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The letrozole phase 1 metabolite carbinol as a novel probe drug for UGT2B7

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Abbreviations used in the manuscript:

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 microsomes

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LC-MS/MS, liquid chromatography - tandem mass spectrometry

UDP-GA, uridine diphosphate-glucuronic acid

UGT, uridine diphosphate (UDP)-glucuronosyltransferases

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Abstract

4,4'-(hydroxymethylene)dibenzonitrile (carbinol) is the main phase 1 metabolite of letrozole, a non-steroidal aromatase inhibitor used for endocrine therapy in postmenopausal breast cancer. We elucidated the contribution of 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 (HLM). LC-MS/MS 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 plasma concentrations of 60 letrozole treated breast cancer patients. We identified UGT2B7 as the predominant UGT isoform involved in carbinol glucuronidation. In HLM and recombinant UGT2B7, we determined K_m values (9.99 and 9.56 μM) and V_{max} values (3,430 and 2,399 pmol/min/mg 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, 2,434 \pm 1,018; *1/*2: n=80, 2,356 \pm 1,372; *2/*2: n=43; 2,251 \pm 1,421 pmol/min/mg protein) nor UGT2B7 protein expression were altered by *UGT2B7*2* genotype. No impact of *UGT2B7*2* on plasma levels of carbinol and carbinol-gluc in 60 letrozole treated patients was found. Taken together, we 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.

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Introduction

Uridine diphosphate (UDP)-glucuronosyltransferases (UGTs) are an important group of enzymes which modulate physiological activity, distribution, and excretion of endogenous and exogenous compounds, including many drugs. Expression and catalytic activity of particular UGT isoenzymes are highly variable due to a variety of factors such as age, diet, diseases, ethnicity, and genetic polymorphisms (Court, 2010; Miners et al., 2010). Since variability in 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 require *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 (FDA, 2012; 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 advance this field of research.

In this study, we set out to identify the UGT isoform(s) responsible for glucuronidation of 4,4'-(hydroxymethylene)-dibenzonitrile (carbinol). Carbinol is the Phase 1 metabolite of letrozole, a non-steroidal aromatase inhibitor used in the adjuvant anti-hormonal treatment of postmenopausal patients with estrogen receptor positive breast cancer (Figure 1). Carbinol-gluc (bis(4-cyanophenyl)methyl

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hexopyranosiduronic acid) is the major metabolite of letrozole as its amount in urine accounts for 65 % of the total dose of administered drug (Sioufi et al., 1997; Pfister et al., 2001). Toward our goal, we used metabolic screening with a battery of recombinant human UGTs, and a panel of HLM, as well as correlative and inhibition experiments. Importantly, 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, the influence of the *UGT2B7*2* allelic variant on carbinol glucuronidation was evaluated.

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Materials and Methods

Chemicals

Carbinol, carbinol-gluc and d₄-carbinol-gluc were synthesized as described previously (Precht et al., 2012). Purity as determined by HPLC-UV analysis exceeded 99%, 98%, and 92% for carbinol, carbinol-gluc, and d₄-carbinol-gluc, respectively. Alamethicin from *Trichoderma viride*, uridine diphosphate glucuronic acid (UDP-GA) trisodium salt, 3'-azido-3'-deoxythymidine (AZT) and 7-hydroxy-4-(trifluoromethyl)coumarin (7-HFC) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). LC-MS-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 Human Liver Microsomes

Recombinant isoenzymes of human UGT enzymes expressed in baculovirus-transfected insect cells (BD Supersomes™) were purchased from BD Gentest (Woburn, MA, USA). Our screened set of isoforms included: UGT1A1, UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT1A8, UGT1A9, UGT1A10, UGT2B4, UGT2B7, UGT2B10, UGT2B15, UGT2B17, and control supersomes. HLM were prepared as described previously from surgically removed liver tissue from patients of both genders (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 Tuebingen (Germany) and written informed consent was obtained from each patient. A pool of HLM was obtained by mixing microsomes from 150 different donors.

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***In vitro* Incubations with Recombinant UGTs and HLM**

The incubation mixtures had a final volume of 100 μ l containing 5 μ g supersomes with recombinant human UGT isoenzymes, 0.1 M sodium phosphate buffer pH 7.4, 5 mM MgCl₂, 1.5% dimethyl sulfoxide, 10mM UDP-GA and carbinol as substrate. According to the manufacturer's guideline 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 μ g/ml (from a stock solution of 0.5 mg/ml in a mixture of 10% DMSO in phosphate buffer) was added. The mixture without substrate and UDP-GA was pre-incubated on ice for 30 min, subsequently warmed to 37°C and then carbinol was added, with an additional 10 min of pre-incubation. The reaction was started by adding UDP-GA. After 30 min, the reaction was stopped by adding 100 μ l of ice-cold 1% acetic acid in acetonitrile containing 1 μ M of d₄-carbinol-gluc as internal standard. To complete protein precipitation, the samples were kept on ice for 10 min. The precipitated protein was separated by centrifugation (5 min, 13,200 rpm) and 20 μ l of the supernatant was diluted with 180 μ l of 0.1% acetic acid prior to LC-MS/MS analysis. Using supersomes and HLM the impact of alamethicin on carbinol glucuronidation was investigated.

For the determination of K_m and V_{max} , 7 μ g of supersomes or HLM were used in order to increase product formation especially in samples with low concentrations of carbinol. Carbinol in the range of concentrations of 0 – 40 μ M were used for supersomes and HLM. The study of the individual carbinol-glucuronidation activity of 148 HLM was performed after 15 min incubation to avoid excessive substrate consumption in the HLM with highest activity. For each experimental batch, HLM glucuronidation activity was corrected for the activity of pooled HLM that were used as biological control. For inhibition studies, *in vitro* incubations were performed as

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described above, in the presence of 2.5 mM AZT. AZT was added prior to the pre-incubation 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 (HLM inactivated at 95°C for 10 min). Final carbinol-gluc concentrations ranged from 0.01 μ M to 10 μ M.

LC-MS/MS Analysis

Chromatographic separation was carried out using a 1200 rapid resolution LC-system (Agilent) on a ZORBAX Eclipse XDB-C18 column (particle size 1.8 μ m, 4.6x50 mm, Agilent Technologies, Waldbronn, Germany) 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 (MRM) mode using a 6460 triple quadrupole mass spectrometer (Agilent) equipped with a Jet Stream electrospray source (Agilent).

Activity of Recombinant UGT Enzymes

The activity of the recombinant UGTs was tested using 100 μ M 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

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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 min retention time.

Western Blot Analysis of UGT2B7 Expression in HLM

The relative UGT2B7 protein content of HLM was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) immunoblotting. 40 µg of microsomal protein per lane were separated on 10% polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Whatman[®] Protran[®] BA 85, GE Healthcare, Freiburg, Germany) by electroblotting. 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 1:1,000 dilution of a polyclonal rabbit anti-UGT2B7 antibody (WB-UGT2B7, BD Gentest, Woburn, MA, USA) in 1% skim milk/TBST buffer. After thorough washing, the membranes were incubated with a labeled secondary antibody, IRDye[®]800CW conjugated goat polyclonal anti-rabbit IgG (LI-COR Biosciences, Lincoln, Nebraska, US) in a dilution of 1:10,000 in 1% skim milk/TBST buffer. The immunoblots were scanned at 800 nm wavelength using the Odyssey[®] Clx Infrared Imaging System (LI-COR), and the images were quantified using the Odyssey Application Software Version 3.0.30. A calibration curve of pooled HLM was included to each membrane (10, 20, 40, 80 µg protein). The amount of UGT2B7 protein in HLM was determined relative to pooled HLM by comparing absolute intensities of the bands and presented as arbitrary units (fold amount of the lowest UGT2B7 expression) per µg protein. Samples with low amounts of UGT2B7 protein were re-quantified with an extended calibration curve (5, 10, 20, 40, 80 µg protein) and by separation of 80 µg of protein.

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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 six 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 Tuebingen, 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 (MALDI-TOF MS)-based genotyping assay and the MassARRAY technology (Sequenom, San Diego, CA, USA) as described previously (Mürdter et al., 2011).

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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). The data were fitted using linear regression with 1/X weighting. Samples were accordingly quantified by their peak area ratios. K_m and V_{max} of Michaelis-Menten kinetics were calculated by curve fitting using Prism 5.04 for Windows (GraphPad Software Inc., La Jolla, CA, USA). 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>).

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Results

Metabolic Screening with a Panel of Recombinant Human UGT

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 isoform-specific contribution of UGT2B7 to the glucuronidation of carbinol. In recombinant UGT2B7 incubated with 10 μM carbinol substrate, consumption was ~20% after 30 min. This equates to a glucuronidation activity of $V = 1450 \pm 142$ pmol/min/mg protein (mean \pm SD) (Figure 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 non-specific UGT substrate 7-HFC.

Enzyme Kinetics Analysis with UGT2B7 Supersomes and HLM

Since UGT2B7 was the major UGT isoform catalyzing carbinol glucuronidation in a panel of supersomes, enzyme kinetics were further investigated. Prior to 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 min incubation time for both HLM 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 HLM followed Michaelis-Menten kinetics with apparent $K_m = 9.56 \pm 1.16$ μM

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(mean \pm SD) and $V_{max} = 2,399 \pm 113$ pmol/min/mg protein, $r^2 = 0.979$, and $K_m = 9.99 \pm 1.12$ μ M and $V_{max} = 3,430 \pm 152$ pmol/min/mg protein, $r^2 = 0.979$, respectively (Figure 3). The Eadie-Hofstee plots of both experiments were monophasic indicating the involvement of only one UGT isoform in the glucuronidation of carbinol in HLM (insets in Figure 3).

In the absence of alamethicin total activity was decreased by 1.5- and 1.4-fold using UGT2B7-supersomes and HLM, respectively. For both supersomes and HLM data, K_m -values were not affected by the presence or absence of alamethicin consistent with alamethicin's pore-forming function not influencing UGT enzymes. Our results are in line with previously reported data in HLM indicating 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 HLM was determined semi-quantitatively by Western blot analysis; a representative blot is shown in Figure 4. The intensity of the bands for all samples was within the range of the calibration samples (pooled HLM). Carbinol glucuronidation activity (*in vitro*-assay) in 148 HLM significantly correlated with UGT2B7 protein content as determined by Western blot (Spearman correlation analysis; $r_s = 0.5088$, **** $P < 0.0001$). Variability in carbinol glucuronidation activity and UGT2B7 protein content was 42-fold and 20-fold, respectively. From the 148 HLM donors, 25 were diagnosed with cholestasis (Nies et al., 2013). As this non-genetic factor may influence UGT2B7 expression and function, we compared both groups. Both expression and activity were significantly reduced in HLM from cholestatic donors (8.7 ± 4.0 UGT2B7 arbitrary units / μ g protein versus 6.9 ± 4.8

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arbitrary units / μg protein [mean \pm SD], $*P = 0.0151$ and $2,494 \pm 1,282$ pmol/min/mg protein versus $1,803 \pm 1,360$ pmol/min/mg protein, $**P = 0.0035$, respectively).

Incubations with UGT2B7 supersomes or pooled HLM with AZT, a competitive UGT2B7 inhibitor (Chen et al., 2010), resulted in 67% and 64% decreased formation rates of carbinol-gluc, respectively.

Effect of the *UGT2B72 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 population, on the glucuronidation activity towards 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 Hardy Weinberg Equilibrium ($p = 0.32$). The calculated minor allele frequency (MAF) was similar to the MAF reported for individuals of European descent in the dbSNP database (0.44 v. 0.5) (Sherry et al., 2001).

In 148 HLM from donors genotyped for *UGT2B7*, the *UGT2B7**2 polymorphism does not alter the UGT2B7 protein content as shown in Figure 6a (Kruskal-Wallis Test).

Activities of carbinol-glucuronidation in 148 HLM from donors genotyped for *UGT2B7* were $2,434 \pm 1,018$ pmol/min/mg protein, $2,356 \pm 1,372$ pmol/min/mg protein, and $2,251 \pm 1,421$ pmol/min/mg protein ($n = 25, 80, 43$, mean \pm SD) for the *UGT2B7* genotypes *1/*1, *1/*2, and *2/*2, respectively, as displayed in Figure 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 SD) for the genotype groups *1/*1, *1/*2, and *2/*2 were 343 ± 85 nM ($n=12$), 373 ± 169 nM

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(n=31), and 406 ± 134 nM (n=17), respectively. Carbinol plasma levels were 0.37 ± 0.13 nM, 0.35 ± 0.11 nM, 0.47 ± 0.37 nM, carbinol-gluc plasma levels were 31.9 ± 13.3 nM, 35.5 ± 17.4 nM, 37.7 ± 14.7 nM, and resulting metabolic ratios were 99.9 ± 54.5 , 108.3 ± 48.9 , 105.6 ± 58.2 , respectively (Figure 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.

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Discussion

4,4'-(hydroxymethylene)dibenzonitrile (carbinol) is the main phase 1 metabolite of letrozole, a non-steroidal aromatase inhibitor used in the adjuvant endocrine treatment of postmenopausal breast cancer. Carbinol is excreted from the system as glucuronide, though 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 UGT, correlation experiments with a panel of HLM from 148 liver donors, and inhibition experiments with the known UGT2B7 competitive inhibitor AZT. Metabolic screening of a tested compound against a panel of recombinant human individual UGT isoforms was instrumental to identify the major isoform catalyzing carbinol-gluc biosynthesis as UGT2B7, with much smaller contribution by UGT1A1, UGT1A9, UGT2B4, and UGT2B17. The glucuronidation of carbinol appears to be highly isoform-specific: we observed only negligible contributions of UGT1A1, 1A9, 2B4 and 2B17 (~ 0.12%, 0.08%, 0.17%, and 0.02%, respectively).

Next, we used a collection of 148 human liver tissues to assess carbinol conjugation catalyzed by HLM. Correlation analysis of carbinol glucuronidation activity determined by LC-MS/MS, and UGT2B7 protein expression in 148 HLM 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 HLM. This observation

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

Importantly, 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 6 α -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 physiological 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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In vitro experiments on carbinol conjugation catalyzed by HLM revealed about 20 and 42-fold variability in the level of UGT2B7 polypeptide, and carbinol glucuronidation rate, respectively (Figure 5a). The frequency distribution of carbinol glucuronidation activity appears monomodal but does not follow Gaussian distribution as determined by the Shapiro-Wilk normality test (Figure 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 and/or proteolytic degradation of UGT2B7 could be a possible mechanism for the observed variability in enzymatic activity, a hypothesis which is currently under investigation in our lab.

UGT2B7 is a highly polymorphic gene with more than 70 identified SNPs, about 50 of these SNPs result in missense, nonsense, and frame shift 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 individuals 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). Though parts of the 3D structure of UGT2B7 have been solved (Miley et al., 2007), the structure-activity relationships with the substrates from these three groups still remain to be elucidated.

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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:CT polymorphism. Our analysis demonstrated that the *UGT2B7*2* genotype alters neither UGT2B7 protein expression nor the carbinol glucuronidation activity of HLM strongly suggesting that carbinol is conjugated by both UGT2B7*1 and UGT2B7*2 polypeptides (Figure 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 showing 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 non-steroidal 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 substrate for evaluating UGT2B7 activity. *In vitro* experiments with HLM and *in vivo* analysis of patient's plasma levels indicate that the *UGT2B7*2*

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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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Authorship Contributions

Participated in research design: Klein, Mürdter, Precht, Schroth, and Schwab

Conducted experiments: Precht

Performed data analysis: Klein, Mürdter, Precht, Schwab

Wrote or contributed to the writing of the manuscript: Brauch, Krynetskiy, Mürdter,
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Footnotes

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Figure legends

Figure 1: Letrozole is metabolized to a carbinol metabolite which undergoes subsequent glucuronidation (Sioufi et al., 1997; Murai et al., 2009; Lazarus and Sun, 2010), the UGT isoform involved in carbinol glucuronidation (UGT2B7) has been identified in this study.

Figure 2: To investigate glucuronidation of carbinol by recombinant human UGTs, a panel of 13 supersomes expressing different UGT isoforms were incubated for 30 min with 10 μ M carbinol. The formation of carbinol-gluc was quantified by LC-MS/MS. The figure includes data from three independent experiments (mean \pm SD); the inset shows the y-axis at 250-fold magnification. UGT2B7 glucuronidation activity was $V = 1450 \pm 142$ pmol/min/mg protein (mean \pm SD). UGT1A1, UGT1A9, UGT2B4, and UGT2B17 account for approximately 0.12%, 0.08%, 0.17%, and 0.02% of the formation rate of UGT2B7, respectively.

Figure 3: Enzyme kinetic analyses of *in vitro* glucuronidation of carbinol catalysed by (A) UGT2B7 supersomes and (B) pooled HLM. 7 μ g supersomes or HLM were incubated for 30 min with several concentrations of carbinol. Quantification of carbinol-gluc was done by LC-MS/MS. Data are presented as means of duplicates. In (A), two and in (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 $K_m = 9.56 \pm 1.16$ μ M (mean \pm SD), $V_{max} = 2,399 \pm 113$ pmol/min/mg protein, $r^2 = 0.979$. (B) In the presence of HLM, the kinetic parameters of carbinol glucuronidations were: $K_m = 9.99 \pm 1.12$ μ M, $V_{max} = 3,430 \pm 152$ pmol/min/mg protein, $r^2 = 0.979$.

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Figure 4: A representative western blot of HLM for UGT2B7 protein quantification including 16 HLM samples, 40 µg/lane, (dotted line), and pooled HLM (80, 40, 20 and 10 µg per lane) as calibration curve (solid line) is shown. Proteins were separated on 10% polyacrylamide gels, transferred to nitrocellulose membranes and stained 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 HLM are presented in Figure 5.

Figure 5: (A) The correlation between *in vitro* carbinol glucuronidation activity and respective UGT2B7 protein content (determined by Western blot) for 148 human liver microsomes is shown. Arbitrary units were defined as fold-variation in expression in comparison to the HLM sample with lowest UGT2B7 expression. Samples from livers with cholestasis are shown as open circles. The carbinol glucuronidation activity significantly correlated with UGT2B7 protein content (Spearman correlation analysis; $r_s = 0.5088$, **** $P < 0.0001$). (B) The frequency distribution of carbinol glucuronidation activity appears monomodal but does not follow Gaussian distribution as determined by the Shapiro-Wilk normality test (**** $P < 0.0001$).

Figure 6: (A) UGT2B7 protein content (determined by western blot) of 148 HLM was stratified by *UGT2B7* genotype. Genotyping for *UGT2B7*2* was done using a MALDI-TOF MS based assay. The presence of the *UGT2B7*2* allele does not alter the UGT2B7 protein content (Kruskal-Wallis Test). (B) *In vitro* carbinol glucuronidation activity of 148 HLM was grouped by *UGT2B7* genotype. Glucuronidation activities are $2,434 \pm 1,018$ pmol/min/mg protein, $2,356 \pm 1,372$ pmol/min/mg protein, and $2,251 \pm 1,421$ pmol/min/mg protein ($n = 25, 80, 43$, mean \pm SD) for the *UGT2B7*

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genotypes $*1/*1$, $*1/*2$, and $*2/*2$, respectively. The presence of the *UGT2B7**2 allele does not influence carbinol glucuronidation activity in HLM.

Figure 7: Carbinol and carbinol-gluc steady state plasma levels of 60

postmenopausal letrozole patients were quantified by LC-MS/MS. The data are stratified according to *UGT2B7* genotype ($*1/*1$, $*1/*2$, $*2/*2$). Steady state plasma levels of (A) carbinol and (B) carbinol-gluc as well as (C) the metabolic ratio (carbinol-gluc / carbinol) do not statistically differ between *UGT2B7* genotype groups (*P*-values indicated are calculated by Kruskal-Wallis Test).

Tables

Table 1: Comparison of K_m -values of reported selective UGT2B7 substrates in HLM.

Substrate	apparent K_m in HLM [μM]	Other UGT activities (% of UGT2B7 activity)	Reference
Carbinol	10	negligible contribution of UGT1A1, 1A9, 2B4, 2B17 (max. 0.17%)	our results
AZT	1,400	UGT2B4 (~18%), 2B17 (~16%)	(Court et al., 2003)
Morphine	1,988	n.d.	(Innocenti et al., 2001)
(\rightarrow 3-Glucuronide)	4,300	UGT1A9, 2B17 (~10%), 2B4 (~11%) 1A3, 1A10, 2B15 (all low activity)	(Court et al., 2003)
	n.d. ^a	UGT1A1, 1A3, 1A8, 2B15 (all low), 1A9 (~6%), 2B4 (~13%)	(Ohno et al., 2008)
	n.d.	UGT1A1, 1A3, 1A6, 1A8, 1A9, 1A10, 2B7	(Stone et al., 2003)
Morphine	1,869	n.d.	(Innocenti et al., 2001)
(\rightarrow 6-Glucuronide)	2,400	UGT2B4 (25-30%) at both substrate conc.; UGT1A1 (12%), 1A3 (24%) at high conc.	(Court et al., 2003)
	n.d.	none	(Stone et al., 2003)
	n.d.	UGT1A1 (~100%), 1A8 (~250%) at 50 μM conc.	(Ohno et al., 2008)

		UGT2B4 (~33%) at 2.5 mM conc.	
MPA → AcMPAG	370	UGT1A1 (~4%)	(Picard et al., 2005)
6-α-OH- Progesteron	94	UGT1A3, 1A10 both < 0.2%	(Bowalgaha et al., 2007)
21-α-OH- Progesteron	34 / 1.22 ^b	UGT1A10	(Bowalgaha et al., 2007)
Denopamine	2,870	“very low”, less than 1.6% or no activity	(Kaji and Kume, 2005)
Naloxone	870	UGT 1A1, 1A3, 1A6, 1A10, 2B4, 2B15 (all low activity), UGT1A8 ~ 10%	(Soars et al., 2001; Di Marco et al., 2005)
Epirubicin	568	none (UGT2B4 not tested)	(Innocenti et al., 2001)
Chloramphenicol → CP-1-O-Gluc	408	UGT1A9 (~5%), many others (all low activity)	(Chen et al., 2010)

^a n.d. = not determined; ^b = best described by the Hill equation with positive cooperativity

Table 2: Studies on the influence of *UGT2B7*2* (rs7439366)

Type of study	System	Substrate	Effect	Reference
No effect of UGT2B7*2				
Functional	Stably expressed UGT2B7*1/*2 in HK293 cells	morphine (3- and 6-glucuronide formation) (+) and (-) menthol, androsterone, codeine	No influence on glucuronidation activity	(Coffman et al., 1998)
Functional	stably transfected HK293 cells	Epirubicin	No influence on glucuronidation activity	(Innocenti et al., 2001)
Functional	stably transfected HEK293 cells	mycophenolic acid (to AcMPAG ^a)	No influence on glucuronidation activity	(Bernard et al., 2006)
Association	53 HLM	morphine, AZT, codeine	No influence on glucuronidation activity and protein expression	(Court et al., 2003)
Association	HLM (28 / 26 for morphine)	androsterone, menthol and morphine (-3-) glucuronidation	No influence on glucuronidation activity	(Bhasker et al., 2000)
In vivo, association	70 cancer patients treated with	morphine	No effect on the metabolic ratios of morphine-3- and 6-glucuronide in	(Holthe et al., 2002)

	morphine		plasma	
Increased activity of UGT2B7*2				
Functional	stably expressed UGT2B7*1/*2 in HK293 cells	buprenorphine	10-fold higher glucuronidation activity for *2	(Coffman et al., 1998)
In vivo, association	86 patients treated with morphine	morphine	Lower morphine levels in homozygotes for *2; Lower levels of morphine-3- and - 6-glucuronide in homozygotes for *1	(Sawyer et al., 2003)
Decreased activity of UGT2B7*2				
Functional	stably transfected HK293 cells	AZT	glucuronidation efficiency (V_{max}/K_m) 1.9- fold higher for <i>UGT2B7*1</i> than for the *2 variant	(Barbier et al., 2000)
Functional	recombinant enzyme, baculovirus infected insect-cells	flurbiprofen	>14-fold decreased glucuronidation efficiency (V_{max}/K_m) for <i>UGT2B7*2</i>	(Wang et al., 2011)

^aAcMPAG = mycophenolic acid acyl glucuronide

Figure 1

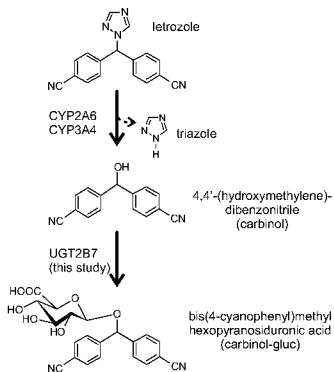


Figure 2

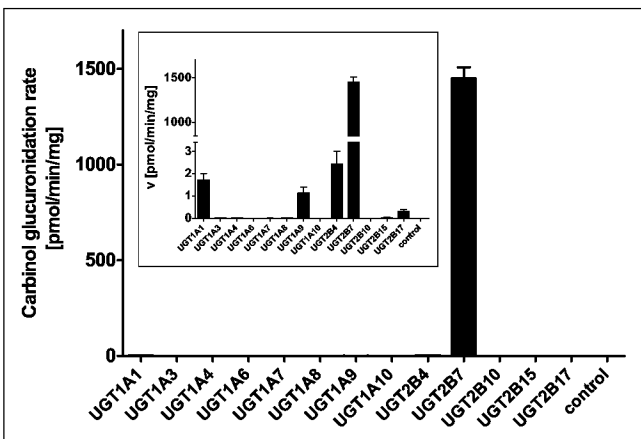


Figure 3

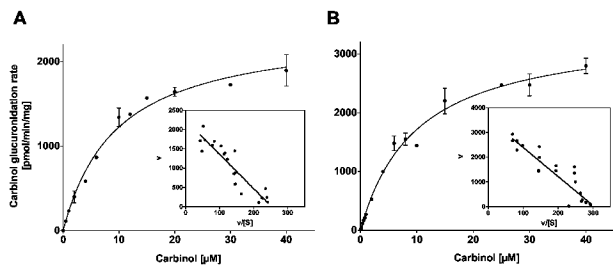


Figure 4



Figure 5

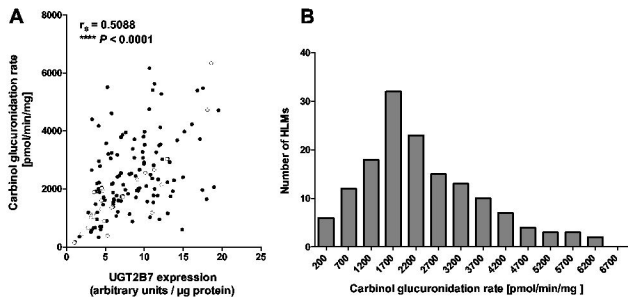


Figure 6

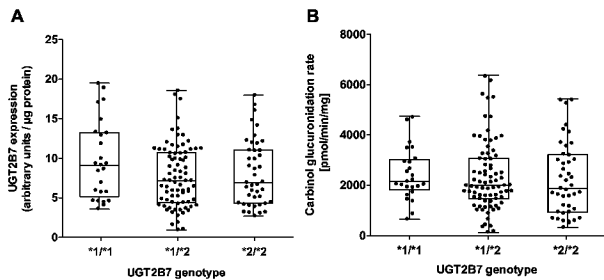


Figure 7

