Identification and Quantification of Novel Major Metabolites of the Steroidal Aromatase Inhibitor, Exemestane

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ABSTRACT

Exemestane (EXE) is an aromatase inhibitor used for the prevention and treatment of estrogen receptor–positive breast cancer. Although the known major metabolic pathway for EXE is reduction to form the active 17β-dihydro-EXE (17β-DHE) and subsequent glucuronidation to 17β-hydroxy-EXE-17-O-β-D-glucuronide (17β-DHE-Gluc), previous studies have suggested that other major metabolites exist for exemestane. In the present study, a liquid chromatography–mass spectrometry (LC-MS) approach was used to acquire accurate mass data in MS2 mode, in which precursor ion and fragment ion data were obtained simultaneously to screen novel phase II EXE metabolites in urine specimens from women taking EXE. Two major metabolites predicted to be cysteine conjugates of EXE and 17β-DHE by elemental composition were identified. The structures of the two metabolites were confirmed to be 6-methylcysteinylandrosta-1,4-diene-3,17-dione (6-EXE-cys) and 6-methylcysteinylandrosta-1,4-diene-17β-hydroxy-3-one (6-17β-DHE-cys) after comparison with their chemically synthesized counterparts. Both underwent biosynthesis in vitro in three stepwise enzymatic reactions, with the first involving glutathione conjugation. The cysteine conjugates of EXE and 17β-DHE were subsequently quantified by liquid chromatography–mass spectrometry in the urine and matched plasma samples of 132 subjects taking EXE. The combined 6-EXE-cys plus 6-17β-DHE-cys made up 77% of total EXE metabolites in urine (vs. 1.7%, 0.14%, and 21% for EXE, 17β-DHE, and 17β-DHE-Gluc, respectively) and 35% in plasma (vs. 17%, 12%, and 36% for EXE, 17β-DHE, and 17β-DHE-Gluc, respectively). Therefore, cysteine conjugates of EXE and 17β-DHE appear to be major metabolites of EXE in both urine and plasma.

Introduction

Breast cancer is the most frequently diagnosed cancer in the United States (Howlader et al., 2016). An estimated 252,710 new cases of female breast cancer were diagnosed in the United States in 2017 with approximately 75% of these being estrogen receptor positive (ER+) (Osborne and Schiff, 2011; Siegel et al., 2017). In postmenopausal women, treatment of early-stage ER+ breast cancer has focused primarily on the elimination of estrogen-induced tumor cell growth. Aromatase inhibitors (AIs), similar to the steroidal substrate analog exemestane (EXE), act to inhibit aromatase activity by blocking the biosynthesis of estrone and estradiol, thereby preventing estrogen-induced tumor cell growth (Miller, 1999; Campos, 2004; Santen et al., 2009). Clinical trials have shown that the use of AIs increases disease-free survival and decreases the occurrence of contralateral breast cancer as compared with the use of selective estrogen receptor modulators, such as tamoxifen (Howell et al., 2005; Ferretti et al., 2006; Eisen et al., 2008; Forbes et al., 2008). EXE is widely used for the adjuvant treatment and prevention of breast cancer in postmenopausal women (Wang and Chen, 2006; Hong et al., 2007; Deeks and Scott, 2009; Petkov et al., 2009). Although AIs such as EXE represent an improvement in treatment and prevention of breast cancer, considerable interindividual variation exists in patients’ responses to these drugs (Paridaens et al., 2003; Chia et al., 2008; Campos et al., 2009; Glück, 2010). The causes for this interindividual variability have not been clearly elucidated.

Phase I metabolites of EXE have been identified in vivo. In addition to the major active phase I metabolite 17β-dihydro-EXE (17β-DHE), several minor metabolites with much lower activities are formed, including 6β-hydroxy-6β-hydroxymethylandrostana-1,4-diene-3,17-dione; 6β-hydroxyandrosta-1,4-diene-3,17-dione; 3β-hydroxy-5α-androst-1-en-6-methylene-17-one; 6β-17β-dihydroxy-6β-hydroxymethylandrosta-1,4-diene-3-one; and 6β-17β-dihydroxyandrosta-1,4-diene-3-one (Evans et al., 1992; Cavalcanti Gde et al., 2011; de Albuquerque Cavalcanti et al., 2011). Cytosolic aldo-keto reductase 1Cs and carbonyl reductase 1 are
Materials and Methods

Chemicals and Materials. EXE was purchased from Sigma-Aldrich (St. Louis, MO), 17b-DHE, 17b-DHE-Gluc, 17b-OH-EXE-d3 (D17b-DHE), 17b-OH-EXE-d3-17-O-β-D-glucoronide (17b-DHE-Gluc) levels (e.g., up to 29-fold in plasma) were associated with increasing numbers of the UGT2B17 deletion allele in women taking EXE, only a small (1.3-fold) increase in plasma 17b-DHE was observed in the same women (Luo et al., 2018). This suggests that other metabolic or excretion pathways may also play a role in EXE metabolism.

The goal of the present study was to characterize other major phase II metabolites of EXE. Results are presented demonstrating the existence of novel phase II cysteine conjugate EXE metabolites in both the urine and plasma of women taking EXE.

Subjects and Samples. One hundred and thirty-two postmenopausal breast cancer patients (one Hispanic, two African Americans, two Asians, and 127 Caucasians; age range: 55–89 years) with ER+ breast tumors were recruited into this study from the Breast Oncology Clinic at the Penn State Hershey Cancer Institute. Approval was obtained from the Institutional Review Board at Penn State University with informed consent obtained from all subjects. Subjects took a single pill (25 mg) of EXE daily (orally) for at least 28 consecutive days and 1 ml aliquot of each urine sample was stored at –80°C until analysis. As a control for EXE metabolism, specimens obtained from women not taking EXE who were recruited into other studies at Penn State University College of Medicine (Hershey, PA; n = 10) were also examined to exclude false positives (Ashmore et al., 2013). Sample Preparation for Identification of EXE Metabolites. Ten urine specimens from subjects taking EXE and 10 urine specimens from control subjects who did not take EXE were selected for analysis. One hundred microliters of 100% methanol was added to a 50-μl aliquot of each urine sample to extract EXE and its metabolites. After vortexing and subsequent centrifugation at 16,100g for 10 minutes at 4°C, the supernatant was transferred to a fresh sample vial for analysis by UPLC-MS.

UPLC-MS Conditions for Screening of EXE Metabolites in Urine. For the simultaneous identification of potential novel phase II metabolites and analysis of known metabolites (including EXE, 17b-DHE, and 17b-DHE-Gluc), urine samples were prepared as described earlier and analyzed using a UPLC-MS system (Waters, Milford, MA) consisting of an ACQUITY UPLC pump, an ACQUITY QTRAP (flow through needle) sample manager, an ACQUITY UPLC BEH column C18 (2.1 x 100 mm, 1.7-μm particle size), and a XEVO G2-S Quadrupole Time of Flight (QTOF) mass spectrometer. UPLC was performed at a flow rate of 0.4 ml/min with solvent A (5 mM ammonium formate and 0.01% formic acid in water) and solvent B (100% acetonitrile) using the following conditions for urine specimens: 0.5 minutes at 25% solvent B, a linear gradient to 100% solvent B in 4 minutes, 1.5 minutes at 100% solvent B followed by re-equilibration with 25% solvent B for 2 minutes. The injection volume of each prepared urine sample was 2 μl. The column temperature was 35°C. The Waters XEVO G2-S QTOF MS was equipped with an electrospray ionization 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. Ultra-pure argon was used as the collision gas with a flow rate of 0.1 l/h. The source and desolvation gas temperatures were 120°C and 500°C, respectively, and the dwell time for each ion was 300 ms. The mass spectrometer was operated in MS2 mode (MassLynx; Waters) as a non-targeted method for metabolite identification (Plumb et al., 2006). In this method, two interleaved scan functions were used for data acquisition, with both functions collecting data over the same mass range [m/z] = 50–1250. The first scan acquired data using a low collision energy and collected information on the intact (parent) ions in each sample. The second scan acquired data with ramped collision energy from low to high and collected the fragment (daughter) ion data of the ions in the preceding scan (Bateman et al., 2007). Chemical Synthesis of 6-Methylcysteinylandrosta-1,4-diene-3,17-dione and 6-Methylcysteinylandrosta-1,4-diene-17b-hydroxy-3-one. The EXE and 17b-DHE used for the chemical synthesis of 6-methylcysteinylandrosta-1,4-diene-3,17-dione (6-EXE-cys) and 6-methylcysteinylandrosta-1,4-diene-17b-hydroxy-3-one (6-17b-DHE-cys) were synthesized as previously described (Platt et al., 2016). Nuclear magnetic resonance (NMR) spectra were recorded with a Bruker (Billerica, MA) Avance instrument with 500 MHz for hydrogen and 125 MHz for carbon. The abbreviations for NMR data are the following: s, singlet; d, doublet; dd, doublet of doublets; m, multiplet; J, coupling constant; δ, chemical shift. Chemical shifts were measured based on the residual proton in NMR solvent, and product purity was determined by UPLC spectrum monitored at 254 nm.

1. 6-EXE-cys. EXE (8.0 mg, 0.027 mmol) and l-cysteine (9.7 mg, 0.080 mmol) were added to a 10-ml round-bottom flask under the protection of argon, followed by the addition of degassed 1.25 N potassium hydroxide in 25% methylanol (1 ml). The mixture was stirred at ambient temperature for 24 hours, and the pH was then adjusted to 5.0 with 2 N cold HCl. The reaction mixture was subsequently applied on a preparative C-18 reverse-phase TLC plate developing in a chamber containing 20% acetonitrile in water. The product band determined by 254 nm UV visualization at Rf = 0.3 was scratched off from the TLC plate with a spatula into a clean 100-ml round-bottom flask. Pure methanol (40 ml) was added to extract the product. After filtration of the methanol-extracted mixture through a fritted funnel and flushing with methanol, the solvent was removed by a rotary evaporator. The residue was washed with 100% hexane (2 x 1 ml) and 100% diethyl ether (2 x 1 ml) and dried to afford the product (1.5 mg, Y = 13%) as an off-white solid. The fragment (daughter) ion data of the ions in the preceding scan (Bateman et al., 2007).
Biosynthesis of 6-EXE-cys, 6-17β-DHE-cys, and Deuterium-Labeled Internal Standards. 6-EXE-cys, 6-17β-DHE-cys, and their corresponding deuterium-labeled internal standards were biosynthesized in a three-step process involving an initial GSH conjugation reaction of EXE or 17β-DHE, removal of glutamic acid from the GSH conjugates using purified active γ-GT, and subsequent removal of glycine from the cysteinylglycine conjugate using HCl as a source of dipeptidase enzyme.

1. **GSH conjugation of EXE and 17β-DHE.** Pooled HCL (1 mg) was added to a reaction containing 100 mM potassium phosphate (pH 7.4) and 250 μM EXE or 355 μM 17β-DHE in a total volume of 1 ml. The reaction was preincubated at 37°C for 3 minutes prior to the addition of 50 μM of 100 mM GSH (final GSH concentration = 5 mM) (Lash et al., 1999; Zarth et al., 2015; Shi et al., 2016). After 2 hours at 37°C, the reaction was stopped with an equal reaction volume of ice-cold acetonitrile and centrifuged at 16,100 × g for 10 minutes at 4°C. Aliquots (50 μl) of the supernatant were injected onto the ACQUITY UPLC BEH C18 column (2.1 × 100 mm, 1.7-μm particle size) for separation by UPLC. The UPLC conditions were the same as those described earlier for screening of EXE metabolites. Conjugate-containing fractions were collected at UPLC retention times of 0.8–2.0 minutes for the EXE-GSH conjugate or 0.5–2.0 minutes for the 17β-DHE-GSH conjugate prior to their concentration and removal of organic solvent by speedvac.

2. **γ-GT-mediated removal of γ-glutamyl from the GSH conjugates of EXE and 17β-DHE.** The EXE-GSH and 17β-DHE-GSH conjugates collected earlier were digested at 37°C for 15 minutes with 1.0 U/ml γ-GT, 100 mM potassium phosphate (pH 7.4), 20 mM glycylglycine, and 5 mM diithiothreitol in a total volume of 600 μl as previously described (Lash et al., 1999; Del Corso et al., 2006; Grillo et al., 2008). The reaction was stopped with an equal reaction volume of ice-cold acetonitrile and centrifuged at 16,100 × g for 10 minutes at 4°C, and aliquots (50 μl) of the supernatant were injected onto the same UPLC system described earlier. Conjugate-containing fractions were collected at UPLC retention times of 1.0–1.5 minutes for the EXE-cysteinylglycine conjugate and 0.5–1.0 minute for the 17β-DHE-cysteinylglycine conjugate prior to their concentration to half their original volume by speedvac.

3. **Dipeptidase-mediated removal of glycine from cysteinylglycine conjugates.** Collected EXE-cysteinylglycine or 17β-DHE-cysteinylglycine conjugates were digested with 1.0 mg/ml pooled HLC in 100 mM potassium phosphate buffer (pH 7.4), 0.2 mM MnCl₂, and 5 mM diithiothreitol at 37°C in a final reaction volume of 200 μl (Hirota et al., 1986; Josch et al., 1998; Cappiello et al., 2004; Del Corso et al., 2006). The reaction was stopped with an equal reaction volume of ice-cold acetonitrile and centrifuged at 16,100 × g for 10 minutes at 4°C, and aliquots (50 μl) of the supernatant were injected onto the same UPLC system described earlier.

4. **Biosynthesis of D₆-6-EXE-cys and D₆-17β-DHE-cys.** In enzymatic reactions identical to those described in steps 1–3, D₆-EXE or D₆-17β-DHE was used as the starting material at 330 μM in 1-ml reactions. Collected D₆-6-EXE-cys and D₆-17β-DHE-cys conjugates were dried by speedvac and dissolved in pure methanol. The concentrations for the stock solutions of D₆-6-EXE-cys and D₆-17β-DHE-cys, estimated by comparing MS peak area with the chemically synthesized standard, were 2.5 and 10 μg/ml, respectively.

**Sample Preparation for Quantification of EXE and Its Metabolites in Urine and Plasma.** To quantify in vivo levels of EXE and its metabolites, the sample preparation method was modified from that described earlier for metabolite identification. For EXE metabolite analysis in plasma, a 2.5-μl aliquot of each plasma sample was first spiked with 2.5 μl of a mixture of deuterium-labeled internal standards in methanol [D₁-EXE (0.17 μM), D₆-17β-DHE (1.7 μM), 17β-OH-EXE-d₃-17-O-β-D-glucuronide (0.52 μM), D₆-6-17β-DHE-cys (0.06 μM), and D₆-17β-DHE-cys (0.25 μM)]. Twenty microliters of pure methanol was then added to extract EXE and its metabolites to precipitate proteins. After vortexing and subsequent centrifugation at 16,100g for 10 minutes at 4°C, 15 μl of the supernatant was transferred to a sample vial and mixed with 15 μl of water prior to analysis by UPLC-MS.

For EXE metabolite analysis in urine, a 2.5-μl aliquot of each urine sample was first spiked with 2.5 μl of the same deuterium-labeled internal standard mixture described earlier. Ten microliters of 75% methanol was then added to extract EXE and its metabolites. After vortexing and subsequent centrifugation at 16,100g for 10 minutes at 4°C, 10 μl of the supernatant was transferred to a sample vial and mixed with 5 μl of water prior to analysis by UPLC-MS.

**UPLC-MS Conditions for Quantification of EXE and Its Metabolites in Urine and Plasma.** For the simultaneous quantification of EXE, 17β-DHE, 17β-DHE-Gluc, 6-EXE-cys, and 6-17β-DHE-cys, urine and plasma samples were prepared as described earlier. Using the same UPLC-MS system described earlier, UPLC was performed with solvent A (5 mM ammonium formate and 0.01% formic acid in water) and solvent B (100% acetonitrile) using the following conditions for both urine and plasma specimens: 0–1 minutes at 25% solvent B, a linear gradient to 52% solvent B for 1.5–2.5 minutes, 2.5–4.5 minutes at 52% solvent B, a linear gradient to 95% solvent B from 4.5 to 5 minutes, 5–9.5 minutes at 95% solvent B, followed by a linear gradient to initial conditions of 25% solvent B from 9.5 to 10 minutes. This was followed by a 1-minute run at 25% solvent B to equilibrate the column to initial conditions before the next sample injection. The flow rate was 0.4 ml/min from 0 to 5 minutes, 0.8 ml/min from 5 to 9.5 minutes, and back to 0.4 ml/min from 9.5 to 10 minutes. The injection volume of each prepared urine and plasma sample was 5 μl. The column temperature was 35°C. The Waters XEVO G2-S QTOF MS was operated in tandem mass spectrometry (MS/MS) mode, with the electrospray ionization probe operated in the positive-ion mode and a capillary voltage at 0.6 kV. The cone and desolvation gas flow rates were maintained at 50 and 800 l/h, respectively, and the collision gas flow rate was 0.1 l/h. The source and desolvation gas temperatures were 120°C and 500°C, respectively, and the dwell time for each ion was 300 ms. The ion-related parameters for the 10 transitions monitored are listed in Table 1.

The limits of quantification for EXE, 17β-DHE, 17β-DHE-Gluc, 6-EXE-cys, and 6-17β-DHE-cys were 2.1, 1.6, 12, 0.7, and 7.2 nM in plasma, respectively, and 2.1, 6, 6.3, 1.5, and 7.2 nM in urine, respectively. Standard curves were constructed by plotting the ratio of analyte peak area to peak area of the corresponding internal standard (described earlier) versus concentration of analyte standard. The concentrations of stock standards were 1000 ppm. A serial dilution of standards at concentrations ranging from 1.6 nM to 1.7 μM, 1.6 nM to 1.7 μM, and its metabolites.

| TABLE 1 |
| MS/MS transitions and ion optic parameters for EXE and EXE metabolites |
| Se | SM/SM Transition | Cone Voltage | Collision Energy (eV) |
| EXE | 297.19 > 297.19 | 25 | 10 |
| D₆-EXE | 300.20 > 300.20 | 25 | 10 |
| 17β-DHE | 299.20 > 299.20 | 25 | 10 |
| D₆-17β-DHE | 302.22 > 302.22 | 25 | 10 |
| 17β-DHE-Gluc | 475.23 > 281.19 | 20 | 15 |
| D₆-17β-DHE-Gluc | 478.25 > 284.21 | 20 | 15 |
| 6-EXE-cys | 418.21 > 297.19 | 15 | 15 |
| D₆-6-EXE-cys | 421.22 > 300.20 | 15 | 15 |
| 6-17β-DHE-cys | 420.22 > 299.20 | 15 | 15 |
| D₆-6-17β-DHE-cys | 423.24 > 302.22 | 15 | 15 |

D₆-17β-DHE-Gluc, 17β-OH-EXE-d₃-17-O-β-D-glucuronide.
UPLC-MS using MSE, a nontargeted method for metabolite identification that allows for the scanning of both intact ions (channel 1) and fragment ions (channel 2) simultaneously over the same mass range. As shown by representative chromatographs in Fig. 1, the MS<sub>E</sub> method applied in this study was sensitive enough to detect known EXE metabolites extracted from the intact ion channel 1 including EXE (Fig. 1A; extracted mass = 297.19), 17β-DHE (Fig. 1C; extracted mass = 299.20), and 17β-DHE-Gluc (Fig. 1E; extracted mass = 475.23) in the urine from subjects taking EXE. All peaks corresponded to those observed for EXE, 17β-DHE, and 17β-DHE-Gluc standards (Fig. 1, B, D, and F, respectively); none were observed in the urine of subjects not taking EXE (results not shown).

In screening for novel EXE conjugates, the MS trace of ([m/z]<sup>+</sup>)<sub>parent</sub> = 297.185 for [EXE-H]<sup>+</sup>, the common fragment ion for EXE conjugates, was extracted from fragment ion channel 2 (Fig. 2A). In addition to the expected peak for EXE (retention time 3.20 minutes), two major peaks (termed peaks 1 and 2, retention times = 1.33 and 1.74 minutes, respectively) were observed. Neither of these peaks were detected in urine specimens from control subjects not taking EXE (results not shown). Molecular ions for compounds that correspond to these two fragment peaks were then searched within the intact ion channel 1; the retention times of peaks 1 and 2 in the intact (parent) ion channel 1 (Fig. 2B) matched that observed for peaks 1 and 2 in fragment ion channel 2 (Fig. 2A). The corresponding mass spectrum of the parent ion for peak 1 from the intact (parent) ion channel 1 showed a major peak with an ([m/z]<sup>+</sup>) = 418.2060, presumably the [X-H]<sup>+</sup> ion, where X refers to an unknown EXE conjugate (Fig. 2C). Two additional accurate mass peaks were observed (Fig. 2C), with ([m/z]<sup>+</sup>) = 440.1874 and 456.1615, likely corresponding to the ([m/z]<sup>+</sup>) for [X+Na]<sup>+</sup> and [X+K]<sup>+</sup>, respectively. The accurate mass spectrum for peak 2 (data not shown) was identical to that observed for peak 1 (Fig. 2C), suggesting that the two peaks are isomers of the same EXE conjugate. The trace of 418.206 extracted from the intact (parent) ion scan from channel 1 (Fig. 2C) exhibited two isomers of the same EXE conjugate. The trace of 418.206 extracted from the intact (parent) ion ([m/z]<sup>+</sup>) = 420.2209) trace in channel 1 (Fig. 3B). The mass spectrum extracted for peak 3 from the intact (parent) ion channel 1 is shown in Fig. 3C. The peaks of ([m/z]<sup>+</sup>) = 420.2209, 442.2027, and 458.1770 likely corresponded to the ions of [17β-DHE-cys +Na]<sup>+</sup>, [17β-DHE-cys +K]<sup>+</sup>, and [17β-DHE-cys +Na]<sup>+</sup> and [17β-DHE-cys +K]<sup>+</sup>, respectively. Similar to that observed for EXE-cys conjugates, peak 3 was observed in all 10 urine samples screened by the MS<sub>E</sub>, whereas peak 4 was observed in seven of the 10 urine samples. Again, neither of these peaks were detected in urine samples from control subjects not taking EXE (results not shown). Therefore, compounds responsible for peaks 3 and 4 were predicted to be 17β-DHE-cys isomers (C<sub>23</sub>H<sub>32</sub>N<sub>5</sub>O<sub>4</sub>S).

Data were analyzed using Prism (version 7; GraphPad Software, San Diego, CA). The accurate mass spectrum for peak 2 (data not shown) was identical to that observed for the putative 6-EXE-cys conjugate corresponding to (m/z)+ = 418.2052, which exactly matched those observed for EXE, 17β-DHE, and 17β-DHE-Gluc standards (Fig. 1, B, D, and F, respectively); none were observed in the urine of subjects not taking EXE (results not shown). The retention times for peaks corresponding to chemically synthesized 6-EXE-cys and 6-17β-DHE-cys (Fig. 4, A and B, respectively) matched those observed for the predicted EXE-cys and 17β-DHE-cys peaks 1 and 3, respectively (Figs. 2D and 3B, respectively), detected in the urine of subjects taking EXE. In addition, coelution experiments were performed by adding chemically synthesized 6-EXE-cys and 6-17β-DHE-cys into urine specimens of EXE-treated subjects. LC-MS analysis showed increases in peak size for each compound (results not shown), further demonstrating that the urinary peaks are identical to the chemically synthesized standards. Together, these data suggest that the MS peaks 1 and 3 (Figs. 2D and 3B, respectively) from urine of women taking EXE corresponded to 6-EXE-cys and 6-17β-DHE-cys.

Bioavailability of Cysteine Conjugates of EXE and 17β-DHE. To confirm the structures of the predicted cysteine conjugates of EXE and 17β-DHE identified earlier, 6-EXE-cys and 6-17β-DHE-cys were synthesized chemically. As described in the Materials and Methods, the structures of both chemically synthesized cysteine conjugates were confirmed by NMR, and the purity of both conjugates was confirmed by LC-MS to be >95% (results not shown). The retention times for peaks corresponding to chemically synthesized 6-EXE-cys and 6-17β-DHE-cys (Fig. 4, A and B, respectively) were identical to those observed for the predicted EXE-cys and 17β-DHE-cys conjugates. The detection of the urinary peaks corresponded to both synthetic standards. Together, these data suggest that the MS peaks 1 and 3 (Figs. 2D and 3B, respectively) from urine of women taking EXE corresponded to 6-EXE-cys and 6-17β-DHE-cys.
was similar to that observed for the chemically synthesized 6-17β-DHE-cys conjugate (Fig. 4B) and to that observed for the putative 17β-DHE-cys conjugate corresponding to peak 3 in the urine of subjects taking EXE (Fig. 3B). In addition, a similar pattern was observed for both the enzymatically synthesized D3-labeled 6-EXE-cys and 6-17β-DHE-cys conjugates (Fig. 4, E and F, respectively). All cysteine conjugates were confirmed by analysis of corresponding mass spectra (results not shown).

Fig. 1. UPLC/MS² analysis of EXE metabolites in urine from a subject taking EXE (A, C, and E) versus chemical standards (B, D, and F). (A and B) Extracted ion chromatogram of 297.185 (EXE) from channel 1 (intact ion). (C and D) Extracted ion chromatogram of 299.201 (17β-DHE) from channel 1. (E and F) Extracted ion chromatogram of 475.23 (17β-DHE-Gluc) from channel 1. TOF, Time of Flight.
Quantification of EXE and Its Metabolites In Vivo. For quantification of EXE and its metabolites in vivo, EXE metabolites were analyzed by UPLC-MS/MS for all urine samples and matched plasma samples from 132 postmenopausal breast cancer patients who had taken 25 mg of EXE per day for at least 4 weeks. As shown for representative MS chromatograms of EXE metabolites from a subject taking EXE (Fig. 5), the retention times were 4.02 minutes for EXE, 3.73 minutes for 17β-DHE, 2.65 minutes for 17β-DHE-Gluc, 1.34 minutes for 6-EXE-cys, and 0.88 minutes for 6-17β-DHE-cys for both urine and plasma using this UPLC-MS/MS method. In all cases, the retention times of each peak were similar to that of their corresponding internal standard peaks (see Figs. 1 and 4). Although the predicted isomers of EXE-cys and 17β-DHE-cys (peaks 2 and 4 in Figs. 2D and 3B, respectively) were not quantified due to a lack of accurate standards, they may be present at similar or higher levels than that observed for 6-EXE-cys and 6-17β-DHE-cys conjugates for plasma EXE plus 17β-DHE were roughly equivalent to the levels of plasma 17β-DHE-Gluc, with the average percentage of TEM ranging from 35% to 36% for both (6-EXE-cys + 6-17β-DHE-cys) and 17β-DHE-Gluc. The percentages of 17β-DHE-Gluc and 6-EXE-cys in TEM (36% and 23%, respectively) were highest when compared with other EXE metabolites in the plasma of EXE-treated subjects, ranging from 12% to 17% for 17β-DHE, 6-17β-DHE-cys, and EXE.

Although a similar trend was observed for urinary EXE metabolites, the levels of urinary 17β-DHE-Gluc were 5.5-fold less than the combined cysteine conjugates for urinary EXE plus 17β-DHE, which were the major urinary metabolites in women taking EXE (Table 2). 17β-DHE-Gluc comprised, on average, 21% of total quantified urinary EXE metabolites versus the combined 6-EXE-cys plus 6-17β-DHE-cys conjugates, which comprised 77% of total quantified urinary EXE metabolites. The levels of urinary 17β-DHE-Gluc (1.4 nmol/mg creatinine) were slightly lower than those observed for 6-17β-DHE-cys (1.8 nmol/mg creatinine), which was 3.3-fold lower than that observed in plasma, with the average percentage of 17β-DHE in total quantified EXE metabolites (TEM) observed at 3-fold lower levels than that observed for 17β-DHE-Gluc. The mean levels of the combined cysteine conjugates for plasma EXE plus 17β-DHE were roughly equivalent to the levels of plasma 17β-DHE-Gluc, with the average percentage of TEM ranging from 35% to 36% for both (6-EXE-cys + 6-17β-DHE-cys) and 17β-DHE-Gluc. The percentages of 17β-DHE-Gluc and 6-EXE-cys in TEM (36% and 23%, respectively) were highest when compared with other EXE metabolites in the plasma of EXE-treated subjects, ranging from 12% to 17% for 17β-DHE, 6-17β-DHE-cys, and EXE.
for the major urinary metabolite 6-EXE-cys. The mean levels of urinary 6-EXE-cys (mean = 5.9 nmol/mg creatinine) were almost 900-fold higher than the urinary metabolite observed at the lowest level, 17β-DHE (mean = 0.0066 nmol/mg creatinine).

Discussion

Previous studies indicated that the major mode of metabolism of EXE is by reduction to form 17β-DHE and UGT2B17-mediated glucuronidation to form 17β-DHE-Gluc (Sun et al., 2010; Luo et al., 2018). In the present study, two novel major EXE metabolites were identified—the cysteine conjugates of EXE (6-EXE-cys) and 17β-DHE (6-17β-DHE-cys). The mean levels of the combined 6-EXE-cys plus 6-17β-DHE-cys were 5.5-fold higher than those observed for 17β-DHE-Gluc in urine and were similar to the levels of 17β-DHE-Gluc observed in plasma.

6-EXE-cys formed the major cysteine conjugate of EXE, comprising 77% and 79% of the total mean cysteine conjugate levels in urine and plasma, respectively. This suggests that the formation of cysteine conjugates is the major excretion pathway for EXE in humans, with 6-EXE-cys the major urinary EXE metabolite (see Scheme 1).

Although the stereochemistry at the 6-position was not assigned in the current paper, we speculate the stereo orientation for the 6-substitute is “α” for both synthesized 6-EXE-cys and 6-17β-DHE-cys based on the proton coupling constant between the proton at the 4-position and the proton at the 6-position of both EXE-cys and 17β-DHE-cys conjugates. For 6-substituted steroids, long-distance coupling between H-4 and H-6 in 1H NMR will be observed only when the 6-substituent is in the α position (Chin and Warren, 1972; Schneider et al., 1973; Numazawa and Oshibe, 1994; Numazawa and Yamaguchi, 1998). In an analysis of a series of 6-substituted phenylaliphatic steroids, Numazawa and Yamaguchi (1998) demonstrated that proton coupling between the hydrogens at the 4- and 6-positions was only observed for the 6α-substituted, but not the 6β-substituted, phenylaliphatic steroids. Similar C-4 proton signals have been reported for a series of 6-alkyl-, 6-bromo-, and 6-(bromoacetoxy)androstenediones (Numazawa and Oshibe, 1994; Numazawa and Yamaguchi, 1998). In addition, the EXE derivative 6α-chloromethylandrosta-1,4-dien-3,17-dione exhibits similar long-distance coupling between H-4 and H-6 (Görlitzer et al., 2006). These data are consistent with the proton coupling pattern of 6-EXE-cys and 6-17β-DHE-cys observed by 1H NMR in the present study (results not shown), suggesting that both conjugates were in the α-position. However, X-ray crystallography analysis of the two conjugates will provide a more definitive validation of their structure.

Previous in vitro studies in a panel of human liver microsomes demonstrated that deletion of the UGT2B17 gene resulted in significant decreases in 17β-DHE-Gluc formation (Sun et al., 2010). Although drastic decreases in urinary and plasma 17β-DHE-Gluc levels were also associated with increasing numbers of the UGT2B17 deletion allele in women taking EXE (e.g., up to 29-fold in plasma), only a small (1.3-fold) corresponding increase in plasma 17β-DHE was observed in the same women (Luo et al., 2018). In the present study, 17β-DHE-Gluc was demonstrated to comprise 36% of the total quantified EXE metabolites in plasma, levels that were approximately equal to that observed for both of the cysteine conjugates combined (which comprised 35% of the total quantified plasma EXE metabolites) and only slightly more than that observed for 6-EXE-cys alone (which comprised 23% of total quantified plasma EXE metabolites). In addition, 17β-DHE-Gluc comprised only 21% of the total quantified EXE metabolites in urine. These values correspond with the fact that either no alterations or only small increases
in urinary or plasma EXE and 17β-DHE were observed in subjects taking EXE who were homozygous for the UGT2B17 deletion polymorphism (i.e., with no active UGT2B17) in previous studies (Luo et al., 2018).

A three-step metabolism pathway similar to the first three steps of the mercapturic acid synthesis pathway was shown to be a viable in vivo mechanism of EXE and 17β-DHE cysteine conjugate formation in the
present studies. The three steps include an initial GSH conjugation reaction catalyzed by GSTs to form EXE-GSH or 17β-DHE–GSH, a second reaction catalyzed by γ-GT to remove the glutamyl moiety from the glutathione conjugate to form EXE-cysteinylglycine or 17β-DHE–cysteinylglycine, and a final reaction where the glycyl moiety is removed by dipeptidase to form the EXE-cys or 17β-DHE-cys conjugates.

In addition to the identified 6-EXE-cys (peak 1 in Fig. 2D) and 6-17β-DHE-cys conjugates (peaks 3 in Fig. 3B), secondary peaks likely corresponding to EXE-cys (peak 2 in Fig. 2D) and 17β-DHE-cys isomers (peaks 4 in Fig. 3B) of unknown structure were observed in the urine of women taking EXE in the present study. EXE and 17β-DHE are α-β unsaturated ketones with several double bonds for GSH metabolism.

### TABLE 2

<table>
<thead>
<tr>
<th></th>
<th>Plasma</th>
<th>Urine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Mean ± S.E.</strong></td>
<td><strong>Range</strong></td>
</tr>
<tr>
<td><strong>EXE</strong></td>
<td>14 ± 1.7</td>
<td>0–105</td>
</tr>
<tr>
<td><strong>17β-DHE</strong></td>
<td>2.5 ± 0.19</td>
<td>0.11–14</td>
</tr>
<tr>
<td><strong>17β-DHE-Gluc</strong></td>
<td>30 ± 4.6</td>
<td>0.32–358</td>
</tr>
<tr>
<td><strong>6-EXE-cys</strong></td>
<td>22 ± 2.9</td>
<td>0.020–226</td>
</tr>
<tr>
<td><strong>6-17β-DHE-cys</strong></td>
<td>5.9 ± 0.58</td>
<td>0–31</td>
</tr>
</tbody>
</table>

\^ TEM = EXE + 17β-DHE + 17β-DHE-Gluc + 6-EXE-cys + 6-DHE-cys. The percentage of TEM was calculated for EXE or each EXE metabolite for every individual subject, with the mean then calculated for each metabolite of all 132 subjects.
conjugation. Therefore, there are several possible positions for the initial GSH conjugation step to occur. Chemical synthesis of other potential isomers of EXE-cys and 17β-DHE-cys conjugates is currently underway to confirm their structures.

Similar to phase II metabolism by glucuronidation, GSH conjugation of either EXE or 17β-DHE likely increases their rate of excretion and eliminates their antiaromatase activities. Significant variations in levels of 6-EXE-cys and 6-17β-DHE-cys were observed in the urine and plasma samples of the 132 subjects. As shown in Table 2, 6-EXE-cys and 6-17β-DHE-cys ranged from 0.020 to 226 nM and 0 to 31 nM in plasma, respectively, and from 0.0033 to 50 nM and 0.0033 to 10 nM in urine, respectively. Interestingly, both the unidentified EXE cysteine conjugate isomer (peak 2 in Fig. 2D) and the unidentified 17β-DHE cysteine conjugate isomer (peak 4 in Fig. 3B) were observed in the urine.
of only seven of the 10 subjects examined in this study. There is significant genetic variability in the GST enzymes (Hayes et al., 2005), including common copy number variants observed for both GSTM1 and GSTT1 (Seidgard et al., 1990; Bell et al., 1993; Arruda et al., 1998; Bailey et al., 1998; Roth et al., 2000; Hayes et al., 2005). The frequencies of homozygous deletion genotypes of GSTM1 and GSTT1 are about 50% and 14% in Caucasians, and the homozygous deletion genotypes of both GSTM1 and GSTT1 were reported to be associated with risk for a variety of cancers and may be linked to alterations in drug metabolism (Hengstler et al., 1998). For example, GSTs are involved in the metabolism of azathioprine (AZA) to mercaptopurine, with 6-methylmercaptopurine riboside a major metabolite of mercaptopurine. The levels of 6-methylmercaptopurine riboside were 2-fold lower in AZA users exhibiting the GSTM1-null genotype than AZA users carrying one or two copies of GSTM1 (Broekman et al., 2018). The deletion of GSTM1 was also associated with reduced response to AZA therapy (Stocco et al., 2014). This suggests that if such GSTs are similarly involved in the metabolism of EXE, copy number variants or other functional polymorphisms could potentially play an important role in the metabolism and efficacy of EXE. Although functional polymorphisms in both the γ-GT and dipeptidase enzymes could similarly modify the levels of EXE-cys and 17β-DHE-cys observed in the plasma and/or urine of subjects taking EXE, they do not metabolize the functional parent compound, EXE, or its major active metabolite, 17β-DHE, and are, therefore, less likely to be important in overall patient response to EXE.

As described earlier, quantification of EXE-cys and 17β-DHE-cys conjugates was performed only for peaks corresponding to the known 6-cysteine conjugates (peaks 1 and 3 in Figs. 2D and 3B, respectively). Although their structure is presently unknown, other peaks corresponding to other EXE-cys and 17β-DHE-cys isomers were identified in the present study, potentially at levels similar to or higher than those observed for 6-EXE-cys and 6-17β-DHE-cys. Therefore, these conjugates likely comprise an even larger proportion of EXE metabolites in the plasma and urine of women taking EXE, further increasing the importance of cysteine conjugate formation and decreasing the overall importance of glucuronidation in the metabolism of EXE. In addition, this further supports previous results demonstrating only small changes in plasma 17β-DHE and no change in urinary 17β-DHE in subjects deficient in DHE glucuronidation capacity since the glucuronide comprises a relatively low percentage of EXE metabolites in vivo (Luo et al., 2018).

A potential limitation of the present study was that the study subjects examined were primarily Caucasians (only five non-Caucasians out of 132 female subjects). Although we did not find observable differences in the levels of EXE or its metabolites between the Caucasian subjects and the five non-Caucasian subjects examined in this study, it is possible that the patterns observed for Caucasian females may not be 100% generalizable to all populations. Additional studies of EXE metabolism in other racial groups will be necessary to better examine this. Another potential limitation of the current study was that the intermediate products of the first and second steps for the proposed three-step pathway for EXE-cys and 17β-DHE-cys formation were not detected in urine samples, suggesting that they are at low levels if present. This also suggests that the final two intermediate enzymatic reaction steps with γ-glutamyl transferase and cysteinylglycine dipeptidase to form the EXE-cys and 17β-DHE-cys conjugates are highly efficient.

In conclusion, two novel EXE phase II metabolites were identified in vivo in women taking EXE. The two metabolites—cysteine conjugates of EXE and 17β-DHE—are the major metabolites of EXE found in the urine of subjects taking EXE, comprising, on average, 77% of total quantified urinary EXE metabolites, and were at levels similar to that observed for the other major EXE metabolite, 17β-DHE-Gluc, in the plasma of the same subjects. Although their exact structure is presently unknown, other EXE and 17β-DHE cysteine conjugates were also identified, further supporting cysteine conjugate formation as the major metabolism pathway for EXE in vivo. The pathways involved in EXE-cys or 17β-DHE-cys formation could, therefore, potentially play an important role in the pharmacokinetics and pharmacodynamics of EXE.

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Authorship Contributions

Participated in research design: Luo, Chen, Truica, Lazarus.
Conducted experiments: Luo, Chen, Baird, Xia.
Performed data analysis: Luo, Chen, Xia, Lazarus.
Wrote or contributed to the writing of the manuscript: Luo, Chen, Truica, Xia, Lazarus.

References
