Potential role of UGT1A4 promoter SNPs in anastrozole pharmacogenomics

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Running title page

Running title: Anastrozole glucuronidation

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Abstract

Anastrozole belongs to the non-steroidal triazole-derivative group of aromatase inhibitors. Recently, clinical trials demonstrated improved antitumoral efficacy and a favorable toxicity with third-generation aromatase inhibitors as compared to tamoxifen. Anastrozole is predominantly metabolized by Phase I oxidation with the potential for further Phase II glucuronidation. It also, however, is subject to direct N-glucuronidation by UDP-glucuronosyltransferase 1A4 (UGT1A4). Anastrozole pharmacokinetics vary widely among patients, but pharmacogenomic studies of patients treated with anastrozole are sparse. In this study, we examined individual variability in the glucuronidation of anastrozole and its association with UGT1A4 promoter and coding region polymorphisms. In vitro assays using liver microsomal preparations from individual subjects (n=96) demonstrated 235-fold variability in anastrozole glucuronidation. Anastrozole glucuronidation was correlated (r = 0.99, p <0.0001) with lamotrigine glucuronidation (a diagnostic substrate for UGT1A4) and with UGT1A4 mRNA expression levels in human liver microsomes (r = 0.99, p<0.0001). Recombinant UGT1A4 catalyzed anastrozole glucuronidation which was inhibited by hecogenin (IC50 = 15 µM), a UGT1A4 specific inhibitor. The promoter region of UGT1A4 is polymorphic, and compared to those homozygous for the common allele, lower enzymatic activity was observed in microsomes from individuals heterozygous for -163G<A, -219T<G and -217C<T (p = 0.009, 0.014 and 0.009, respectively). These results indicate that variability in glucuronidation could contribute to response to anastrozole in the treatment of breast cancer.
Introduction

Breast cancer is the most frequently diagnosed cancer in women and the second most frequent cause of cancer-related death. In developed countries, around 75% of all breast cancers occur in postmenopausal women, of which about 80% are Estrogen-receptor positive (Anderson et al., 2002). Until recently, tamoxifen (TAM) has been the adjuvant treatment of choice for both pre- and postmenopausal women with estrogen-receptor-positive early breast cancer (Kamdem et al., 2010). Tumor recurrence and mortality in women with estrogen-receptor-positive breast cancer are significantly decreased after 5 years of adjuvant TAM. Nonetheless, yearly recurrence rates remain above 2%, with the Early Breast Cancer Trialists’ Collaborative Group study showing a 15-year recurrence of more than 30% (EBCTCG, 2005). Additionally, a small proportion of women have serious side-effects, including increased incidence of endometrial cancer, thromboembolism and cerebrovascular events (Wysowski et al., 2002; Braithwaite et al., 2003; Cuzick, 2003; EBCTCG, 2005; Lewis, 2007). Thus, alternative hormonal therapies have been sought for adjuvant treatment of breast cancer.

In the past decade, a number of aromatase inhibitors (AIs) have been developed as an alternate approach to TAM for the treatment of estrogen receptor–positive breast cancer. The current third-generation AIs (anastrozole, exemestane and letrozole) are highly specific to the aromatase enzyme and have fewer side effects than previous generations of AIs (Fabian, 2007). Anastrozole binds reversibly to the aromatase enzyme and inhibits the conversion of androgens to estrogens in peripheral tissues outside the central nervous system (CNS) and a few CNS sites in various regions within the brain (Simpson, 2003). Evidence from several clinical trials indicates that
anastrozole may be superior to TAM as a first-line therapy for postmenopausal women with metastatic breast cancer (Ferretti et al., 2006). Results from at least eight major clinical trials indicate that anastrozole alone is associated with longer disease-free survival than therapy with TAM alone (Eisen et al., 2008), which supports the use of anastrozole as a first-line therapy or as a second-line therapy after treatment with TAM.

Although anastrozole has demonstrated some superiority relative to TAM (Needleman and Tobias, 2008), many patients still experience a recurrence of breast cancer. In addition, there is substantial inter-individual variability with respect to tolerability, and musculoskeletal complaints can be so severe that some patients withdraw from therapy. This variability is consistent with possible differences among patients in drug pharmacokinetics and/or pharmacodynamics, potentially driven by host genetic variability. These factors, if understood, would offer the potential for individualizing treatment and ensuring that patients receive optimal therapy.

Anastrozole is predominantly modified by hepatic metabolism via oxidation by CYP3A4 into hydroxyl anastrozole, which may further undergo glucuronidation by UGT1A4 into hydroxyl anastrozole glucuronide (Dowsett et al., 2001; Kamdem et al., 2010). Anastrozole can also undergo direct glucuronidation catalyzed by UGT1A4 into anastrozole N-glucuronide (Kamdem et al., 2010; Lazarus and Sun, 2010). UGT1A4 is a conjugative detoxification enzyme that facilitates water solubility and excretion of various substrates (Jakoby and Ziegler, 1990). The variability of human glucuronidation activity by UGT1A family is influenced by the presence of single nucleotide polymorphisms (SNPs), which have been identified not only in the coding regions of the UGT1A unique first exons and the common exons 2–5, but also within the 5′-flanking
regions (Guillemette et al., 2000a; Guillemette et al., 2000b; Strassburg et al., 2002; Ehmer et al., 2004; Wiener et al., 2004; Lankisch et al., 2005; Benoit-Biancamano et al., 2009). These SNPs have been shown to alter glucuronidation activity and/or have been associated with the risk of cancer, toxicity, response to therapy and unwanted drug side effects (Ando et al., 1998; Guillemette et al., 2000a; Vogel et al., 2001; Strassburg et al., 2002; Wiener et al., 2004; Benoit-Biancamano et al., 2009). Therefore, it is conceivable that differences in anastrozole glucuronidation may contribute to the overall variability in treatment effect experienced by patients. Previous studies suggest that UGT1A4 coding SNPs may not contribute to variability in response to anastrozole, but the potential impact of UGT1A4 promoter SNPs on anastrozole glucuronidation has not been explored. To address this issue, we examined the glucuronidation of anastrozole in human liver microsomes and analyzed the effect of genetic variants of UGT1A4 on anastrozole glucuronidation.

Materials and Methods

Chemicals and Reagents.

Anastrozole (2,2’-[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis(2-methylpropanenitrile) was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Lamotrigine (6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine), hecogenin, alamethicin, magnesium chloride, Tris-HCl and UDP glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (St Louis, MO). Baculovirus- expressed human UGT1A4 was purchased from BD Gentest Corp. (Woburn, MA) Rabbit antihuman UGT1A4 primary and secondary antibodies were purchased from Santa Cruz Biotechnology, Inc.
Precast 10% acrylamide gels were obtained from Bio-Rad Laboratories (Hercules, CA). Chemiluminescence reagents were obtained from GE Healthcare (Piscataway, NJ). All other reagents were of HPLC grade or of the highest grade commercially available.

**Human liver microsomes (HLMs).**

The HLMs used for this study have been described previously (Edavana et al., 2012). Briefly, human liver specimens (n= 96, from non-cancerous subjects, collected and frozen between 0 -3 hrs of surgical excision) were obtained from the Cooperative Human Tissue Network (CHTN). All liver specimens were from Caucasian/ African American/ unknown donors ranging in age from 26 to 102 years donors, with 54 male, 36 female and 6 unknown. African Americans were excluded from this study due to low numbers that precluded racial comparisons. All liver specimens were snap-frozen upon harvest and were confirmed as histologically normal tissue by CHTN. Microsomes were prepared from human liver tissue as previously described (Turesky et al., 1991), and stored frozen at -80°C until assayed. Liver microsomal protein levels were determined using the Bradford method (Bradford, 1976) with bovine serum albumin as a standard. We also included a mixture of liver microsomes pooled from 24 individual donors ( BD Gentest Corp., Woburn, MA). Pooling was based on CYP activity as per manufacturer data sheet.

**Quantitative Real-Time PCR.**

Total RNA from human liver samples was isolated using TRIzol (Invitrogen, Carlsbad, CA) and used as a template for cDNA synthesis with Superscript II (Invitrogen, Grand Island, NY). Quantitative RT-PCR was performed using a Prism
7900HT Sequence Detection System and SYBR Green PCR MasterMix (Applied Biosystems, Foster City, CA). The following primers were used: 5′ AGAGAGGTGTCA GTGGTGATCT-3′ (forward) and 5′-AACAGCCACACGGATGCATA-3′ (reverse) for UGT1A4 and 5′-ATCCTGGCCTCGCTGTCC-3′ (forward) and 5′-CTCCTGCTTGGCTGATCCACAT-3′ for β-actin (reverse). The PCR conditions were as follows: an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 1 min. Amplified products were monitored by measuring the increase of fluorescence intensity from the SYBR Green dye that binds to the double-stranded DNA amplification product. The dissociation curves for each reaction were examined to ensure amplification of a single PCR product in the reaction. The -fold change in mRNA levels were determined after normalizing the gene expression levels by those of β-actin (2^(-ΔΔCt) method) (Schmittgen and Livak, 2008).

**Glucuronidation of anastrozole and lamotrigine by HLMs.**

The glucuronidation of anastrozole and lamotrigine were measured in pooled HLMs, recombinant UGT1A4, and in microsomes from 96 individual human livers. Enzymatic assays were performed according to standard procedure (Benoit-Biancamano et al., 2009; Kamdem et al., 2010). Microsomes were pre-incubated with alamethicin (25 µg ml^-1 in final incubation) on ice for 15 min. Alamethicin was dissolved in 90/10 incubation buffer/ethanol. The final concentration of ethanol in the reaction was less than 0.1%. Incubations (200 µl) contained substrate (anastrozole or lamotrigine, 100 µM), 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, 100µg ml^-1 microsomes/20µg ml^-1 recombinant UGT1A4 and 5 mM UDPGA, and were carried out at 37°C for 90 min.
The reactions were terminated by the addition of 20µl acetonitrile, followed by centrifugation at 13000 g for 15 min. Aliquots (1 µl) were then analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Incubations without UDPGA, anastrozole, lamotrigine or microsomes were carried out as controls to ensure that produced metabolites were microsome- and UDPGA dependent. Metabolite quantitation was performed with an Aquity UPLC (Waters, Milford, MA) interfaced source to an TSQ-QuantumUltra triple quadrupole (ThemoScientific, SanJose, CA) using a heat assisted electrospray ionization source (HESI). An Aquity UPLC BEH (Bridged Ethane-Silicon Hybrid) C18 column was operated with a linear gradient from 5% acetonitrile/0.1% formic acid to 80% acetonitrile/0.1% formic acid in 5 min at 0.4 mL/min. Anastrozole and anastrozole glucuronide were acquired by monitoring the ion transition of \( m/z \) 294.0 to 225.2 and \( m/z \) 470.2 to 225.2, respectively. Lamotrigine and lamotrigine glucuronide were acquired by monitoring the ion transition of \( m/z \) 256 to 166.018 and \( m/z \) 432 to 345.000, respectively. Due to the absence of authentic standards for anastrozole glucuronide and lamotrigine glucuronide, quantification of the glucuronide was accomplished using a standard curve for anastrozole and lamotrigine.

**Inhibition of anastrozole glucuronidation**

Anastrozole (100 µM) was incubated in the absence or presence of different concentrations of hecogenin (0 – 100 µM), a known UGT1A4 inhibitor (Uchaipichat et al., 2006). Incubations were performed for 90 min using 100µg protein ml\(^{-1}\) recombinant UGT1A4 or 0.5 mg protein ml\(^{-1}\) of pooled HLM.

**Genotyping**
Genotyping for UGT1A4 SNPs was performed in 96 samples. Genotyping for UGT1A4 5'sequences (-36G>A, -163G>A, -217T>G and -219C>T) and exon1 (70C>A and 142 T>G) SNPs were performed as described previously (Saeki et al., 2005; Benoit-Biancamano et al., 2009). All amplification was performed using 1U of Taq Polymerase. Sequencing was performed using an ABI Prism 3700 DNA analyzer (Applied Biosystems, Foster City, CA).

**Statistical Analysis**

Both parametric and non-parametric tests were performed to examine the correlation of anastrozole glucuronidation with UGT1A4 mRNA, lamotrigine glucuronidation and UGT1A4 SNPs. Also, UGT1A4 mRNA was correlated with UGT1A4 SNPs. In parametric one-way ANOVA, non-Gaussian distributed variables were log transformed and analysis was implemented using ‘PROC GLM’. Non-parametric one-way ANOVA was executed using ‘PROC NPAR1WAY’. A p< 0.05 (2-sided) was considered to be statistically significant and all analyses were performed using SAS software (version 9.2, Statistical Analysis Systems, Cary, NC).
Results

Analysis of anastrozole glucuronidation in human liver microsomes:

Inhibition by hecogenin

Chemical structures of anastrozole, lamotrigine and their glucuronides are presented in Figure 1. Representative ion chromatograms show that the retention times of anastrozole glucuronide and anastrozole were 2.87 min, and 3.95 min, respectively (Figure 2A). The retention times of lamotrigine glucuronide and lamotrigine were 1.35 min, and 1.63 min, respectively (Figure 2B). Hecogenin was examined due to its ability to inhibit anastrozole glucuronidation (Wiener et al., 2004). Hecogenin at 5 µM inhibited anastrozole glucuronidation activity by 40% in pooled HLMs (IC50=15 µM) and 65% in recombinant UGT1A4 (IC50=4.72 µM). At 50 µM, hecogenin reduced anastrozole glucuronide formation by 75% in pooled HLMs and 90% in recombinant UGT1A4 (Figure 3A and 3B).

Anastrozole glucuronidation by human liver microsomes

Inter-individual variability of anastrozole glucuronidation in 96 samples ranged from 0.023- 5.4 pmol/mg/min protein. There was a significant correlation between UGT1A4 mRNA level and anastrozole glucuronidation (Figure 4A). Anastrozole glucuronide formation was highly correlated with lamotrigine glucuronidation, a known UGT1A4 substrate (Figure 4B).

Effect of UGT1A4 genotype on anastrozole glucuronidation
To determine the contribution of UGT1A4 coding and promoter polymorphisms to anastrozole glucuronidation, dideoxy sequencing was performed on genomic DNA from liver specimens of 96 human subjects. Previous studies suggested that coding region SNPs 70C>A and 142 T>G can alter UGT1A4 catalytic activities (Ehmer et al., 2004; Wiener et al., 2004; Benoit-Biancamano et al., 2009), but no significant association between these SNPs with anastrozole glucuronidation (Figure 5A) and UGT1A4 mRNA (Figure 5B) were found in the current study. We then examined SNPs in the promoter region of UGT1A4. Sequencing analysis of UGT1A4 promoter region revealed the existence of four different SNPs of variable frequencies [-36G>A (0.07), -163G>A (0.13), -217T>G (0.08), and -219C>T (0.16)]. SNPs -163G<A (p=0.009), -217T<G (p=0.009) and -219C<T (p=0.014) were significantly correlated with anastrozole glucuronidation (Figure 6). Also, SNPs -163G<A (p=0.009), -217T<G (p=0.009) and -219C<T (p=0.013) were significantly correlated with UGT1A4 mRNA expression levels. Microsomes from samples heterozygous for the T<G allele at position -217 exhibited significantly increased glucuronidation activity toward anastrozole compared to microsomes from samples heterozygous for the G<A and C<T at positions -163 and -219 respectively. Except SNPs 142T>G and -163G<A, none of the SNPs are consistent with Hardy Weinberg equilibrium, possibly due to the presence of unknown population substructure. There was no evidence of linkage of the UGT1A4 promoter SNPs with each other or with the coding region SNPs. Thus, the correlations of individual SNPs with UGT1A4 activity were examined separately (Table1).
Discussion

Aromatase inhibitors have been approved as a first-line treatment option for hormone-dependent advanced breast cancer in postmenopausal patients. However, emerging data suggest high inter-individual variability in the beneficial and adverse effects of anastrozole and other aromatase inhibitor drugs (Sanford and Plosker, 2008).

Anastrozole is metabolized and cleared mainly through N-dealkylation, hydroxylation via the CYP3A4 enzyme (Dowsett et al., 2001; Antoniou and Tseng, 2005), and glucuronidation via the UGT1A4 enzyme (Kamdem et al., 2010). The major metabolites of anastrozole identified in urine and plasma are triazole, OH-anastrozole, the glucuronide of OH-anastrozole, and the N-glucuronide of anastrozole (AstraZeneca, 2007). N-glucuronidation of anastrozole is catalyzed predominantly by UGT1A4 (and to a lesser extent by UGT2B7 and UGT1A3) (Kamdem et al., 2010; Lazarus and Sun, 2010), resulting in deactivation and elimination of the active drug. Therefore, it is possible that variability in anastrozole glucuronidation due to genetic factors may contribute to the overall inter-individual variability in efficacy and tolerability experienced by patients.

In this study, recombinant UGT1A4 glucuronidated anastrozole, consistent with other studies (Kamdem et al., 2010; Lazarus and Sun, 2010). However, UGT1A4 may not be the only hepatic UGT participating in the glucuronidation of anastrozole. UGT2B7 and UGT1A3 present in HLM may also have a minor role in anastrozole glucuronidation (Kamdem et al., 2010; Lazarus and Sun, 2010). Moreover, UGT1A4 is expressed in the liver with extensive inter-individual variability mostly dictated by environmental factors,
age, gender, race, genetic variability and concomitant drug administration (Wilkinson, 2005).

Previous studies have demonstrated that hecogenin is a specific UGT1A4 inhibitor (Uchaipichat et al., 2006; Kamdem et al., 2010). In the current study, hecogenin significantly inhibited anastrozole glucuronidation in both HLM (IC$_{50}$=15 µM) and recombinant UGT1A4 (IC$_{50}$=4.72 µM). These results were consistent with those of Kamdem et al., 2010 (Kamdem et al., 2010), but not with Lazarus and Sun, 2010 (Lazarus and Sun, 2010), and Erickson et al., 2012 (Erickson-Ridout et al., 2012), where clozapine (a substrate of UGT1A4) glucuronidation was inhibited by 90% with 10µM hecogenin. Interindividual variability of UGT1A4, and possibly a minor role of UGT1A3 and UGT2B7 in anastrozole glucuronidation, may have an effect on inhibition studies. Variations in substrate concentration and purity may also have an effect on experimental results. Moreover, Zhou et al. (Zhou et al., 2010) suggest that multiple aglycone binding sites exist within UGT1A4, which may result in atypical kinetics (both homotropic and heterotropic) in a substrate-dependent fashion.

Anastrozole glucuronidation was highly correlated with lamotrigine glucuronidation (a diagnostic substrate for UGT1A4) ($r$=0.99, $p$<0.0001), but some recent studies suggest that lamotrigine may not be entirely specific for UGT1A4 (Erickson-Ridout et al., 2011). However, these results support the predominant (but not the sole) role of UGT1A4 in the direct glucuronidation of anastrozole.

UGT1A4 is expressed primarily in the liver, but it is also expressed in biliary tissue, colon, and breast (Strassburg et al., 1997; Chouinard et al., 2006).
Glucuronidation activities in different human tissues have been shown to exhibit a high degree of inter-individual variation which cannot be explained by unique tissue specificity of \textit{UGT1A4} gene expression (Strassburg et al., 2000; Shipkova et al., 2001). A potential contributor to variability is the presence of SNPs within the promoter and coding regions of the \textit{UGT1A4} gene, leading to quantitative or qualitative alterations of specific catalytic activities between individuals. Our data on UGT1A4 mRNA expression levels in 96 human subjects showed marked inter-individual variability, suggesting that genetic variants in \textit{UGT1A4} could impact anastrozole pharmacogenomics. We examined the association between \textit{UGT1A4} promoter and coding SNPs and variability in anastrozole glucuronidation. Previous UGT1A4 genotype-phenotype correlations have focused primarily on the coding region SNPs (\textit{UGT1A4} 70C>A and 142 T>G). Consistent with previous studies (Kamdem et al., 2010; Lazarus and Sun, 2010), we found no statistically significant association between these genetic variants and anastrozole glucuronidation formation rates. However, there are reports of an association of coding region SNPs with differential metabolic activity toward mutagenic amines and endogenous steroids, altering hepatic metabolism and detoxification (Ehmer et al., 2004; Benoit-Biancamano et al., 2009; Gulcebi et al., 2011; Zhou et al., 2011). More recently, functional SNPs in the promoter elements located within the 5'-upstream region of \textit{UGT1A4} have been described (Saeki et al., 2005; Erichsen et al., 2008; Benoit-Biancamano et al., 2009; Menard et al., 2009). UGT1A4 expression and induction were reduced in the presence of SNPs at positions -219 and -163 (Erichsen et al., 2008). We observed a similar decrease in anastrozole glucuronidation and UGT mRNA expression levels in liver microsomes from individuals heterozygous for -163G<A
and -219C<T alleles. On the other hand, microsomes from individuals heterozygous for the -217T<G allele exhibited significantly higher glucuronidation activity towards anastrozole as well as increased UGT1A4 transcriptional activity. These promoter SNPs (-163G<A, -217 T<G and -219C<T) were significantly associated with both anastrozole glucuronidation and UGT1A4 mRNA expression levels. Benoit-Biancamano et al., reported a strong linkage disequilibrium of promoter SNPs(-163A, -219T, -419A and -463T) with coding region variant 142 T, but in our study none of these UGT1A4 promoter SNPs were in linkage disequilibrium either with each other or with the coding region SNPs (Benoit-Biancamano et al., 2009). Examination of SNP haplotypes in relation to enzymatic activity would be optimal, but our sample size was insufficient to examine the effect of multiple SNPs in a statistically robust manner.

Studies have demonstrated a consistent association of UGT1A4 genotype with cancer risk, toxicities and drug response (Innocenti et al., 2001; Nakajima and Yokoi, 2005; Figueroa et al., 2008; Murdter et al., 2011). Improvement of the genetic model predictive of UGT1A4 phenotype could provide a biomarker for predicting cancer risk and therapeutic efficacy. To our knowledge, this study describes for the first time the association of UGT1A4 promoter SNPs and anastrozole glucuronidation. Limitations to the study include the unavailability of authentic standards to examine the isomeric selectivity of anastrozole glucuronidation and thus, stereospecific genotype-phenotype analyses. Nonetheless, we have demonstrated a significant impact of UGT1A4 genotype on anastrozole glucuronidation, which highlights the importance of UGT1A4 genotype in anastrozole pharmacogenomics studies.
Authorship contribution

Participated in research design: Edavana, Kadlubar

Conducted experiments: Edavana, Williams

Contributed new reagents or analytical tools: Boysen

Performed data analysis: Edavana, Dhakal

Wrote or contributed to the writing of the manuscript: Edavana, Kadlubar, Penney, Yao-Borengasser, Boysen.
References


Footnotes

This work was supported by the National Institutes of Health National Cancer Institute [R01CA118981].
Figure Legends

Figure 1. Chemical structures of anastrozole and lamotrigine. Anastrozole, 2,2’-[5-(1H-1,2,4-triazol-1-ylmethyl)-1,3-phenylene]bis(2-methylpropanenitrile), (1A) and lamotrigine, 6-(2,3-dichlorophenyl)-1,2,4-triazine-3,5-diamine (1B) and potential sites for glucuronidation.

Figure 2. Mass spectrometry of anastrozole, lamotrigine and their glucuronides. Representative extracted ion chromatogram of anastrozole (2A), lamotrigine (2B) and their glucuronides produced by human liver microsomal incubations.

Figure 3. Inhibition of anastrozole glucuronidation by hecogenin. IC₅₀ values were calculated in HLM (15µM, 4A) and recombinant UGT1A4 (4.72µM, 4B). Each microsomal incubation with inhibitor was carried out in triplicate. Means of the experiments are designated by (+) sign.

Figure 4. Correlation plot of anastrozole glucuronidation with UGT1A4 mRNA (5A) and lamotrigine glucuronidation (5B). The “r” and p values specify Spearman’s correlation coefficient and statistical significance, respectively.

Figure 5. Influence of UGT1A4 coding region SNPs on anastrozole glucuronidation (6A) and UGT1A4 mRNA expression levels (6B). The (+) sign and line inside each box indicate mean and median, and the upper and lower limits of the box are 75th and 25th percentiles, respectively. The vertical bars above and below
represent the maximum and minimum values, respectively. The solid circles outside the box are outlier values. P values indicate the statistical significance of Kruskal Wallis tests (non-parametric one way ANOVA).

**Figure 6. Influence of UGT1A4 promoter SNPs on anastrozole glucuronidation activity in human liver microsomes.**

The (+) sign and line inside each box indicate mean and median, and the upper and lower limits of the box are 75th and 25th percentiles, respectively. The vertical bars above and below designate the maximum and minimum values, respectively. The solid circles outside the box are outlier values. P values indicate the statistical significance of rank sum tests (non-parametric two-sample t-test).
# Tables

## Table 1. Analysis of anastrozole glucuronidation and UGT mRNA expression levels by UGT1A4 variants

<table>
<thead>
<tr>
<th>Frequency (%)</th>
<th>Anastrozole glucuronidation (pmol/µg/min) median (SD(^1))</th>
<th>UGT1A4 mRNA median (SD(^1))</th>
<th>(P) for HWE(^2)</th>
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<tr>
<td>70C&gt;A</td>
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<td>CC</td>
<td>48 (18.7)</td>
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<td>1.4 (0.3)</td>
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<td>142T&gt;G</td>
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<td>1.2 (0.4)</td>
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<td>-36G&gt;A</td>
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<td>81 (89)</td>
<td>0.3 (1.2)</td>
<td>1.4 (1.4)</td>
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<td>1.0 (1.4)</td>
<td>1.5 (0.3)</td>
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<td>73 (79.3)</td>
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<td>1.4 (0.1)</td>
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<td>3 (3.3)</td>
<td>1.9 (1.2)</td>
<td>1.7 (0.3)</td>
</tr>
<tr>
<td>-219C&gt;T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>69 (74.2)</td>
<td>0.4 (1.1)</td>
<td>1.4 (0.3)</td>
</tr>
<tr>
<td>CT</td>
<td>19 (20.4)</td>
<td>0.9 (0.7)</td>
<td>1.0 (0.4)</td>
</tr>
<tr>
<td>TT</td>
<td>5 (5.4)</td>
<td>1.0 (2.2)</td>
<td>1.2 (0.6)</td>
</tr>
</tbody>
</table>

\(^1\)Standard deviation  
\(^2\)Hardy-Weinberg Equilibrium
Figure 1

A  Anastrozole

\[
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\end{array}
\]

\[
\begin{array}{c}
\text{N} \\
\text{N} \\
\text{Cl} \\
\text{Cl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{Anastrozole N-glucuronide} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\end{array}
\]

\[
\begin{array}{c}
\text{glucuronide} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\end{array}
\]

\[
\begin{array}{c}
\text{UGT} \\
\text{UDPGA} \\
\text{UGT} \\
\text{UDPGA} \\
\end{array}
\]

B  Lamotrigine

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{Cl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{lamotrigine N2-glucuronide} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{H}_2\text{N} \\
\text{N} \\
\text{N} \\
\text{N} \\
\text{Cl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{glucuronide} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\text{Cl} \\
\end{array}
\]
Figure 4

A

Anastrozole glucuronidation (pmol/µg/min)

UGT1A4 mRNA

r = 0.99
P < 0.0001

B

Anastrozole glucuronidation (pmol/µg/min)

Lamotrigine glucuronidation (pmol/µg/min)

r = 0.99
P < 0.0001
Figure 5

A

B