Glucuronidation of Active Tamoxifen Metabolites by the Human UDP Glucuronosyltransferases

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ABSTRACT:
Tamoxifen (TAM) is an antiestrogen that has been widely used in the treatment and prevention of breast cancer in women. One of the major mechanisms of metabolism and elimination of TAM and its major active metabolites 4-hydroxytamoxifen (4-OH-TAM) and 4-OH-N-desmethyl-TAM (endoxifen; 4-hydroxy-N-desmethyl-tamoxifen) is via glucuronidation. Although limited studies have been performed characterizing the glucuronidation of 4-OH-TAM, no studies have been performed on endoxifen. In the present study, characterization of the glucuronidating activities of human UDP glucuronosyltransferases (UGTs) against isomers of 4-OH-TAM and endoxifen was performed. Using homogenates of individual UGT-overexpressing cell lines, UGTs 2B7 – 1A8 > UGT1A10 exhibited the highest overall O-glucuronidating activity against trans-4-OH-TAM as determined by $V_{\text{max}}/K_M$ with the hepatic enzyme UGT2B7 exhibiting the highest binding affinity and lowest $K_M$ (3.7 $\mu$M). As determined by $V_{\text{max}}/K_M$, UGT1A10 exhibited the highest overall O-glucuronidating activity against cis-4-OH-TAM, 10-fold higher than the next-most active UGTs 1A1 and 2B7, but with UGT1A7 exhibiting the lowest $K_M$. Although both N- and O-glucuronidation occurred for 4-OH-TAM in human liver microsomes, only O-glucuronidation activity was observed for endoxifen; no endoxifen-N-glucuronidation was observed for any UGT tested. UGTs 1A10 – 1A8 > UGT2B7 exhibited the highest overall glucuronidating activities as determined by $V_{\text{max}}/K_M$ for trans-endoxifen, with the extrahepatic enzyme UGT1A10 exhibiting the highest binding affinity and lowest $K_M$ (38.9 $\mu$M). Similar to that observed for cis-4-OH-TAM, UGT1A10 also exhibited the highest activity for cis-endoxifen. These data suggest that several UGTs, including UGTs 1A10, 2B7, and 1A8 play an important role in the metabolism of 4-OH-TAM and endoxifen.
pathways into several metabolites after oral administration, including the hydroxylated TAM metabolites 4-OH-TAM and 4-hydroxy-N-desmethylTAM (endoxifen). Since both trans-4-OH-TAM and endoxifen exhibit up to 100-fold the levels of antiestrogenic activity compared with TAM and other TAM metabolites (Jordan et al., 1977; Lim et al., 2005), it is thought that they may be the major contributors to the antiestrogenic properties of TAM. Although cis-4-OH-TAM is thought to possess some estrogen agonist activity, this isomer exhibits significant antiestrogenic activity in vitro when in the presence of estradiol (Robertson et al., 1982; Murphy et al., 1990).

An important route of elimination and detoxification of TAM and its metabolites is via glucuronidation. TAM is excreted predominantly through the bile, a process largely facilitated by TAM conjugation to glucuronic acid during the glucuronidation process (Lien et al., 1989), and TAM glucuronides have been identified in the urine of TAM-treated patients (Poon et al., 1993). Most of the 4-OH-TAM found in the bile of TAM-treated patients was as a glucuronide conjugate (Lien et al., 1989). The fact that TAM metabolites are found in their unconjugated form in feces is likely due to β-glucuronidase-catalyzed deglucuronidation within the microflora that colonize within the small intestine (Lien et al., 1989). TAM glucuronide conjugates have been identified in the serum of TAM-treated patients (Lien et al., 1989), and it has been suggested that glucuronidation within target tissues such as the adipose tissue of the breast may also be important in terms of TAM metabolism and overall TAM activity (Nowell et al., 2005).

Micromoles from human liver specimens exhibit high glucuronidating activities toward TAM to form TAM-N'--glucuronide and both the trans- and cis-isomers of 4-OH-TAM to form 4-OH-TAM-N'-- and 4-OH-TAM-O-glucuronides (Sun et al., 2006). One of the UGTs involved in the glucuronidation of TAM and its metabolites is the hepatic enzyme UGT1A4 (Nishiyama et al., 2002; Kaku et al., 2004), which catalyzes the formation of a quaternary ammonium-linked glucuronide with the N,N-dimethylamino alkyl side chain of TAM or 4-OH-TAM (Kaku et al., 2004). This pattern of ammonium-linked glucuronidation is consistent with the glucuronidation activity of UGT1A4 against primary, secondary, and tertiary amines present in a variety of carcinogenic compounds, androgens, progestins, and plant steroids (Wiener et al., 2004a). In a screening of selected UGT-overexpressing baculosomes, UGTs 1A1, 1A3, 1A4, 1A8, 1A9, 2B7, and 2B15 were shown to exhibit activity against 4-OH-TAM (Kaku et al., 2004). However, a comprehensive characterization and kinetic analysis of the glucuronidating enzymes responsible for O-glucuronidation of TAM metabolites have not yet been performed, with no studies having yet been performed analyzing endoxifen glucuronidation. The goal of the present study was to characterize the glucuronidation of the TAM metabolites 4-OH-TAM and endoxifen and to identify the UGTs active in this pathway.

**Materials and Methods**

**Chemicals and Materials.** trans-4-OH-TAM (98% pure), trans-4-OH-TAM/cis-4-OH-TAM mixture (70:30% ratio), UDPGA, alamethicin, β-glucuronidase, anti-calnexin antibody, and bovine serum albumin were purchased from Sigma-Aldrich (St. Louis, MO). Ethylchloroformate, toluene, ethylene-diamine, NADPH, and diphenhydramine were purchased from Fisher Scientific (Pittsburgh, PA). NADPH was prepared by adding dithiothreitol (10–20 mg protein/ml) at ~80°C. Microsomal protein concentrations were measured using the BCA assay. All protocols involving the analysis of tissue specimens were approved by the institutional review board at the Penn State College of Medicine and in accordance with assurances filed with and approved by the United States Department of Health and Human Services.

Two new UGT-overexpressing cell lines were generated for the experiments outlined in this study. The UGT1A3 wild-type cDNA was synthesized by standard reverse transcription using normal human liver total RNA and inserted into the pcDNA3.1/V5-His-TOPO plasmid. The sense and antisense primers used for PCR amplification of UGT1A3 were UGT1A3/sense (sense, 5'-AAAGCGAATTTGACGGACAC3') and UGT1A3/antisense (antisense, 5'-GGAAATGTCAAGGAAATTGTC3'), corresponding to nucleotides ~61 to ~42 and +1635 to +1665, respectively, relative to the UGT1A3 translation start site. A UGT1A8-overexpressing cell line was previously received from Dr. Thomas Tephly (University of Iowa, Iowa City, IA). Although this cell line was used in previous studies of substrate glucuronidation experiments (Dellinger et al., 2006), it was recently discovered that two polymorphic variants within the UGT1A8 sequence were identified in this cell line; a T→C change at nucleotide +362, and a C→G change at nucleotide +518 (GenBank accession no. NM_019076), both resulting in missense amino acid changes. A wild-type UGT1A8-overexpressing cell line was generated by reverse transcription of total RNA from the previously received cell line and subsequent PCR amplification from the cDNA using the sense and antisense primers IAB8/sense (sense, 5'-TTCTCTCATGCTCAGCGACAG3') and IAB8/antisense (antisense, 5'-CTCAATGGTTCCATTTCGG3'), corresponding to nucleotides ~7 to +15 and +1570 to +1594, respectively, relative to the translation start site. PCR amplification for both UGTs 1A3 and 1A8 was performed in a GeneAmp 9700 thermocycler (Applied Biosystems, Foster City, CA) as follows: 1 cycle of 94°C for 2 min, 35 cycles of 94°C for 30 s, 55°C for 30 s, and 68°C for 4 min, followed by a final cycle of 7 min at 68°C.

Site-directed mutagenesis (SDM) using the QuikChange SDM kit (Stratagene, La Jolla, CA) was performed for UGT1A8 to change the variant base at nucleotide +362 from the polymorphic C to the wild-type T using the primer set IAB8WTs and IAB8WTs (sense, 5'-TTTAACTTAATTTTCGCAAGGAGG3' and antisense, 5'-CTTCCTCGAATTCGCAAGGAATAAATGTTAAA3'), corresponding to nucleotides +349 to +377 relative to the translation start site; the underlined bases indicate the base-pair changes. Primer set 1A8P173S and 1A8P173AS (sense, 5'-AGTTAAA-3' and antisense, 5'-AGTTAAA-3') and 1A8AS (antisense, 5'-AGTTAAA-3') were used to change the variant base at nucleotide +518 from the polymorphic T to the wild-type C using the QuikChange SDM kit (Stratagene, La Jolla, CA) as follows: 1 cycle of 95°C for 4 min, 25 cycles of 95°C for 30 s, 55°C for 1 min, and 68°C for 16 min, and a final cycle of 68°C for 7 min. Due to the high homology between UGT1A3 and UGT1A4, the PCR product of UGT1A3 amplification was digested with the restriction enzyme XbaI before gel extraction to remove coamplified UGT1A4 PCR product. The specific PCR products of UGT1A3 (1662 base pairs) and UGT1A8 (1601 base pairs) were purified using the QIAEX II gel extraction kit after electrophoresis in 1.5% agarose and subsequently subcloned into the pcDNA3.1/V5-His-TOPO mammalian expression vector using standard methodologies. Conformation of insert orientation was performed by restriction enzyme digestion, and UGT1A3 and UGT1A8 wild-type sequences were confirmed by dideoxy sequencing of the entire PCR-amplified UGT1A3 or UGT1A8 cDNA product using two vector primers (T7 and BGH; Integrated DNA Technologies, Inc., Coralville, IA), a UGT1A3 internal antisense primer (UGT1A3intAS; 5'-GATAAATGTTTCTCCACCACCG3' and antisense, 5'-GATAAATGTTTCTCCACCACCG3'), corresponding to nucleotides 1028 to +1046 relative to the UGT1A3 translation start site) and a UGT1A8 internal antisense primer (1A8intAS; 5'-GATAAATGTTTCTCCACCACCG3' and antisense, 5'-GATAAATGTTTCTCCACCACCG3'), corresponding to nucleotides 1594 to +1620 relative to the UGT1A8 translation start site).
to nucleotides +125 to +147 relative to the UGT1A8 translation start site). The cloned UGT1A3 and 1A8 inserts were compared with sequences described in GenBank, and they were confirmed to be 100% homologous to the wild-type UGT1A3 and UGT1A8 sequences.

**UGT1A3**- and UGT1A8-overexpressing human embryonic kidney fibroblast (HEK293) cell lines were generated by standard electroporation techniques in a GenePulser Xcell (Bio-Rad, Hercules, CA) using 10 μg of pcDNA3.1/V5-His-TOPO/UGT plasmid DNA with 5 × 10^7 HEK293 cells (in 0.5 ml) in serum-free DMEM media, with electroporation at 250 V and 1000 μF. After transfection, HEK293 cells were grown in 5% CO2 to 80% confluence in DMEM supplemented with 4.5 mM glucose, 10 mM HEPES, 10% fetal bovine serum, 100 μM penicillin, 100 μg/ml streptomycin, and 2.7 mM KCl (pH 7.4) in a humidified incubator at 37°C. Cells were stored at 80°C in 100-μl aliquots. Total homogenate protein concentrations were measured using the BCA protein assay.

**Western Blot Analysis.** Levels of UGT1A protein were determined as described previously (Dellinger et al., 2007) by our laboratory. UGT2B expression in UGT-overexpressing cell lines was assessed by Western blot analysis using a newly synthesized affinity-purified chicken anti-UGT2B antibody generated against the peptide CWKDQFQYSEVLGRPTTL, which is common to all human UGT2B family members (Poonco Rabbit Farm, Candaensis, PA). Antibodies were used in a 1:5000 dilution. UGTs 2B7, 2B15 and 2B17 were quantified against 200 to 250 ng of human UGT2B protein (supplied in the Western blotting kit provided by Gentest) by densitometric analysis using a newly synthesized affinity-purified chicken anti-UGT2B antibody against the peptide CWKDQFQYSEVLGRPTTL, which is common to all human UGT2B family members (Poonco Rabbit Farm, Candaensis, PA). Antibodies were used in a 1:5000 dilution. UGTs 2B7, 2B15 and 2B17 were quantified against 200 to 250 ng of human UGT2B protein (supplied in the Western blotting kit provided by Gentest) by densitometric analysis of X-ray film exposures (1 s–2 min) of Western blots using a GS-800 densitometer with Quantity One software (Bio-Rad). All cell homogenate protein levels were normalized to the levels of β-actin observed in each lane (quantified by densitometric analysis of Western blots as described above). Determinations of aglycone-glucuronide formation in UGT2B7-, UGT2B15-, and UGT2B17-overexpressing cell lines were calculated relative to the levels of UGT expression in the respective cell lines. X-ray film bands were always below densitometer saturation levels as indicated by the densitometer software. Densitometric results were always consistent irrespective of the exposure time. Western blot and subsequent densitometric analysis were performed in triplicate on three separate occasions, by using the same UGT-containing cell homogenates used for activity assays, with relative UGT protein levels expressed as the mean of these experiments.

**Synthesis of Endoxifen.** Using 4-OH-TAM (70:30 mixture; Sigma-Aldrich) as substrate, two steps were required for its conversion to endoxifen.

**Synthesis of cis/trans-4-OH-N-ethoxycarbonyl-N-methylTAM (cis/trans-endoxifen).** To 7.22 (m, 7H).

**Synthesis of cis/trans-4-OH-N-desmethylTAM (cis/trans-endoxifen).** To
7.4. trans-endoxifen-O-glucuronide and cis-endoxifen-O-glucuronides were confirmed by their stability to 1 M HCl treatment and their sensitivity to β-glucuronidase treatment. For kinetic assays of trans- or cis-endoxifen-O-glucuronide formation, the pH of buffer A was kept at 5.0 for better resolution, and all of the analytical conditions were the same as those for 4-OH-TAM as described above.

As controls, glucuronidation assays were regularly performed using human HLMs (as a positive control for glucuronidation activity) and untransfected HEK293 cell homogenate protein (as a negative control for glucuronidation activity) as described previously (Fang et al., 2002; Wiener et al., 2004a). Experiments were always performed in triplicate in independent assays.

LC/MS Identification of 4-OH-TAM-O-Glucuronides and Endoxifen-O-Glucuronides. trans- and cis-4-OH-TAM-O-glucuronides were collected after separation by HPLC using a Gold 126 Solvent Module system (Beckman Coulter), and they were subsequently identified using a Shimadzu LC/MS 2010 EV system. trans- and cis-4-OH-TAM-O-glucuronides were loaded onto a reverse phase column (4.6 × 50 mm, Shimadzu C18; Shimadzu, Tokyo, Japan), and they were analyzed at a flow rate of 0.2 ml/min by applying a linear mobile phase gradient from 10 to 80% (v/v) methanol/H2O over 30 min. By using a positive mode, the electrospray voltage of 1.5 kV was applied.

trans- and cis-endoxifen-O-glucuronides were identified using an liquid chromatography-tandem mass spectrometry system (Applied Biosystems). trans- and cis-endoxifen-O-glucuronides were loaded onto a C18 column (2.1 × 150 mm, 3 μm, Aquasil C18; Thermo, Bellefonte, PA), and they were subsequently analyzed at a flow rate of 0.2 ml/min by applying a mobile phase gradient from 5% to 100% (v/v) methanol/acetic acid. By using a positive mode, the electrospray voltage of 2.5 kV was applied.

Statistical Analysis. The Student’s t test (two-sided) was used for comparing rates and kinetic values of glucuronide formation for individual UGT1A and UGT2B enzymes against the different substrates examined in this study. Kinetic constants were determined using GraphPad Prism4 software (GraphPad Software Inc., San Diego, CA).

Results

Synthesis of Endoxifen. Previous reports examining endoxifen metabolism and activity involved multistep synthesis (Burke and Koch, 2004). In the present study, a two-step synthesis of endoxifen by the demethylation of 4-OH-TAM has been developed as outlined in Fig. 1. Treatment of 4-OH-TAM (1) with ethylchloroformate in refluxing toluene gave the intermediate (2) as an isomeric mixture of cis/trans-isomers. The treatment of the mixture (2) with hydrazine hydrate in the presence of ethylene glycol and potassium hydroxide led to a mixture (60:40) of geometrical cis/trans-isomers of endoxifen (3).

The regioisomeric mixture (3) was characterized on the basis of 1H NMR and mass spectra. The MS spectrum of the trans/cis-mixture (3) showed a clear [M + H] + peak at m/z 374 (the molecular weight of endoxifen is 373). The ratio of the mixture was assigned on the basis of the integration of the –NCH3 protons by NMR (δ 2.35, trans-isomer and 2.40, cis-isomer) and HPLC of the crude mixture. The trans- and cis-configuration was assigned to the separated isomers of endoxifen on the basis of their 1H NMR chemical shift profile in accordance with the literature reports (Shani et al., 1985; Ogawa et al., 1991). The chemical shift of triplets for –OCH2 proton in 1H NMR spectra appeared at δ 3.87 and 4.08 ppm, respectively. Based on previous studies (Shani et al., 1985; Ogawa et al., 1991), the chemical shifts of the cis-isomer, where the –OCH2 substituent is situated trans to the aryl ring, are shifted to the lower field than those in the trans-form. Based on this information, the isomer having the triplet at δ 4.08 was assigned as the cis-isomer (40%) and the isomer with δ 3.87 was identified as the trans-isomer (60%).

Separation and Collection of 4-OH-TAM and Endoxifen Isomers. As described under Materials and Methods, isocratic HPLC elution programs were developed to efficiently separate 4-OH-TAM and endoxifen isomers. Due to relatively high level of intraconversion between trans- and cis-4-OH-TAM as well as trans- and cis-endoxifen under acidic condition, the separation buffer was maintained at pH 7.4 using triethylamine. As shown in Fig. 2, the trans- (peak 1) and cis-isomers (peak 2) of 4-OH-TAM eluted in HPLC of a 70%: 30% mix of trans/cis-4-OH-TAM (Fig. 2A), 98% pure trans-4-OH-TAM purchased from Sigma-Aldrich (Fig. 2B), and collected pure cis-4-OH-TAM (Fig. 2C), at retention times of 16.6 and 19.9 min, respectively. Two peaks were also observed for the newly synthesized, NMR-confirmed endoxifen mixture, eluting at 8.5 min (peak 3) and 10.5 min (peak 4) with a peak 3:peak 4 area ratio of 60:40 (Fig. 2D). Collected peaks 3 (Fig. 2E) and 4 (Fig. 2F) were characterized and confirmed by proton NMR data, which indicated that peaks 3 and 4 were trans- and cis-endoxifen, respectively. Therefore, the pattern of shorter retention time for the trans- versus cis-isomers was consistent for both TAM metabolites by HPLC. The purified, collected trans- and cis-4-OH-TAM and trans- and cis-endoxifen were stable in ethanol, and they were used for glucuronidation assays described below.

Characterization of 4-OH-TAM-O-Glucuronides. Previous studies have demonstrated that HLMs catalyze the glucuronidation of 4-OH-TAM to both the O-glucuronide and N'-glucuronide forms for both its trans- and cis-isomers and that UGT1A4 is the major enzyme responsible for forming 4-OH-TAM-N'-glucuronide (Sun et al., 2006). To identify the UGTs responsible for O-glucuronidation of 4-OH-TAM isomers, cell lines overexpressing UGTs 1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17 were examined. Similar to that observed in previous study (Sun et al., 2006), the O- and N'-glucuronides of trans-4-OH-TAM formed in assays with HLMs eluted with elution times of 18.2 min (peak 1) and 23 min (peak 2), respectively (Fig. 3A); a similar pattern was observed for cis-4-OH-TAM, with retention times of 18.6 min (peak 4) and 22.5 min (peak 5) for the respective O- and N'-glucuronides (Fig. 3B). In assays with UGT-overexpressing cell homogenates, single glucuronide peaks corresponding to the retention times of peak 1 and peak 4 were observed in assays with trans- and cis-4-OH-TAM, respectively, for several UGTs, including UGT2B7 (Fig. 3, C and D). Similar to that observed previously (Sun et al., 2006), peaks 1 and 4 were not alkali-sensitive but were sensitive to treatment with β-glucu-
FIG. 2. Separation and collection of 4-OH-TAM and endoxifen isomers by HPLC. Separation and collection were performed by HPLC as described under Materials and Methods. A 70:30 mixture of trans-4-OH-TAM/cis-4-OH-TAM purchased from Sigma-Aldrich (A); 98% pure trans-4-OH-TAM purchased from Sigma-Aldrich (B); cis-4-OH-TAM purified from 70:30 mixture shown in A (C); 60:40 mixture of newly synthesized (see Fig. 1) trans-endoxifen/cis-endoxifen (D); trans-endoxifen purified from 60:40 mixture shown in D (E); and cis-endoxifen purified from 60:40 mixture shown in D (F). Peak 1, trans-4-OH-TAM; peak 2, cis-4-OH-TAM; peak 3, trans-endoxifen; and peak 4, cis-endoxifen.

FIG. 3. HPLC and MS analysis of 4-OH-TAM-glucuronides. Glucuronidation assays were performed and 4-OH-TAM-glucuronides separated by HPLC as described under Materials and Methods. HLMs (10 µg of protein; A and B) or UGT2B7-overexpressing cell homogenates (500 µg of protein; C and D) were incubated at 37°C for 60 min with trans-4-OH-TAM (50 µM for A and 16 µM for C), or with cis-4-OH-TAM (50 µM for B and 25 µM for D), and 4 mM UDPGA before analysis by HPLC. Peak 1, trans-4-OH-TAM-O-glucuronide; peak 2, trans-4-OH-TAM-N'-glucuronide; peak 3, trans-4-OH-TAM; peak 4, cis-4-OH-TAM-O-glucuronide; peak 5, cis-4-OH-TAM-N'-glucuronide; and peak 6, cis-4-OH-TAM. E, MS analysis of trans-4-OH-TAM-O-glucuronide formed by HLMs.
GLUCURONIDATION OF TAMOXIFEN METABOLITES

Characterization of Endoxifen Glucuronides. Single putative glucuronide peaks with retention times of 17.9 min (peak 1) and 18.4 min (peak 3) were observed in assays of HLMs (Fig. 4, A and B) or UGT-overexpressing cell homogenates including UGT1A10 (Fig. 4, C and D) with either trans-endoxifen or cis-endoxifen, respectively. Both peaks were stable after incubations with 1 M HCl overnight, but they were sensitive to treatment with β-glucuronidase (data not shown), suggesting that both glucuronide peaks were the O-glucuronide conjugates of trans- and cis-endoxifen, respectively. The products corresponding to the predicted trans- and cis-4-OH-TAM-o-glucuronides were collected, and then they were analyzed by LC-tandem mass spectrometry detection (Fig. 4E). The mass spectrum for the trans-endoxifen-O-glucuronide (with a calculated molecular weight of 549.2) showed a clear [M+H] ion at m/z 550.2 and a fragment ion at m/z 374.2, the latter corresponding to the parent drug endoxifen +H with the loss of the glucuronic acid moiety (molecular weight = 176 g/mol; Fig. 4E). A virtually identical pattern was observed for cis-endoxifen-O-glucuronide with a clear [M+H] ion at m/z 550.7 (data not shown). These data suggest that the glucuronides derived from both isomers were in fact monoglucuronides.

Since endoxifen is a secondary amine, potential endoxifen-N-glucuronides may be sensitive to acid treatment. For optimal resolution of 4-OH-TAM and endoxifen isomers, the HPLC gradient used in the present kinetic study was pH 5.0. It is possible that endoxifen-N-
glucuronides were deconjugated during the HPLC elution process at this pH, since N-glucuronides of secondary amines are susceptible to cleavage under mildly acidic conditions (Babu et al., 1992). When assays were run on a HPLC gradient at pH 7.4, only single glucuronide peaks corresponding to peaks 1 and 3 (for trans- and cis-endoxifen, respectively) were observed for both HLMs and UGT-overexpressing cells, and these glucuronide peaks were stable in 1 M HCl but sensitive to β-glucuronidase treatment (data not shown). These data indicated that these single peaks were in fact endoxifen-O-glucuronides and that no endoxifen-N-glucuronides formation was observed in assays with endoxifen.

**Kinetic Analysis of 4-OH-TAM and Endoxifen Glucuronidation by Human UGTS.** In addition to the newly made UGT1A3 and UGT1A8 cell lines described in this report, cell lines overexpressing UGTs 1A1, 1A4, 1A6, 1A7, 1A9, 1A10, 2B4, 2B7, 2B10, 2B11, 2B15, and 2B17 were used for this analysis as described previously (Babu et al., 1992; G. Chen, R. Dellinger, D. Sun, T. Spratt, and P. Lazarus, manuscript submitted for publication). Of the UGTs tested in this study, UGTs 1A3, 1A7, 1A9, 1A10, 2B7, 2B15, and 2B17 exhibited detectable levels of O-glucuronidating activity against both 4-OH-TAM isomers (Table 1). UGT1A4 selectively exhibited O-glucuronidation activity against both 4-OH-TAM isomers in this assay. Of the UGTs tested in this study, UGT1A3 was the only enzyme exhibiting detectable N-glucuronidation activity against both 4-OH-TAM isomers as well as a previous study (Sun et al., 2006). UGTs 1A6, 2B4, 2B10, and 2B11 did not exhibit detectable glucuronidating activity against either 4-OH-TAM isomer based upon trans- and cis-4-OH-TAM (Table 1). UGT1A4 was shown to be the major enzyme involved in N-glucuronidation of TAM and both detectable N-glucuronidation activity was observed by UGT1A3 against cis-4-OH-TAM.

Of the UGT-overexpressing cell lines tested, the highest overall O-glucuronidation activities against trans-endoxifen by UGT family 1A enzymes as determined by Western blot analysis (Dellinger et al., 2007), the order of O-glucuronidation activity of the trans-4-OH-TAM isomer based upon V_{\text{max}}/K_{M} was 1A8 > 1A10 > 1A1 > 1A3 > 1A9 ≥ 1A7. A newly designed anti-UGT2B antibody (described under Materials and Methods) was reactive against UGTs 2B7, 2B15, and 2B17 (Fig. 5). After normalizing for UGT2B protein expression, the order of O-glucuronidation activity of the trans-4-OH-TAM isomer based upon V_{\text{max}}/K_{M} was 2B7 ≥ 2B17 for the cis-isomer of 4-OH-TAM. The order of O-glucuronidation activity based upon V_{\text{max}}/K_{M} was 1A10 > 1A8 > 1A3 > 1A7 > 1A9 ≥ 1A7 for the UGT1A family and 2B7 > 2B15 > 2B17 for the UGT2B family. Overall, the UGT2B family members exhibited the lowest K_{M} values against 4-OH-TAM, with UGTs 2B7 (3.7 and 14.8 μM) and 2B17 (40.6 and 18 μM) exhibiting high affinity for the trans- and cis-isomers, respectively. Of the family 1A UGTs, 1A8 exhibited the lowest K_{M} (22.7 μM) against trans-4-OH-TAM and 1A7 exhibited the lowest (7.4 μM) against cis-4-OH-TAM.

![Fig. 5](image_url)

**TABLE 1**

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<td>μM</td>
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Of the UGTs tested in this study, UGT1A3 was the only enzyme exhibiting both O- and N-glucuronidation activity against 4-OH-TAM. The K_{M} and V_{\text{max}}/K_{M} of UGT1A3 for trans-4-OH-TAM-N-glucuronide formation were 193 μM and 0.005 μl·min\(^{-1}\)·μg\(^{-1}\); no N-glucuronide formation was observed by any UGT screened in this study, UGT1A4-overexpressing cells against both isomers of endoxifen. The highest overall O-glucuronidation activity against trans-endoxifen was 2B7. The order of O-glucuronidation activity against cis-endoxifen by UGT2B enzymes was 2B15 > 2B7 > 2B17. Overall, UGT1A10 exhibited the lowest K_{M} against both trans- (39.9 μM) and cis-endoxifen (11.2 μM). No endoxifen-N-glucuronide formation was observed by any UGT screened in this study including UGT1A4. As exhibited for 4-OH-TAM isomers, UGTs 1A6, 2B4, 2B10, and 2B11 exhibited no detectable glucuronidation activity against either endoxifen isomer.

**Discussion**

Limited studies had previously been reported identifying the UGT enzymes involved in TAM metabolism. UGT1A4 was shown to be the major enzyme involved in N-glucuronidation of TAM and both

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**TABLE 1**

Kinetic analysis of UGTs-induced O-glucuronidation of trans- and cis-4-OH-TAM

All data are the mean ± S.D. based on three independent experiments. Homogenates from cells overexpressing UGTs 1A6, 2B4, 2B10, or 2B11 exhibited no detectable glucuronidating activity against 4-OH-TAM.
isomers of 4-OH-TAM (Sun et al., 2006). The \( N^+ \)-glucuronidation activity exhibited by UGT1A4 to form a quaternary ammonium-linked glucuronide with the \( N,N \)-dimethylamino alkyl side chain of TAM is consistent with the activity spectrum of UGT1A4 to produce \( N^- \)-glucuronidated metabolites with other compounds (Green and Tephly, 1998; Ren et al., 1999; Wiener et al., 2004a). In the present study, UGT1A3 also exhibited \( N^- \)-glucuronidation activity, but only for the trans-isomer of 4-OH-TAM and at relatively low levels. This is consistent with the dual activities observed for UGT1A3 against other substrates (Green and Tephly, 1998).

Although UGT2B15 was shown in previous studies to be active against cis-4-OH-TAM (Ogura et al., 2006), the present study is the first to perform a comprehensive analysis of O-glucuronidation of 4-OH-TAM, and its the first to examine endoxifen glucuronidation. In addition, this is the first report to examine the pure trans- and cis-endoxifen isomers independently. Using an optimized synthesis strategy based on the methodology described by Kitagawa et al. (2000), large amounts of endoxifen were synthesized for these studies, with the trans- and cis-isomers separated and purified for kinetic analysis. Both isomers of endoxifen are \( O^- \)-glucuronidated; however, unlike 4-OH-TAM, no \( N^- \)-glucuronidation of endoxifen isomers was detected in our assays for either liver microsomes or individually overexpressed UGTs, including UGT1A4, suggesting that the demethylation of the electrophilic amine on the 4-OH-TAM side chain results in a lack of \( N^- \)-glucuronidation by UGTs.

Kinetic analysis suggests that UGT2B7 is the major hepatic enzyme responsible for the \( O^- \)-glucuronidation of the trans-isomer of both 4-OH-TAM and endoxifen. This was reflected by a relatively high \( V_{\text{max}}/K_{\text{M}} \) and low apparent \( K_{\text{M}} \) compared with other hepatic UGTs against the two TAM metabolites. Of the extrahepatic UGTs, both 1A8 and 1A10 exhibited significant \( O^- \)-glucuronidation activity against the trans-isomer of 4-OH-TAM and endoxifen, with UGT1A10 exhibiting the lowest \( K_{\text{M}} \) value of any UGT against trans-endoxifen.

Like that observed for the trans-isomers, UGT1A10 also exhibited significant levels of \( O^- \)-glucuronidation activity against cis-4-OH-TAM and cis-endoxifen, exhibiting 10- to 24-fold higher levels of activity as determined by \( V_{\text{max}}/K_{\text{M}} \) than any other UGT examined. However, some regioisomeric specificity was apparent in terms of UGT activity relationships against the different isomers of the two TAM metabolites. UGT1A9, which exhibited only low levels of activity against their respective trans-isomers, exhibited \( O^- \)-glucuronidation activity that was similar to or greater than that observed for UGT1A8 against the cis-isomers of both 4-OH-TAM or endoxifen. Similarly, UGTs 2B15 and 2B17, which exhibited low or undetectable levels of activity against both trans-4-OH-TAM and trans-endoxifen, exhibited similar or greater \( O^- \)-glucuronidation activity against the cis-isomers of both TAM metabolites to that exhibited by UGT2B7, activities that were similar to the levels observed for UGT1A8 against the cis-tAM metabolites. The \( K_{\text{M}} \) values of UGTs 2B15 and 2B17 were among the lowest observed for any UGT against cis-4-OH-TAM. In addition, although exhibiting low \( O^- \)-glucuronidation activity against the trans-isomers and cis-endoxifen, UGT1A1 exhibited a level of activity that was among the highest of all hepatic UGTs as determined by \( V_{\text{max}}/K_{\text{M}} \) against cis-4-OH-TAM. Therefore, although it is likely that UGTs 1A10 and 2B7 play an important overall role in hepatic and potentially extrahepatic elimination of 4-OH-TAM and endoxifen, there is significant cross-reactivity with other UGTs for the different isomeric forms of the two TAM metabolites.

Although several of the UGTs highly active against 4-OH-TAM and endoxifen isomers are hepatic, including UGT2B7, several are also expressed in target tissues for TAM response, including breast (Lepine et al., 2004; our unpublished results), including UGT2B7, and the extrahepatic UGTs 1A8 and 1A10. The presence of all three in breast could be important in the local elimination of TAM metabolites in this target tissue.

In summary, results from this study indicate that UGTs 2B7, 1A8, and 1A10 were highly active against trans-4-OH-TAM and trans-endoxifen. UGTs 1A10, 1A8, 2B7, and 2B15 were highly active against cis-4-OH-TAM, whereas UGT1A10 exhibited the highest \( O^- \)-glucuronidation activity against cis-endoxifen. These UGTs are expressed in human liver and/or breast, and alterations in their expression or activity, including genetic variation, could potentially affect TAM-related treatment efficacy. Studies are currently underway examining functional polymorphisms in these active enzymes and their potential role in overall response to TAM.

### References


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