most widely expressed GST (found in erythrocytes, placenta, lung, brain, muscle, liver, and more) and has two prevalent nonsynonymous SNPs that directly influence enzyme activity (Strange et al., 2000; Suzuki et al., 1987). The nonsynonymous SNPs change the 105th amino acid from isoleucine to valine (Ile/Val 105; rs1695) and 114th amino acid from alanine to valine (Ala/Val 114; rs1138272). Altering the 105th position affects the geometry of the substrate binding site of GSTP1. Consequently, this modification results in an approximately three-fold difference in substrate affinity *in vitro* (Ali-Osman et al., 1997; Hu et al., 1997; Zimniak et al., 1994). The 114th amino acid is positioned outside the active site but may still influence GSTP1 activity and substrate access to the site (Ali-Osman et al., 1997; Hu et al., 1997; Parker et al., 2008).

Structural changes may affect the interaction of heavy metals with GSTP1, but to our knowledge these relationships have not been tested. Epidemiological studies found associations between GST polymorphisms (including *GSTP1* 105 and 114) and inter-individual differences in metabolism and elimination of Hg and arsenic as assessed by biomarkers (Custodio et al., 2004; Gundacker et al., 2009; Marcos et al., 2006), but influence of genotype on GSTP1 interaction with metals at the protein level are unknown.

Here, we genetically engineered *E. coli* to express two key *GSTP1* polymorphisms (Ile/Val 105 and Ala/Val 114- four combinations). The goal was to use an *in vitro* platform to assess the functional effects of these polymorphisms on enzyme kinetics and to characterize the impact of heavy metal exposure (HgCl₂, MeHg, selenium, lead, arsenic, cadmium, and manganese) on enzyme function. Such a screening approach has previously been used in cancer biology to supplement epidemiological results. We propose a similar scheme to improve mechanistic understanding of environmental epidemiological and toxicological studies. Our work is in line with a recent US National Research Council (NRC, 2007) recommendation to develop and validate high throughput *in vitro* screening assays for toxicity testing purposes that may improve understanding of inter-individual variability and risk.

MATERIALS AND METHODS

Synthesis of GSTP1 Variant Proteins

Four variants of the human *GSTP1* gene were genetically engineered using commonly employed methods (Chang et al., 1999). The most prevalent version of *GSTP1* ('IA' with genotype Ile105 Ala114) was obtained from Harvard University (Plasmid ID HSCD00000618). Two PCR reactions (referred to as Steps 1 and 2) using primers detailed in Supplemental Table 1 were used to mutate GSTP1 IA into GSTP1 VA (Val105 Ala114). The QuikChange II Site-directed Mutagenesis Kit (Stratagene, CA, USA) was used to mutate *GSTP1* to IV (Ile105 Val114) and VV (Val105 Val114) with primers listed in Supplemental Table 1. Reactions were carried out with a Mastercycler Gradient thermocycler (Eppendorf, NY, USA). The four *GSTP1* genes were cloned into pET100/D-TOPO vectors with N-terminal polyhistidine tags according to the manufacturer's protocol (Invitrogen, CA, USA). DNA sequencing was performed with a 3730 XL Sequencer (Applied Biosystems, CA, USA) to ensure proper sequence of each mutant. Constructs were propagated in TOP10 *E. coli*, and expressed in BL21 Star (DE3) *E. coli* following induction by isopropyl β-D-1-thiogalactopyranoside (IPTG). GSTP1 proteins were purified with nickel-affinity chromatography using PerfectPro Ni-NTA resin (5Prime, MD, USA) and were stored in 0.1 M potassium phosphate buffer (pH 6.6) at -20 °C. Protein concentration was determined via the Bradford method (Bradford, 1976).

GST Assays

Enzyme activity was assessed according to the method of Habig et al. (1974) by measuring GSH conjugation with 1-chloro-2,4-dinitrobenzene (CDNB). The HTS 7000 Plus Bioassay Reader (Perkin Elmer, MA, USA) measured absorption at 340 nm signifying product formation. Briefly, all reactions took place in 0.1 M potassium phosphate buffer containing 5 μg/mL protein, 0.01–10 mM GSH, and 0.01–3 mM CDNB. Each trial consisted of one microplate with three replicate wells for every substrate concentration in the range. Enzyme kinetics (V_{max} , K_m) were calculated for each trial by holding GSH (10 mM) or CDNB (2 mM) constant and varying the concentration of the other substrate. Final kinetic parameters are based on the mean of four to six successful kinetic trials. Several failed trials, mainly due to microplate reader malfunction, were excluded.

Metal Inhibition

Enzyme activity of GSTP1 allozymes was measured after incubation with 1 nM-1 mM of methylmercury (II) chloride (MeHg), mercury (II) chloride (HgCl₂, 10 pM-320 μM), manganese (II) chloride, lead (II) acetate, cadmium (II) chloride (100 pM-1 mM), sodium selenate, sodium selenite, or sodium arsenate (1 nM-10 mM). Dose ranges for heavy metals, specifically MeHg and HgCl₂, were selected to encompass inhibitory concentrations of soluble GSTs observed in previous experiments performed by us (data not published) and

others (Almar and Dierickx, 1990). Assays contained 5 µg/mL purified protein and fixed GSH and CDNB concentrations set to approximately the K_m values for a given GSTP1 allozyme. These substrate concentrations enable comparison of metal inhibition across allozymes as each variant is treated with substrate concentrations associated with 50% of its maximum enzyme activity. All metals, purchased from Sigma Aldrich or Fisher Scientific, were administered from 50–200 mM stock solutions in distilled water with the exception of MeHg which was dissolved in 100% dimethylsulfoxide. MeHg reactions and controls contained a final concentration of 3% dimethylsulfoxide for dissolving purposes. Enzymes were incubated with metal for ten minutes prior to addition of the substrates, GSH and CDNB, and subsequent measurement of product formation. Two to six successful trials (six to eighteen total replicates) of each Hg treatment were analyzed depending on the allozyme. GSTP1 IA and VA were used for method development and as such had the most inhibition assay trials. One to three trials of all other heavy metal treatments were analyzed. Fewer trials were performed due to lack of GSTP1 inhibition at any concentration by cadmium, lead, arsenic, and manganese.

Statistical Analyses

Enzyme activity data was curve fitted using a non-linear regression program (GraphPad Prism Version 3.02, GraphPad Software Inc., CA, USA) to calculate the maximum velocity of substrate formation (V_{max}) and the Michaelis constant (K_m) for substrate affinity according to the following equation:

$$Y = (V_{max})(S)/(K_m + S)$$

Y represents enzyme activity (µmol/min/mg) and S represents substrate concentration (mM). The concentrations of metal inhibiting 50% of enzyme activity (IC_{50}) and inhibition constants (K_i) were calculated using GraphPad Prism.

All statistical analyses were performed with PASW Statistics v. 18 (SPSS, Chicago, IL). Mean kinetic and inhibition parameters were compared among GSTP1 allozymes using ANOVA tests followed by pairwise comparisons with the Tukey method. For variables with unequal variances according to Levene's test, Welch ANOVA and Dunnett's T3 test were used instead. In all statistical tests, p-values <0.05 were considered statistically significant. All data are presented as mean ± SEM.

RESULTS

GSTP1 Gene Mutation and Protein Synthesis

The human *GSTP1* gene (IA) was mutated to encode *GSTP1* VA, IV, and VV. DNA sequencing confirmed the desired sequence in all four variants before expression via pet-TOPO- d-100 plasmids in BL21 Star *E. coli* cells. Proteins were purified with nickel-resin affinity chromatography, which yielded approximately 1 mg purified allozyme per batch. SDS-PAGE 2D gel electrophoresis and Coomassie Blue staining confirmed single bands around 25 kDa in the purified fractions used for enzyme activity assays.

Enzyme Activity of GSTP1 Allozymes

Enzyme activity with varying levels of the substrates CDNB and GSH revealed the four allozymes to have different kinetic properties (Table 1, Figure 1). GSTP1 IA ($K_m \pm SEM = 0.33 \pm 0.07$ mM) and IV ($K_m = 0.28 \pm 0.02$ mM) had significantly greater affinity for CDNB compared with GSTP1 VA and VV ($K_m = 1.15 \pm 0.07$, 0.63 ± 0.1 mM, respectively). VA had the least affinity for CDNB, but the highest V_{max} suggesting it requires more

available substrate to catalyze efficiently. Overall, IA had significantly better catalytic efficiency compared to the three less frequent allozymes as assessed by k_{cat}/K_m (IA: $98.2 \pm 14 \text{ mM}^{-1}\text{s}^{-1}$ vs. VA: 35.9 ± 6.8 , IV 55 ± 9.8 , VV $43.4 \pm 9.3 \text{ mM}^{-1}\text{s}^{-1}$). Kinetic parameters with varying levels of GSH were similar among the four GSTP1 allozymes. ANOVA revealed a significant difference among the allozymes' K_m values for GSH, though the sole significant pairwise difference was between GSTP1 VA ($0.93 \pm 0.11 \text{ mM}$) and IV ($0.48 \pm 0.15 \text{ mM}$; $p=0.03$ for comparison).

GSTP1 and Mercury Inhibition

Four GSTP1 variants were incubated with a range of HgCl_2 (10 pM- 320 μM) and MeHg (1 nM- 1mM) doses prior to addition of substrates, and genotype influenced sensitivity towards both Hg forms (Table 2, $p<0.05$ for Welch ANOVA comparing IC_{50} and K_i values of all allozymes). HgCl_2 was the most potent inhibitor of all GSTP1 allozymes ($p<0.01$, test not shown) compared with MeHg treatment. Sensitivity of the GSTP1 allozymes followed the same trend for HgCl_2 and MeHg inhibition (most sensitive first: VA > IA > VV > IV). Pairwise comparisons revealed significant differences between IC_{50} and inhibitory constants (K_i) accounting for GSH concentration and allozyme substrate affinity, of the GSTP1 VA variant treated with HgCl_2 and the respective IC_{50} and K_i values for GSTP1 IA (Dunnett's T3 test, $p<0.05$). Furthermore, according to Dunnett's T3 test, IC_{50} and K_i (accounting for CDNB concentration) values for HgCl_2 were significantly different from one another when comparing GSTP1 VA with VV ($p<0.05$). MeHg inhibition varied among allozymes to a lesser extent as only IC_{50} and K_i (CDNB) values for GSTP1 VA and VV were significantly different from one another using Dunnett's T3 test ($p<0.05$). GSTP1 IV was the most resistant allozyme to MeHg inhibition ($\text{IC}_{50} \pm \text{SEM} = 480 \pm 88.4 \mu\text{M}$) compared with three other variants and was five times less sensitive than GSTP1 VA ($\text{IC}_{50} = 93.9 \pm 18.0 \mu\text{M}$).

GSTP1 and Other Heavy Metals

The inhibitory effects of two inorganic selenium compounds (sodium selenate, sodium selenite) were tested on four GSTP1 allozymes. GSTP1 was not inhibited by sodium selenate concentrations as high as 1 mM, while sodium selenite had similar potency to HgCl_2 (see Table 3), and potency did not vary significantly by allozyme. GSTP1 enzyme activity, regardless of amino acid sequence, did not reach 50% inhibition by lead acetate, manganese chloride, or cadmium chloride at concentrations up to 1 mM or by sodium arsenate at concentrations up to 10 mM.

DISCUSSION

Genetic polymorphisms in *GSTP1*, which encodes an important detoxification enzyme that catalyzes the conjugation of GSH with many endogenous and exogenous electrophilic substrates, have been linked in epidemiological studies to altered metabolism and biomarker levels of toxicants such as methylmercury (Custodio et al. 2004; Engström et al., 2008; Goodrich et al., 2011; Gundacker et al. 2009). We assessed the kinetic properties of four GSTP1 variants encoded by two key nonsynonymous SNPs in *GSTP1*, Ile/Val 105 and Ala/Val 114, on protein function with common substrates *in vitro* in the presence and absence of mercury (HgCl_2 , MeHg), selenium, arsenic, lead, manganese and cadmium. Kinetic parameters and inhibition by HgCl_2 and MeHg significantly differed ($p<0.05$) among the four recombinant allozymes suggesting a role for both polymorphisms in altering enzyme function.

In most human populations, the major *GSTP1* alleles are Ile105 (frequency >50%) and Ala114 (>90%; NCBI). The two most common allozymes of GSTP1 in human populations are GSTP1 IA and VA, though GSTP1 IV and VV exist to a limited extent (estimated $\leq 5\%$

based on allele frequencies in HapMap populations). The 105 and 114 loci are in linkage disequilibrium in many populations (Moyer et al., 2008). Previous biochemical characterization of GSTP1 allozymes have focused on GSTP1 IA and VA, and the few studies incorporating GSTP1 IV and VV had inconsistent results (Ali-Osman et al., 1997; Hu et al., 1998; Johansson et al., 1998; Moyer et al., 2008; Zimniak et al., 1994). In this study, GSTP1 IA and IV had the greatest affinity for the electrophilic substrate, CDNB, and their affinities were 3–4 fold greater than that of GSTP1 VA. Previous biochemical studies of GSTP1 allozymes found similar trends with GSTP1 IA having a greater affinity for CDNB (1.6–3.7 fold lower K_m) compared with GSTP1 VA (Ali-Osman et al., 1997; Hu et al., 1997; Johansson et al., 1998; Moyer et al., 2008; Zimniak et al., 1994). GSTP1 IA was the most efficient at catalyzing the conjugation of GSH with CDNB (see k_{cat}/K_m , Table 1). Similar to previous studies, the affinity of GSTP1 IA, VA, and VV for GSH were near identical (Ali-Osman et al., 1997; Zimniak et al., 1994). Interestingly, GSTP1 IV had a significantly greater affinity for GSH compared to GSTP1 VA. Overall, allozymes with Ile105 (GSTP1 IA, IV) performed better in kinetic assays with varying CDNB concentrations compared to allozymes with Val105 as noted when comparing K_m and k_{cat}/K_m between GSTP1 IA and VA or IV and VV.

Crystallography and structural 3D modeling have shown that residue 105 is found in the H-site of GSTP1, the site of electrophilic substrate binding (Oakley et al., 1997; Parker et al., 2008). Substituting valine for isoleucine in this position alters the hydrophobicity and shape of the H-site (Ali-Osman et al., 1997; Zimniak et al., 1994) and interaction with the catalytically important residue, Tyr109 (Johansson et al., 1998; Parker et al., 2008). This substitution decreases substrate (CDNB) affinity and catalytic activity of GSTP1 as observed in the present study. The impact on substrate affinity and catalytic efficiency from substituting valine for alanine at residue 114 is less pronounced. While residue 114 is found outside the H-site, it has the potential to influence the secondary and tertiary structure of GSTP1 as it partakes in a superhelical structure impacting the H-site shape (Ali-Osman et al., 1997; Parker et al., 2008). Furthermore, 114 may be involved in a hydrophobic clamp at the opening of the solvent channel that leads to both the G- (GSH binding) and H-sites (Hu et al., 1997). Therefore, while residue 105 directly impacts the H-site, amino acid substitution at position 114 may also indirectly influence active site shape and substrate access.

Along with comparing the enzymatic activity of four GSTP1 variants, this study assessed the impact of genotype and heavy metal exposure on GSTP1. Four versions of GSTP1 were significantly inhibited by $HgCl_2$ and MeHg (Table 2). $HgCl_2$ was the more potent inhibitor, and this is consistent with other *in vitro* studies that found enzymes and neurochemical receptors to be more sensitive to $HgCl_2$ compared with MeHg (Allen et al., 2001; Basu et al., 2005). The observed IC_{50} s for $HgCl_2$ (range across allozymes: 24.1 to 172 μM) and MeHg (93.9 to 480 μM) were within the range (10 to 300 μM) of IC_{50} s observed for human, rat, and calf GSTs following $HgCl_2$ (Almar and Dierickx, 1990; Dierickx, 1982; Poon and Chu, 2000; Reddy et al., 1981) and MeHg treatment (Reddy et al., 1981).

While Dierickx (1982) observed differential inhibition of rat GSTs depending on GST isozyme, this is the first study to show that genotype of a specific GST, GSTP1, may also influence mercury sensitivity. For both $HgCl_2$ and MeHg treatments, allozymes ranked according to sensitivity with the most sensitive first were as follows: GSTP1 VA>IA>VV>IV. This trend suggests that the Ile105 and Val114 residues may confer some protection against inhibition by Hg. Inhibition of GSTP1 activity by Hg likely occurs via two mechanisms: directly by binding to GSTP1 thiol groups and indirectly by conjugation with available GSH. GSTP1 contains four cysteine residues, including one residue (Cys48) enzyme inhibitors are known to bind to and one (Cys102) near the H-site (Oakley et al.,

1997). Studies on mercury inhibition of GSTs, including the present, have observed that increasing GSH concentration or adding cysteine partially decreases inhibition, irrespective of CDNB concentration (Almar and Dierckx, 1990; Dierckx 1982). Amino acid substitutions at residues 105 and 114 could influence both purposed inhibitory mechanisms. Structural alterations accompanying the sequence change may affect the positioning of the cysteine residues, and thus impact the ability of Hg to bind to and inhibit the enzyme. As previously discussed, substitution of Val for Ile105 changes the structure of the H-site and subsequently affinity for CDNB and catalytic efficiency. The impaired enzymatic activity of GSTP1 VA and VV compared to GSTP1 IA and IV, respectively, may hinder their ability to utilize available GSH efficiently when Hg binds to GSH and/or binds to and inhibits the enzyme itself.

Sodium selenite inhibited GSTP1 activity with similar potency to HgCl_2 while sodium selenate was not inhibitory (up to 1 mM). Selenium exhibits hormesis as some selenium is needed in the body (e.g., for selenoprotein synthesis) but high levels are toxic (Chiang et al., 2010). Unlike sodium selenate, selenite is known to bind thiols, including GSH, and GSH binding may constitute the main mechanism of GST inhibition observed here (Żbikowska et al., 1997). *In vitro* treatment of pig GSTs found selenite inhibited activity while selenate had no effect as observed in the present study (Żbikowska et al., 1997). *GSTP1* genotype did not alter sensitivity to selenite inhibition. GSTP1 activity, regardless of genotype, was not significantly inhibited (>50%) after incubation with arsenic, lead, manganese, or cadmium. Previous studies revealed arsenic treatment to have no effect on GST activity in human erythrocytes (Poon and Chu, 2000) or to increase GST activity in rat hepatocytes treated *in vitro* (Kojima et al., 2006).

GSTP1 specifically has been shown to play an important role in increased arsenic excretion in a strain of Chinese Hamster Ovarian cells, stemming from increased expression (Wang et al., 1993). The lack of inhibition of recombinant GSTP1 in the present study is consistent with previous studies suggesting no direct interaction between arsenic and GSTs. Though rat studies have implicated lead in increasing GST expression and subsequent activity (Daggett et al., 1998), lead has not been shown to directly inhibit GST enzyme activity (Dierckx, 1982) as seen here with human GSTP1. Consistent with GSTs isolated from rats and calves, recombinant human GSTP1, regardless of genotype, was not inhibited by manganese chloride (Dierckx, 1982; Reddy et al., 1981).

Cadmium inhibited 50% of GST activity *in vitro* at concentrations between 10 and 750 μM in calf liver (Reddy et al. 1981), human erythrocytes (Poon and Chu, 2000) and of GSTP1 isolated from a hepatoma cell line (Almar and Dierckx, 1990) while 200 μM decreased the activity of GSTs isolated from rat liver by 37% (Dierckx, 1982). In the present study, the IC_{50} for all GSTP1 allozymes was greater than 1 mM, though significant inhibition at 1 mM compared to untreated controls was observed for all variants (data not shown, 81–88% of specific activity of control, $p < 0.05$). Species differences among GSTs and experimental conditions (substrate concentrations, source and concentration of GSTs, GST isoforms) may contribute to the differences observed.

GSTP1 105 and 114 polymorphisms, which are linked to differences in enzyme function and Hg sensitivity *in vitro*, are significantly associated with altered mercury biomarker levels (hair and blood), representing primarily MeHg exposure, at the population level. Several studies found significant associations between Val105 and/or Val114 alleles and lower biomarker levels (Engström et al., 2008; Goodrich et al., 2011) while other studies linked higher biomarker levels to Val alleles (Custodio et al., 2004; Gundacker et al., 2009). Multiple factors may underlie the discrepant results including Hg exposure level, specific biomarkers analyzed, statistical methodologies employed (e.g., ANOVA vs. linear

regression; modeling single SNP vs. SNP combinations), frequency of *GSTP1* 105 and 114 SNPs in the study populations, and linkage with other SNPs influencing *GSTP1* expression. Alternatively, given multiple testing in many of these epidemiological studies and the inconsistent direction of relationships between *GSTP1* genotype and Hg biomarker levels observed across studies, the significant associations could be false positives. In arsenic exposed populations, deletion polymorphisms in *GSTT1* and *GSTM1* have been shown to alter the proportion of methylated arsenic metabolites in urine. For example, *GSTM1* deletion genotype was associated with a higher proportion of monomethylated arsenic (MMA), a particularly toxic metabolite (Engström et al., 2007). However, *GSTP1* Val105 was only associated with a near-significant shift in the urinary metabolic profile (Marcos et al., 2006).

Limited epidemiological associations and *in vitro* results suggest that *GSTP1* 105 and *GSTP1* 114 genotypes have the potential to influence Hg toxicokinetics. While the relationships between *GSTP1* genotype, Hg biomarker levels, and ensuing toxicity merit future study, several considerations must be employed when interpreting this gene-environment data. Glutathione-Hg conjugation may be influenced by many factors including glutathione availability and oxidation state, Hg exposure (species, dose, duration), exposures to other thiol-reactive compounds, and overall functionality of GSTs. While Hg spontaneously reacts with GSH intracellularly, GSTs can also aid this important Hg detoxification process by way of reaction catalysis and conjugate transport (Ballatori and Clarkson, 1985). Furthermore, GST inhibition by Hg at high doses, as observed in this study, may act as a temporary detoxification mechanism, preventing Hg from inhibiting other key intracellular proteins. Expression of GST increases following Hg exposure, and in rats, the *GSTP1* equivalent experiences the most pronounced upregulation (11-fold; Brambila et al., 2002). While *GSTP1* may be of particular importance to the Hg toxicokinetic pathway, it is one of several GSTs which can interact with Hg. Likewise, *GSTP1* 105 and 114 are two of a multitude of polymorphisms in *GSTP1*, many of which are suspected to alter enzyme activity or gene expression (Moyer et al., 2008). Thus, while *GSTP1* Ile/Val 105 and Ala/Val 114 may significantly influence enzyme activity and sensitivity to Hg inhibition *in vitro*, genotype at two loci in one GST isozyme would be expected to have a very minor impact, if any, on Hg toxicokinetics *in vivo*.

In a broader context, the combined genotype across dozens of key polymorphic sites that affect expression or enzymatic activity of several GST isozymes (and of other key proteins in the Hg toxicokinetic pathway) has the potential to impact the wide inter-individual variability observed in heavy metal metabolism. *In vitro* characterization of protein products encoded by polymorphisms may provide a better understanding of epidemiological links between polymorphisms and interaction with metals or other toxic exposures (Li and Woods, 2009; Moyer et al., 2008). Nevertheless, careful consideration of a multitude of factors (e.g., toxicant exposure source and dose, protein expression, SNP prevalence) and synthesis of *in vitro* and epidemiological data related to all functional SNPs in the pathway must be employed when interpreting gene-environment data and applying it to risk assessment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Adamis PD, Gomes DS, Pinto ML, Panek AD, Eleutherio EC. The role of glutathione transferases in cadmium stress. *Toxicol Lett*. 2004; 154:81–88. [PubMed: 15475181]
- Allen JW, Mutkus LA, Aschner M. Mercuric chloride, but not methylmercury, inhibits glutamine synthetase activity in primary cultures of cortical astrocytes. *Brain Res*. 2001; 891:148–157. [PubMed: 11164818]
- Ali-Osman F, Akande O, Antoun G, Mao JX, Buolamwini J. Molecular cloning, characterization, and expression in *Escherichia coli* of full length cDNAs of three human glutathione S-transferase Pi gene variants. *J Biol Chem*. 1997; 272:10004–10012. [PubMed: 9092542]
- Almar MM, Dierickx PJ. In vitro interaction of mercury, copper (II) and cadmium with human glutathione transferase Pi. *Res Commun Chem Path Pharmacol*. 1990; 69:99–102. [PubMed: 2218073]
- Ballatori N, Clarkson TW. Biliary secretion of glutathione and glutathione-metal complexes. *Fund Appl Toxicol*. 1985; 5:816–831.
- Basu N, Stamler CJ, Loua KM, Chan HM. An interspecies comparison of mercury inhibition on muscarinic acetylcholine receptor binding in the cerebral cortex and cerebellum. *Toxicol Appl Pharm*. 2005; 205:71–76.
- Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 1976; 72:248–254. [PubMed: 942051]
- Brambila E, Liu J, Morgan DL, Beliles RP, Waalkes MP. Effect of mercury vapor exposure on metallothionein and glutathione S-transferase gene expression in the kidney of nonpregnant, pregnant, and neonatal rats. *J Toxicol Environ Health A*. 2002; 65:1273–1288. [PubMed: 12167210]
- Chang M, Bolton JL, Blond SY. Expression and purification of hexahistidine-tagged human glutathione S-transferase P1-1 in *Escherichia coli*. *Protein Expr Purif*. 1999; 17:443–448. [PubMed: 10600464]
- Chiang EC, Shen S, Kengeri SS, Xu H, Combs GF, Morris JS, et al. Defining the optimal selenium dose for prostate cancer risk reduction: Insights from the U-shaped relationship between selenium status, DNA damage, and apoptosis. *Dose Response*. 2009; 8:285–300. [PubMed: 20877485]
- Cote ML, Kardia SLR, Wenzlaff AS, Land SJ, Schwartz AG. Combinations of glutathione S-transferase genotypes and risk of early-onset lung cancer in Caucasians and African Americans: a population-based study. *Carcinogenesis*. 2005; 26:811–819. [PubMed: 15661806]
- Custodio HM, Broberg K, Wennberg M, Jansson JH, Vessby B, Hallmans G, et al. Polymorphisms in glutathione-related genes affect methylmercury retention. *Arch Environ Health*. 2004; 59:588–595. [PubMed: 16599007]
- Custodio HM, Harari R, Gerhardsson L, Skerfving S, Broberg K. Genetic influences on the retention of inorganic mercury. *Arch Environ Occup Health*. 2005; 60:17–23. [PubMed: 16961004]
- Daggett DA, Oberley TD, Nelson SA, Wright LS, Kornguth SE, Siegel FL. Effects of lead on rat kidney and liver: GST expression and oxidative stress. *Toxicology*. 1998; 128:191–206. [PubMed: 9750042]
- Dierickx PJ. In vitro inhibition of the soluble glutathione S-transferases from rat liver by heavy metals. *Enzyme*. 1982; 27:25–32. [PubMed: 7067686]
- Engström KS, Broberg K, Concha G, Nermell B, Warholm M, Vahter M. Genetic polymorphisms influencing arsenic metabolism: evidence from Argentina. *Environ Health Perspect*. 2007; 115:599–605. [PubMed: 17450230]
- Engström KS, Stromberg U, Lundh T, Johannsson I, Vessby B, Hallmans G, et al. Genetic variation in glutathione-related genes and body burden of methylmercury. *Environ Health Perspect*. 2008; 116:734–739. [PubMed: 18560528]
- Goodrich JM, Wang Y, Gillespie B, Werner R, Franzblau A, Basu N. Glutathione enzyme and selenoprotein polymorphisms associate with mercury biomarker levels in Michigan dental professionals. *Toxicol Appl Pharmacol*. 2011; 257:301–308.

- Gundacker C, Wittmann KJ, Kukuckova M, Komarnicki G, Hikkel I, Gencik M. Genetic background of lead and mercury metabolism in a group of medical students in Austria. *Environ Res.* 2009; 109:786–796. [PubMed: 19515364]
- Habig WH, Pabst MJ, Jakoby WB. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. *J Biol Chem.* 1974; 249:7130–7139. [PubMed: 4436300]
- Hayes JD, Flanagan JU, Jowsey IR. Glutathione s-transferases. *Ann Rev Pharmacol Toxicol.* 2005; 45:51–88. [PubMed: 15822171]
- Hu X, Xia H, Srivastava SK, Herzog C, Awasthi YC, Ji X, et al. Activity of four allelic forms of glutathione s-transferase hGSTP1-1 for diol epoxides of polycyclic aromatic hydrocarbons. *Biochem Biophys Res Commun.* 1997; 238:397–402. [PubMed: 9299520]
- Ingelman-Sundberg M. Pharmacogenetics of cytochrome P450 and its applications in drug therapy: the past, present, and future. *TRENDS in Pharmacological Science.* 2004; 25:193–200.
- Ishimoto TM, Ali-Osman F. Allelic variants of the human glutathione S-transferase P1 gene confer differential cytoprotection against anticancer agents in *Escherichia coli*. *Pharmacogenetics.* 2002; 12:543–553. [PubMed: 12360105]
- Johansson A, Stenberg G, Widersten M, Mannervik B. Structure-activity relationships and thermal stability of human glutathione transferase P1-1 governed by the H-site residue 105. *J Mol Biol.* 1998; 278:687–698. [PubMed: 9600848]
- Kojima C, Qu W, Waalkes MP, Himeno S, Sakurai T. Chronic exposure to methylated arsenicals stimulates arsenic excretion pathways and induces arsenic tolerance in rat liver cells. *Toxicol Sci.* 2006; 91:70–81. [PubMed: 16436460]
- Li T, Woods JS. Cloning, expression and biochemical properties of CPOX4, a genetic variant of coproporphyrinogen oxidase that affects susceptibility to mercury toxicity in humans. *Toxicol Sci.* 2009; 109:228–236. [PubMed: 19339664]
- Marcos R, Martínez V, Hernández A, Creus A, Sekaran C, Tokunaga H, et al. Metabolic profiles in workers occupationally exposed to arsenic: role of GST polymorphisms. *JOEM.* 2006; 48:334–341. [PubMed: 16531839]
- Moyer AM, Salavaggione OE, Wu TY, Moon I, Eckloff BW, Hildebrandt AT, et al. Glutathione S-transferase P1: gene sequence variation and functional genomic studies. *Cancer Res.* 2008; 68:4791–4801. [PubMed: 18559526]
- National Research Council (NRC). Toxicity Testing in the 21st Century: A Vision and a Strategy. The National Academies Press; Washington, D.C: 2007.
- Oakley AJ, Lo Bello M, Battistoni A, Ricci G, Rossjohn J, Villar HO, et al. The structures of human glutathione transferase P1-1 in complex with glutathione and various inhibitors at high resolution. *J Mol Biol.* 1997; 274:84–100. [PubMed: 9398518]
- Omenn GS. Prospects for pharmacogenetics and ecogenetics in the new millennium. *Drug Metab Dispos.* 2001; 29:611–614. [PubMed: 11259362]
- Parker LJ, Ciccone S, Italiano LC, Primavera A, Oakley AJ, Morton CJ, et al. The anti-cancer drug chlorambucil as a substrate for the human polymorphic enzyme glutathione transferase P1-1: kinetic properties and crystallographic characterisation of allelic variants. *J Mol Biol.* 2008; 380:131–144. [PubMed: 18511072]
- Poon R, Chu I. Effects of trivalent antimony on human erythrocyte glutathione S- transferases. *J Biochem Mol Toxicol.* 2000; 14:169–176. [PubMed: 10711633]
- Reddy CC, Scholz RW, Massaro EJ. Cadmium, methylmercury, mercury and lead inhibition of calf liver glutathione S-transferase exhibiting selenium-independent glutathione peroxidase activity. *Toxicol Appl Pharmacol.* 1981; 61:460–468. [PubMed: 7330884]
- Srivastava SK, Singhal SS, Hu X, Awasthi YC, Zimniak P, Singh SV. Differential catalytic efficiency of allelic variants of human glutathione S-transferase Pi in catalyzing the glutathione conjugation of thiotepa. *Arch Biochem Biophys.* 1999; 366:89–94. [PubMed: 10334868]
- Suzuki T, Coggan M, Shaw DC, Board PG. Electrophoretic and immunological analysis of human glutathione S-transferase isozymes. *Ann Hum Genet.* 1987; 51:95–106. [PubMed: 3118757]
- Strange RC, Jones PW, Fryer AA. Glutathione s-transferase: genetics and role in toxicology. *Toxicol Lett.* 2000; 112–113:357–363.

- Wang HF, Lee TC. Glutathione S-transferase pi facilitates the excretion of arsenic from arsenic-resistant Chinese hamster ovary cells. *Biochem Biophys Res Commun.* 1993; 192:1093–1099. [PubMed: 8099479]
- Żbikowska HM, Wachowicz B, Krajewski T. Comparative effects of selenite and selenate on the glutathione-related enzymes activity in pig blood platelets. *Biol Trace Elem Res.* 1997; 57:259–269. [PubMed: 9359992]
- Zimniak P, Nanduri B, Pikula S, Bendorowicz-Pickula J, Singhal SS, Srivastava SK, et al. Naturally occurring human glutathione s-transferase GSTP1-1 isoforms with isoleucine and valine in position 104 differ in enzymatic properties. *Eur J Biochem.* 1994; 224:893–899. [PubMed: 7925413]

RESEARCH HIGHLIGHTS

- We compare kinetic parameters and metal inhibition of four GSTP1 allozymes *in vitro*.
- GSTP1 Ile105 Ala114 had the best substrate affinity and catalytic efficiency.
- Selenium, methylmercury, and inorganic Hg inhibited GSTP1 activity.
- GSTP1 allozymes varied in sensitivity to methylmercury and inorganic Hg inhibition.

Figure 1. Specific activity of four GSTP1 variants with increasing concentration of the electrophilic substrate CDNB is displayed below. Data points represent mean $\pm$ SEM specific activity from four or five trials (each trial consists of three replicates).

Table 1

Kinetic parameters for conjugation of CDNB and GSH by four GSTP1 variants. Data represent mean $\pm$ SEM based on four to six trials (one trial has three replicates). For a given row, the p-value is reported for the ANOVA test^a comparing the kinetic parameter among GSTP1 alleles.

Substrate	Amino Acid	GSTP1 IA	GSTP1 VA	GSTP1 IV	GSTP1 VV	p value
CDNB	Ile105 Ala114	Ile105 Ala114	Val105 Ala114	Ile105 Val114	Val105 Val114	
	K_m (mM)	0.33 $\pm$ 0.07	1.15 $\pm$ 0.07 [‡]	0.28 $\pm$ 0.02	0.63 $\pm$ 0.1 [*]	<0.001
	V_{max} (μ mol/min/mg)	68.1 $\pm$ 8.2	93.4 $\pm$ 16.8	33.0 $\pm$ 3.7 [*]	57.0 $\pm$ 5.0	0.006
	K_{cat}/K_m (mM ⁻¹ s ⁻¹)	98.2 $\pm$ 14	35.9 $\pm$ 6.8 [‡]	55.0 $\pm$ 9.8 [*]	43.4 $\pm$ 9.3 [*]	0.003
GSH	K_m (mM)	0.90 $\pm$ 0.02	0.93 $\pm$ 0.11	0.48 $\pm$ 0.15	0.81 $\pm$ 0.08	0.04
	V_{max} (μ mol/min/mg)	70.2 $\pm$ 14.1	59.1 $\pm$ 11.1	47.6 $\pm$ 5.1	42.6 $\pm$ 7.2	0.41
	K_{cat}/K_m (mM ⁻¹ s ⁻¹)	33.6 $\pm$ 6.1	28.3 $\pm$ 4.8	52.5 $\pm$ 11.2	23.4 $\pm$ 4.1	0.05

Pairwise comparisons with the reference GSTP1 (IA) were made in each row using the Tukey method^a, and significance is indicated:

* p<0.05,

[‡] p<0.01,

^{‡‡} p<0.001.

^a For V_{max} (both CDNB and GSH), p-value is reported from Welch ANOVA due to unequal variances. For these parameters, pairwise comparison used Dunnett's T3 test instead of the Tukey method.

Table 2

Inhibitory concentrations (IC_{50}) and constants (K_i) of two Hg species on four GSTP1 variants (mean $\pm$ SEM based on two to six trials with replicates each). The p-value for the Welch ANOVA test, used to compare means in each row, is indicated.

		GSTP1 IA	GSTP1 VA	GSTP1 IV	GSTP1 VV	p value
HgCl₂	IC_{50} (μ M)	53.7 $\pm$ 7.0	24.1 $\pm$ 3.2 *	172 $\pm$ 32.9	96.5 $\pm$ 11.2	0.02
	K_i [CDNB] (μ M)	24.7 $\pm$ 3.2	12.6 $\pm$ 2.0	81.5 $\pm$ 15.6	48.4 $\pm$ 5.5	0.03
	K_i [GSH] (μ M)	29.3 $\pm$ 3.8	12.8 $\pm$ 1.5 *	61.4 $\pm$ 11.8	45.6 $\pm$ 5.2	0.02
MeHg	IC_{50} (μ M)	135 $\pm$ 23.1	93.9 $\pm$ 18.0	480 $\pm$ 88.4	196 $\pm$ 5.7	0.03
	K_i [CDNB] (μ M)	61.9 $\pm$ 10.6	48.8 $\pm$ 9.4	227 $\pm$ 41.9	98.1 $\pm$ 2.9	0.04
	K_i [GSH] (μ M)	73.5 $\pm$ 12.6	51.2 $\pm$ 9.8	171 $\pm$ 31.6	92.4 $\pm$ 2.7	0.08

For all variables, comparisons of allozymes to the reference, GSTP1 IA, were made with Dunnett's T3 test, and significance is labeled as follows:

* $p < 0.05$.

Table 3

Inhibitory concentrations (IC_{50}) of five heavy metals on four GSTP1 variants (mean $\pm$ SEM based on three to nine replicates from one to three trials).

Metal	Form	IC_{50} (μ M) GSTP1 IA	IC_{50} (μ M) GSTP1 VA	IC_{50} (μ M) GSTP1 IV	IC_{50} (μ M) GSTP1 VV
As	sodium arsenate	>10,000	>10,000	>10,000	>10,000
Pb	lead (II) acetate	>1000	>1000	>1000	>1000
Mn	manganese (II) chloride	>1000	>1000	>1000	>1000
Se	sodium selenite	52.6 ± 3.4	62.8 ± 10.5	60.7 ± 4.9	43.7 ± 4.1
Cd	cadmium (II) chloride	>1000	>1000	>1000	>1000