HHS Public Access

Author manuscript

Chem Biol Interact. Author manuscript; available in PMC 2016 December 05.

Published in final edited form as:

Chem Biol Interact. 2015 December 5; 242: 390–395. doi:10.1016/j.cbi.2015.11.005.

Benzene Oxide is a Substrate for Glutathione *S*-Transferases

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Abstract

Benzene is a known human carcinogen which must be activated to benzene oxide (BO) to exert its carcinogenic potential. BO can be detoxified *in vivo* by reaction with glutathione and excretion in the urine as *S*-phenylmercapturic acid. This process may be catalyzed by glutathione *S*-transferases (GSTs), but kinetic data for this reaction have not been published. Therefore, we incubated GSTA1, GSTT1, GSTM1, and GSTP1 with glutathione and BO and quantified the formation of *S*-phenylglutathione. Kinetic parameters were determined for GSTT1 and GSTP1. At 37 °C, the putative K_m and V_{max} values for GSTT1 were 420 μM and 450 fmol/s, respectively, while those for GSTP1 were 3600 μM and 3100 fmol/s. GSTA1 and GSTM1 did not exhibit sufficient activity for determination of kinetic parameters. We conclude that GSTT1 is a critical enzyme in the detoxification of BO and that GSTP1 may also play an important role, while GSTA1 and GSTM1 seem to be less important.

Keywords

benzene oxide; glutathione *S*-transferase; GSTT1; GSTP1; kinetics; detoxification

1. Introduction

Benzene causes acute myeloid leukemia and other hematopoietic diseases [1, 2]. Although the mechanism of leukemogenesis is not fully understood, cytochrome P450-mediated metabolism of benzene is absolutely required for mutagenicity in *S. typhimurium* and for toxicity in mice [3–6]. Oxidation of benzene, catalyzed primarily by P450 2E1, yields benzene oxide (BO), which is in equilibrium with oxepin (Scheme 1). BO is reactive and unstable, readily rearranging to phenol. Urinary phenol and phenol conjugates account for

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Conflict of interest statement

The authors declare no conflict of interest.

about 40% of total benzene exposure, suggesting that activation to BO is a major metabolic pathway in humans [7]. The detoxification of BO *in vivo* proceeds via reaction with glutathione (GSH), resulting in excretion of *S*-phenylmercapturic acid (SPMA) in the urine (Scheme 1).

We have recently completed a biomarker study of urinary SPMA in more than 2200 smokers from five ethnic groups and, in a genome-wide association study (GWAS), evaluated genetic associations with more than 11 million genotyped or imputed polymorphisms in these individuals [8]. In the GWAS, we observed highly significant associations at chromosome 22q11 ($P = 6.0 \times 10^{-107}$) which could be explained by deletion of glutathione *S*-transferase theta 1 (*GSTT1*), with weaker associations with *GSTM1* on chromosome 1p13. Differences in *GSTT1* copy number explained approximately 21% of SPMA variation in this large multi-ethnic cohort. While previous studies have observed an effect of *GSTT1* on SPMA levels [9], ours was by far the largest reported to date.

We are not aware of any reports in the literature on the catalysis of BO conjugation with GSH by specific glutathione *S*-transferases (GSTs). Formation of *S*-phenylglutathione (SPG) is the requisite step in the detoxification of BO and excretion of SPMA *in vivo*, as illustrated in Scheme 1. Thus SPMA excretion reflects exposure to benzene, its activation to BO, and the detoxification of BO. Other GSTs may also catalyze this conjugation, but the relative importance of each isozyme has not been reported. The enzymes investigated in this work are GSTA1, GSTT1, GSTM1, and GSTP1.

GSTs are highly polymorphic in the general population. *GSTT1* or *GSTM1* deletion is observed in 9% to 76% of the population, with differences between ethnic groups [10]. *GSTA1* or *GSTP1* deletion has not been reported, but polymorphisms which alter their expression or catalytic activity have been described [11]. The biological consequences of GST polymorphisms on the detoxification of BO have been of interest in occupationally-exposed workers. For workers with occupational exposure to benzene, there is a 2- to 4-fold increased risk of chronic benzene poisoning among *GSTT1*-null individuals [12, 13], and risk of chronic benzene poisoning is weakly correlated with *GSTP1* polymorphisms [14]. These GST isozymes clearly play a role in the detoxification of benzene, but their activity toward BO has not yet been directly evaluated.

Previous reports have been mixed regarding the importance of GST isozymes in the excretion of SPMA [9]. The most consistent correlation between genotype and SPMA excretion has been observed for *GSTT1*. Many but not all studies have demonstrated that the *GSTT1*-null genotype results in lower levels of urinary SPMA, and thus less efficient detoxification of BO [15–18]. Fewer studies have shown a significant effect of the *GSTM1*-null genotype [19–22]. *GSTA1* polymorphisms have not been studied extensively, but a mutation which lowers GSTA1 expression has been shown to affect SPMA excretion [23, 24]. The most studied *GSTP1* polymorphism results in an I105V mutation in the active site, which impacts substrate specificity but in general has not affected the excretion of SPMA [25–28]. The results from our GWAS study encouraged us to directly examine the catalysis of BO detoxification by different GSTs in order to clarify their respective roles.

2. Materials and Methods

CAUTION: Benzene is a known human carcinogen, and BO is its activated form; use extreme care when handling.

2.1. Chemicals and Synthesis

Recombinant human GSTA1, GSTT1, GSTM1, and GSTP1 were purchased from MyBioSource (San Diego, CA, USA). All other reagents were purchased from Sigma-Aldrich. BO was synthesized essentially as described previously [29] in 97% purity (3% phenol).

S-Phenylglutathione was synthesized by adding BO (31 mg, 0.33 mmol) to GSH (111 mg, 0.36 mmol) in a solution of MeOH (4 mL) and 1 N NaOH (1 mL). Previous reports suggest that product yield increases under basic conditions, which should increase the nucleophilicity of the thiol and increase the stability of BO in solution [29, 30]. After 30 min, the pH was adjusted to ~2 with 1 N HCl (1.2 mL) in order to dehydrate the intermediate, yielding SPG. The product was concentrated *in vacuo* and purified by HPLC, resulting in 35.6 mg of SPG as a white powder (0.093 mmol, 28% yield). ^{1}H NMR (500 MHz, D_2O) δ ppm 1.97 (m, 2 H, -$\underline{CH_2}$-CH_2-CO-) 2.23 – 2.37 (m, 2 H, -CH_2-$\underline{CH_2}$-CO-) 3.24 (dd, *J* = 14.5, 8.1 Hz, 1 H, -CH_2-S-) 3.42 (dd, *J* = 14.6, 4.9 Hz, 1 H, -CH_2'-S-) 3.48 (d, *J* = 17.4 Hz, 1 H, -ND-CH_2-COO) 3.54 (d, *J* = 17.4 Hz, 1 H, -ND-CH_2'-COO) 3.61 (m, 1 H, D_2N-CH-COO) 4.47 (dd, *J* = 8.1, 4.7 Hz, 1 H, -$\underline{CH}$-CH_2-S-) 7.23 – 7.29 (m, 1 H, -S-Ph) 7.32 (m, 2 H, S-Ph) 7.41 (m, 2 H, S-Ph). ^{13}C NMR (126 MHz, D_2O) δ ppm 26.3, 31.4, 34.9, 43.3, 53.3, 54.2, 127.5, 129.4 (2 C), 130.8 (2 C), 133.4, 171.4, 174.8, 176.1, 181.5. COSY and HSQC spectra were consistent with the above assignments (Supplemental Data S1).

2.2. Benzene Oxide Stability Studies

The half-life of BO in 0.1 M phosphate buffer (pH 7.4) at 37 °C was measured on a Cary 100 spectrophotometer (Agilent Technologies) based on the absorbance at 315 nm (Supplemental Data S2). Additionally, BO stability was investigated by ^{1}H NMR on an Ascend 500 MHz spectrometer (Bruker Corporation) in various organic solvents over time under ambient light. BO was stored neat (20 °C, < 1 day), in MeOH (< 1 day, 20 °C), in Et_2O (4 °C, > 60 days), in $CDCl_3$ (−20 °C, > 75 days), or in dioxane (4 to 20 °C, > 90 days, as 1% or 10% BO solutions).

2.3. Enzyme Incubation Conditions

All buffers were prepared on the same day as the assay. The incubation buffer (pH 7.4) was prepared by combining solutions of 0.1 M K_2HPO_4 (80 mL) and KH_2PO_4 (20 mL). A stock solution of GSH (10 mM) was also prepared in phosphate buffer and adjusted to pH 7.4. The incubations were performed using a rapid quench-flow instrument (model RQF-3, KinTek Corporation). BO was stored at 4 °C as 1% or 10% solutions in dioxane, and this was added to phosphate buffer (1 mL final volume) immediately before the reaction such that the organic solvent was ≤ 1% of the volume. Final concentrations of BO were 50 μM, 100 μM, 500 μM, 1 mM, and 5 mM. Final concentration of GSH was 5 mM, and 1.7 mU of enzyme was added to each incubation. The final volume of the reaction was 34 μL. We did not have

an isotopically labelled standard of SPG, so SPMA (0.4 pmol) and [D_5]SPMA (3 pmol) were added as internal standards to account for variation in sample mixing, recovery, and detection. The quench-flow instrument was adjusted to 25 °C or 37 °C using a heated water recirculating bath (Isotemp 1016S, Fisher Scientific). The enzymes were pre-incubated with GSH before introducing BO. The incubation time was 7 s, and the reaction was quenched with trichloroacetic acid (TCA). The collected product mixture was desalted and purified by solid-phase extraction (Strata-X, 30 mg, Phenomenex), concentrated *in vacuo* to dryness, and reconstituted in 20 μL 10% MeOH in H_2O. Analyses at 25 °C were performed in four replicates (Supplemental Data S3) and analyses at 37 °C were performed in triplicate.

The amount of enzyme added was based on the reported GSH conjugation activity with a model substrate. The specific activity of GSTA1 and GSTP1 with 1-chloro-2,4-dinitrobenzene (CDNB) was 6.5 U/mg, and the specific activity of GSTM1 with CDNB was 50 U/mg. GSTT1 does not catalyze GSH conjugation with CDNB, so *p*-nitrobenzyl chloride was used, yielding 29.4 U/mg reported specific activity. To achieve 1.7 mU activity, the masses of GSTA1, GSTT1, GSTM1, and GSTP1 added were 0.26, 0.057, 0.034, and 0.26 μg, respectively.

2.4. Analysis of S-Phenylglutathione

The samples were analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The chromatography was carried out on a Synergi Polar-RP column (150 × 0.5 mm, 4 μm, 80 Å, Phenomenex) under isocratic conditions (65% A, 35% B, 15 μL/min, 8 μL injection volume) where A was 0.1% aq. HCOOH and B was MeOH. The MS conditions were as follows: the ionization source was positive-mode electrospray ionization, the selected reaction monitoring transition (1.0 amu isolation width, 35 V collision energy) for SPG was *m/z* 384.1 $[M + H]^+ \rightarrow$109.0 $[PhS]^+$, which was the major fragment observed. SPMA and [D_5]SPMA fragmented similarly, with *m/z* transitions of 240.1 → 109.0 for SPMA and 245.1 → 114.1 for [D_5]SPMA. The analyte was quantified by a linear calibration curve applying the peak area ratio of SPG to [D_5]SPMA and normalizing to the amount of [D_5]SPMA standard added. The limit of detection was assessed by dilution of a standard solution of SPG in water until the signal-to-noise ratio was approximately 3:1. The limit of detection for this method is approximately 2 fmol on-column. The calibration curve was linear between 2 fmol and 1040 fmol on-column.

2.5. Calculations

Nonenzymatic SPG formation was determined by incubating GSH with BO in the absence of enzyme. Enzymatic product formation was then determined by subtracting the nonenzymatic SPG formation from the total product formation. The resulting data were analyzed by nonlinear regression using Microsoft Excel [31].

3. Results

3.1 Benzene Oxide Stability Studies

BO is unstable in its pure form and in protic solvents; it rapidly rearranges to phenol and other products. Under our incubation conditions (37 °C, pH 7.4 phosphate buffer), the

degradation to phenol followed first-order kinetics, with a half-life of 5.2 min (rate constant = 0.133 min^{-1}). This is comparable to what has been reported previously in aqueous media [29]. BO also degrades rapidly in MeOH (< 1 day, 20 °C). However, BO is stable when diluted in aprotic solvents. No degradation was observed in the first 2–3 months when BO was stored in Et_2O, $CDCl_3$, or dioxane. The solution in dioxane was reevaluated by ^1H NMR (500 MHz, $CDCl_3$) again after 7 months of storage at 4 °C. The NMR spectrum showed 53% degradation to phenol and 2% degradation to another product, which was likely muconaldehyde, resulting from spontaneous oxidation of oxepin [32, 33]. Observed chemical shifts of phenol were δ ppm 7.2 (2 H), 6.9 (1 H), and 6.8 (2 H), and those of muconaldehyde were 10.3 (1 H), 9.7 (1 H), and 7.5 (2 H), with the signal from the remaining protons masked by phenol or BO; 6.3 (2 H), 5.9 (2 H), and 5.1 ppm (2 H). The observed degradation to phenol may have been catalyzed by the presence of water as the solution absorbed moisture over time, which was observed by NMR.

3.2 SPG Formation is Catalyzed by GSTs

The initial reaction product of BO with GSH is a hydroxycyclohexadiene species, which can be dehydrated to *S*-phenylglutathione (SPG), restoring aromaticity (Scheme 1). Previous studies have shown that this intermediate is relatively stable under neutral conditions, but readily dehydrates when exposed to acid [29, 30]. The rearrangement of BO to phenol is also catalyzed by acid. Thus the TCA quench serves three purposes: to denature the enzymes, to catalyze the dehydration of the intermediate, and to catalyze the degradation of BO to phenol.

Both GSTT1 and GSTP1 catalyzed the conjugation of BO with GSH with reproducible Michaelis-Menten kinetics (Figure 1). GSTT1 had a lower putative K_m than GSTP1 (Table 1), however, GSTP1 had a higher V_{max}. The amount of enzyme added was determined by reported GSH conjugation activity with CDNB for GSTP1 or *p*-nitrobenzyl chloride for GSTT1 (1.7 mU each), not by concentration of enzyme. For this reason, direct comparison of enzyme efficiencies should be made with caution and we decided not to express activity normalized to mg protein. We also verified that SPG formation is linear with respect to time and enzyme concentration for both GSTT1 and GSTP1 under the conditions used here. The concentration of BO remained approximately constant during the 7 s reaction; we estimate that < 2% degraded to phenol and < 1% reacted to form SPG based on the half-life of BO and the product formation.

GSTM1 and GSTA1 showed minimal activity in conjugating BO with GSH (Figure 1). In these reactions, nonenzymatic product formation accounted for > 80% of total product formation, whereas in the GSTT1 and GSTP1 incubations, nonenzymatic product formation accounted for only 20–40% of the total. After accounting for nonenzymatic SPG formation, the remaining enzymatic formation of SPG by GSTM1 and GSTA1 was small or not detected, which made calculation of kinetic parameters impossible for these two enzymes.

The incubations were also performed at 25 °C. As expected, the enzymatic activity was lower at the lower temperature. The K_m and V_{max} values for GSTT1 were 1100 μM and 360 fmol/s, respectively, and those for GSTP1 were 6300 μM and 3300 fmol/s at 25 °C. Similar to the 37 °C incubations, kinetic parameters for GSTM1 and GSTA1 could not be

determined at 25 °C. The spontaneous product formation was also lower at 25 °C, and it accounted for a smaller percentage of the total product formation.

4. Discussion

All human tissues express GSTs, but each tissue has a unique expression profile of GST isozymes [34]. Both the expression and the activity of each isozyme will impact the relative importance of GSTs in BO detoxification. Benzene is oxidized to BO primarily by P450 2E1 in the liver, where GSTA1 is highly expressed, GSTP1 is minimally expressed, and GSTT1 and GSTM1 expression levels are intermediate [35–38]. Benzene can also be oxidized to BO by CYP2F1 in the lung, where GSTP1 expression predominates [35, 39]. The half-life of BO is 5–6 min, which affords sufficient time for BO to leave the site of oxidation and enter the blood stream, thus GSTs that are more widely expressed may still play an important role in BO detoxification. The kidney, for example, may be an important site of metabolism because it expresses high levels of GSTT1 and plays a critical role in further metabolism of glutathione conjugates to mercapturic acids [37, 40].

The *in vitro* study of BO presented a unique set of challenges. The primary challenge is that BO is unstable in aqueous solution. The incubation time must then be restricted to only a few seconds, or else the concentration of BO will decrease over the course of the incubation. To perform 7-second incubations, a rapid quench flow instrument was required. However, this instrument restricted the total incubation volume to only 34 μL. Due to the short incubation time and the small reaction volume, total SPG formation was below the limit of detection (~2 fmol on-column) by LC-MS/MS when concentrations of BO were below 50 μM. Finally, the spontaneous reaction between BO and GSH must be assessed and subtracted from the total SPG formation, which introduced more variability between replicates in the calculations of enzymatic SPG formation.

The concentrations of BO used here were necessary to enable detection of SPG, but the physiological concentrations of BO are significantly lower. In a study performed in rats, a single dose of 400 mg/kg benzene resulted in only 90 nM BO circulating in the blood, although intracellular BO concentrations could not be assessed [41]. Humans are exposed to lower concentrations of benzene, so it is likely that the GSTs investigated here will not be saturated by the concentration of BO *in vivo*. At the lowest concentration of BO, rates of SPG formation were comparable for GSTT1 (29 ± 14 fmol/s) and GSTP1 (38 ± 9 fmol/s) at 37 °C, and rates were low for GSTA1 and GSTM1 (< 1 fmol/s). This suggests that, at physiologically relevant doses of benzene, GSTT1 and GSTP1 may both play major roles in the detoxification of BO.

Our results for GSTT1 are consistent with previous studies relating genetic polymorphisms to chronic benzene poisoning and SPMA excretion in humans exposed to benzene. GSTT1 had the lowest K_m value of the isozymes tested here. The data obtained from our GWAS study show that *GSTT1* deletion is strongly correlated with SPMA excretion ($P = 6.0 \times 10^{-107}$) and accounts for 21% of total variation in urinary SPMA. Taken together, the data strongly suggest that GSTT1 is an important enzyme in catalyzing the detoxification of BO by conjugation with GSH. We also analyzed the importance of *GSTM1* copy number in our

GWAS study. Our results show that deletion of *GSTM1* accounted for only 1.5% of variation in urinary SPMA, which is consistent with these *in vitro* data that suggest a less important role for GSTM1 in the enzymatic detoxification of BO.

Polymorphism data for *GSTP1* suggest a lesser impact on SPMA formation *in vivo* and seem to have only a weak correlation with risk of chronic benzene poisoning [14]. However, a *GSTP1*-null genotype has not been identified, so the majority of studies investigate the correlation between SPMA excretion and an I105V point mutation in the active site of GSTP1. It is not known if this mutation will affect the activity of GSTP1 in conjugating BO to GSH. The *in vitro* biochemical characterization is necessary to evaluate GSTP1 activity, as it is more difficult to observe the importance of GSTP1 in humans, where the vast majority if not all individuals express functional enzyme.

Another way to assess the importance of GSTP1 *in vivo* is by inducing enzyme expression. In a recent phase II clinical trial in humans, subjects were administered a beverage derived from broccoli sprouts containing a high concentration of sulforaphane [42]. Sulforaphane is a potent inducer of the Nrf2 pathway, which results in the upregulation of a number of detoxification enzymes, including the GSTP family [43]. The results of this trial demonstrated that treatment with sulforaphane significantly increased the detoxification of BO, reflected by increased excretion of SPMA. The subjects were also genotyped for *GSTT1* and *GSTM1* deletion, but the increase in SPMA excretion was observed in all groups receiving sulforaphane treatment, regardless of *GSTT1* or *GSTM1* status [42]. We believe that upregulation of GSTP1 expression was responsible for the enhanced detoxification of BO observed in these individuals.

5. Conclusion

GSTT1 and GSTP1 catalyze BO conjugation with GSH at a higher rate than either GSTM1 or GSTA1 *in vitro* and are likely more important in the BO detoxification process *in vivo*. Significant catalytic activity was not detected for GSTM1 or GSTA1 *in vitro* due to competing nonenzymatic product formation and lower enzymatic activity. This is the first study to determine the kinetic parameters of GST conjugation of BO, a critical intermediate in benzene carcinogenesis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Bob Carlson for editorial assistance. We thank the laboratories of Natalia Tretyakova and Lisa Peterson for partially furnishing equipment used in this study. This work was supported by the U.S. National Institutes of Health, National Cancer Institute [Grant CA- 138338].

Abbreviations

BO	benzene oxide

GSTs	glutathione *S*-transferases
GSTA1	glutathione *S*-transferase alpha 1
GSTM1	glutathione *S*-transferase mu 1
GSTP1	glutathione *S*-transferase pi 1
GSTT1	glutathione *S*-transferase theta 1
SPMA	*S*-phenylmercapturic acid
SPG	*S*-phenylglutathione

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Highlights

- Benzene oxide detoxification is catalyzed by glutathione *S*-transferases.
- GSTT1 and GSTP1 catalyzed the conjugation of benzene oxide with glutathione.
- GSTM1 and GSTA1 did not catalyze the conjugation of benzene oxide with glutathione.

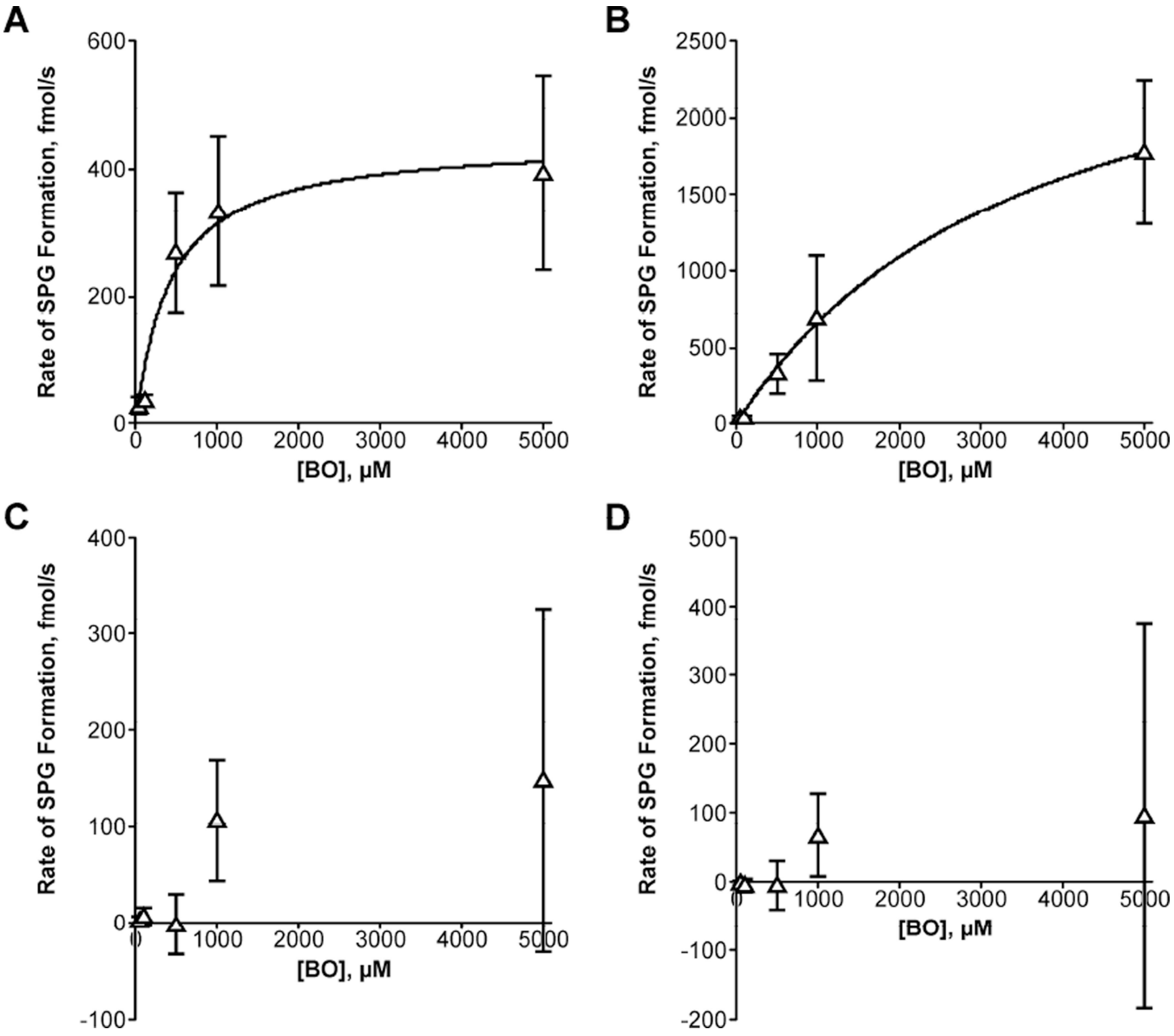
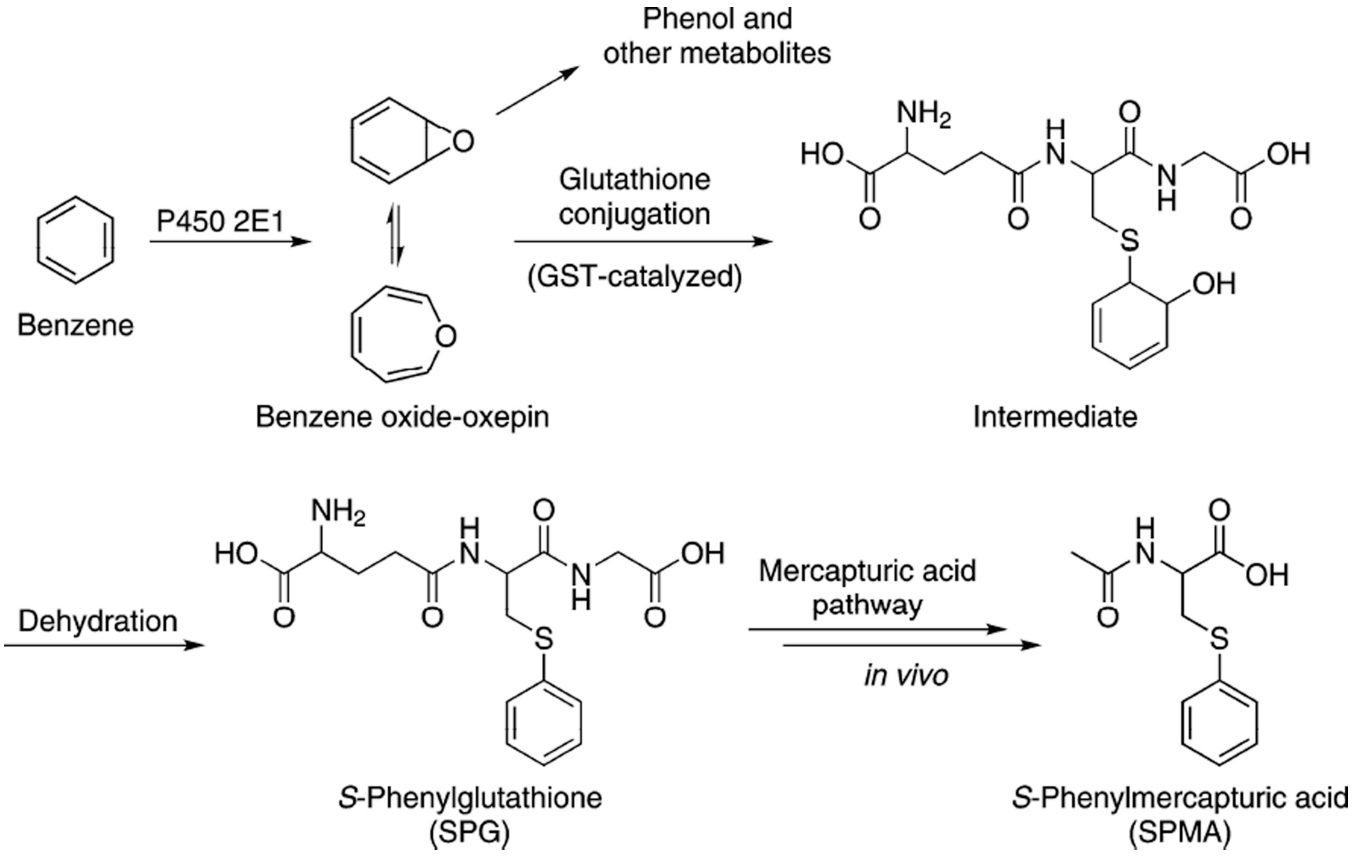


Figure 1.

Rate of SPG formation as a function of BO concentration. Experimental data are shown for five BO concentrations at 37 °C with (A) GSTT1, (B) GSTP1, (C) GSTM1, and (D) GSTA1. Data points and error bars represent the mean ± standard deviation. Calculated best-fit curves from nonlinear regression analyses for GSTT1 and GSTP1 are overlaid. Concentration of enzyme was normalized to 1.7 mU activity toward a model substrate.



Scheme 1.

Metabolism of benzene *in vivo* resulting in the excretion of SPMA in the urine.

Table 1

Kinetic parameters for GSTT1 and GSTP1

	K_m (μM)	V_{max} (fmol/s)
GSTT1	420	450
GSTP1	3600	3100

Incubations were performed at 37 °C with 1.7 mU (0.057 μg) GSTT1 and 1.7 mU (0.26 μg) GSTP1