The Letrozole Phase 1 Metabolite Carbinol as a Novel Probe Drug for UGT2B7

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ABSTRACT

Carbinol [4,4'-hydroxymethylene]dibenzonitrile is the main phase 1 metabolite of letrozole, a nonsteroidal aromatase inhibitor used for endocrine therapy in postmenopausal breast cancer. We elucidated the contribution of UDP-glucuronosyltransferase (UGT) isoforms on the glucuronidation of carbinol. Identification of UGT isoforms was performed using a panel of recombinant human UGT enzymes. Kinetic studies were done in recombinant human UGT2B7 and pooled human liver microsomes (HLMs). A liquid chromatography–tandem mass spectrometry method was used for detection of metabolites. To assess the impact of UGT2B7*2, we determined the carbinol glucuronidation activity using HLM as well as UGT2B7 protein expression in 148 human livers. Moreover, we analyzed the plasma concentrations of 60 letrozole-treated breast cancer patients. We identified UGT2B7 as the predominant UGT isoform involved in carbinol glucuronidation. In HLMs and recombinant UGT2B7, we determined $K_m$ values (9.99 and 5.96 $\mu$M) and $V_{max}$ values (3430 and 2399 pmol/min per milligram of protein), respectively. In the set of 148 human livers, carbinol glucuronidation activity significantly correlated with UGT2B7 protein as determined by Western blotting ($r_s = 0.5088, P < 0.0001$). Neither carbinol glucuronidation activity (*1/*1: $n = 25$, 2434 ± 1018; *1/*2: $n = 80$, 2356 ± 1372; *2/*2: $n = 43$, 2251 ± 1421 pmol/min per milligram of protein) nor UGT2B7 protein expression was altered by the UGT2B7*2 genotype. No impact of UGT2B7*2 on plasma levels of carbinol and carbinol-gluc [bis(4-cyanophenyl)methyl hexopyranosiduronic acid] in 60 letrozole-treated patients was found. Taken together, these findings suggest carbinol as a novel in vitro probe substrate for UGT2B7. In vitro and in vivo data suggest a lack of influence of the UGT2B7*2 polymorphism on carbinol glucuronidation.

Introduction

The UDP-glucuronosyltransferases (UGTs) are an important group of enzymes that modulate physiologic activity, distribution, and excretion of endogenous and exogenous compounds, including many drugs. Expression and catalytic activity of particular UGT isoenzymes are highly variable owing to a variety of factors, such as age, diet, diseases, ethnicity, and genetic polymorphisms (Court, 2010; Miners et al., 2010). Since variability in the expression of specific UGT isoforms, and resulting variations in plasma levels, can impact therapy outcome, especially for drugs with narrow therapeutic ranges, an assessment of the level and catalytic activity of UGTs is of utmost importance during clinical studies and in the course of pharmacotherapy.

In addition, the development of new drugs requires in vitro identification of UGT enzymes involved in the metabolism, as well as an assessment of the drugs’ potential for inhibition or induction of individual UGT isoforms, a likely cause for drug-drug interactions. Because multiple allelic variants of UGT-encoding genes exist, the effects of genetic variations on enzymatic activity should also be characterized (Food and Drug Administration, 2012, http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm292362.pdf; Meyer et al., 2013; Saladores et al., 2013). In practice, the enzyme kinetics study of UGTs is limited by the availability of selective probe substrates for the characterization of individual UGT isoforms (Court, 2005). Therefore, the identification of novel appropriate probe substrates is important to the advancement of this field of research.

In this study, we set out to identify the UGT isoform(s) responsible for the glucuronidation of 4,4'-[(hydroxymethylene)dibenzo nitrile (carbinol). Carbinol is the phase 1 metabolite of letrozole, a nonsteroidal aromatase inhibitor used in the adjuvant antihormonal treatment of postmenopausal patients with estrogen receptor–positive breast cancer (Fig. 1). Carbinol-gluc [bis(4-cyanophenyl)methyl hexopyranosiduronic acid] is the major metabolite of letrozole, its amount in urine accounting for 65% of the total dose of administered drug (Sioufi et al., 1997;

ABBREVIATIONS: AZT, 3'-azido-3'-deoxythymidine; carbinol, 4,4'-hydroxymethylene)dibenzonitrile; carbinol-gluc, bis(4-cyanophenyl)methyl hexopyranosiduronic acid; 7-HFC, 7-hydroxy-4-( trifluoromethyl)coumarin; HLM, human liver microsome; LC-MS/MS, liquid chromatography–tandem mass spectrometry; TBST, Tris-buffered saline with 0.1% Tween 20; UDP-GA, UDP-glucuronic acid; UGT, UDP-glucuronosyltransferase.
Pfister et al., 2001). Toward our goal, we used metabolic screening with a battery of recombinant human UGTs and a panel of human liver microsomes (HLMs), as well as correlative and inhibition experiments. Our enzyme kinetic studies demonstrated the superior affinity and specificity of carbinol as a probe substrate for the human UGT2B7 isoform. Based on our findings, we investigated carbinol glucuronidation in a panel of 60 postmenopausal breast cancer patients receiving letrozole treatment who were genetically characterized for the presence of UGT2B7 allelic variants. In this way, we evaluated the influence of the UGT2B7*2 allelic variant on carbinol glucuronidation.

Materials and Methods

Chemicals

Carbinol, carbinol-gluc, and d₅-carbinol-gluc were synthesized as described previously (Precht et al., 2012). Purity, as determined by high-performance liquid chromatography-UV analysis, exceeded 99, 98, and 92% for carbinol, carbinol-gluc, and d₅-carbinol-gluc, respectively.

Alamethicin from *Trichoderma viride*, UDP glucuronic acid (UDP-GA) trisodium salt, 3'-azido-3'-deoxythyridine (AZT), and 7-hydroxy-4-(trifluoromethyl)coumarin (7-HFC) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Liquid chromatography-mass spectrometry-grade acetonitrile was purchased from Carl Roth GmbH & Co KG (Karlsruhe, Germany). All other chemicals were of analytical grade. Sample handling was performed in polypropylene labware.

Recombinant UGTs and HLMs

Recombinant isoenzymes of human UGT enzymes expressed in baculovirus-transfected insect cells (BD Supersomes) were purchased from BD Gentest (Woburn, MA). Our screened set of isoforms included the following: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17, and control supersomes. HLMs were prepared as described previously from surgically removed liver tissue from patients of both sexes (Nies et al., 2009, 2013). The study was approved by the ethics committees of the medical faculty of the Charité, Humboldt-University Berlin (Germany), and the University of Tübingen (Germany), and written informed consent was obtained from each patient. A pool of HLMs was obtained by mixing microsomes from 150 different donors.

In Vitro Incubations with Recombinant UGTs and HLMs

The incubation mixtures had a final volume of 100 μl containing 5 μg of supersomes with recombinant human UGT isoenzymes, 0.1 M sodium phosphate buffer (pH 7.4), 5 mM MgCl₂, 1.5% dimethy sulfoxide, 10 mM UDP-GA, and carbinol as substrate. According to the manufacturer’s guidelines and well established data from previously published papers (Kaji and Kume, 2005; Ohno et al., 2008; Donato et al., 2010), alamethicin at a concentration of 25 μg/ml (from a stock solution of 0.5 mg/ml in a mixture of 10% dimethy sulfoxide in phosphate buffer) was added. The mixture without substrate and UDP-GA was preincubated on ice for 30 minutes and subsequently warmed to 37°C; then carbinol was added, with an additional 10 minutes of preincubation. The reaction was started by adding UDP-GA. After 30 minutes, the reaction was stopped by adding 100 μl of ice-cold 1% acetic acid in acetonitrile containing 1 μM of d₅-carbinol-gluc as internal standard. To complete protein precipitation, the samples were kept on ice for 10 minutes. The precipitated protein was separated by centrifugation (5 minutes, 16,400 g), and 20 μl of the supernatant was diluted with 180 μl of 0.1% acetic acid before liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. Using supersomes and HLMs, the impact of alamethicin on carbinol glucuronidation was investigated.

For the determination of Kaz and Vₘₐₓ, 7 μg of supersomes or HLM was used to increase product formation, especially in samples with low concentrations of carbinol. Carbinol in the range of concentrations of 0–40 μM was used for supersomes and HLMs. The study of the individual carbinol-glucuronidation activity of 148 HLMs was performed after 15 minutes of incubation to avoid excessive substrate consumption in the HLMs with the greatest activity. For each experimental batch, HLM glucuronidation activity was corrected for the activity of pooled HLMs that were used as biologic control. For inhibition studies, in vitro incubations were performed as described already, in the presence of 2.5 mM AZT. AZT was added before the preincubation on ice as aqueous solution (25 mg/ml).

Quantification of Carbinol-Gluc

Calibration. For the quantification of carbinol-gluc, a recently developed LC-MS/MS method for the determination of letrozole, carbinol, and carbinol-gluc in human plasma was adapted (Precht et al., 2012). Sample preparation was done by protein precipitation with acetonitrile. Eight calibration samples were prepared in sodium phosphate buffer containing 0.05 mg/ml microsomal protein (HLMs inactivated at 95°C for 10 minutes). Final carbinol-gluc concentration ranged from 0.01 to 10 μM.

LC-MS/MS Analysis. Chromatographic separation was carried out using a 1200 rapid-resolution LC-system (Agilent Technologies, Waldbronn, Germany) on a ZORBAX Eclipse XDB-C18 column (particle size 1.8 μm, 4.6 × 50 mm; Agilent Technologies) maintained at 40°C and a gradient of acetonitrile (20–70%) in 0.1% acetic acid in water.

MS analysis was performed in the multiple reaction monitoring mode using a 6460 triple quadrupole MS (Agilent Technologies) equipped with a Jet Stream electrospray source (Agilent Technologies).

Activity of Recombinant UGT Enzymes

The activity of the recombinant UGTs was tested using 100 μM 7-HFC as a substrate (Kaji and Kume, 2005). 7-HFC stock solution (5 mM dissolved in ethanol) was freshly prepared before each experiment. LC-MS/MS analysis of 7-HFC–glucuronide formation was done using the previously described method for the quantification of carbinol-gluc with the additional transition 405.1 → 229.1, resulting in peaks at ~2.8 minutes of retention time.

Western Blot Analysis of UGT2B7 Expression in HLMs

The relative UGT2B7 protein content of HLMs was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting; 40 μg of microsomal protein per lane was separated on 10% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Whatman Protran BA
85; GE Healthcare, Freiburg, Germany) by electrophoresing. Sample loading and transfer efficiency were checked by Ponceau S staining. The membranes were blocked with 5% skim milk in Tris-buffered saline with 0.1% Tween 20 (TBST buffer) and incubated with a 1:1000 dilution of a polyclonal rabbit anti-UGT2B7 antibody (WB-UGT2B7; BD Gentest) in 1% skim milk/TBST buffer. After thorough washing, the membranes were incubated with a labeled secondary antibody, IRDye800CW-conjugated goat polyclonal anti-rabbit IgG (LI-COR Biosciences, Lincoln, NE) in a dilution of 1:10,000 in 1% skim milk/TBST buffer. The immunoblots were scanned at a wavelength of 800 nm using the Odyssey Clix Infrared Imaging System (LI-COR), and the images were quantified using the Odyssey Application Software version 3.0. A calibration curve of pooled HLM was included to each membrane (10, 20, 40, 80 μg of protein). The amount of UGT2B7 protein in HLMs was determined relative to pooled HLMs by comparing absolute intensities of the bands and presented as arbitrary units (-fold amount of the lowest UGT2B7 expression) per microgram of protein. Samples with low amounts of UGT2B7 protein were requantified with an extended calibration curve (5, 10, 20, 40, 80 μg of protein) and by separation of 80 μg of protein.

Plasma Samples of Letrozole Patients

Plasma samples were taken from 60 postmenopausal women diagnosed with early breast cancer who received letrozole treatment (2.5 mg/day) for 6 months. The patients are participating in an ongoing observational trial investigating the outcome predictors of adjuvant endocrine therapy (German Registry of Clinical Studies: DRKS00000605). The study was approved by the ethics committee of the University of Tübingen, Germany, and the German Federal Institute for Drugs and Medical Devices (BfArM), Bonn, Germany. All participants gave informed consent. In the plasma samples, letrozole and its metabolites carbinol and carbinol-gluc were quantified by LC-MS/MS, as previously described (Precht et al., 2012).

Genotyping for UGT2B7*2

Genomic DNA was isolated from whole blood samples of patients treated with letrozole, as well as of patients with corresponding liver samples (Nies et al., 2009, 2013) using both the QIAamp DNA Blood BioRobot MDx Kit and the QIAamp DNA Blood Mini Kit System (Qiagen, Hilden, Germany).

DNA samples were analyzed for UGT2B7*2 using a multiplex matrix-assisted laser desorption/ionization time-of-flight mass spectrometry–based genotyping assay and the MassARRAY technology (Sequenom, San Diego, CA) as described previously (Mürdter et al., 2011).

Data Analysis

Calibration curves of the LC-MS/MS method were obtained by plotting peak area ratios of carbinol-gluc and the internal standard against known analyte concentrations using the Mass Hunter Quantitative Analysis Software B.04.00 (Agilent Technologies). The data were fitted using linear regression with 1/x weighting. Samples were accordingly quantified by their peak area ratios. Km and Vmax of Michaelis-Menten kinetics were calculated by curve fitting using Prism 5.04 for Windows (GraphPad Software, Inc., La Jolla, CA). Prism 5.04 was also used to detect and remove outliers. The distribution of UGT2B7 genotypes was tested for Hardy–Weinberg equilibrium based on Fisher’s exact test (Institute of Human Genetics, Munich, Germany; http://ihg.gsf.de/cgi-bin/hw/hwa1.pl).

Results

Metabolic Screening with a Panel of Recombinant Human UGTs. In vitro incubations with 13 recombinant UGT isoforms (BD Supersomes), including UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17, and control supersomes revealed the highly isofrom-specific contribution of UGT2B7 to the glucuronidation of carbinol. In recombinant UGT2B7 incubated with 10 μM carbinol substrate, consumption was ~20% after 30 minutes, which equates to a glucuronidation activity of V = 1450 ± 142 pmol/min per milligram of protein (mean ± S.D.) (Fig. 2). The inset shows the negligible contribution of the other UGT isoforms to carbinol glucuronidation by UGT1A1, UGT1A9, UGT2B4, and UGT2B17, accounting for approximately 0.12, 0.08, 0.17, and 0.02% of the formation rate of UGT2B7, respectively. The other isoforms did not contribute to a detectable product formation. Among the included isoforms, UGT2B7 was the only relevant UGT isoform involved in the glucuronidation of carbinol. Glucuronidation activity for all O-glucuronidating UGT isoforms was confirmed by incubation with the nonspecific UGT substrate 7-HFC.

Enzyme Kinetics Analysis with UGT2B7 Supersomes and HLMs. Since UGT2B7 was the major UGT isoform catalyzing carbinol glucuronidation in a panel of supersomes, enzyme kinetics was further investigated. Before the in vitro experiments, we determined the linear ranges of incubation time and protein amount. Glucuronide formation was linear up to 0.2 mg/ml microsomal protein and 60 minutes’ incubation time for both HLMs and supersomes (data not shown). To keep substrate turnover below 20%, we chose lower protein concentrations and incubation times. Enzyme kinetic analysis of carbinol glucuronidation in UGT2B7 supersomes and HLMs followed Michaelis-Menten kinetics with apparent Km = 9.56 ± 1.16 μM (mean ± S.D.) (Fig. 2). The inset shows the y-axis at 250-fold magnification. UGT2B7 glucuronidation activity was V = 1450 ± 142 pmol/min per milligram of protein (mean ± S.D.). UGT1A1, UGT1A9, UGT2B4, and UGT2B17 account for approximately 0.12, 0.08, 0.17, and 0.02% of the formation rate of UGT2B7, respectively.
In the absence of alamethicin, the total activity was decreased by 1.5- and 1.4-fold using UGT2B7 supersomes and HLMs, respectively. For both supersomes and HLM data, \( K_m \) values were not affected by the presence or absence of alamethicin, which is consistent with alamethicin’s pore-forming function not influencing UGT enzymes. Our results are in line with previously reported data in HLMs that indicated a 2- to 3-fold increased activity for various substrates in the presence of alamethicin (Fisher et al., 2000; Walsky et al., 2012).

**Correlation of UGT2B7 Expression with Glucuronidation Activity and Inhibition Studies.** UGT2B7 protein expression in 148 HLMs was determined semi-quantitatively by Western blot analysis; a representative blot is shown in Fig. 4. The intensity of the bands for all samples was within the range of the calibration samples (pooled HLMs). Carbinol glucuronidation activity (in vivo assay) in 148 HLMs significantly correlated with UGT2B7 protein content as determined by Western blot (Spearman correlation analysis, \( r_s = 0.5088; P < 0.0001 \); Fig. 5). Variability in carbinol glucuronidation activity and UGT2B7 protein content was 42- and 20-fold, respectively. From the 148 HLMs, 25 were diagnosed as having cholestasis (Nies et al., 2013). As this nongenetic factor may influence UGT2B7 expression and function, we compared both groups. Both expression and activity were significantly reduced in HLMs from cholestatic donors [8.7 \pm 1360 pmol/min per milligram of protein, 2356 \pm 312 pmol/min per milligram of protein and 2251 \pm 1421 pmol/min per milligram of protein \((n = 25, 80, 43; \text{mean} \pm \text{S.D.})\) for the UGT2B7 genotypes *1/*1, *1/*2, and *2/*2, respectively, as displayed in Fig. 6B. Steady-state plasma levels of letrozole, carbinol, and carbinol-gluc of 60 postmenopausal breast cancer patients treated with letrozole (2.5 mg per day) were stratified according to UGT2B7*2 genotype. Letrozole plasma levels (mean \pm S.D.) for the genotype groups *1/*1, *1/*2, and *2/*2 were 343 \pm 85 nM \((n = 12)\), 373 \pm 169 nM \((n = 31)\), and 406 \pm 134 nM \((n = 17)\), respectively. Carbinol plasma levels were 0.37 \pm 0.13 nM, 0.35 \pm 0.11 nM, 0.47 \pm 0.37 nM, carbinol gluc plasma levels were 31.9 \pm 13.3 nM, 35.5 \pm 17.4 nM, 37.7 \pm 14.7 nM, and resulting metabolic ratios were 99.9 \pm 54.5, 108.3 \pm 48.9, 105.6 \pm 58.2, respectively (Fig. 7). Kruskal-Wallis testing revealed that for all compounds, the plasma levels and resulting metabolic ratios did not differ significantly between UGT2B7 genotype groups, indicating that the UGT2B7*2 genotype does not influence the steady-state plasma levels of carbinol-gluc, carbinol, or the parent drug letrozole.

**Effect of the UGT2B7*2 Polymorphism on Carbinol Glucuronidation In Vitro and In Vivo and on Hepatic UGT2B7 Protein Expression.** We investigated the effect of the UGT2B7*2 allele, a common UGT2B7 variant in the Caucasian and Asian populations, on the glucuronidation activity toward carbinol. This was done both in vitro (in the presence of HLM) and in vivo using plasma samples of patients to analyze steady-state plasma levels of carbinol-gluc. The genotype distribution in donors of the liver bank did not significantly deviate from the Hardy-Weinberg equilibrium \( (P = 0.32) \). The calculated minor allele frequency was similar to that reported for individuals of European descent in the Single-Nucleotide Polymorphism Database (0.44 versus 0.5) (Sherry et al., 2001). In 148 HLMs, from donors genotyped for UGT2B7, the UGT2B7*2 polymorphism does not alter the UGT2B7 protein content as shown in Fig. 6A (Kruskal-Wallis test). Activities of carbinol-glucuronidation in 148 HLMs from donors genotyped for UGT2B7 were 2434 \pm 1018 pmol/min per milligram of protein, 2356 \pm 1372 pmol/min per milligram of protein and 2251 \pm 1421 pmol/min per milligram of protein \((n = 25, 80, 43; \text{mean} \pm \text{S.D.})\) for the UGT2B7 genotypes *1/*1, *1/*2, and *2/*2, respectively, as displayed in Fig. 6B.

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![Fig. 3](image-url) Enzyme kinetic analyses of in vitro glucuronidation of carbinol catalyzed by (A) UGT2B7 supersomes and (B) pooled HLMs; 7-μg supersomes or HLMs were incubated for 30 minutes with several concentrations of carbinol. Quantification of carbinol-gluc was done by LC-MS/MS. Data are presented as means of duplicates. (A) Two and (B) three outliers were removed using Prism 5.04. In cases of missing error bars, data points are almost identical. The insets show Eadie-Hofstee plots of the experimental data. (A) Carbinol glucuronidation catalyzed by UGT2B7 supersomes follows Michaelis-Menten kinetics with apparent \( K_m = 9.56 \pm 1.16 \mu M \) (mean \pm S.D.), \( V_{max} = 2399 \pm 113 \text{ pmol/min per milligram of protein, } r^2 = 0.979 \) (B) In the presence of HLMs, the kinetic parameters of carbinol glucuronidations were \( K_m = 9.99 \pm 1.12 \mu M \), \( V_{max} = 3430 \pm 152 \text{ pmol/min per milligram of protein, } r^2 = 0.979 \).
Protein expression in 148 HLMs as determined by immunoblot analysis and glucuronidation activity as determined by LC-MS/MS and UGT2B7 binol conjugation catalyzed by HLMs. Correlation analysis of carbinol; UGT2B17 (0.12, 0.08, 0.17, and 0.02%, respectively).

We observed only negligible contributions of UGT1A1, 1A9, 2B4, and 2B17. The glucuronidation of carbinol appears to be highly isoform-specific: smaller contribution by UGT1A1, UGT1A9, UGT2B4, and UGT2B17. Isoform catalyzing carbinol-gluc biosynthesis as UGT2B7, with much smaller contribution by UGT1A1, UGT1A9, UGT2B4, and UGT2B17. This finding suggests carbinol as an excellent specific substrate for phenotypic analysis of UGT2B7 enzymatic activity. Because physiologic activity of carbinol is strongly reduced compared with letrozole, it also has a potential as an in vivo probe for clinical studies.

**Discussion**

Carbinol is the main phase 1 metabolite of letrozole, a nonsteroidal aromatase inhibitor used in the adjuvant endocrine treatment of postmenopausal breast cancer. Carbinol is excreted from the system as glucuronide; however, the specific UGT isoform responsible for carbinol conjugation remained unknown. The primary aim of the present study was to identify the UGT isoform(s) involved in the glucuronidation of carbinol to the renally excreted carbinol-glucuronide.

To this end, we used a combination of several methods, including in vitro metabolic screening with recombinant human UGTs, correlation experiments with a panel of HLM from 148 liver donors, and inhibition experiments with a rabbit anti-UGT2B7 antibody. Detection was performed after incubation with a IRDye800-labeled secondary antibody using the Odyssey Clx Infrared Imaging System. Quantification data for 148 HLMs are presented in Fig. 5.

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Next, we used a collection of 148 human liver tissues to assess carbinol conjugation catalyzed by HLMs. Correlation analysis of carbinol glucuronidation activity as determined by LC-MS/MS and UGT2B7 protein expression in 148 HLMs as determined by immunoblot analysis revealed a highly significant association ($P < 0.0001$), again suggesting that carbinol glucuronidation is mainly mediated by UGT2B7. AZT, a previously characterized UGT2B7 inhibitor, considerably decreased carbinol glucuronidation in both UGT2B7 supersomes and pooled HLMs. This observation confirmed our assumption that UGT2B7 mediates the phase 2 metabolism of letrozole.

These experiments, for the first time, comprehensively identified the UGT2B7 isoform as a major isoform catalyzing the glucuronidation of carbinol. Four other isoforms—UGT1A1, UGT1A9, UGT2B4, and UGT2B17—revealed a measurable, but about 1000-fold lower, activity toward carbinol and evidently do not contribute much to its metabolic transformation.

We further investigated the kinetic parameters of carbinol glucuronidation in recombinant human UGT enzymes and HLMs. Our results showed that the apparent $K_m$ for carbinol glucuronidation in pooled HLMs ($K_m = 9.99 \pm 1.12 \mu M$) was strikingly similar to the apparent $K_m$ in recombinant UGT2B7 enzyme ($K_m = 9.56 \pm 1.16 \mu M$). The similarity of $K_m$ values between HLMs and recombinant enzymes has been highlighted previously as an attribute of ideal UGT-selective probes (Court, 2005). Additionally, the monophasic Eadie-Hofstee plot of carbinol glucuronidation in HLMs indicates the involvement of only one UGT isoform. Taken together, these experiments prove our notion that carbinol is almost exclusively glucuronidated by UGT2B7.

In our experiments, carbinol appeared to be a highly selective substrate for UGT2B7, with high affinity and conversion rate in vitro. In comparison, many UGT2B7-selective probe drugs, summarized in Table 1, manifest much lower affinity and are metabolized by additional UGT isoforms, which makes interpretation of in vitro and in vivo results cumbersome. Among the drugs listed in Table 1, only 6α-progesterone and epirubicin have comparable selectivity, with much lower affinity to UGT2B7. This finding suggests carbinol as an excellent specific substrate for phenotypic analysis of UGT2B7 enzymatic activity. Because physiologic activity of carbinol is strongly reduced compared with letrozole, it also has a potential as an in vivo probe for clinical studies.

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Next, we used a collection of 148 human liver tissues to assess carbinol conjugation catalyzed by HLMs. Correlation analysis of carbinol glucuronidation activity as determined by LC-MS/MS and UGT2B7 protein expression in 148 HLMs as determined by immunoblot analysis revealed a highly significant association ($P < 0.0001$), again suggesting that carbinol glucuronidation is mainly mediated by UGT2B7. AZT, a previously characterized UGT2B7 inhibitor, considerably decreased carbinol glucuronidation in both UGT2B7 supersomes and pooled HLMs. This observation confirmed our assumption that UGT2B7 mediates the phase 2 metabolism of letrozole.

These experiments, for the first time, comprehensively identified the UGT2B7 isoform as a major isoform catalyzing the glucuronidation of carbinol. Four other isoforms—UGT1A1, UGT1A9, UGT2B4, and UGT2B17—revealed a measurable, but about 1000-fold lower, activity toward carbinol and evidently do not contribute much to its metabolic transformation.

We further investigated the kinetic parameters of carbinol glucuronidation in recombinant human UGT enzymes and HLMs. Our results showed that the apparent $K_m$ for carbinol glucuronidation in pooled HLMs ($K_m = 9.99 \pm 1.12 \mu M$) was strikingly similar to the apparent $K_m$ in recombinant UGT2B7 enzyme ($K_m = 9.56 \pm 1.16 \mu M$). The similarity of $K_m$ values between HLMs and recombinant enzymes has been highlighted previously as an attribute of ideal UGT-selective probes (Court, 2005). Additionally, the monophasic Eadie-Hofstee plot of carbinol glucuronidation in HLMs indicates the involvement of only one UGT isoform. Taken together, these experiments prove our notion that carbinol is almost exclusively glucuronidated by UGT2B7.

In our experiments, carbinol appeared to be a highly selective substrate for UGT2B7, with high affinity and conversion rate in vitro. In comparison, many UGT2B7-selective probe drugs, summarized in Table 1, manifest much lower affinity and are metabolized by additional UGT isoforms, which makes interpretation of in vitro and in vivo results cumbersome. Among the drugs listed in Table 1, only 6α-progesterone and epirubicin have comparable selectivity, with much lower affinity to UGT2B7. This finding suggests carbinol as an excellent specific substrate for phenotypic analysis of UGT2B7 enzymatic activity. Because physiologic activity of carbinol is strongly reduced compared with letrozole, it also has a potential as an in vivo probe for clinical studies.
In vitro experiments on carbinol conjugation catalyzed by HLMs revealed about 20- and 42-fold variability in the level of UGT2B7 polypeptide and carbinol glucuronidation rates, respectively (Fig. 5A). The frequency distribution of carbinol glucuronidation activity appears monomodal but does not follow Gaussian distribution as determined by the Shapiro-Wilk normality test (Fig. 5B). The molecular mechanism for such variability remains to be elucidated. Our preliminary data showed no correlation between the UGT2B7 mRNA and polypeptide levels, thus ruling out transcriptional regulation of UGT2B7 activity (Precht et al., unpublished data). On the other hand, the correlation between the UGT2B7 polypeptide level and its enzymatic activity evidences against the allosteric mechanism of UGT2B7 regulation. We speculate that post-translational modification or proteolytic degradation of UGT2B7 could be a possible mechanism for the observed variability in enzymatic activity, a hypothesis that is currently under investigation in our laboratory.

UGT2B7 is a highly polymorphic gene with more than 70 identified single nucleotide polymorphisms, about 50 of which result in missense, nonsense, and frameshift mutations. The most frequent missense mutation, rs7439366, defines the allelic variant UGT2B7*2 (rs7439366; 802C>T), and causes an amino acid change (H268Y) (Jin et al., 1993). UGT2B7*2 has an allele frequency of 50% in persons of European descent (Bhasker et al., 2000). The functionality of the UGT2B7*2 variant has been thoroughly assessed in vitro and in vivo with a variety of substrates, which can be classified into three groups: those conjugated equally well by *1 and *2 allelic variants, those preferably metabolized by the *1 variant, and those preferably metabolized by the *2 variant (Table 2). Although parts of the three-dimensional structure of UGT2B7 have been solved (Miley et al., 2007), the structure-activity relationships with the substrates from these three groups remain to be elucidated.

To assess the effects of the H268Y polymorphism on carbinol glucuronidation activity by UGT2B7, we performed in vitro experiments with HLM from donors genotyped with respect to the rs7439366 polymorphism. Our analysis demonstrated that the UGT2B7*2 genotype alters neither UGT2B7 protein expression nor the carbinol glucuronidation of HLMs, strongly suggesting that carbinol is conjugated by both UGT2B7*1 and UGT2B7*2 polypeptides (Fig. 6).

We further tested this proposition in 60 postmenopausal women receiving treatment with letrozole who were genotyped with respect to *1 and *2 alleles. Plasma concentrations of carbinol, carbinol-gluc, and their metabolic ratio were determined. No statistically significant difference was found between groups of patients with *1/*1, *1/*2, or *2/*2 genotype. The results of this experiment supported the conclusion that UGT2B7 genetic polymorphism at rs7439366 does not contribute to variability in carbinol glucuronidation.
Summarizing our in vitro results from HLM incubations stratified for UGT2B7*1 and *2 and in vivo plasma levels of letrozole patients genotyped for UGT2B7*1 and *2, we conclude that the common polymorphism UGT2B7*2 (rs7439366, 802CT, H268Y) has no influence on carbinol glucuronidation activity. This is in line with other studies that have shown no influence of the UGT2B7*2 polymorphism on glucuronidation activity for several substrates, such as morphine, epirubicin, and mycophenolic acid (Table 2).

In conclusion, our study demonstrated that carbinol, the phase 1 metabolite of the nonsteroidal aromatase inhibitor letrozole, is a highly specific substrate of the UGT2B7 isoform. This compound is therefore suggested as a high-affinity in vitro and in vivo probe.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Apparent $K_m$ in HLMs</th>
<th>Other UGT Activities (% of UGT2B7 Activity)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbinol</td>
<td>10</td>
<td>Negligible contribution of UGT1A1, 1A9, 2B4, 2B17 (max. 0.17%)</td>
<td>Our results</td>
</tr>
<tr>
<td>AZT</td>
<td>1400</td>
<td>UGT2B4 (~18%), 2B17 (~16%)</td>
<td>Court et al., 2003</td>
</tr>
<tr>
<td>Morphine (→ 3-glucuronide)</td>
<td>1988</td>
<td>UGT1A9, 2B4 (~10%), 2B4 (~11%) 1A3, 1A10, 2B15 (all low activity)</td>
<td>Innocenti et al., 2001</td>
</tr>
<tr>
<td>Morphine (→ 6-glucuronide)</td>
<td>4300</td>
<td>UGT1A1, 1A3, 1A8, 1A9, 1A10, 2B7</td>
<td>Court et al., 2003</td>
</tr>
<tr>
<td>MPA → AcMPAG</td>
<td>370</td>
<td>UGT1A1 (~4%)</td>
<td>Picard et al., 2005</td>
</tr>
<tr>
<td>6-α-OH-progesterone</td>
<td>94</td>
<td>UGT1A3, 1A10 both &lt; 0.2%</td>
<td>Bowalgaha et al., 2007</td>
</tr>
<tr>
<td>21-α-OH-progesterone</td>
<td>34 / 1.22a</td>
<td>UGT1A10</td>
<td>Bowalgaha et al., 2007</td>
</tr>
<tr>
<td>Denopamine</td>
<td>2.870</td>
<td>“Very low,” &lt;1.6% or no activity</td>
<td>Kaji and Kume, 2005</td>
</tr>
<tr>
<td>Naloxone</td>
<td>870</td>
<td>UGT1A1, 1A3, 1A6, 1A10, 2B4, 2B15 (all low activity), UGT1A8 ~10%</td>
<td>Soars et al., 2001; Di Marco et al., 2005</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>568</td>
<td>None (UGT2B4 not tested)</td>
<td>Innocenti et al., 2001</td>
</tr>
<tr>
<td>Chloramphenicol → CP-1-O-Gluc</td>
<td>408</td>
<td>UGT1A9 (~5%), many others (all low activity)</td>
<td>Chen et al., 2010</td>
</tr>
</tbody>
</table>

conc., concentration; max., maximum; n.d., not determined.

aBest described by the Hill equation with positive cooperativity.

<table>
<thead>
<tr>
<th>Type of Study</th>
<th>System</th>
<th>Substrate</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>No effect of UGT2B7*2</td>
<td>Functional</td>
<td>Stably expressed UGT2B7*1/*2 in HEK293 cells</td>
<td>Morphine (3- and 6-glucuronide formation), (+) and (−) menthol, androsterone, codeine</td>
<td>No influence on glucuronidation activity</td>
</tr>
<tr>
<td>Functional</td>
<td>Functional Association</td>
<td>Stably transfected HEK293 cells</td>
<td>Morphine, AZT, codeine</td>
<td>No influence on glucuronidation activity</td>
</tr>
<tr>
<td>Association</td>
<td>HLMs (28 / 26 for morphine)</td>
<td>Androsterone, menthol and morphine (3-glucuronide)</td>
<td>No influence on glucuronidation activity</td>
<td>Bhasker et al., 2000</td>
</tr>
<tr>
<td>In vivo, association</td>
<td>70 Cancer patients treated with morphine</td>
<td>Morphine</td>
<td>No effect on the metabolic ratios of morphine-3- and 6-glucuronide in plasma</td>
<td>Holthe et al., 2002</td>
</tr>
<tr>
<td>Increased activity of UGT2B7*2</td>
<td>Functional</td>
<td>Stably expressed UGT2B7*1/*2 in HEK293 cells</td>
<td>Buprenorphine</td>
<td>10-Fold higher glucuronidation activity for *2</td>
</tr>
<tr>
<td></td>
<td>In vivo, association</td>
<td>86 Patients treated with morphine</td>
<td>Morphine</td>
<td>Lower morphine levels in homozygotes for *2; Lower levels of morphine-3- and 6-glucuronide in homozygotes for *1</td>
</tr>
<tr>
<td>Decreased activity of UGT2B7*2</td>
<td>Functional</td>
<td>Stably transfected HEK293 cells</td>
<td>AZT</td>
<td>Glucuronidation efficiency ($V_{max}/K_m$) 1.9-fold higher for UGT2B7*1 than for the *2 variant</td>
</tr>
<tr>
<td>Functional</td>
<td>Recombinant enzyme, baculovirus infected insect-cells</td>
<td>Flurbiprofen</td>
<td>&gt;14-Fold decreased glucuronidation efficiency ($V_{max}/K_m$) for UGT2B7*2</td>
<td>Wang et al., 2011</td>
</tr>
</tbody>
</table>

AcMPAG, mycophenolic acid acyl glucuronide; HEK, human embryonic kidney.
substrate for evaluating UGT2B7 activity. In vitro experiments with HLMs and in vivo analysis of patients’ plasma levels indicate that the UGT2B7*2 polymorphism does not influence the enzyme’s activity with regard to carbinol glucuronidation. Therefore, carbinol may serve as a probe insensitive to the most common allelic variant of UGT2B7.

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Performed data analysis: Klein, Mürder, Precht, Schwab.
Wrote or contributed to the writing of the manuscript: Brauch, Krynetskiy, Mürder, Precht, Schrot, Schwab.

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Chen M, LeDuc B, Kerr S, Howe D, and Williams DA (2010) Identification of human UGT2B7 as a probe insensitive to the most common allelic variant of UGT2B7 with regard to carbinol glucuronidation. Therefore, carbinol may serve as a probe insensitive to the most common allelic variant of UGT2B7.

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