Characterization of Cytosolic Glutathione S-Transferases Involved in the Metabolism of the Aromatase Inhibitor, Exemestane^S

Irina Teslenko, Christy J.W. Watson, Zuping Xia, Gang Chen, and Philip Lazarus*

Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, Washington

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ABSTRACT

Exemestane (EXE) is a hormonal therapy used to treat estrogen receptor-positive breast cancer by inhibiting the final step of estrogen biosynthesis catalyzed by the enzyme aromatase. Cysteine conjugates of EXE and its active metabolite 17 β -dihydro-EXE (DHE) are the major metabolites found in both the urine and plasma of patients taking EXE. The initial step in cysteine conjugate formation is glutathione conjugation catalyzed by the glutathione S-transferase (GST) family of enzymes. The goal of the present study was to identify cytosolic hepatic GSTs active in the GST-mediated metabolism of EXE and 17 β -DHE. Twelve recombinant cytosolic hepatic GSTs were screened for their activity against EXE and 17 β -DHE, and glutathionylated EXE and 17 β -DHE conjugates were detected by ultra-performance liquid chromatography tandem mass spectrometry. GST α (GSTA) isoform 1, GST μ (GSTM) isoform 3 and isoform 1 were active against EXE, whereas only GSTA1 exhibited activity against 17 β -DHE. GSTM1 exhibited the highest affinity against EXE with a Michaelis-Menten constant (K_M) value that was 3.8- and 7.1-fold lower than that observed for GSTA1 and GSTM3, respectively. Of the three GSTs, GSTM3 exhibited the highest intrinsic clearance against

EXE (intrinsic clearance = 0.14 nl·min⁻¹·mg⁻¹). The K_M values observed for human liver cytosol against EXE (46 μ M) and 17 β -DHE (77 μ M) were similar to those observed for recombinant GSTA1 (53 and 30 μ M, respectively). Western blot analysis revealed that GSTA1 and GSTM1 composed 4.3% and 0.57%, respectively, of total protein in human liver cytosol; GSTM3 was not detected. These data suggest that GSTA1 is the major hepatic cytosolic enzyme involved in the clearance of EXE and its major active metabolite, 17 β -DHE.

SIGNIFICANCE STATEMENT

Most previous studies related to the metabolism of the aromatase inhibitor exemestane (EXE) have focused mainly on phase I metabolic pathways and the glucuronidation phase II metabolic pathway. However, recent studies have indicated that glutathionylation is the major metabolic pathway for EXE. The present study is the first to characterize hepatic glutathione S-transferase (GST) activity against EXE and 17 β -dihydro-EXE and to identify GST α 1 and GST μ 1 as the major cytosolic GSTs involved in the hepatic metabolism of EXE.

Introduction

Breast cancer is the most commonly diagnosed cancer in women and, despite an overall improvement in breast cancer therapy, continues to be their second leading cause of death (American Cancer Society, 2021). More than 75% of diagnosed breast cancers are found to be expressing estrogen receptors (ERs) with estrogen contributing to tumor growth and proliferation (Howlader et al., 1975–2013; Osborne and Schiff, 2011). Hormone therapy is most commonly prescribed for early-stage, ER-positive breast cancer in postmenopausal women, including treatments with selective estrogen receptor modulators or aromatase

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inhibitors [Untch and Thomssen, 2010; Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2015]. The mechanism of action of selective estrogen receptor modulators, including tamoxifen (TAM), is manifested through blocking the ER to prevent the binding of estrogen, whereas aromatase inhibitors, including exemestane (EXE), act through suicide inhibition of the enzyme aromatase and disrupt androgen conversion to estrogen in the final step of estrogen biosynthesis (Miller, 1999; Campos, 2004; Eisen et al., 2008; Patel and Bihani, 2018). When compared with tamoxifen, EXE demonstrates a higher clinical efficacy and safety profile (Kieback et al., 2010; Goss et al., 2011). Moreover, long-term use of EXE demonstrated a 65% reduction in the incidence of invasive breast cancer among high-risk but healthy postmenopausal women (Goss et al., 2011). In addition, unlike that observed for TAM, EXE has not been associated with higher risk for thromboembolism or gynecologic events, such as endometrial cancers, polyps, and fibroids (Coombes et al., 2007; Goss et al., 2011). However, although EXE demonstrates improved treatment efficacy as compared with TAM, the overall response rate of 46% is still relatively low (Paridaens et al., 2003; Paridaens et al., 2008). Additionally, compared with TAM, EXE has

ABBREVIATIONS: CDNB, 1-chloro-2,4-dinitrobenzene; CL_{INT} , intrinsic clearance; DHE, dihydro-EXE; DHE-GS, S-(androsta-1,4-diene-17 β -ol-3-on-6 α -ylmethyl)-L-glutathione; ER, estrogen receptor; ESI, electrospray ionization; EXE, exemestane; EXE-GS, S-(androsta-1,4-diene-3,17-dion-6 α -ylmethyl)-L-glutathione; Gluc, glucuronide; GSH, glutathione; GST, glutathione S-transferase; GSTA, GST α ; GSTK, GST κ ; GSTM, GST μ ; GSTO, GST ω ; GSTP, GST π ; GSTT, GST θ ; GSTZ, GST ζ ; HLC, human liver cytosol; HRMS, high-resolution mass spectrum; K_M , Michaelis-Menten constant; LC-MS, liquid chromatography–mass spectrometry; m/z, mass-to-charge ratio; pTPM, protein-coding transcripts per million; TAM, tamoxifen; UGT, UDP-glucuronosyltransferase; UPLC, ultra-pressure liquid chromatography; UPLC/MS, UPLC–mass spectrometry.

been associated with a higher incidence of hot flashes, musculoskeletal pain, and lower bone mineral density (Coombes et al., 2007).

EXE is extensively metabolized in patients with <1% and <10% remaining as unmetabolized EXE in urine and plasma, respectively (Pfizer, 2018). A major route of EXE metabolism is the formation of 17 β -dihydroexemestane (DHE), an active metabolite catalyzed by cytochrome P450s, aldoketoreductases, and carbonyl reductases (Kamdem et al., 2011; Platt et al., 2016; Peterson et al., 2017). 17 β -DHE is further conjugated through the glucuronidation pathway to form the inactive 17 β -DHE-glucuronide (Gluc) by UDP-glucuronosyltransferase (UGT) 2B17 (Sun et al., 2010; Luo et al., 2017).

Recently, two novel cysteine (Cys) conjugates of EXE and 17 β -DHE were identified: 6 α -EXE-Cys and 6 α -17 β -DHE-Cys, which combined composed 77% and 35% of the total urinary and plasma EXE metabolites, respectively, in subjects taking EXE (Luo et al., 2018). Cysteine conjugates are formed through a three-step metabolic pathway, in which the first step involves conjugation with the tripeptide glutathione (GSH; γ -Glu-Cys-Gly) catalyzed by the glutathione-S-transferase (GST) family of enzymes (Hinchman and Ballatori, 1994; Hayes et al., 2005). The glutathione conjugates are subsequently metabolized by γ -glutamyl transferase to remove the glutamyl moiety and by dipeptidase to remove the glycyl moiety, which ultimately forms the cysteine conjugate that is rapidly excreted. As the first step in this conjugation pathway, GST-mediated glutathionylation is likely a key step in EXE and 17 β -DHE metabolism and excretion (Luo et al., 2018; Hanna and Anders, 2019). The ultimate cysteine conjugates render EXE and DHE more water-soluble and more easily excreted; although it is assumed that these conjugates also render EXE and DHE less active, this has not yet been directly tested.

GSTs are a multifunctional superfamily of enzymes involved in both catalytic and signaling processes. Cytosolic GSTs compose the largest subfamily of GSTs and are traditionally recognized as the major GSTs involved in phase II metabolism (Habig et al., 1974b; Hayes et al., 2005; Jancova et al., 2010). They are largely expressed in human liver and detoxify a variety of endogenous compounds and exogenous xenobiotics, including environmental pollutants, carcinogens, many drugs, and chemotherapeutic agents (Dostalek and Stark, 2012; Hanna and Anders, 2019). GSTs are also highly polymorphic and have been associated with an increased risk for a variety of cancers, variability in drug toxicity, cancer resistance, and altered drug metabolism (Perera et al., 2002; Elhasid et al., 2010; Josephy, 2010; Allocati et al., 2018). The goal of the present study was to identify the hepatic cytosolic GSTs important in the metabolism of EXE and 17 β -DHE.

Materials and Methods

Chemicals and Materials. EXE and 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from Sigma-Aldrich (St. Louis, MO), and 17 β -OH-EXE-d3 (D₃-17 β -DHE) and EXE-19-d3 (D₃-EXE) were purchased from Toronto Research Chemicals (North York, ON, Canada). L-Glutathione (reduced) was obtained from Alfa Aesar (Haverhill, MA). Oasis HLB cartridges (1 cc) were purchased from Waters (Milford, MA). Liquid chromatography–mass spectrometry (LC-MS)–grade formic acid and acetonitrile were obtained from Thermo Fisher Scientific (Waltham, MA), and LC-MS–grade ammonium formate was purchased from Sigma-Aldrich. Hexane, diethyl ether, potassium hydroxide, and methanol were purchased from VWR (Radnor, PA) at American Chemical Society quality or higher. Milli-Q water was used for the preparation of all solutions.

Pooled human liver cytosol (HLC; 50 subjects) was purchased from Xenotech (Kansas City, KS). Recombinant human GST α (GSTA) 1, GSTA4, GST κ (GSTK) 1, GST μ (GSTM) 2, and GSTM4 were obtained from ProSpec Protein Specialists (East Brunswick, NJ), GSTM1 and GSTM3 were purchased from Novus Bio (Centennial, CO), and GSTA2 was purchased from Sigma-Aldrich; all purchased recombinant GSTs were >80% pure as indicated by the

manufacturer. Luria broth was purchased from Invitrogen (Carlsbad, CA), and isopropyl-1-thio- β -D-galactopyranoside and ampicillin were purchased from Sigma-Aldrich. B-PER Complete Protein Extraction Reagent, Ni-NTA affinity purification columns, dextran desalting columns, Pierce BCA protein assay kits, Pierce silver staining kits, SuperSignal West Pico Plus Chemiluminescent Substrate, and BL21 (DE3) and BL21 (DE3)pLysS competent *Escherichia coli* were purchased from Thermo Fisher Scientific. Monoclonal GSTA1 and GSTM1 and polyclonal GSTM3 antibody (mouse Ig) were obtained from Novus Bio (Centennial, CO), and goat anti-mouse IgG secondary antibody (horseradish peroxidase) was purchased from Sigma-Aldrich. All other chemicals were purchased from Thermo Fisher Scientific unless otherwise specified.

Synthesis of EXE and DHE. EXE and 17 β -DHE were synthesized as previously described (Platt et al., 2016). The NMR spectra of the two compounds were recorded on a Bruker Avance I instrument with 500 MHz for proton and 125 MHz for carbon. Chemical shifts were measured based on the residual proton in the NMR deuterium solvent. High-resolution mass spectrum (HRMS) was acquired using a Xevo G2-S QToF mass spectrometer (Waters) with a positive electrospray ionization (ESI). The chemical purity was determined on an Acquity H Class UPLC (Waters) with an ACQUITY UPLC BEH C18 column (2.1 $\times$ 100 mm; Waters) monitored at 254 nm using a photodiode array detector.

Synthesis of S-(Androsta-1,4-diene-3,17-dione-6 α -ylmethyl)-L-glutathione. EXE (11.7 mg, 0.039 mmol) and L-glutathione (33.4 mg, 0.109 mmol) were added to a 10-ml round-bottom flask under the protection of argon, which was followed by the addition of degassed 1.25 N potassium hydroxide (1 ml). The mixture was stirred at 4°C for 24 hours. Water (10 ml) was added to the reaction mixture, which was then washed with dichloromethane (3 $\times$ 8 ml). The pH of the water phase was adjusted to $\sim$ 3 with 2 N HCl, and it was then stored in a refrigerator overnight. The aqueous sample was loaded onto conditioned Oasis HLB 1 cc cartridges (14 aliquots), which was followed by sequential elution with water (1.5 ml) and methanol (1.5 ml). The collected methanol solutions were combined, concentrated, and dried to afford S-(androsta-1,4-diene-3,17-dione-6 α -ylmethyl)-L-glutathione (EXE-GS) (10.6 mg, Y = 45%) as a white solid. ¹H NMR (D₂O) δ 7.33 (d, J = 10.1 Hz, 1 H), 6.19 (dd, J₁ = 10.1, J₂ = 1.8 Hz, 1 H), 6.00 (bs, 1 H), 4.50 (dd, J₁ = 8.8 Hz, J₂ = 5.1 Hz, 1 H), 3.83 (s, 2 H), 3.69 (dd, J₁ = J₂ = 6.4 Hz, 1 H), 3.23 (s, 1 H), 3.00 (dd, J₁ = 14.2 Hz, J₂ = 5.1 Hz, 1 H), 2.87 (dd, J₁ = 12.8 Hz, J₂ = 6.3 Hz, 1 H), 2.81 (dd, J₁ = 14.2 Hz, J₂ = 8.8 Hz, 1 H), 2.69 (m, 1 H), 2.60 (dd, J₁ = 12.8 Hz, J₂ = 6.9 Hz, 1 H), 2.45 (m, 3 H), 2.16–2.20 (m, 1 H), 2.00–2.09 (m, 3 H), 2.18–2.20 (m, 2 H), 1.54–1.75 (m, 4 H), 1.07–1.26 (m, 2 H), 1.19 (s, 3 H), 0.90–0.94 (m 1 H), 0.86 (s, 3 H), 0.74 (dd, J₁ = 24.4 Hz, J₂ = 12.3 Hz, 1 H). ¹³C NMR (D₂O) δ 228.1, 188.9, 174.8, 174.7, 173.8, 173.6, 172.5, 161.5, 125.2, 120.1, 53.8, 53.4, 53.2, 49.6, 48.8, 48.4, 45.0, 41.7, 39.3, 38.5, 35.7, 34.3, 33.8, 31.3, 30.6, 26.1, 22.0, 21.4, 17.7, 13.2. HRMS (ESI) mass-to-charge ratio (m/z) calculated C₃₀H₄₂N₃O₈S [MH]⁺ 604.2687; found 604.2693; purity > 95%.

Synthesis of S-(Androsta-1,4-diene-17 β -ol-3-on-6 α -ylmethyl)-L-Glutathione. S-(androsta-1,4-diene-17 β -ol-3-on-6 α -ylmethyl)-L-glutathione (DHE-GS) was synthesized as described above for EXE-GS using 17 β -DHE (10.6 mg, 0.036 mmol) and L-glutathione (37.3 mg, 0.12 mmol) as the starting materials. DHE-GS (2.5 mg, Y = 11%) was purified as a white solid. ¹H NMR (D₂O) δ 7.35 (d, J = 10.2 Hz, 1 H), 6.20 (d, J = 10.2 Hz, 1 H), 5.99 (s, 1 H), 4.49 (dd, J₁ = 8.8 Hz, J₂ = 5.2 Hz, 1 H), 3.81 (s, 2 H), 3.68 (dd, J₁ = J₂ = 6.4 Hz, 1 H), 3.49 (J₁ = J₂ = 8.5 Hz, 1 H), 2.99 (dd, J₁ = 14.1 Hz, J₂ = 5.0 Hz, 1 H), 2.78–2.88 (m, 2 H), 2.55–2.68 (m, 2 H), 2.44 (m, 2 H), 2.06 (m, 3 H), 1.89 (m, 1 H), 1.62–1.77 (m, 4 H), 1.21–1.49 (m, 3 H), 1.18 (s, 3 H), 0.74–0.96 (m, 3 H), 0.69 (s, 3 H), 0.61 (dd, J₁ = 24.4, J₂ = 12.2, 1 H); ¹³C NMR (D₂O) δ 188.9, 175.5, 174.7, 174.0, 173.6, 172.4, 162.0, 125.1, 119.9, 81.1, 53.9, 53.8, 53.2, 49.4, 45.2, 42.7, 41.9, 39.5, 39.4, 35.9, 34.8, 33.9, 33.8, 31.3, 28.7, 26.1, 22.9, 22.4, 17.8, 10.6. HRMS (ESI) m/z calculated C₃₀H₄₄N₃O₈S [MH]⁺ 606.2844, found 606.2900; purity = 94%.

Biosynthesis of D₃-EXE-GS and D₃-17 β -DHE-GS. D₃-EXE-GS and D₃-17 β -DHE-GS, internal standards for LC-MS analysis of glutathione conjugates of EXE and 17 β -DHE, were generated in an enzymatic reaction containing pooled HLC (200 μ g), 100 mM potassium phosphate (KH₂PO₄) at pH 7.4, and 100 ppm D₃-EXE or 100 ppm D₃-17 β -DHE in a total volume of 200 μ l. The reaction was incubated at 37°C for 3 minutes before the addition of 5 mM GSH (Lash et al., 1999; Zarth et al., 2015; Shi et al., 2016). Final mixtures were incubated for 2 hours at 37°C and stopped with 200 μ l cold acetonitrile. Mixtures were subsequently centrifuged at 16,100 $\times$ g for 10 minutes at 4°C. Supernatants

were collected, and aliquots of 50 μ l were injected onto the ACQUITY UPLC BEH C18 column (2.1 $\times$ 100 mm; Waters). The ultra-pressure liquid chromatography (UPLC) conditions used were as described below. Fractions containing EXE-GS conjugates were collected at UPLC retention times of 1.2–2 minutes, whereas DHE-GS conjugates were collected at 0.8–1.8 minutes.

Recombinant Protein Production. The Human Protein Atlas was queried (April 19, 2018), and 12 cytosolic human GSTs were identified to be hepatically expressed: GSTA1, GSTA2, GSTA4, GSTK1, GSTM1, GSTM2, GSTM3, GSTM4, GST ω (GSTO) 1, GST π (GSTP) 1, GST ζ (GSTZ) 1, and GST θ (GSTT) 1. GSTA1, GSTA4, GSTK1, GSTM2, GSTM4, GSTM1, GSTM3, and GSTA2 were purchased commercially; GSTT1, GSTP1, GSTZ1, and GSTO1 were not found to be commercially available and were cloned and expressed as described below.

In addition to the noncommercially available GSTs (GSTT1, GSTP1, GSTZ1, and GSTO1), the GSTs that exhibited initial activity against EXE and 17 β -DHE (GSTA1, GSTM1, and GSTM3) were also cloned and expressed as codon-optimized, recombinant proteins. All GSTs were cloned with a C-terminal His-tag. Codon-optimized plasmids encoding wild-type human GSTA1, GSTM3, GSTT1, GSTZ1, GSTP1, and GSTO1 were commercially synthesized by GenScript (Piscataway, NJ) in the pET-15b vector and transformed into chemically competent BL21 (DE3) *E. coli*. GSTM1 codon-optimized plasmid was similarly commercially cloned into the pET-15b vector but was transformed into BL21 (DE3)pLysS *E. coli*. Transformed *E. coli* cells were grown under ampicillin selection, and the DNA sequence of cloned genes were confirmed with Sanger sequencing (Genewiz, South Plainfield, NJ). Selected bacterial colonies were grown overnight in 10 ml of LB supplemented with 100 μ g/ml of ampicillin at 37°C in a tabletop shaker (250 rpm). A total of 90 ml of fresh LB medium containing 100 μ g/ml of ampicillin was inoculated with the overnight culture and grown an additional 2.5 hours to reach an optical density value of 600. The expression of GST protein was induced by the addition of isopropyl-1-thio- β -D-galactopyranoside to a final concentration of 0.5 mM. After induction, cells were grown for an additional 4 hours at 37°C and then harvested by centrifugation. The cytosolic fraction was collected after cell lysis with 150 μ l B-PER Complete Bacterial Protein Extraction Reagent and centrifugation per the manufacturer's protocols.

His-tagged GST proteins were purified from the cytosol by nickel affinity chromatography. Ni-NTA resin spin columns were equilibrated with binding buffer containing 50 mM sodium phosphate (pH 8.0), 300 mM NaCl, and 10 mM imidazole. Each cytosolic lysate was mixed with an equal volume of binding buffer, loaded onto the column, and incubated for 20 minutes at 4°C for maximal binding. After incubation, the columns were centrifuged at 700 $\times$ g for 2 minutes, and the flow through was discarded. Columns were subsequently washed with 20, 60, and 100 mM of imidazole in PBS (pH 7.4), and each of the elutions were collected; GSTP1 His-tagged protein was washed with 20, 60, 100, 150, and 200 mM of imidazole. Using dextran desalting columns, the imidazole was removed, and purified proteins were subsequently stored in PBS (pH 7.4) buffer. A total of 125 ng of protein for each fraction was loaded onto 15% SDS-PAGE gel, and all were found to be greater than 90% pure by silver staining (Supplemental Fig. 1). As performed for commercially purchased recombinant GST proteins, the activity of purified recombinant proteins was verified using the CDNB assay described below. Pure recombinant enzymes were stored in 200- μ l aliquots in 25% glycerol at –20°C.

GST Activity Assays. The enzymatic activities of recombinant GSTs were verified using a spectrophotometric assay against CDNB, a known substrate for all cytosolic GSTs (Habig et al., 1974a). Reactions were performed using 1 mM CDNB and 1 mM GSH in 100 mM sodium phosphate buffer at 30°C for 6 minutes. The absorbance of the CDNB-glutathione conjugate product was read at 340 nm (Habig et al., 1974b).

To screen for conjugation activity against EXE and 17 β -DHE, activity assays were performed for the eight commercially purchased GSTs (GSTA1, GSTA4, GSTK1, GSTM2, GSTM4, GSTM1, GSTM3, and GSTA2) and the four non-commercially available GSTs that were cloned as recombinant proteins in our laboratory (GSTT1, GSTP1, GSTO1, and GSTZ1). GST recombinant protein (2.5 ng/ μ l) was mixed with 250 μ M of EXE or 17 β -DHE in a 25- μ l reaction containing 100 mM potassium phosphate. The reaction was preincubated at 37°C for 3 minutes prior to the addition of 5 mM GSH to start the incubation (1 hour, 37°C). Incubations were terminated by adding an aliquot (2.5 μ l) to ice-cold acetonitrile (5 μ l) spiked with 2.5 μ l of D₃-EXE-GS or D₃-17 β -DHE-GS internal standard. After vortexing and centrifuging at 16,100 $\times$ g for 10 minutes

at 4°C, supernatants (10 μ l) were transferred to a glass sample vial containing 10 μ l water. Reactions containing HLC were performed as a positive control, and reactions without enzyme were performed as a negative control.

For enzyme kinetic analysis, EXE-GS formation was examined as described above in reactions incubated for 1 hour at 37°C using 2.5–8.0 ng/ μ l of purified recombinant GSTA1, GSTM1, or GSTM3 protein and varying concentrations of EXE (2–250 μ M). Similar kinetic assays were also performed with pooled HLC (1 μ g/ μ l). 17 β -DHE-GS conjugate formation was examined using recombinant GSTA1 (5 ng/ μ l) or pooled HLC protein (2 μ g/ μ l) as described above for EXE, using 17 β -DHE (2–250 μ M) as the substrate. Reaction mixtures were processed as described above and loaded onto the ultra-pressure liquid chromatography–mass spectrometry (UPLC/MS) to monitor glutathione (GS) conjugate formation. EXE-GS and 17 β -DHE-GS concentrations were quantified using a standard curve generated from a serial dilution of known amounts of chemically synthesized EXE-GS or 17 β -DHE-GS (synthesis described above).

UPLC/MS Analysis of GS Conjugates. EXE-GS and 17 β -DHE-GS conjugate formation was monitored in individual reactions using a UPLC/MS system (Waters) consisting of an Acquity UPLC, Acquity UPLC BEH C18 column (2.1 $\times$ 100 mm), and Xevo G2-S QToF mass spectrometer. The UPLC flow rate was 0.4 ml/min with a column temperature of 35°C. UPLC conditions for EXE-GS separation were as follows: 0.5 minutes at 25%:75% of mobile phase B (100% acetonitrile): mobile phase A (5 mM ammonium formate and 0.01% formic acid), a linear gradient to 100% B in 4 minutes, and 1.5 minutes at 100% B, which was followed by re-equilibrium with 25% B for 2 minutes. The UPLC conditions for DHE-GS separation were 2 minutes at 20% mobile phase B, a linear gradient to 100% B in 4 minutes, and 1.5 minutes at 100% B, which was followed by re-equilibrium with 20% B for 2 minutes. The Waters Xevo G2-S QToF MS was operated in tandem mass spectrometry mode, and the ESI probe operated in the positive-ion mode with a capillary voltage at 0.6 kV. Nitrogen was used as both the cone and desolvation gases with flow rates maintained at 50 and 800 l/h, respectively. The collision energy for EXE-GS and DHE-GS detection was optimized at 25 V with a cone voltage of 30 V. Glutathione conjugate formation was detected using the following mass transitions (m/z): 604.2692→297.184, 607.281→300.203, 606.2849→299.2, and 609.3037→302.203 to monitor EXE-GS, D₃-EXE-GS, 17 β -DHE-GS, and D₃-17 β -DHE-GS, respectively.

Recombinant GSTA1, GSTM1, and GSTM3 Quantification in HLC. Western blot analysis was performed to quantify the amount of GSTA1, GSTM1, and GSTM3 protein present in commercial-pooled HLC. Serial dilutions of HLC (25–6.25 μ g of total protein) and purified recombinant protein (GSTA1 = 1–0.125 μ g, GSTM1 = 0.25–0.03 μ g, or GSTM3 = 1–0.03 μ g) were loaded on three separate 15% SDS-polyacrylamide gels and, after electrophoresis, were transferred to polyvinylidene fluoride) membranes using an iBlot system (Thermo Fisher Scientific). The membranes were blocked overnight at 4°C in blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 50 mM Tris-Cl, pH 7.6; 0.9% NaCl, 0.1% Tween 20), and subsequently washed three times (10 minutes each) with Tris-buffered saline/Tween 20. For GSTA1, GSTM1, and GSTM3, membranes were probed with mouse monoclonal GSTA1 antibody (1:400 dilution), mouse monoclonal GSTM1 antibody (1:1,000 dilution), and mouse polyclonal GSTM3 antibody (1:250), respectively, for 2 hours in blocking buffer, which was followed by a goat anti-mouse secondary antibody (1:10,000 dilution) in blocking buffer for 1 hour. Bands on the membranes probed with anti-GSTM1 and anti-GSTA1 were visualized with the Novex ECL Chemiluminescent Kit, whereas the membrane probed with anti-GSTM3 was visualized with SuperSignal Femto Maximum Sensitivity Substrate. The densitometry analysis of images at 400-second exposure time were performed using Image J software (National Institutes of Health, Bethesda, MD). Western blot experiments were performed in triplicate.

Enzyme kinetic analysis. Kinetic parameters were determined utilizing the Michaelis-Menten equation using GraphPad Prism software (version 6.01). $V_{\max}$ values were calculated as nmol/min/mg of pure recombinant protein. All reported values were results of three independent experiments.

Results

Identification of EXE-GS and DHE-GS Conjugates. Chemically synthesized standard EXE-GS and 17 β -DHE-GS at 10 ppm were used to confirm the identity of EXE-GS and 17 β -DHE-GS conjugates in the

Fig. 1. UV and UPLC/MS chromatograms of EXE-GS and DHE-GS conjugates. (A) EXE-GS standard with the parent ion spectrum (insert) for the peak at 1.20 minutes; (B) EXE-GS conjugate formation by HLC with the parent ion spectrum (insert) for the peak at 1.20 minutes; (C) EXE-GS conjugate formation with no enzyme added; (D) 17 β -DHE-GS standard with the parent ion spectrum (insert) for the peak at 0.80 minutes; (E) 17 β -DHE-GS conjugate formation by HLC with the parent ion spectrum (insert) for the peak at 0.90 minute; (F) 17 β -DHE-GS conjugate formation with no enzyme added.

screening assays. As shown by representative chromatograms in Fig. 1, Fig. 1A depicts the EXE-GS standard with one major UV peak (254 nm) at 1.2 minutes and a corresponding parent ion spectrum with mass to charge ratio (m/z)⁺ of 604.27, which matches with the predicted [EXE-GS+H]⁺ ion (C₃₀H₄₂N₃O₈S) (m/z)⁺ = 604.28. The chemical structure of the synthesized EXE-GS was confirmed by NMR (described in *Materials and Methods*) and found to be 95% pure. A representative UV chromatogram of a glutathione conjugation assay utilizing HLC and EXE as a substrate (Fig. 1B) shows the EXE-GS parent ion of (m/z)⁺ = 604.28 at 1.2 minutes. A comparable UV trace of the 17 β -DHE-GS standard is shown in Fig. 1D with the main peak detected by UV at 0.80 minutes and the corresponding parent ion (m/z)⁺ = 606.28 matching with the predicted [17 β -DHE-GS+H]⁺ ion (C₃₀H₄₄N₃O₈S) [(m/z)⁺ = 606.28]. The synthesized 17 β -DHE-GS standard was also confirmed by NMR with an estimated purity of 94%. Fig. 1E shows glutathione conjugate formation in HLC with 17 β -DHE as the substrate. The peak at 0.90 minutes in Fig. 1E has an (m/z)⁺ = 606.28, and matched with the synthesized standard in Fig. 1D, which confirmed the identity of the peak as the glutathione conjugate of 17 β -DHE.

A total of 12 recombinant hepatic cytosolic GSTs (GSTA1, GSTA2, GSTA4, GSTK1, GSTM1, GSTM2, GSTM3, GSTM4, GSTO1, GSTP1, GSTZ1, and GSTT1) were screened for activity against EXE and its active metabolite 17 β -DHE. All 12 GSTs were active against the universal GST substrate, CDNB (Supplemental Fig. 2). A sensitive tandem mass spectrometry method (described above) was developed to detect glutathione conjugates of EXE and 17 β -DHE. As shown in Fig. 2, GSTA1, GSTM1, and GSTM3 exhibited significant activity against EXE in screening assays (panel A) with GSTA1 > GSTM3 $\approx$ GSTM1. None of the other recombinant cytosolic GST enzymes tested (GSTA2, GSTA4, GSTK1, GSTM2, GSTM4, GSTO1, GSTP1, GSTZ1, and GSTT1) exhibited activity against EXE. Interestingly, only GSTA1 exhibited activity against 17 β -DHE among the 12 cytosolic GSTs tested (Fig. 2B).

Representative Michaelis-Menten plots of EXE-GS and 17 β -DHE-GS formation are shown in Fig. 3. Kinetic analysis of EXE-GS formation by recombinant GSTA1, GSTM1, and GSTM3 enzymes demonstrated that GSTM1 exhibited the highest affinity for EXE [Michaelis-Menten constant (K_M) = 14 $\pm$ 4.1 μ M] followed by GSTA1 (K_M = 53 $\pm$ 17 μ M) and GSTM3 (K_M = 99 $\pm$ 6.0 μ M; Table 1). The apparent K_M (46 $\pm$ 12 μ M) for EXE-GS formation in HLC was similar to that observed for GSTA1. GSTM3 exhibited the highest rate of EXE-GS formation (V_{max} = 14 $\pm$ 1.4 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹), which was approximately 3- and 33-fold higher than that observed for GSTA1 (V_{max} = 4.7 $\pm$ 2.9 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹) and GSTM1 (V_{max} = 0.43 $\pm$ 0.29 nmol $\cdot$ min⁻¹ $\cdot$ mg⁻¹), respectively. Overall, recombinant GSTM3 exhibited a marginally higher intrinsic clearance (CL_{INT}) of EXE (CL_{INT} = 0.14 $\pm$ 0.092 nl $\cdot$ min⁻¹ $\cdot$ mg⁻¹) compared with GSTA1 (CL_{INT} = 0.085 $\pm$ 0.043 nl $\cdot$ min⁻¹ $\cdot$ mg⁻¹) but exhibited a CL_{INT} that was 4.4-fold higher than that observed for GSTM1 (CL_{INT} = 0.032 $\pm$ 0.017 nl $\cdot$ min⁻¹ $\cdot$ mg⁻¹). Similar to that observed for EXE-GS formation, the K_M observed for 17-DHE in HLC (51 $\pm$ 14 μ M) was similar to that observed for GSTA1 (32 $\pm$ 12 μ M).

Relative Expression of GSTA1, GSTM1, and GSTM3 in Pooled HLC. Quantitative Western blot analysis was performed to determine the relative amounts of each active GST present in pooled HLC. Commercial antibodies for GSTA1, GSTM1, and GSTM3 were first verified for specificity and cross-reactivity (Supplemental Fig. 3). Western blots containing serial dilutions of HLC and purified recombinant GSTA1, GSTM1, and GSTM3 proteins were then probed with each specific antibody (representative images shown in Fig. 4). Densitometry analysis of three independent experiments, each containing pure recombinant protein as a standard, estimated that GSTA1 and GSTM1 composed 0.043 $\pm$ 0.0051 and 0.0057 $\pm$ 0.00043 μ g of GST per microgram of HLC, respectively (Table 2). No GSTM3 proteins were detected in HLC by Western blot analysis.

Fig. 2. Cytosolic GST activities against EXE and 17β-DHE. (A) EXE-GS formation by recombinant GSTs; (B) 17β-DHE-GS formation by recombinant GSTs. The GSTs were screened by incubating GSH with EXE (250 μM) using 2.5 ng/μl of purified recombinant protein. GS conjugates of EXE and DHE were detected using a Waters Xevo G2-S QToF mass spectrometer and expressed in ppm as described in *Materials and Methods*. Asterisks represent those GST enzymes exhibiting glutathionylation activities against EXE or 17β-DHE that were >3x the mock control.

Discussion

Glutathionylation is an important step in the xenobiotic detoxification pathway of many compounds and might play an important role in the rate of excretion and elimination of EXE, thereby contributing to the variation observed in EXE treatment efficacy and side effects (Jancova et al., 2010). As shown in previous studies, cysteine conjugates of EXE compose 77% of total urinary metabolites, whereas in plasma, cysteine conjugate levels are similar to another major metabolite, 17β-DHE-Gluc

(Luo et al., 2018). These data suggest that the formation of cysteine conjugates is a major route of metabolism of EXE. Cysteine conjugates are formed through a three-step pathway similar to that observed in the mercapturic acid synthesis pathway with the first step, GSH conjugation, catalyzed by the GST family of enzymes (Hanna and Anders, 2019). Glutathione conjugates are typically inactive, less toxic, more water soluble, and more readily excreted than parent unconjugated compound (Allocati et al., 2018). Subsequently, the glutamyl moiety is removed by γ-glutamyltransferases, the glycyl moiety is removed by

Fig. 3. Representative Michaelis-Menten curves of recombinant GSTs and HLC for the conjugation of EXE to EXE-GS (A) and DHE to DHE-GS (B). For EXE conjugation, experiments were performed using 1 μg/μl of HLC protein, 2.5 ng/μl of purified recombinant GSTA1, or 8 ng/μl of purified recombinant GSTM1 or GSTM3 with varying concentrations (2–250 μM) of EXE. For 17β-DHE conjugation, experiments were performed using 2 μg/μl of HLC protein or 5 ng/μl purified recombinant GSTA1 with varying concentrations (2–250 μM) of 17β-DHE.

TABLE 1
Kinetic analysis of GSTs against EXE and 17β-DHE
K_M, V_{max}, and CL_{INT} are expressed as the mean ± S.D. of three independent experiments.

	EXE-GS			17β-DHE-GS		
	<i>K_M</i> μ <i>M</i>	<i>V_{max}</i> nmol·min ⁻¹ ·mg ^{-1a}	<i>CL_{INT}</i> nl·min ⁻¹ ·mg ^{-1a}	<i>K_M</i> μ <i>M</i>	<i>V_{max}</i> nmol·min ⁻¹ ·mg ^{-1a}	<i>CL_{INT}</i> nl·min ⁻¹ ·mg ^{-1a}
HLC	46 ± 12	0.044 ± 0.0067	0.0018 ± 0.00010	51 ± 14	0.0020 ± 0.00084	0.000037 ± 0.0000082
GSTA1	53 ± 17	4.7 ± 2.9	0.085 ± 0.043	32 ± 12	0.48 ± 0.13	0.017 ± 0.0067
GSTM3	99 ± 6.0	14 ± 1.4	0.14 ± 0.092		Not active	
GSTM1	14 ± 4.1	0.43 ± 0.29	0.032 ± 0.017		Not active	

^aData are expressed as milligrams of total cytosolic protein.

dipeptidases, and cysteine conjugates are excreted in the urine (Hanna and Anders, 2019). This three-step pathway has been verified for EXE through in vitro biosynthesis, in which the resulting enzymatically synthesized cysteine conjugate of EXE matches both chemically synthesized EXE-Cys, and through ex vivo analysis of urinary EXE-Cys detected in subjects taking EXE (Luo et al., 2018). In the present study, we demonstrate that GSTA1, GSTM1, and GSTM3 are the major enzymes forming the EXE-GS conjugate and that GSTA1 is the primary GST leading to the formation of 17β-DHE-GS (see Fig. 5). The α class of GSTs is the major form of the enzyme expressed in hepatocytes, and they play a critical role in cellular defense against oxidative stress by catalyzing glutathione peroxidase reactions (e.g., fatty acid hydroperoxides and phospholipid hydroperoxides) and detoxification of xenobiotics, such as environmental carcinogens (e.g., polycyclic aromatic hydrocarbons) and therapeutic drugs (busulfan, cyclophosphamide, and chlorambucil) (Czerwinski et al., 1996; Coles and Kadlubar, 2005; Bertholee et al., 2017; Attia et al., 2021). GSTA1 is quickly released into the bloodstream during hepatocellular damage, and serum GSTA1 concentration has been used as a biomarker for hepatic or renal injury (Mikstacki et al., 2016; Li et al., 2017). In addition to being involved in various catalytic processes, GSTA1 was also reported to be a modulator of the c-Jun N-terminal kinase signaling pathway by protein-protein interactions. c-Jun N-terminal kinase functions as a critical control point in the mitogen-activated protein kinase pathways during cellular stress (Adnan et al., 2012; Pljesa-Ercegovac et al., 2018), which suggests that GSTA1 plays multiple important roles in the cell. GSTA1 is the major GST expressed in liver (Coles and Kadlubar, 2005) and is the only GST that showed activity against both EXE and 17β-DHE. According to the Human Protein Atlas, hepatic GSTA1 mRNA expression is 1,958 protein-coding transcripts per million (pTPM), which is 5.5-fold higher than that observed for GSTM1 (353 pTPM) and 169-fold higher than GSTM3 (12 pTPM) [http://www.proteinatlas.org; queried on July 10, 2021; (Uhlén et al., 2015)]. Additional sources have suggested that GSTA1 composes 2%–5% of total soluble protein in

hepatocytes (Beckett and Hayes, 1993; Hayes and Pulford, 1995). In the present study, Western blot analysis of pooled HLC showed that GSTA1 composes 4.3% of the total cytosolic protein, a value consistent with these previous reports. The affinities for EXE (*K_M* = 46 ± 12 μM) and 17β-DHE (*K_M* = 51 ± 14 μM) in HLC glutathione conjugation were similar to those observed for purified GSTA1 (*K_M* = 53 ± 17 μM and 32 ± 12 μM, respectively), which supports a role for GSTA1 as the major GST active in the metabolism of both EXE and 17β-DHE in human liver.

The rate of GS conjugate formation (i.e., *V_{max}*) and *CL_{INT}* by recombinant GSTA1 for EXE was 9.7- and 5-fold higher, respectively, than that observed for 17β-DHE-GS. These data are consistent with previous metabolomic studies demonstrating that EXE-Cys is the major metabolite observed in the urine of women taking EXE. In these subjects, the mean levels of EXE-Cys in the urine (5.9 ± 0.58 nmol/mg creatinine) and plasma (22 ± 2.9 nM) were 3.3- and 3.7-fold higher, respectively, than that observed for 17β-DHE-Cys (Luo et al., 2018). 17β-DHE-Gluc, another major metabolite of EXE, is found at comparable levels to EXE-Cys in plasma but was 4.2-fold lower in urine in patients taking EXE (Luo et al., 2018). These data further suggest that cysteine conjugate formation through GS conjugation of EXE is the primary route of elimination of EXE via phase II metabolism.

Consistent with other GST isoforms, GSTM1 catalyzes the glutathionylation of a variety of different electrophilic compounds including carcinogens, environmental toxins, therapeutic drugs [busulfan, acetaminophen, azathioprine, cisplatin, etc. (Czerwinski et al., 1996; Peters et al., 2000; Arakawa et al., 2012)], and products of oxidative stress (Marinković et al., 2013). Similar to that observed for GSTA1, GSTM1 is also involved in the mitogen-activated protein kinase signal transduction pathway through complexes with apoptosis signal-regulating kinase 1 and repression of apoptotic cell death (Cho et al., 2001), which supports its important role in multiple cellular processes. The data from the

Fig. 4. Western blot quantification of GSTA1, GSTM3, and GSTM1 in HLC. (A) Serial dilution of HLC (25–6.25 μg of total protein) and purified recombinant GSTA1 (1–0.125 μg) used as standard, probed with a GSTA1-specific antibody; (B) serial dilution of HLC (25–6.25 μg of total protein) and purified recombinant GSTM1 (0.25–0.03 μg) used as standard, probed with a GSTM1-specific antibody; (C) serial dilution of HLC (25–6.25 μg of total protein) and purified recombinant GSTM3 (1–0.03 μg) used as standard, probed with a GSTM3-specific antibody. Western blots were conducted as described in *Materials and Methods*. rGST, recombinant GST.

TABLE 2

Mean levels of GSTA1, GSTM1, and GSTM3 in human liver cytosols

GSTA1	GSTM1	GSTM3
0.043 ± 0.0051 ^a	0.0057 ± 0.00043 ^a	Not detected

^aData are expressed as micrograms of GST per micrograms of liver cytosolic protein.

present study suggest that GSTM1 also plays an important role in the hepatic clearance of EXE. GSTM1 exhibited the highest affinity of any GST enzyme for EXE with a K_M value of 14 μM , which is 3.7-fold lower than GSTA1 ($K_M = 53 \pm 17 \mu\text{M}$). However, GSTM1 composed only 0.57% of all hepatic cytosolic protein as determined by quantitative Western blot analysis, a level that was >7.5-fold lower than that observed for GSTA1 in the same experiments, suggesting that its role in hepatic EXE metabolism may be secondary to GSTA1.

The third isoform, GSTM3, shows the highest intrinsic clearance values for EXE in vitro (0.14 $\text{nl}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) among the three GSTs. However, it likely plays a minimal role in the hepatic clearance of EXE since it was not detected in HLC. These data are consistent with that observed in the Human Protein Atlas database, which showed that GSTM3 mRNA levels are 169-fold less abundant than GSTA1 and 29-fold less abundant than GSTM1 in liver [http://www.proteinatlas.org; queried on July 10, 2021; (Uhlén et al., 2015)]. GSTM3 might, however, contribute to the first-pass metabolism of EXE since its expression is about 2-fold higher than GSTM1 in the small intestine (http://www.proteinatlas.org; queried on July 10, 2021).

EXE reduction to 17 β -DHE during phase I metabolism and the subsequent glucuronidation of 17 β -DHE by UGT2B17 has been extensively studied (Sun et al., 2010; Kamdem et al., 2011; Platt et al., 2016; Peterson et al., 2017). Deletion of UGT2B17 was associated with decreased 17 β -DHE-Gluc metabolite levels and increased levels of 17 β -DHE (Luo et al., 2017). Recent studies examining the potential correlation between the UGT2B17 deletion and commonly reported side effects (fatigue, hot flashes, and joint pain) in

postmenopausal women taking EXE found that the UGT2B17 gene deletion is associated with a higher risk of severe fatigue, hot flashes, and joint pain (Ho et al., 2020). Interestingly, common functional polymorphisms also exist for several GSTs including GSTA1 and GSTM1. GSTA1 has three linked, functional single-nucleotide polymorphisms in the promoter region resulting in two allelic variants, GSTA1*A and GSTA1*B, with the GSTA1*B minor allele frequency at 0.43–0.49 in the Caucasian population (Mikstacki et al., 2016; Michaud et al., 2019). The promoter single-nucleotide polymorphism in position –52 (G>A) has functional importance since it impairs the binding of the specificity protein 1 transcription factor, reduces promoter activity, and is associated with altered hepatic GSTA1 expression and increased risk for a variety of cancers (Coles et al., 2001; Coles and Kadlubar, 2005). The GSTA1*B genotype was also associated with interindividual variability in busulfan clearance in children undergoing hematopoietic stem cell transplantation, which led to the higher incidence of hematopoietic stem cell transplantation-related toxicities such as sinusoidal obstruction syndrome, acute graft-versus-host disease, and other treatment-related toxicities (Ansari et al., 2017). In addition, GSTM1 has a whole-gene deletion polymorphism that is highly prevalent (minor allele frequency = 48%–57%) in the Caucasian population and has been associated with increased risk for a variety of cancers (Geisler and Olshan, 2001). The GSTM1 null genotype has also been linked with alterations in drug metabolism and clinical efficacy. For example, the GSTM1 null genotype has been associated with lower efficacy in patients taking azathioprine as an immunosuppressant during inflammatory bowel disease (Lucafò et al., 2019). These GST functional variants may therefore contribute to the variability in hepatic EXE metabolism in the population.

In conclusion, in vitro studies suggest that GSTA1 is the major isoform responsible for the hepatic metabolism of EXE and 17 β -DHE. GSTM1 contributes to the hepatic clearance of EXE but not 17 β -DHE. Although GSTM3 is active against EXE, it does not contribute significantly to hepatic clearance, given its negligible expression in the liver. Further studies examining the

Fig. 5. Schematic of exemestane metabolism. AKR, aldoketoreductase; CBR, carbonyl reductase; CYP, cytochrome P450; GGT, γ -glutamyl transferase.

role of functional polymorphisms in GSTA1 and GSTM1 on EXE metabolism should be performed to better evaluate the role of these enzymes on individual differences in EXE efficacy, side effects, and overall treatment outcomes.

Authorship Contributions

Participated in research design: Teslenko, Lazarus.

Conducted experiments: Teslenko.

Contributed new reagents or analytic tools: Xia.

Performed data analysis: Teslenko, Chen, Lazarus.

Wrote or contributed to the writing of the manuscript: Teslenko, Watson, Xia, Chen, Lazarus.

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Address correspondence to: Dr. Philip Lazarus, Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University, 412 E. Spokane Falls Blvd., PBS Building Room 431, P.O. Box 1495, Spokane, WA 99202-2131. E-mail: phil.lazarus@wsu.edu