Influence of Glutathione-S-Transferase A1*B Allele on the Metabolism of the Aromatase Inhibitor, Exemestane, in Human Liver Cytosols and in Patients Treated With Exemestane

Irina Teslenko, Julia Trudeau, Shaman Luo, Christy J.W. Watson, Gang Chen, Cristina I. Truica, and Philip Lazarus

Department of Pharmaceutical Sciences, College of Pharmacy and Pharmaceutical Sciences, Washington State University, Spokane, Washington (I.T., J.T., S.L., C.J.W.W., G.C., P.L.) and Penn State University, College of Medicine, Division of Hematology and Oncology, Department of Medicine, Hershey, Pennsylvania (C.I.T.)

Received March 21, 2022; accepted June 21, 2022

ABSTRACT
Exemestane (EXE) is used to treat postmenopausal women diagnosed with estrogen receptor positive (ER+) breast cancer. A major mode of metabolism of EXE and its active metabolite, 17β-dihydroexemestane, is via glutathionylation by glutathione-S-transferase (GST) enzymes. The goal of the present study was to investigate the effects of genetic variation in EXE-metabolizing GST enzymes on overall EXE metabolism. Ex vivo assays examining human liver cytosols from 75 subjects revealed the GSTA1 *B*B genotype was associated with significant decreases in S-(androsta-1,4-diene-3,17-dion-6-ol-3-on-6-ylmethyl)-L-glutathione (P = 0.034) and S-(androsta-1,4-diene-17β-ol-3-on-6-ylmethyl)-L-glutathione (P = 0.014) formation. In the plasma of 68 ER+ breast cancer patients treated with EXE, the GSTA1 *B*B genotype was associated with significant decreases in both EXE-cysteine (cys) (29%, Ptrend = 0.0056) and 17β-DHE-cys (34%, P = 0.032) as compared with patients with the GSTA1*A*A genotype, with significant decreases in EXE-cys (Ptrend = 0.0067) and 17β-DHE-cys (Ptrend = 0.028) observed in patients with increasing numbers of the GSTA1*B allele. A near-significant (Ptrend = 0.060) trend was also observed for urinary EXE-cys levels from the same patients. In contrast, plasma and urinary 17β-DHE-Gluc levels were significantly increased (36%, P = 0.00097 and 52%, P = 0.0089; respectively) in patients with the GSTA1*B*B genotype. No significant correlations were observed between the GSTM1 null genotype and EXE metabolism and may play a role in interindividual variability in overall response to EXE.

SIGNIFICANCE STATEMENT
The present study is the first comprehensive pharmacogenomic investigation examining the role of genetic variability in GST enzymes on exemestane metabolism. The GSTA1*B*B genotype was found to contribute to interindividual differences in the metabolism of EXE both ex vivo and in clinical samples from patients taking EXE for the treatment of ER+ breast cancer. Since GSTA1 is a major hepatic phase II metabolizing enzyme in EXE metabolism, the GSTA1*B allele may be an important biomarker for treatment outcomes and toxicities.

Introduction
Breast cancer accounts for 30% of all malignancies in women and continues to be the leading cause of cancer-related death in women worldwide (Loibl et al., 2021). Additionally, over their lifetime, women in the United States have a greater than 12% chance of being diagnosed with breast cancer (Akram et al., 2017). Primarily because of earlier detection and more effective systemic treatments (Harbeck and Gnant, 2017; Jahan et al., 2021), a steady decrease in mortality has been observed since the 1970s; however, variability exists in patient clinical response and survival, suggesting a need for treatment optimization and implementation of pharmacogenomics to personalize current standards of care (Vianna-Jorge et al., 2013).

Endocrine therapy is a cornerstone for the systemic treatment of estrogen receptor positive (ER+) breast cancer (Zelnak and O’Regan, 2015). The two major classes of endocrine therapies for postmenopausal women are selective ER modulators [e.g., tamoxifen] and aromatase inhibitors [AIs; e.g., exemestane (EXE)] (Coombes et al., 2007). Although tamoxifen has been the
characterized single nucleotide polymorphisms (SNPs) in high
linkage disequilibrium in the promoter region (–567 T>G, –69
C>T, and –52 G>A), (Morel et al., 2002) resulting in the
GSTA1*A and GSTA1*B alleles. The GSTA1*B allele has been
linked to lower hepatic expression of GSTA1 and altered metab-
olism of certain medications (Hayes and Strange, 2000; Ansari
et al., 2017). In Caucasian populations, the GSTA1*B allele has a
minor allele frequency of 0.43–0.49 (Mikstacki et al., 2016;
Michaud et al., 2019). Additionally, a polymorphic copy number
variant of GSTM1 has been identified (minor allele frequency of
0.48–0.57 in Caucasian populations) (Geisler and Olshan,
2001) and has been shown to influence drug metabolism
(Lucàfo et al., 2019b). Genetic variations in these two enzymes
could potentially alter overall patient response and toxicities re-
lated to EXE treatment. The primary objective of this study
was to investigate the effect of polymorphisms in GSTA1 and
GSTM1 on both glutathione conjugation activity in human liver
tissue and in the formation of major phase II metabolites found
in the plasma and urine of patients taking EXE.

Materials and Methods

Chemicals and Materials. EXE was purchased from Sigma-
Aldrich (St Louis, MO) and gamma-L-glutamyl-L-cysteinyl-glycine
(glutathione; GSH) was obtained from Alfa Aesar (Haverhill, 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 (St Louis, MO). An Acquity UHPLC BEH C18 col-
umn (2.1 x 100 mm) was purchased from Waters (Milford, MA). Pooled
human liver cytosol (HLC) was obtained from Xenotech (Kansas City,
KS). Pierce BCA protein assay kits, PureLink Genomic DNA Isolation
kits, TaqMan Copy Number Reference Assays (RNaP, Human;
catalog #A30064), GSTM1 TaqMan Copy Number Variant Assays
(catalog # 4400291), and GSTA1 TaqMan SNP Genotyping Assays (cat-
alog # 4351374) were purchased from Thermo-Fisher Scientific. All
other chemicals were purchased from Thermo-Fisher Scientific unless
otherwise specified.

Human Liver Specimens. Normal adjacent human liver tissue
specimens and corresponding genomic DNA samples were procured
from the Tissue Procurement Core at the H. Lee Moffitt Cancer Center
(Tampa, FL) from 75 patients undergoing hepatocarcinoma surgery
(Coughtrie et al., 1987; Yokota et al., 1989). Tissues were flash frozen
within 2 hours of removal. The majority of subjects (>90%, n = 70)
were Caucasian, with 8% (n = 5) of Hispanic descent; 36% (n = 27)
were female and the average age was 63 years. Cytosolic fractions
were prepared from each tissue sample using differential centrifugation
methods described previously (Dellinger et al., 2007; Ashmore et al.,
2018) and stored at –80°C. All procedures involving tissue specimens
have been approved by the H. Lee Moffitt Cancer Center’s Institutional
Review Board and are in compliance with assurances submitted to and
approved by the US Department of Health and Human Services.

Clinical Study Subjects. A total of 132 postmenopausal women
diagnosed with breast cancer at the Penn State Hershey Medical Cen-
ter (Hershey, PA) were recruited for this study. The Penn State Institu-
tional Review Board approved the study with informed consent
obtained from all individuals and all specimens deidentiﬁed. The major-
ity of the subjects (94%, n = 124) were Caucasian, and their age range
was 35–89 years. All subjects were women who had ER+ tumors and
agreed to orally ingest 25 mg of EXE daily for at least 28 days. Patients
receiving EXE simultaneously with adjuvant chemotherapy, other ad-
juvant endocrine treatments, or chronic corticosteroid or megestrol ace-
tate therapy were excluded from the study. Included as control subjects
were 10 healthy volunteers (all Caucasian, ages 45–70 years) who were
not taking EXE. On day 28 of the study, subjects were instructed to pro-
vide blood (10 cc) and urine (up to 50 ml) at a time point 4–6 hours after
taking their last daily EXE dose. Differential centrifugation (1300 g for 15 minutes) was performed to fractionate the whole blood; aliquots of plasma, buffy coat, and urine were stored at −80 °C until analysis (Luo et al., 2017; Luo et al., 2018), with genomic DNA extracted from corresponding buffy coats using standard protocols as previously described (Luo et al., 2018).

**Glutathione Activity Assays.** Glutathione conjugation activity assays were performed with the cytosolic fraction (HLC) of the human liver specimens obtained from the H. Lee Moffitt Cancer Center. Reactions were performed in duplicate in a total volume of 25 μL, containing 1 μL of HLC protein, 100 mM potassium phosphate buffer, and 250 μM of either EXE or 17β-DHE (Platt et al., 2016). Preincubation at 37 °C for 3 minutes was followed by the addition of 5 mM GSH to initiate the reaction (1 hour, 37 °C). Reactions were quenched with a mixture of ice-cold acetonitrile (25 μL) and deuterium labeled S-(androsta-1,4-diene-3,17-dion-6-ylmethyl)-L-glutathione (D1-EXE-GS) and S-(androsta-1,4-diene-17/α-3-on-6-ylmethyl)-L-glutathione (D2-DHE-GS) internal standards (Teslenko et al., 2021). After thorough mixing by vortex and centrifugation at 16,100 g (10 minutes at 4 °C), supernatants (10 μL) were transferred to glass vials containing 10 μL of water. As a positive control, reactions using commercial pooled (50 subjects) HLC protein were performed. Negative control reactions contained all components except an enzyme source. GSH conjugate metabolites of EXE and 17β-DHE were quantified against standard curves with known concentrations of S-(androsta-1,4-diene-3,17-dion-6-ylmethyl)-L-glutathione (EXE-GS) or 17β-DHE-GS. Chemical synthesis of the EXE-GS and 17β-DHE-GS standards were previously described (Teslenko et al., 2021). Rates of EXE-GS and 17β-DHE-GS conjugation were calculated in nmol·min⁻¹·mg cytosolic protein⁻¹. Total HLC protein concentration was determined via the Pierce BCA assay.

**Ultra-High Pressure Liquid Chromatography-Mass Spectrometry Conditions for GS Conjugate Detection.** A validated method for monitoring EXE-GS and 17β-DHE-GS conjugate formation was used to quantify GSH conjugates (Teslenko et al., 2021). Briefly, GSH conjugates were detected using an LC-MS system (Waters Acquity UPLC/XEVO G2-S QToF) with analyte separation achieved using a HPLC (Luo et al., 2018). Quantification of GSH conjugates was performed using an LC-MS system (Waters Acquity UPLC/XEVO G2-S QToF) with analyte separation achieved using a HPLC (Luo et al., 2018). Quantification of GSH conjugates was performed using an LC-MS system (Waters Acquity UPLC/XEVO G2-S QToF) with analyte separation achieved using a HPLC (Luo et al., 2018).

**Data Analysis.** Glutathione conjugate formation with EXE and 17β-DHE ranged from 0.16 to 1.44 nmol·min⁻¹·mg cytosolic protein⁻¹ and with 17β-DHE from 0.062 to 0.23 nmol·min⁻¹·mg cytosolic protein⁻¹. Genomic DNA corresponding to the 75 liver tissue specimens was genotyped for common GSTA1 and GSTM1 polymorphisms. The GSTA1 locus was genotyped for the wild type GSTA1*A allele (−567T, −69C, and −52G) and the variant GSTA1*B allele (−567G, −69T, and −52A) (Morel et al., 2002; Coles and Kadlubar, 2005b; Suvakov et al., 2014). The panel contained 29 samples that were homozygous wild type *A*A, 35 samples that were heterozygous *A*B, and 11 that were homozygous polymorphic *B*B. The GSTA1*B minor allele frequency (0.39) was close to reported frequencies in Caucasian populations (Mikstacki et al., 2016; Michaud et al., 2019) and GSTA1* null frequency (0.39) was close to reported frequencies in Caucasians (0.48–0.57) (Geisler and Olshan, 2001) and GSTM1 genotypes were not in Hardy-Weinberg equilibrium. Additionally, the GSTM1 deletion polymorphism was genotyped, with GSTM1 *0*0 corresponding to the null genotype with no functional copies and GSTM1 *1*1 corresponding to a wild-type genotype with two functional copies. Five samples were wild type *1*1, whereas 35 specimens were homozygous null *0*0, and another 35 specimens were heterozygous *1*0. The minor allele frequency of the GSTM1*0* allele of 0.70 in this population was higher than previously reported values in Caucasians (0.48–0.57) (Geisler and Olshan, 2001) and GSTM1 genotypes were not in Hardy-Weinberg equilibrium.

There was a significant (P trend = 0.025) decrease in EXE-GS formation with increasing numbers of the GSTA1*B allele, with a rate of 0.82 ± 0.27 nmol·min⁻¹·mg cytosolic protein⁻¹ observed for specimens with the *A*A genotype, 0.76 ± 0.25 nmol·min⁻¹·mg cytosolic protein⁻¹ observed for specimens with the *A*B genotype, and 0.61 ± 0.25 nmol·min⁻¹·mg cytosolic protein⁻¹ observed for specimens with the *B*B genotype.

**TABLE 1**

<table>
<thead>
<tr>
<th>Mean ± SD (nmol·min⁻¹·mg HLC protein⁻¹)</th>
<th>Range (nmol·min⁻¹·mg HLC protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EXE-GS</td>
<td>0.76 ± 0.27</td>
</tr>
<tr>
<td>17β-DHE-GS</td>
<td>0.12 ± 0.28</td>
</tr>
</tbody>
</table>

SD, standard deviation.
A n = 75 HLC specimens.
cytosolic protein$^{-1}$ observed for specimens with the $^B*B$ genotype (Fig. 1A). The rates of 17$\beta$-DHE-GS formation showed a similarly significant ($P_{\text{trend}} = 0.0076$) decrease with increasing numbers of the GSTA1$^B*B$ allele: 0.13 ± 0.032 nmol min$^{-1}$mg cytosolic protein$^{-1}$ for specimens with the $^A*A$ genotype, 0.12 ± 0.025 nmol min$^{-1}$mg cytosolic protein$^{-1}$ for specimens with the $^A*B$ genotype, and 0.11 ± 0.02 nmol min$^{-1}$mg cytosolic protein$^{-1}$ for specimens with the $^B*B$ genotype. Both EXE-GS and 17$\beta$-DHE-GS rates of formation were significantly lower ($P = 0.034$ and $P = 0.014$, respectively) for the $^B*B$ genotype specimens as compared with the wild-type $^A*A$ genotype specimens.

Since GSTA1 was the only enzyme previously shown to excrete EXE metabolites (Teslenko et al., 2021), only EXE-GS rates of formation were analyzed against the GSTM1 null genotype. After stratifying by GSTM1 genotype, no significant differences in EXE-GS formation were observed in the HLC from the same human liver specimens (Fig. 1B).

**Phase II Plasma and Urine Metabolites Versus GST Genotype in Subjects Taking EXE.** The levels of major phase II EXE metabolites in both plasma and urine are shown in Table 2. In plasma, the major phase II metabolites were 17$\beta$-DHE-Gluc (35% of total EXE metabolites), EXE-cys (32% of total EXE metabolites), and 17$\beta$-DHE-cys (9.5% of total EXE metabolites). A similar pattern was observed in urine, with EXE-cys, 17$\beta$-DHE-cys, and 17$\beta$-DHE-Gluc comprising 63%, 19%, and 16% of the total EXE metabolites, respectively.

The frequency of GSTA1 genotypes in this population was 29% for $^A*A$ subjects ($n = 20$), 46% for $^A*B$ subjects ($n = 31$), and 25% for $^B*B$ subjects ($n = 17$). The minor allele frequency of the GSTA1$^B*B$ allele of 0.48 was, as expected, close to previously reported values in Caucasians (Mikstacki et al., 2016; Michaud et al., 2019) and in Hardy Weinberg equilibrium. GSTM1 genotyping results indicated that 42 subjects exhibited the GSTM1 null genotype (62%), 18 subjects had one copy of GSTM1 (26%), whereas only six subjects had both copies present (9%). In addition, two subjects (3%) had three copies of the GSTM1 gene. The minor allele frequency of the GSTM1$^*0$ allele of 0.78 in this population was higher than previously reported values in Caucasians (0.48–0.57) (Geisler and Olshan, 2001) and the GSTM1 genotypes were not in Hardy-Weinberg equilibrium.

The level of each plasma or urinary metabolite was expressed as a fraction of total exemestane metabolites (TEM). TEM included EXE and both phase I (17$\beta$-DHE) and phase II metabolites (17$\beta$-DHE-Gluc, EXE-cys, and 17$\beta$-DHE-cys). Subjects with the GSTA1 $^B*B$ genotype exhibited significantly lower relative levels of EXE-cys ($P = 0.0056$) and 17$\beta$-DHE-cys ($P = 0.032$) metabolites in plasma (0.25 EXE-cys/TEM and 0.073 17$\beta$-DHE-cys/TEM) as compared with those with the GSTA1 $^A*A$ genotype (0.35 EXE-cys/TEM and 0.11 17$\beta$-DHE-cys/TEM; Fig. 2A). The observed decreases corresponded to 29% for plasma EXE-cys levels and 34% for plasma 17$\beta$-DHE-cys levels. A significant trend toward decreasing levels of EXE-cys ($P_{\text{trend}} = 0.0067$)
TABLE 2
Major EXE metabolite concentrations in the plasma and urine of subjects taking EXE

<table>
<thead>
<tr>
<th></th>
<th>Plasma (nM)</th>
<th>Urine (nmol/mg creatinine)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>EXE</td>
<td>24 ± 22</td>
<td>3–104</td>
</tr>
<tr>
<td>17β-DHE</td>
<td>3.3 ± 2.4</td>
<td>0.87–14</td>
</tr>
<tr>
<td>17β-DHE-Gluc</td>
<td>50 ± 64</td>
<td>0.39–358</td>
</tr>
<tr>
<td>EXE-cys</td>
<td>39 ± 37</td>
<td>4.3–226</td>
</tr>
<tr>
<td>17β-DHE-cys</td>
<td>10 ± 6.8</td>
<td>1.2–31</td>
</tr>
</tbody>
</table>


and 17β-DHE-cys ($P_{trend} = 0.028$) was observed in plasma in subjects with increasing numbers of the GSTA1*B allele. In contrast, the relative plasma 17β-DHE-Gluc levels were significantly ($P = 0.013$) higher for subjects with the GSTA1 *A*B genotype (0.47 17β-DHE-Gluc/TEM) as compared with subjects with the GSTA1 *A*A genotype (0.30 17β-DHE-Gluc/TEM); a significant ($P_{trend} = 0.00097$) trend of increasing plasma 17β-DHE-Gluc levels was observed in subjects with increasing numbers of the GSTA1*B allele. No significant differences were observed for the levels of plasma EXE or 17β-DHE in the plasma of subjects after stratification by GSTA1 genotype (results not shown). Additionally, no significant correlations were observed between plasma phase II EXE metabolite levels and GSTM1 genotypes (Fig. 2B). Although the two patients with three copies of the GSTM1 gene were not included in the association analysis of GSTM1 genotype versus plasma or urinary EXE metabolites, their mean EXE-cys levels were 24 ± 4.8 nM and 7.1 ± 7.1 nmol per mg creatinine, respectively, which were lower than the 35 ± 26 nM and 9.2 ± 2.8 nmol per mg creatinine observed in plasma and urine, respectively, for patients who were homozygous wild-type for GSTM1 (results not shown).

Similarly, there was a nonsignificant trend ($P_{trend} = 0.060$) toward decreasing levels of urinary EXE-cys in subjects with increasing numbers of the GSTA1*B allele (Fig. 3A), with urinary EXE-cys levels 15% lower for subjects with the GSTA1 *B*B genotype (0.56 EXE-cys/TEM) as compared with subjects with the GSTA1 *A*A genotype (0.66 EXE-cys/TEM). Also, similar to that observed in plasma, a significant ($P = 0.026$) 52% higher level of urinary 17β-DHE-Gluc was observed in subjects with the GSTA1 *B*B genotype (0.25 17β-DHE-Gluc/TEM) as compared with subjects with the GSTA1 *A*A genotype (0.12 17β-DHE-Gluc/TEM), and a significant ($P_{trend} = 0.0089$) trend in urinary 17β-DHE-Gluc to a significantly lower level ($P_{trend} = 0.00083$) of 17β-DHE-Gluc/TEM in subjects with the GSTA1*B allele.

Fig. 2. Association between GST genotype and plasma EXE metabolites. The levels of EXE and its metabolites (17β-DHE, EXE-cys, 17β-DHE-cys, and 17β-DHE-Gluc) were determined in the plasma of 68 patients taking EXE, then stratified by GSTA1 and GSTM1 genotype. EXE and metabolite levels are presented as a fraction of total EXE + EXE metabolites (TEM = EXE + 17β-DHE + EXE-cys + 17β-DHE-cys + 17β-DHE-Gluc). (A) EXE-cys/TEM, 17β-DHE-cys/TEM and 17β-DHE-Gluc/TEM versus GSTA1 genotype. GSTA1*A represents the wild-type GSTA1 allele, whereas GSTA1*B represents polymorphic GSTA1 allele. (B) EXE-cys/TEM and 17β-DHE-Gluc/TEM versus GSTM1 copy number variant genotype. The GSTM1*1 represents the wild-type gene, whereas the GSTM1*0 corresponds to the GSTM1 deletion variant. Since GSTM1 is not active against 17β-DHE, the 17β-DHE-cys fraction versus GSTM1 genotype was not included in this analysis. Within each genotype, the middle bars indicate the mean, whereas the upper and lower bars represent the standard error of the mean. Statistical analysis included unpaired $t$ test and ANOVA test for trend.
levels was observed in subjects with increasing numbers of the GSTA1*B allele. Also, similar to the results observed in the plasma, no significant correlations were observed between urinary phase II EXE metabolite levels and GSTM1 genotypes (Fig. 3B), and no correlation between urinary EXE, 17β-DHE, or 17β-DHE-cys levels and GSTA1 genotype was observed (results not shown).

**Discussion**

Despite the increased clinical benefits of AI therapy, up to 56% of patients do not respond, with patient compliance rates low because of adverse events, such as hot flashes, fatigue, and myalgia (Paridaens et al., 2003; Paridaens et al., 2008; Goss et al., 2011; Hadji et al., 2013; Blok et al., 2018). Genetic variation in key EXE-metabolizing enzymes may be an important factor that contributes to individual variation in the metabolism, efficacy, and adverse events in patients taking EXE, and identifying pharmacogenomic factors contributing to this variation can help personalize treatment (Ingle, 2013), potentially resulting in improved compliance and patient outcomes. Previous studies have shown that the glucuronidation of the major active EXE metabolite, 17β-DHE, by UGT2B17 is an important phase II metabolic pathway for EXE (Luo et al., 2017), and that the UGT2B17 deletion was associated with altered 17β-DHE and 17β-DHE-Gluc levels in the plasma and urine of women taking EXE (Luo et al., 2017) and influences EXE pharmacokinetics (Chen et al., 2016). However, more recent studies have shown that glutathionylation via GST enzymes is also a major phase II elimination pathway for EXE, with GSTA1 the major glutathione S-transferase involved in the hepatic metabolism of EXE and 17β-DHE (Luo et al., 2018; Teslenko et al., 2021). GST-mediated glutathione-conjugated products of EXE and 17β-DHE are further metabolized to cysteine conjugates by γ-glutamyl transferase and dipeptidases, resulting in the major EXE-cys and 17β-DHE-cys metabolites observed in the plasma and urine of patients taking EXE (Luo et al., 2018).

In the present study, we demonstrated that the GSTA1*B allele was associated with significant decreases in HLC GSH conjugation activity for both EXE and 17β-DHE, with a significant decrease in both EXE-cys and 17β-DHE-cys observed in specimens from subjects with the GSTA1*B genotype as compared with specimens from subjects with the GSTA1*A*A genotype. This pattern was also observed in the plasma of subjects taking EXE. Subjects with the GSTA1*B*B genotype exhibited significant decreases in plasma EXE-cys and 17β-DHE-cys levels, respectively, as compared with subjects with the GSTA1*A*A genotype, with statistically significant linear trends indicating decreasing levels of plasma EXE-cys and 17β-DHE-cys with increasing numbers of the GSTA1*B allele.

The data in the present study are consistent with previous functional studies demonstrating that the GSTA1*B allele is associated with decreased GSTA1 expression based on results from luciferase reporter assays (Morel et al., 2002). Previous studies demonstrated that the lower transcriptional activity...
observed for the GSTA1*B allele was caused by the G > A substitution at the -52 location interfering with the binding of the ubiquitous transcription factor Sp1, resulting in four-fold lower GSTA1 hepatic expression as compared with wild-type GSTA1 encoded by the GSTA1*A allele (Morel et al., 2002; Coles and Kadlubar, 2005a). Consistent with these previous studies, the homozygous GSTA1*B*B genotype was shown to be associated with the altered metabolism, efficacy, and toxicity of several agents, including cyclophosphamide, busulfan, and azathioprine (Anasari et al., 2017; Lucafo et al., 2019a; Michaud et al., 2019; Attia et al., 2021), and multiple studies have linked the GSTA1*B*B genotype to increased risk of colorectal, prostate, breast, and bladder cancer (Deng et al., 2015).

A similar, though nonsignificant, pattern was observed in the urine from the same subjects taking EXE, where a 15% decrease in urinary EXE-cys levels were observed in subjects with the GSTA1*B*B genotype. This decrease is roughly 1.5-fold less than the decrease observed in plasma EXE-cys levels for the same subjects. This lack of statistical significance may be attributed to the extra-hepatic GSTM3 enzyme, which was previously shown to exhibit the highest intrinsic clearance of all GST enzymes tested against EXE in vitro (Teslenko et al., 2021). According to the human protein atlas, GSTM3 is highly expressed in the human kidney (http://www.proteinatlas.org; queried on February 20th, 2022; (Uhlen et al., 2015)). This suggests that although this enzyme may not be playing a major role in EXE conjugation in the liver, it could be playing a more important role in kidney-related urinary metabolism. Interestingly, the GSTM3 gene has a 3 base pair deletion polymorphism in intron 6 (resulting in the GSTM3*B allele (rs200126965) with a low minor allele frequency of 2--4% (Wang et al., 2020). GSTM3 genotyping analysis was performed in the present study, resulting in the identification of only one*A/B heterozygote and no homozygous GSTM3*B*B subjects in this population (results not shown).

Most interestingly, in contrast to the decreases observed for EXE-cys and 17b-DHE-cys, 17b-DHE-Gluc levels were significantly increased by 36%--52% in both the plasma and urine from patients with the GSTA1*B*B genotype. This suggests that increases in GSTA1 conjugation lead to a corresponding shift in phase II metabolism toward the glucuronidation pathway. Given that both EXE-cys and 17b-DHE-cys are active metabolites of EXE (unpublished results), this corresponding increase in the inactive 17b-DHE-Gluc in subjects with the GSTA1*B*B genotype would result in decreased levels of total active EXE in these subjects.

No significant correlation between GSTM1 genotype and either HLC EXE-GS rate of formation or EXE-cys and 17b-DHE-Gluc levels in both plasma and urine from patients taking EXE were observed in the present study. These data suggest that GSTM1 genotype has little influence on EXE metabolism in vivo and are consistent with previous studies showing that compared with GSTA1, the GSTM1 intrinsic clearance is 2.6-fold lower, and its hepatic expression is 7.5-fold lower (Teslenko et al., 2021). Interestingly, the GSTM1 null genotype was not in Hardy-Weinberg equilibrium in the populations examined in this study. A likely explanation is that the GSTM1 null genotype was shown in previous studies to be associated with risk for both hepatocellular carcinoma as well as breast cancer (Qi et al., 2016; Li et al., 2019). The higher-than-expected GSTM1 null genotype frequency observed in the present studies is consistent with the fact that the liver specimens used in this study were originally from patients who had hepatocarcinoma, and the patients taking EXE who participated in this study were diagnosed with breast cancer.

In summary, this is the first comprehensive pharmacogenomic study to assess the effects of major GST polymorphisms on EXE metabolism both ex vivo and in patients taking EXE for ER+ breast cancer. These studies demonstrate an association with the GSTA1*B allele and decreases in (1) EXE-GS and 17b-DHE-GS formation in liver specimens ex vivo and (2) in EXE-cys and 17b-DHE-cys levels in vivo in patients taking EXE. Decreases in these metabolite levels also corresponded with increases in the relative levels of 17b-DHE-Gluc in patients homozygous for GSTA1*B allele, indicating an increased importance of the glucuronidation pathway in EXE metabolism in these individuals. Further studies evaluating the role of the GSTA1*B*B genotype in EXE efficacy, side effects, and overall treatment outcomes should be performed to fully elucidate the importance of this genotype in patients taking EXE.

**Authorship Contributions**

**Participated in research design:** Teslenko, Lazarus.

**Conducted experiments:** Teslenko, Trudeau, Luo, Chen, Truica, Lazarus.

**Performed data analysis:** Teslenko, Chen, Truica, Lazarus.

**Wrote or contributed to the writing of the manuscript:** Teslenko, Watson, Truica, Lazarus.

**References**


