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Structure-toxicity relationship study of *para*-halogenated styrene analogues in CYP2E1 transgenic cells

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Abstract

Styrene is one of the most important industrial intermediates consumed in the world and is mainly used as a monomer for reinforced plastics and rubber. Styrene has been found to be hepatotoxic and pneumotoxic in humans and experimental animals. The toxicity of styrene is suggested to be metabolism-dependent. Styrene-7,8-oxide has been considered as the major metabolite responsible for styrene-induced cytotoxicity. The objective of the study was to investigate the correlation between cytotoxicity of styrene and chemical and biochemical properties of the vinyl group of styrene by development of structure activity relationships (SAR). 4-Fluorostyrene, 4-chlorostyrene and 4-bromostyrene were selected for the SAR study. Cytotoxicity of styrene and the halogenated styrene derivatives with an order of 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene ≈ styrene was observed in CYP2E1 transgenic cells. Similar orders in the efficiency of the metabolism of styrene and the halogenated styrene analogues to their oxides and in the electrophilicity of the corresponding oxides were observed. Additionally, the order of the potency of cellular glutathione depletion and the degree of protein adduction induced by styrene and the halogenated styrenes were consistent with that of their cytotoxicities. The wild-type cells were less susceptible to the toxicity of the corresponding model compounds than CYP2E1 cells. The present study provided insight into the roles of the biochemical and chemical properties of styrene in its cytotoxicity.

Keywords

Styrene; Styrene Oxide; Bioactivation; Protein Adduct

1. Introduction

Styrene is a high production volume chemical with around 20-30 million tons produced each year to make products such as rubber, plastic, fiberglass, pipes, automobile parts and food

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Conflict of interests

The authors declare that there are no conflicts of interests.

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containers. Occupational exposures to styrene occur mainly in the reinforced plastic industry (IARC, 1994; IARC, 2002; Leibman, 1995; Scott and Preston, 1994). Human exposure to styrene may also result from cigarette smoke, engine exhausts, heating systems, newly installed carpets, and painting (Hodgson *et al.*, 1993; Teixeira *et al.*, 2010; Huff *et al.*, 2011). Styrene has also been detected in the foods and drinking water stored in polystyrene containers (Fleming-Jones and Smith, 2003; Tang *et al.*, 2000). Animal studies showed that intraperitoneal administration of styrene in mice caused elevated activities of γ -glutamyltranspeptidase (GGT) and lactate dehydrogenase (LDH) in bronchoalveolar lavage fluid (BALF) (Carlson, 1997; Gadberry *et al.*, 1996). Multifocal necrosis and cell loss in bronchiolar epithelium were observed in CD-1 mice after exposure to 40 or 160 ppm inhaled styrene (Green *et al.*, 2001). Upon repeated exposures, there was decreased cytoplasmic staining of Clara cells and cell crowding in the terminal bronchioles (Cruzan *et al.*, 1997). Studies in CD-1 and B6C3F1 mice consistently showed cell crowding, decreased staining, and increased cell replication in the Clara cells of the mouse bronchiolar epithelium (Cruzan *et al.*, 1997; Green *et al.*, 2001). Green *et al.* reported increased proliferation in Clara cells after mice were administered orally with styrene (Green *et al.*, 2001). Recently, Harvilchuck *et al.* found elevated reactive oxygen species in Clara cells after exposure to styrene (Harvilchuck *et al.*, 2009).

Styrene is primarily metabolized by P450 isozymes such as CYP2F2 and CYP2E1 (Guengerich *et al.*, 1991; Nakajima *et al.*, 1994; Carlson, 1997; Green *et al.*, 2001; Cruzan *et al.*, 2012) to form a chemically active metabolite, styrene-7,8-oxide (**5**, Scheme 1), which has been suggested to be responsible for styrene toxicity in mice (Bond, 1989). Styrene oxide as an electrophilic species can react with the nucleophilic sites of biomolecules to form adducts. Various DNA adducts have been identified in humans exposed to styrene (Maki-Paakkanen *et al.*, 1991). Styrene was found to induce glutathione depletion in both the liver and lung (Carlson *et al.*, 2006). Styrene oxide derived albumin and hemoglobin modifications were detected in workers exposed to styrene occupationally (Christakopoulos *et al.*, 1993; Fustinoni *et al.*, 1998; Johanson *et al.*, 2000; Osterman-Golkar *et al.*, 1995; Vodicka *et al.*, 1999; Vodicka *et al.*, 2001; Vodicka *et al.*, 2003), and the protein modifications correlated with the levels of styrene exposure (Vodicka *et al.*, 1999). Lanosa *et al.* found a close correlation between metabolic activation of styrene and the sensory irritation response to styrene (Lanosa *et al.*, 2010). Recently, our group reported cellular protein modification by styrene oxide in cultured micro-dissected mouse airways and cells after exposure to styrene (Yuan *et al.*, 2010). Mörbt *et al.* also identified modified thioredoxin reductase by styrene oxide in cultured A297 cells incubated with styrene (Mörbt *et al.*, 2009).

The objectives of the present study were to develop structure toxicity relationships of selected halogenated styrenes and to investigate the role of the vinyl group of styrene in cytotoxicity induced by styrene. This allowed us to better understand the biochemical mechanisms of pulmonary toxicity induced by styrene.

2. Materials and methods

2.1 Materials

Styrene (**1**, 99%), 4-fluorostyrene (**2**, 99%), 4-chlorostyrene (**3**, 97%), 4-bromostyrene (**4**, 98%), styrene oxide (**5**, 98%, racemic), 4-fluorostyrene oxide (**6**, 99%), 4-chlorostyrene oxide (**7**, 98%), and 4-bromostyrene oxide (**8**, 98%), trifluoroacetic acid, CDCl_3 , dimethylformamide (DMF), *N,O*-bis(trimethylsilyl)trifluoroacetamide, cysteamine, β -nicotinamide adenine dinucleotide phosphate (NADPH), and glutathione (GSH) were purchased from Sigma Chemical Co. (St. Louis, MO). Ethyl acetate, anhydrous Na_2SO_4 , HPLC-grade acetonitrile and secureseal were obtained from Fisher Scientific Co.

(Pittsburgh, PA). DNase kit and RNase I were from Qiagen (Valencia, CA). ^{14}C -Styrene (β - ^{14}C), ^{14}C -4-fluorostyrene (β - ^{14}C), and ^{14}C -4-bromostyrene (β - ^{14}C) were custom synthesized by American Radiolabeled Chemical, Inc., (St. Louis, MO) with chemical purity of 99+% and radioactive purity of 99+%. CellTiter 96 Aqueous One solution kit was from Promega (Sunnyvale, CA). Trypan Blue stain was purchased from Bio Whittaker (Walkersville, MD). Transgenic cell line expressing CYP2E1 (h2E1) and the wild-type cell line (cHo1, human B-lymphoblastoid) were obtained from BD-Gentest (Palo Alto, CA). The cell lines were used in our earlier styrene toxicity studies (Chung *et al.*, 2006; Yuan *et al.*, 2010). Recombinant human CYP2E1 was purchased from BD-Gentest (Palo Alto, CA).

2.2 Synthesis of styrene, 4-fluorostyrene, 4-chlorostyrene, and 4-bromostyrene oxide-derived glycols (9-12)

The individual oxides (100 mg each) were mixed with 20 mL of 1.0 N HCl containing 40% acetonitrile, followed by stirring at room temperature overnight. The organic solvent was evaporated by a rotary evaporator, and the remaining aqueous was extracted with acetyl acetate (15 mL $\times$ 3). The organic layers were combined, dried over anhydrous Na_2SO_4 , and evaporated to dryness in vacuum. The residues were chromatographed in a silica gel column to give the desired compounds. Styrene glycol (**9**): ^1H -NMR (300 MHz, CDCl_3) δ 3.48-2.82 (br, 2H, OH), 3.65 (dd, J = 8.4 and 11.5 Hz, 1H, CH_2), 3.74 (dd, J = 3.4 and 11.5 Hz, 1H, CH_2), 4.81 (dd, J = 3.4 and 8.4 Hz, 1H, CH), 7.52-7.18 (m, 5H, Ar). 4-Fluorostyrene glycol (**10**): ^1H -NMR (300 MHz, CDCl_3) δ 3.36-2.68 (br, 2H, OH), 3.65 (dd, J = 8.3 and 11.4 Hz, 1H, CH_2), 3.75 (dd, J = 3.4 and 11.4 Hz, 1H, CH_2), 4.82 (dd, J = 3.4 and 8.3 Hz, 1H, CH), 7.07 (dt, J = 2 and 8.5 Hz, 2H, Ar), 7.35 (dd, J = 5.4 and 8.5 Hz, 2H, Ar). 4-Chlorostyrene glycol (**11**): ^1H -NMR (300 MHz, CDCl_3) δ 3.22-2.62 (br, 2H, OH), 3.64 (dd, J = 8.2 and 11.2 Hz, 1H, CH_2), 3.76 (dd, J = 3.2 and 11.2 Hz, 1H, CH_2), 4.82 (dd, J = 3.2 and 8.2 Hz, 1H, CH), 7.32 (d, J = 8.4 Hz, 2H, Ar), 7.36 (d, J = 8.4 Hz, 2H, Ar). 4-Bromostyrene glycol (**12**): ^1H -NMR (300 MHz, CDCl_3) δ 3.32-2.74 (br, 2H, OH), 3.63 (dd, J = 8.2 and 11.4 Hz, 1H, CH_2), 3.77 (dd, J = 3.4 and 11.4 Hz, 1H, CH_2), 4.81 (dd, J = 3.4 and 8.2 Hz, 1H, CH), 7.27 (d, J = 8.4 Hz, 2H, Ar), 7.51 (d, J = 8.4 Hz, 2H, Ar).

2.3 Synthesis of styrene, 4-fluorostyrene, 4-chlorostyrene, and 4-bromostyrene oxide-derived cysteamine adducts (13-20)

General procedure: the oxides (5 mmol) were individually mixed with cysteamine (7.5 mmol) in 10 mL of acetonitrile-water solution (6 : 1) containing triethylamine (10 mmol). The mixtures were stirred at room temperature under an atmosphere of nitrogen for 36 h. The resulting reaction mixtures were diluted with ethyl acetate (30 mL) and then washed with water (10 mL $\times$ 3). The organic layers were combined, dried over anhydrous Na_2SO_4 , and evaporated under vacuum. The residues were subjected to column chromatography on silica gel to afford the following cysteamine adducts.

The reaction of styrene oxide with cysteamine gave a mixture of 2-(2-aminoethylthio)-2-phenylethanol and 2-(2-aminoethylthio)-1-phenylethanol (**13** and **17**, approximate 1:1) as light yellow oil (197 mg, 20% in yield). ^1H -NMR (300 MHz, CDCl_3): δ 2.48-3.04 (m, 6H + 8H), 3.80-3.90 (m, 2H), 3.96 (dd, J = 5.7, 7.9 Hz, 1H), 4.78 (dd, J = 3.6, 9 Hz, 1H), 5.29 (brs, 2H), 7.20-7.45 (m, 5H $\times$ 2); m/z = 198.2 $[\text{M}+\text{H}]^+$.

The reaction of 4-fluorostyrene oxide with cysteamine gave a mixture of 2-(2-aminoethylthio)-2-(4-fluorophenyl)ethanol and 2-(2-aminoethylthio)-1-(4-fluorophenyl)ethanol (**14** and **18**, approximate 1:1) as light yellow oil (194 mg, 18%). ^1H -NMR (300 MHz, CDCl_3): δ 2.45-3.05 (m, 6H + 8H), 3.78-3.90 (m, 2H), 3.95 (dd, J = 6, 7.2 Hz, 1H), 4.78 (dd, J = 3.48, 8.9 Hz, 1H), 5.30 (brs, 2H), 6.94-7.14 (m, 2H $\times$ 2), 7.24-7.42 (m, 2H $\times$ 2); m/z = 216.3 $[\text{M}+\text{H}]^+$.

The reaction of 4-chlorostyrene oxide with cysteamine gave a mixture of 2-(2-aminoethylthio)-2-(4-chlorophenyl)ethanol and 2-(2-aminoethylthio)-1-(4-chlorophenyl)ethanol (**15** and **19**, approximate 1:1) as light yellow oil (150 mg, 13% in yield). ¹H-NMR (300 MHz, CDCl₃): δ 2.50-3.16 (m, 6H + 8H), 3.72-4.06 (m, 2H), 4.12 (dd, *J* = 7.1, 14Hz, 1H), 4.78 (dd, *J* = 3.4, 8.8Hz, 1H), 5.30 (brs, 2H), 7.18-7.42 (m, 4H × 2); *m/z* = 232.1 [M+H]⁺.

The reaction of 4-bromostyrene oxide with cysteamine gave a mixture of 2-(2-aminoethylthio)-2-(4-bromophenyl)ethanol and 2-(2-aminoethylthio)-1-(4-bromophenyl)ethanol (**16** and **20**, approximate 1:1) as light yellow oil (206 mg, 16% in yield). ¹H-NMR (300 MHz, CDCl₃): δ 2.50-3.00 (m, 6H + 8H), 3.79-3.87 (m, 2H), 3.92 (dd, *J* = 5.6, 7.6Hz, 1H), 4.75 (dd, *J* = 3.5, 9.0Hz, 1H), 5.30 (brs, 2H), 7.14-7.36 (m, 2H × 2), 7.40-7.56 (m, 2H × 2); *m/z* = 276.0 [M+H]⁺.

2.4 Instrumentation

The HPLC system consisted of an Agilent 1100 LC binary pump system and an Agilent 1100 Autosampler (Palo Alto, CA). The HPLC system was interfaced with a PE Sciex API 2000 LC/MS/MS System including an electrospray ionization source and a triple quadrupole mass analyzer (Applied Biosystems, Foster City, CA). Data were gathered by software Analyst 1.2. Microplate readers VERSA_{Max} and GeminiXS (Molecular Devices, Sunnyvale, CA) were utilized for cell viability studies and protein assays.

2.5 Cell culture

Parental and h2E1 cells were cultured in RPMI 1640 supplemented with L-glutamine and 10% fetal bovine serum (Hyclone Laboratory, Logan, UT) under 95% air/5% CO₂ at 37 °C, according to the protocol our lab published earlier (Chung *et al.*, 2006; Yuan *et al.*, 2010).

2.6 Cytotoxicity study in vitro

Before treatment, cells were counted with Trypan Blue staining by use of a hemocytometer to reach a density of 2×10^5 cells/mL. Styrene or halogenated styrenes (100 μM) dissolved in DMF were added to 24-well microplates containing CYP2E1 or wild-type cells. The final concentration of the organic solvent was < 0.5%. The resulting plates were sealed with a secureseal to prevent possible evaporation of the chemicals tested. After 24 h incubation, cell viability was determined by CellTiter 96 Aqueous One solution assay.

2.7 Cellular protein covalent binding

Parental and CYP2E1 cells were cultured in 10-cm Petri dishes at a density of 5×10^5 cells/mL (10 mL per dish). The cells were incubated with ¹⁴C-labeled styrene, 4-fluorostyrene, or 4-bromostyrene (chemical concentration: 100 μM; specific radioactivity: 6.25 Ci/mol). After 24 h incubation, the cells were washed three times with PBS (pH 7.4), lysed with 500 μL of lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% Triton) at room temperature for 15 min, and centrifuged at 19,000 *g* for 20 min. The supernatant was collected and incubated with one unit of DNase and RNase I at room temperature for 20 min, followed by extensive dialysis (MWCO: 3.5KDa, Fisher brand) against PBS buffer with 0.1% SDS at room temperature until the radioactivity in the dialysis solution reached the background. An aliquot of 200 μL of the dialyzed sample was mixed with 5 ml of scintillation cocktail solution and the mixture was subjected to radioactivity assessment using a scintillation counter. Protein assay was performed using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Bovine serum albumin was used as a protein standard.

2.8 SDS-PAGE and radioactivity imaging

Protein concentrations of the dialyzed protein samples were measured using bicinchoninic acid protein kit (Pierce Chemical, Rockford, IL), and equal amounts (20 μ g) of protein were loaded and resolved by SDS-polyacrylamide gel electrophoresis using pre-cast 10% Tris-glycine gels (Invitrogen, Carlsbad, CA). The gel was dried in a gel dryer (Bio-Rad, Hercules, CA) at 80 °C for 1 h. And the dried gel was exposed to a tritium storage phosphor screen (GE Healthcare, Piscataway, NJ) for 7 days. The exposed screen was scanned by a Typhoon Trio imager (GE Healthcare, Piscataway, NJ).

2.9 Bioactivation by recombinant human CYP2E1

Enzyme kinetic studies were performed following the protocol established in our lab (Shen *et al.*, 2010). Briefly, a 0.5 mL solution containing recombinant human CYP2E1 (50 pmol), 5 mM $MgCl_2$, and 2 mM NADPH in 50 mM phosphate buffer (pH 7.4) was preincubated in a 37 °C water bath shaker for 3 min. The reaction was initiated by addition of 5.0 μ L of various concentrations of styrene, 4-fluorostyrene, 4-chlorostyrene or 4-bromostyrene (final concentration: 0.1–100 μ M). The incubation continued for 20 min and was quenched by addition of the same volume of ethyl acetate, followed by spiking with deuterated styrene glycol (styrene glycol- d_8 , prepared in our lab) as internal standard (Shen *et al.*, 2010). The resulting mixture was vortexed and the organic phase was collected. The collected extract was dried by blowing with N_2 , and the remaining was silylated by *N,O*-bis(trimethylsilyl)trifluoroacetamide and subjected to GC/MS analysis. The production of the resulting glycols was quantified by comparing their peak areas *vs.* that of the internal standard. V_{max} and K_m values were calculated by non-linear least-squares regression analysis using SigmaPlot software.

2.10 Reactivity of styrene oxide and halogenated styrene oxides to cysteamine

Styrene oxide, 4-fluorostyrene oxide, 4-chlorostyrene oxide and 4-bromostyrene oxide (1.0 μ M) were individually incubated with cysteamine (100 μ M) in phosphate buffer in a 37 °C water bath. A 100 μ L aliquot of the reaction mixture was withdrawn at 1, 3, 7, 15, 30 and 60 min and was immediately mixed with 100 μ L of 1 N HCl, followed by LC/MS analysis. An LC/MS scan operated in a positive and selective ion monitoring (SIM) mode was acquired. The ions with *m/z* 198, 216, 232, and 276 were monitored, and these ions were responsible for the respective $[M+H]^+$ of the corresponding cysteamine adducts. The LC conditions and mass spectrometer parameters were applied as follows: twenty microliter samples were injected into a C_{18} column (3 μ , 2.1×100 mm, Alltech, Deerfield, IL); the samples were eluted from the column with a gradient of solvent A (0.1% aqueous trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid in acetonitrile) at a flow rate of 200 μ L/min, and the flow was directed to the mass spectrometer. Mass spectrometer operating conditions: positive ionization mode, ionization voltage at 5kV, orifice potential at 30 volts, and ion source temperature at 250 °C.

2.11 Cellular GSH contents

CYP2E1 cells were cultured in 6-well plates at a density of 1×10^6 cells/mL. The cells were incubated with styrene, 4-fluorostyrene, or 4-bromostyrene (100 μ M) and harvested at various time points. Cellular GSH were assessed using an ApoGSHTM glutathione detection kit (BioVision, CA). The assay was performed based on the protocol provided by the manufacturer. In brief, the harvested cells were resuspended in 100 μ L cell lysis buffer. After 15 min incubation at room temperature, the supernatants were collected and transferred to a test tube containing 2 μ L of 25 mM monochlorobimane and 2 μ L of the 50 U/mL glutathione *S*-transferase solution. After incubating for 30 min at 37 °C, fluorescence at Ex/Em = 380/460 nm was measured using a GeminiXS fluorescence plate reader.

2.12 Statistics

All values were presented as mean $\pm$ SE. Analysis of Variance (ANOVA) with post hoc pairwise comparison of the means using a Tukey test for multiple comparisons ($p < 0.01$) was performed using PRISM (GraphPad Software, Inc., La Jolla, CA).

3. Results

3.1 Cytotoxicity of styrene and halogenated styrenes

Styrene, 4-fluorostyrene, 4-chlorostyrene, and 4-bromostyrene were selected for the structure-toxicity relationship study, and their toxicities were assessed in CYP2E1 transgenic cells following the procedure we applied early (Chung *et al.*, 2006). Cells were incubated with 100 μ M styrene or the halogenated styrenes. Cell viability was determined after 24 h incubation by CellTiter 96 Aqueous One solution assay. Relative to the group treated with vehicle, 4-chlorostyrene and 4-bromostyrene induced 57.7 % and 39.6 % decreased in cell viability, respectively. Comparing with styrene-treated group, 4-fluorostyrene showed little difference in toxicity to CYP2E1 cells.

Susceptibility of the wild-type cells to styrene and the halogenated styrenes was also evaluated at concentration of 100 μ M. As expected, the wild-type cells were found to be less susceptible to styrene and the corresponding halogenated styrenes than CYP2E1 transgenic cells (Figure 1).

3.2 Protein covalent binding

Cellular protein covalent binding levels were measured by assessment of radioactivity bound to cellular proteins. To minimize the cost of protein covalent binding study, we selected 4-fluorostyrene and 4-bromostyrene to represent halogenated styrenes, and we had 14 C-styrene, 14 C-4-fluorostyrene, and 14 C-4-bromostyrene custom synthesized. CYP2E1 cells were exposed to 100 μ M the individual 14 C-labeled compounds for 24 h. Figure 2 shows the radioactivity bound to proteins obtained from cells after exposure to the individual 14 C-labeled styrenes. The binding load was 2.02, 2.27 and 6.17 nmol/mg protein for styrene, 4-fluorostyrene and 4-bromostyrene, respectively (Table 1). In parallel, the wild-type cells were incubated with individual 14 C-labeled styrene, 4-fluorostyrene and 4-bromostyrene at 100 μ M for 24 h. Less radioactivity bound to cellular proteins was observed in the wild-type cells relative to that in CYP2E1 cells after exposure to the individual radioactive styrenes (Figure 2). Particularly, 5-fold lower radioactivity was bound to proteins in the wild-type cells than CYP2E1 cells treated with 14 C-labeled 4-bromostyrene.

In addition, the radioactive protein samples were resolved by SDS-PAGE, and radioactivity of the resolved proteins was imaged. As expected, much more radioactivity was bound to proteins obtained from cells treated with 4-bromostyrene than that of cells after treatment with styrene or 4-fluorostyrene (Figure 3). In addition, similar patterns in radioactive bands were observed in the gel loaded with protein samples obtained from cells after exposure to radioactive styrene, 4-fluorostyrene or 4-bromostyrene.

3.3 Bioactivation by recombinant CYP2E1

Bioactivation of styrene and the halogenated styrenes to the corresponding oxides was evaluated in a recombinant human CYP2E1 system. The halogenated styrenes were exclusively metabolized to oxides **6-8**, and no phenols were detected. The resulting kinetic parameters were calculated and summarized in Table 2. 4-Bromostyrene and 4-chlorostyrene showed the lowest K_m value, followed by 4-fluorostyrene and styrene. The same observation was found in the order of V_{max} values for 4-bromostyrene, 4-

chlorostyrene, 4-fluorostyrene and styrene. Values of $V_{\max}/K_m$ were calculated and found to follow the order of 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene $\approx$ styrene (Table 1).

3.4 Reactivity to cysteamine

Thio groups (-SH) have been suggested to be mainly responsible for the nucleophilic attacks by styrene oxide in protein covalent modification (Rappaport *et al.*, 1993). To evaluate the potentials of protein adduction by the oxides derived from styrene and the halogenated styrenes, we measured the reactivities of the oxides toward cysteamine. The formation of the cysteamine adducts was monitored by LC/MS at various time points. Authentic cysteamine adducts derived from the oxides were synthesized and characterized. The reaction of cysteamine with styrene oxide and the halogenated styrene oxides produced two regioisomers of the corresponding adducts at a ratio of approximate 1:1 estimated by their $^1\text{H-NMR}$ spectra (Scheme 1). The two regioisomers were eluted out as a single peak in chromatography of LC/MS and could not be separated from each other. The formation of the two regioisomers was assessed by integration of the peak areas responsible for the two isomers of the cysteamine adducts. A time-dependent formation of the corresponding adducts was observed, and the production of the adducts reached a plateau in 15 min. The kinetic data were processed and reaction rate constants were calculated, using XLfit of an Exponential Association Model. The rate constants for production of the cysteamine adducts derived from 4-bromostyrene oxide, 4-chlorostyrene oxide, 4-fluorostyrene oxide and styrene oxide were 29.3 ± 3.19 , 16.1 ± 2.08 , 15.7 ± 1.57 and 13.4 ± 1.88 (min^{-1}), respectively (Table 1).

3.5 Glutathione depletion

Depletion of cellular GSH has often been considered as an important biomarker of early stage cell injury by many electrophilic toxicants. Styrene has been reported to induce GSH depletion *in vitro* and *in vivo* (Carlson *et al.*, 2006; Gamer *et al.*, 2004; Harvilchuck and Carlson, 2008; Turner *et al.*, 2005). We compared the potencies of GSH depletion induced by styrene and the halogenated styrenes. Cellular GSH contents of CYP2E1 cells were monitored at 0, 3, 6, 12, and 24 h after exposure to 100 μM styrene or the halogenated styrenes. As expected, the three halogenated styrenes along with styrene induced GSH depletion (Figure 4). The GSH contents reached the corresponding lowest levels at 3 h, then bounced back, and recovered at 12 h. The group treated with 4-bromostyrene showed the greatest degree in GSH depletion, followed by 4-chlorostyrene and 4-fluorostyrene. Styrene showed similar potency of GSH depletion as 4-fluorostyrene.

4. Discussion

The biochemical mechanisms of styrene-induced cytotoxicity remain unknown. As part of our mechanistic study, we developed structure-activity (toxicity) relationships of selected halogenated styrene analogues. The hypothesis for the study was that cytotoxicities induced by styrene derivatives with similar metabolic pathway are associated with their biochemical and chemical properties. The rationale of the study includes that halogens belong to group 17 of the periodic table and share similar chemical characteristics but with different reactivity. We reasoned that the halogenated styrene analogues would undergo a similar metabolic activation through cytochrome P450 oxidation at different rates. We chose styrene, 4-fluorostyrene, 4-chlorostyrene and 4-bromostyrene for our structure-toxicity relationship study and found that 4-bromostyrene was the most toxic to CYP2E1 cells, followed by 4-chlorostyrene and 4-fluorostyrene. Styrene showed a similar degree of toxicity as 4-fluorostyrene (Figure 1 and Table 1). The results indicate that the cytotoxicity of the halogenated styrene compounds increases with the increase in the periodic number of the halogens substituted at *para* position of styrene. Actually, we chemically synthesized 4-

iodostyrene and observed its cytotoxicity (more toxic than 4-bromostyrene) in CYP2E1 cells. Unfortunately, observed unstableness of 4-iodostyrene made us to give up its further investigation.

Styrene-induced cytotoxicity has been suggested to require metabolic activation. To determine whether the epoxidation of the vinyl group is a key step to initiate styrene toxicity, we determined $V_{\max}/K_m$ of enzymatic epoxidation of styrene and the three halogenated styrene derivatives in a recombinant human CYP2E1. Parameter $V_{\max}/K_m$ represents the catalytic efficiency of a biochemical reaction. The order of $V_{\max}/K_m$ of styrene and the three halogenated styrene derivatives in CYP2E1 was found to be 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene $\approx$ styrene (Table 1), which matches the order of the cytotoxicities induced by the four compounds (Figure 1 and Table 1). It can explain that higher turnover ($V_{\max}/K_m$) of bioactivation of the halogenated styrenes produces more reactive metabolites responsible for their cytotoxicities.

The electrophilicity of electrophilic species is a determining factor in S_N1 (unimolecular nucleophilic substitution) and S_N2 (bimolecular nucleophilic substitution) reactions. We hypothesize that the electrophilicity of oxides derived from styrene and the styrene analogues is another important element for their cytotoxicities. We evaluated the electrophilicities of styrene oxide, 4-fluorostyrene oxide, 4-chlorostyrene oxide and 4-bromostyrene oxide by determining the rates of cysteamine adductions with the corresponding oxides. The order of the electrophilicities of the oxides was 4-bromostyrene oxide > 4-chlorostyrene oxide > 4-fluorostyrene oxide $\approx$ styrene oxide (Table 1). Interestingly, the order of electrophilicities of the oxides was also consistent to that of cytotoxicities induced by the corresponding parent styrene analogues. This implicates a possible correlation between the electrophilicities of the oxides and the cytotoxicities induced by styrene and the halogenated styrenes.

Taken together, two factors, *i.e.* the total amount of the oxides generated and the electrophilicity of the oxides, appear to correlate with the cytotoxicities of styrene and the three halogenated styrene derivatives. Here we introduce a new parameter, namely bioactivation-reactivity (BR) index, which combines the efficiency of reactive metabolite formation with the electrophilicity of the reactive metabolite. This parameter is presented by the value of $k \times V_{\max}/K_m$, and this provides a platform to compare those metabolism-dependent electrophilic species. Parameter k stands for the rate of a reaction of an electrophilic metabolite with a nucleophilic agent. Bioactivation-reactivity indexes of 4-bromostyrene, 4-chlorostyrene, 4-fluorostyrene and styrene were calculated and are listed in Table 1. The order of the BR indexes was 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene $\approx$ styrene, which matches the order of cytotoxicities induced by these compounds.

As an electrophilic species, styrene oxide has been reported to react with nucleophilic biomolecules, such as glutathione (GSH) (Carlson *et al.*, 2006; Gamer *et al.*, 2004; Harvilchuck and Carlson, 2008; Turner *et al.*, 2005), proteins (Christakopoulos *et al.*, 1993; Fustinoni *et al.*, 1998; Johanson *et al.*, 2000; Osterman-Golkar *et al.*, 2000; Vodicka *et al.*, 1999; Vodicka *et al.*, 2001; Vodicka *et al.*, 2003) and nucleic acids (Maki-Paakkanen *et al.*, 1991). Turner *et al.* observed the decrease in GSH in the liver and lung as early as one hour after styrene administration in a time- and dose-dependent manner (Turner *et al.*, 2005). A time-course of GSH depletion was observed in CYP2E1 cells after exposure to styrene and the three halogenated styrene derivatives (Figure 4). 4-Bromostyrene was found to deplete cellular GSH the greatest, followed by 4-chlorostyrene and 4-fluorostyrene/styrene. The order in potency of GSH depletion by styrene and the halogenated styrene analogues matches that of their cytotoxicities as well as their BR indexes (Table 1).

Adduction of albumin and hemoglobin by styrene oxide has been well documented (Christakopoulos *et al.*, 1993; Fustinoni *et al.*, 1998; Johanson *et al.*, 2000; Osterman-Golkar *et al.*, 2000; Vodicka *et al.*, 1999; Vodicka *et al.*, 2001; Vodicka *et al.*, 2003). Recently, our lab reported cellular protein adduction derived by styrene oxide in cultured tissues and cells after exposure to styrene (Yuan *et al.*, 2010). In the present study, we compared the radioactivity bound to cellular proteins obtained from CYP2E1 cells after incubation with ^{14}C -styrene, ^{14}C -4-fluorostyrene or ^{14}C -4-bromostyrene. Both total radioactivity tracing (Figure 2) and electrophoresis-based radioactivity imaging (Figure 3) showed similar order in degree of protein adduction induced by the individual radioactive styrene and halogenated styrene derivatives with those of their bioactivations (BR index), GSH depletion and cytotoxicity. The consistent patterns observed support our hypothesis that the cytotoxicity induced by styrene derivatives, which share a similar bioactivation process correlates their metabolic and biochemical identities. The “amplifying” effect of *para*-substitution of chlorine and bromine, along with the structure-toxicity relationship developed, provides us a unique approach for the mechanistic studies of styrene-induced cytotoxicity. Additionally, it appears that similar cellular proteins were modified by reactive metabolites of styrene, 4-fluorostyrene and 4-bromostyrene (Figure 3). This opens new avenues for investigation of the role of protein modification in styrene-induced cytotoxicity.

Halogens are known electron-withdrawing groups, and the inductive effects of fluorine, chlorine and bromine substituted on styrene may cause decreased electron density of the π -systems, which disfavors the oxidation of styrene. However, the unshared *p*-electrons of halogens can also enhance the aromatic electron density by *p*- π delocalization (Sykes, 1986). It is likely that the effectiveness of the substituted halogen atoms participating in the electron delocalization plays a favorable role in the observed rates of bioactivation of the halogenated styrenes. Besides, the *para*-substituted halogens can alter the reactivity of the epoxide group of styrene oxide through the *p*- π electron delocalization that stabilizes the developing cation of the epoxides of the halogenated styrenes (Scheme 2). We anticipate that the capability of the electron delocalization plays a significant role in the observed reactivity (electrophilicity) of the oxides to cysteamine. The elevated capacity of the electron delocalization increases the probability for chemical reactions of the oxides with cellular biomolecules, which possibly triggers the process of their cytotoxicities.

CYP2E1 has been reported to be one of cytochrome P450 enzymes responsible for the metabolic activation of styrene (Guengerich *et al.*, 1991; Nakajima *et al.*, 1994). Our earlier study demonstrated that CYP2E1 cells are more susceptible to styrene than the wild-type cells (Chung *et al.*, 2006). Similar observation was obtained in CYP2E1 and wild-type cell lines after exposure to the halogenated styrenes (Figure 1). This implicates that the halogenated styrenes were metabolically activated by similar enzyme system.

The halogenated styrenes were used in the present study as model compounds to probe the correlation between metabolic activation of styrene and its cytotoxicity. Little is known about metabolisms and cytotoxicities of the halogenated styrenes. The present metabolism study demonstrated that the three halogenated styrenes were exclusively biotransformed to the corresponding oxides (**6-8**) by CYP2E1. The results showed the consistence with our original hypothesis that the halogenated styrenes undergo similar bioactivation pathway(s) as that of styrene.

In conclusion, the structure-toxicity relationship study showed that *para*-substituted halogenated styrene analogues induced cytotoxicity in an order of 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene and that styrene was as toxic as 4-fluorostyrene. CYP2E1 participated in the bioactivation and cytotoxicity of the halogenated styrenes in CYP2E1 abundant cells. The cytotoxicities induced by styrene and the halogenated styrene analogues

were proportional to the efficiency of their bioactivation to oxides, electrophilicity of the oxides, potency of cellular GSH depletion, and degree of protein adduction. The results support our hypothesis that cytotoxicities induced by latent toxicants with similar metabolic pathways correlate with their biochemical and chemical properties. The novel parameter bioactivation-reactivity index we introduced provides a useful platform for the comparison of latent toxicants (metabolic activation required).

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References

1. Bond JA. Review of the toxicology of styrene. *Crit Rev Toxicol*. 1989; 19:227–249. [PubMed: 2653733]
2. Carlson GP. Comparison of mouse strains for susceptibility to styrene-induced hepatotoxicity and pneumotoxicity. *J Toxicol Environ Health*. 1997; 51:177–187. [PubMed: 9176557]
3. Carlson GP. Effects of inducers and inhibitors on the microsomal metabolism of styrene to styrene oxide in mice. *J Toxicol Environ Health*. 1997; 51:477–488. [PubMed: 9233381]
4. Carlson GP, Turner M, Mantick NA. Effects of styrene and styrene oxide on glutathione-related antioxidant enzymes. *Toxicology*. 2006; 227:217–226. [PubMed: 16971035]
5. Christakopoulos A, Bergmark E, Zorcec V, Norppa H, Maki-Paakkanen J, Osterman-Golkar S. Monitoring occupational exposure to styrene from hemoglobin adducts and metabolites in blood. *Scand J Work Environ Health*. 1993; 19:255–263. [PubMed: 8235514]
6. Chung J-K, Yuan W, Liu G, Zheng J. Investigation of styrene toxicity in CYP2E1 transgenic cells. *Toxicology*. 2006; 226:99–106. [PubMed: 16872732]
7. Cruzan G, Cushman JR, Andrews LS, Granville GC, Miller RR, Hardy CJ, Coombs DW, Mullins PA. Subchronic inhalation studies of styrene in CD rats and CD-1 mice. *Fundam Appl Toxicol*. 1997; 35:152–165. [PubMed: 9038236]
8. Cruzan G, Bus J, Hotchkiss J, Harkema J, Banton M, Sarang S. CYP2F2-generated metabolites, not styrene oxide, are a key event mediating the mode of action of styrene-induced mouse lung tumors. *Regul Toxicol Pharmacol*. 2012; 62:214–220. [PubMed: 22041433]
9. Fleming-Jones ME, Smith RE. Volatile organic compounds in foods: A five-year study. *J Agric Food Chem*. 2003; 51:8120–8217. [PubMed: 14690406]
10. Fustinoni S, Colosio C, Colombi A, Lastrucci L, Yeowell-O'Connell K, Rappaport SM. Albumin and hemoglobin adducts as biomarkers of exposure to styrene in fiberglass-reinforced-plastics workers. *Int Arch Occup Environ Health*. 1998; 71:35–41. [PubMed: 9523247]
11. Gadberry MG, DeNicola DB, Carlson GP. Pneumotoxicity and hepatotoxicity of styrene and styrene oxide. *J Toxicol Environ Health*. 1996; 48:273–294. [PubMed: 8656450]
12. Gamer AO, Leibold E, Deckardt K, Kittel B, Kaufmann W, Tennekens HA, van Ravenzwaay B. The effects of styrene on lung cells in female mice and rats. *Food Chem Toxicol*. 2004; 42:1655–1667. [PubMed: 15304312]
13. Green T, Toghiani A, Foster J. The role of cytochromes P-450 in styrene induced pulmonary toxicity and carcinogenicity. *Toxicology*. 2001; 169:107–117. [PubMed: 11718952]
14. Guengerich FP, Kim D-H, Iwasaki M. Role of human cytochrome P-450IIE1 in the oxidation of many low molecular weight cancer suspects. *Chem Res Toxicol*. 1991; 4:168–179. [PubMed: 1664256]
15. Harvilchuck JA, Carlson GP. Comparison of styrene and its metabolites styrene oxide and 4-vinylphenol on cytotoxicity and glutathione depletion in Clara cells of mice and rats. *Toxicol Lett*. 2008; 183:28–35. [PubMed: 18926891]
16. Harvilchuck JA, Pu X, Klaunig JE, Carlson GP. Indicators of oxidative stress and apoptosis in mouse whole lung and Clara cells following exposure to styrene and its metabolites. *Toxicology*. 2009; 264:171–178. [PubMed: 19666080]

17. Hodgson AT, Wooley JD, Daisey JM. Emissions of volatile organic compounds from new carpets measured in a large-scale environmental chamber. *Air Waste*. 1993; 43:316–324. [PubMed: 8457318]
18. Huff J, Infante PF. Styrene exposure and risk of cancer. *Mutagenesis*. 2011; 26:583–884. [PubMed: 21724974]
19. IARC. Some industrial chemicals. *IARC Monogr Eval Carcinog Risks Hum*. 1994; 60:233–246. [PubMed: 7869572]
20. IARC. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene. *IARC Monogr Eval Carcinog Risks Hum*. 2002; 82:437–522.
21. Johanson G, Ernstgard L, Gullstrand E, Lof A, Osterman-Golkar S, Williams CC, Sumner SC. Styrene oxide in blood, hemoglobin adducts, and urinary metabolites in human volunteers exposed to (13)C(8)-styrene vapors. *Toxicol Appl Pharmacol*. 2000; 168:36–49. [PubMed: 11000098]
22. Lanosa MJ, Willis DN, Jordt S, Morris JB. Role of metabolic activation and the TRPA1 receptor in the sensory irritation response to styrene and naphthalene. *Toxicol Sci*. 2010; 115:589–595. [PubMed: 20176620]
23. Leibman KC. Metabolism and toxicity of styrene. *Environ Health Perspect*. 1975; 11:115–119. [PubMed: 809262]
24. Maki-Paakkanen J, Waller S, Osterman-Golkar S, Norppa H. Single-strand breaks, chromosome aberrations, sister-chromatid exchanges, and micronuclei in blood lymphocytes of workers exposed to styrene during the production of reinforced plastics. *Environ Mol Mutagen*. 1991; 17:27–31. [PubMed: 1991456]
25. Mörbt N, Mögel I, Kalkhof S, Feltens R, Röder-Stolinski C, Zheng J, Vogt C, Lehmann I, von Bergen M. Proteome changes in human bronchoalveolar cells following styrene exposure indicate involvement of oxidative stress in the molecular-response mechanism. *Proteomics*. 2009; 9:4920–4933. [PubMed: 19862763]
26. Nakajima T, Elovaara E, Gonzalez FJ, Gelboin HV, Raunio H, Pelkonen O, Vainio H, Aoyama T. Styrene metabolism by cDNA-expressed human hepatic and pulmonary cytochromes P450. *Chem Res Toxicol*. 1994; 7:891–896. [PubMed: 7696548]
27. Osterman-Golkar S, Christakopoulos A, Zorcec V, Svensson K. Dosimetry of styrene 7,8-oxide in styrene- and styrene oxide-exposed mice and rats by quantification of haemoglobin adducts. *Chem Biol Interact*. 1995; 95:79–87. [PubMed: 7697755]
28. Rappaport SM, Ting D, Jin Z, Yeowell-O'connell K, Waidyanatha S, McDonald T. Application of Raney nickel to measure adducts of styrene oxide with hemoglobin and albumin. *Chem Res Toxicol*. 1993; 6:238–244. [PubMed: 7682851]
29. Scott D, Preston RJ. A critical review of the cytogenetic effects of styrene with an emphasis on human population monitoring: a synopsis. *Crit Rev Toxicol*. 1994; 24(Suppl):S47–8. [PubMed: 7818771]
30. Shen S, Zheng F, Gao L, Zeng S, Zheng J. Detection of phenolic metabolites of styrene in mouse microsomal incubations. *Drug Metab Disp*. 2010; 38:1934–1943.
31. Sykes, P. Electrophilic and nucleophilic substitution in aromatic system. In: Sykes, P., editor. *A guidebook to mechanism in organic chemistry*. Longman Singapore Publishers Pte Ltd; Singapore: 1986. p. 130-177.
32. Tang W, Hemm I, Eisenbrand G. Estimation of human exposure to styrene and ethylbenzene. *Toxicology*. 2000; 144:39–50. [PubMed: 10781869]
33. Teixeira JP, Gaspar J, Coelho P, Costa C, Pinho-Silva S, Costa S, Da Silva S, Laffon B, Pásaro E, Rueff J, Farmer P. Cytogenetic and DNA damage on workers exposed to styrene. *Mutagenesis*. 2010; 25:617–621. [PubMed: 20729469]
34. Turner M, Mantick NA, Carlson GP. Comparison of the depletion of glutathione in mouse liver and lung following administration of styrene and its metabolites styrene oxide and 4-vinylphenol. *Toxicology*. 2005; 206:383–388. [PubMed: 15588928]
35. Vodicka P, Koskinen M, Stetina R, Soucek P, Vodickova L, Matousu Z, Kuricova M, Hemminki K. The role of various biomarkers in the evaluation of styrene genotoxicity. *Cancer Detect Prev*. 2003; 27:275–284. [PubMed: 12893075]

36. Vodicka P, Soucek P, Tates AD, Dusinska M, Sarmanova J, Zamecnikova M, Vodickova L, Koskinen M, de Zwart FA, Natarajan AT, Hemminki K. Association between genetic polymorphisms and biomarkers in styrene-exposed workers. *Mutat Res.* 2001; 482:89–103. [PubMed: 11535253]
37. Vodicka P, Tvrdik T, Osterman-Golkar S, Vodickova L, Peterkova K, Soucek P, Sarmanova J, Farmer PB, Granath F, Lambert B, Hemminki K. An evaluation of styrene genotoxicity using several biomarkers in a 3-year follow-up study of hand-lamination workers. *Mutat Res.* 1999; 445:205–224. [PubMed: 10575431]
- Yuan W, Jin H, Chung J-K, Zheng J. Evidence for cellular protein covalent binding derived from styrene metabolite. *Chem Biol Interact.* 2010; 186:323–330. [PubMed: 20470765]

Highlights

- We investigated the structure-toxicity relationship of halogenated styrenes.
- Cytotoxicity order: 4-bromostyrene > 4-chlorostyrene > 4-fluorostyrene $\approx$ styrene.
- Bioactivation of the styrenes follows the same order of their toxicities.
- GSH depletion and protein covalent binding are consistent with their toxicities.
- The results indicate bioactivation of styrene is correlated with its cytotoxicity.

Figure 1. CYP2E1 and wild-type cells were exposed to vehicle, styrene (ST), 4-fluorostyrene (F-ST), 4-chlorostyrene (Cl-ST) or 4-bromostyrene (Br-ST) at concentration of 100 μ M at 37 $^{\circ}$ C for 24 h. Cell viability was assessed by use of CellTiter 96 Aqueous One solution assay. Control: cells were treated with vehicle. (* indicates significant difference from the styrene group, $p < 0.01$, means $\pm$ SE, $n = 4$)

Figure 2. CYP2E1 and wild-type cells were incubated with ^{14}C -labeled styrene (ST), 4-fluorostyrene (F-ST) or 4-bromostyrene (Br-ST) at concentration of $100\ \mu\text{M}$ at $37\ ^\circ\text{C}$ for 24 h. Cellular proteins were harvested, extensively dialyzed against PBS buffer with 0.1% SDS, and subjected to radioactivity assessment and protein assay (* indicates significant difference from the styrene group, $p < 0.01$, means $\pm$ SE, $n = 4$)

Figure 3.

CYP2E1 cells were incubated with ^{14}C -labeled styrene (ST), 4-fluorostyrene (F-ST) or 4-bromostyrene (Br-ST) at concentration of $100\ \mu\text{M}$ at $37\ ^\circ\text{C}$ for 24 h. Cellular proteins were harvested, extensively dialyzed, and subjected to SDS-polyacrylamide gel electrophoresis. Radioactive protein bands were detected by exposure of gel to a phosphor screen.

Figure 4. CYP2E1 cells were incubated with styrene (ST), 4-fluorostyrene (F-ST), 4-chlorostyrene (Cl-ST) or 4-bromostyrene (Br-ST) at concentration of 100 μ M. Cellular GSH contents were determined at designed time points of exposure. Control: cells were treated with vehicle as 100% for the corresponding time points (* indicates significant difference from styrene or styrene oxide, $p < 0.01$, means $\pm$ SE, $n = 4$).

Scheme 1.

Scheme 2.

Table 1

Summary of cytotoxicity in human CYP2E1 cells and biochemical and chemical properties of styrene (ST), 4-fluorostyrene (F-ST), 4-chlorostyrene (Cl-ST) and 4-bromostyrene (Br-ST) in a recombinant human CYP2E1 system, and of the reactivity of the corresponding oxides to cysteamine.

	Cell viability (% of control after 24 h exposure)	$V_{\max}/K_m$ (mL/min/mg)	Reactivity of oxides to cysteamine (k , min ⁻¹)	Bioactivation index ($k \times V_{\max}/K_m$)	GSH depletion (% of control after 3 h exposure)
ST	80.0 ± 3.0 ^a	0.62 ± 0.03 ^a	13.4 ± 1.88 ^a	8.30 ± 0.57 ^a	83.7 ± 2.1 ^a
F-ST	78.8 ± 3.2 ^a	0.66 ± 0.05 ^a	15.7 ± 1.57 ^a	10.4 ± 0.64 ^a	82.4 ± 2.2 ^a
Cl-ST	57.7 ± 9.9 ^b	0.98 ± 0.04 ^b	16.1 ± 2.08 ^a	15.8 ± 2.03 ^b	71.2 ± 5.8 ^b
Br-ST	39.6 ± 4.1 ^c	1.09 ± 0.05 ^b	29.3 ± 3.19 ^b	31.9 ± 3.48 ^c	61.0 ± 6.5 ^b

Control: vehicle-treated.

Values are expressed as mean ± SD, n = 4.

^a, ^b, ^c values with different superscripts are significantly different from one another (p<0.05).

Table 2

Parameters K_m and V_{max} of metabolism of styrene (ST), 4-fluorostyrene (F-ST), 4-chlorostyrene (Cl-ST) and 4-bromostyrene (Br-ST) to the corresponding oxides in a recombinant human CYP2E1 system.

	ST	F-ST	Cl-ST	Br-ST
K_m (μ M)	56.1 ± 4.26^a	54.2 ± 5.06^a	39.4 ± 1.44^b	39.0 ± 3.78^b
V_{max} (pmol/min/mg)	34.7 ± 1.77^a	36.3 ± 1.94^a	38.5 ± 0.97^a	42.6 ± 3.18^b

Values are expressed as mean + SD.

^{a,b} values with different superscripts are significantly different from one another ($p < 0.05$), $n = 4$.