Glucuronidation of Active Tamoxifen Metabolites by the Human UDP Glucuronosyltransferases

Dongxiao Sun, Arun K. Sharma, Ryan W. Dellinger, Andrea S. Blevins-Primeau, Renee M. Balliet, Gang Chen, Telih Boyiri, Shantu Amin, and Philip Lazarus

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 glucuronidation 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-glucuronidating 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.

1-[(4-(2-Dimethylaminoethoxy)-phenyl]-1,2-diphenylbut-1(Z)-ene (TAM) is a nonsteroidal antiestrogen that has been commonly used for the treatment and prevention of estrogen-dependent breast cancer (Fishier et al., 1998; Osborne, 1998; Cuzick et al., 2003; Howell et al., 2003). Adjuvant TAM treatment increases recurrence-free survival and overall survival in breast cancer patients with hormone receptor-positive tumors irrespective of their nodal status, menopausal status, or age (Chowdhury and Ellis, 2005). In addition to its antiestrogenic properties, which have been related to symptoms such as hot flashes, vaginal bleeding, and pruritus vulvae (Osborne, 1998; Nechushtan and Perez, 2002), TAM also has partial estrogen-agonistic effects that may be linked to reduced risk for ischemic heart disease and osteoporosis (McDonald and Steward, 1991; Rutqvist and Mattsson, 1993) but that may also increase the risk for endometrial cancer (van Leeuwen et al., 1994) and venous thromboembolism (Meier and Jick, 1998). Although TAM is generally well tolerated, there is significant interindividual variability in the clinical efficacy as well as toxicities of TAM. For example, approximately 30% of patients acquire TAM resistance and relapse (EBCTCG, 1998). In addition, the relative risk of endometrial cancers in patients treated with TAM is estimated to be 2- to 3-fold that of controls, with risk increasing with both the duration and cumulative dose of TAM treatment (Fishier et al., 1994; Bergman et al., 2000). The mechanisms underlying variability in response to TAM and to TAM-related toxicities remains obscure. Since there is compelling evidence that TAM is converted to antiestrogenic metabolites that are more potent than TAM itself, one hypothesis is that altered patterns of metabolism of TAM and/or its primary metabolites might contribute to this interindividual variability.

TAM is activated predominantly via cytochrome P450-mediated
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 antidepressive 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 antidepressive properties of TAM. Although cis-4-OH-TAM is thought to possess some estrogen agonist activity, this isomer exhibits significant antidepressive 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 degradation 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).

Microsomes from human liver specimens exhibit high glucuronidating activities toward TAM to form TAM-β-glucuronide and both the trans- and cis-isomers of 4-OH-TAM to form 4-OH-TAM-β-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, adrenals, 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 has 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/3-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). Dulbecco’s modified Eagle’s medium, fetal bovine serum, penicillin-streptomycin, and Geneticin Chemical Co. (Milwaukee, WI). Dulbecco’s modified Eagle’s medium (DMEM), Dulbecco’s phosphate-buffered saline (minus calcium chloride and magnesium chloride), fetal bovine serum, penicillin-streptomycin, and Genetecin (G-418) were purchased from Invitrogen (Carlsbad, CA). The Platinum PfX DNA polymerase and the pcDNA3.1/V5-His-TOPO mammalian expression vector were obtained from Invitrogen, whereas the restriction enzymes DpnI and StuI were purchased from New England Biolabs (Beverly, MA). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL), whereas the QIAEX II gel extraction kit was purchased from QIAGEN (Valencia, CA).

The human UGT1A1 and UGT2B7 Western blotting kits and the anti-UGT1A and anti-UGT2B7 antibodies were purchased from Gentest (Woburn, MA). All other chemicals used were purchased from Fisher Scientific (Pittsburgh, PA) unless otherwise specified.

Tissues and Cell Lines. A description of the normal human liver tissue specimens used for these studies was provided previously (Wiener et al., 2004b). Briefly, tissues were quick-frozen at −70°C within 2 h post surgery. Liver microsomes were prepared through differential centrifugation as described previously (Coughtrie et al., 1986), and they were stored (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 UGT1A3s (sense, 5′-AAAAAGAATAATGACGGCAG-3′) and UGT1A3a (antisense, 5′-GGAAATGATCGGAAATGTTTC-3′), corresponding to nucleotides −61 to −42 and +1635 to +1656, respectively, relative to the UGT1A3 translation start site. A UGT1A8-overexpressing cell line was previously received from Dr. Thomas Tephy (University of Iowa, Iowa City, IA). Although this cell line was used in previous studies of substrate glucuronidation experiments (Del linger et al., 2006), it was recently discovered that two polymorphic variants within the UGT1A8 sequence were identified in this cell line; a C>T change at nucleotide +362, and a C>G change at nucleotide +515 (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 1A8s (sense, 5′-TTCTCATCGTGCAGCAGCAGG-3′) and 1A8as (antisense, 5′-CTCAATGGGTCATTTGTTGGG-3′) 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 (Stratage n, La Jolla, CA) was performed for UGT1A8 to change the variant at nucleotide +362 from the polymeric C to the wild-type T using the primer set 1A8WTS and 1A8WTAS (sense, 5′-TTTAACTTATTTTTCG-CATTGCAGGAG-3′) and antisense, 5′-CTCTTCGATCTGGCGAAT-AGTAAA-3′, respectively; corresponding to nucleotides +349 to +377 relative to the translation start site; the underlined bases indicate the base-pair change and corresponding bases in the primer set 1A8WT and 1A8WTS (sense, 5′-CAGGAGGAAT-AGGTGCCACTATCTTTG-3′) and antisense, 5′-CAAAGATGGTGGCAAG-TATTCCTCTGAGG-3′, respectively, corresponding to nucleotides +506 to +532 relative to the UGT1A8 translation start site) were then used to change nucleotide +518 from G to C. SDM PCR amplification was performed 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, what GI1TA3 and UGT1A8 wild-type sequences were confirmed by dyeoxy 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 (UGTA3intAS; 5′-GATAAAGTTTCCTCCACCCGAC-3′, corresponding to nucleotides 1635 to 1656, respectively, relative to the UGT1A3 translation start site) and a UGT1A8 internal antisense primer (1A8intAS; 5′-GATAAAGTTTCCTCCACCCGAC-3′, corresponding to nucleotides 1594 to 1615, respectively, 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 (HEK)293 cell lines were generated by standard electroporation techniques in a GenePulser Xcell (Bio-Rad, Hercules, CA) using 10 μg of pcDNA3.1/5His-TOPO/UGT plasmid DNA with 5 × 107.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 G-418 (700 μg/ml medium) for the selection of the G-418-resistant cells, with selection medium changed every 3 to 4 days. Individual UGT 1A3- and 1A8-overexpressing cell colonies were selected and monitored for UGT expression via Western blotting analysis (described below). Additional cell lines overexpressing the UGT1A and UGT2B isoforms analyzed in this study have been described previously (Ren et al., 2000; Dellinger et al., 2006; G. Chen, R. Dellinger, D. Sun, T. Spratt, and P. Lazarus, manuscript submitted for publication). All UGT overexpressing cell lines were grown in DMEM as described above. Cells were grown to 80% confluence every 3 to 4 days. Individual UGT 1A3- and 1A8-overexpressing cell lines were generated by standard electroporation techniques described in GenBank, and they were confirmed to be 100% homologous to the published sequence (accession number: GenBank:U93989). The cloned UGT1A3 and 1A8 inserts were compared with sequences determined in GenBank, and they were confirmed to be 100% homologous to the published sequence.

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 measured by Western blot analysis using a newly synthesized affinity-purified chicken anti-UGT2B17 antibody generated against the peptide CWKDQFQYSVELGRPTTL, which is common to all human UGT2B family members (Pooncoo Rabbit Farm, Canadensis, PA). Antibodies were used in a 1:5000 dilution. UGTs 2B7, 2B15 and 2B17 were quantified against 200 to 250 ng of human UGT2B7 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 blot 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. The first step resulted in the formation of the intermediate cis/trans-4-OH-N-ethoxy carbonyl-N-methylTAM, with this intermediate being converted to cis/trans-endoxifen. The synthesis of cis/trans-4-OH-N-ethoxy carbonyl-N-methylTAM, with this intermediate being converted to cis/trans-endoxifen, was characterized and confirmed byBruker Avance 500 MHz NMR spectrometer (Bruker, Newark, DE); 1H NMR (DMSO-d6, 500 MHz, trans-isomer) δ 0.85 (t, J = 7.5 Hz, 3H), 2.35 (s, 3H), 2.40 (q, J = 7.5 Hz, 2H), 2.85 (t, J = 5.5 Hz, 2H), 3.87 (t, J = 5.5 Hz, 2H), 6.58 (d, J = 8.5 Hz, 2H), 6.71 (d, J = 9.0 Hz, 2H), 6.75 (d, J = 8.5 Hz, 2H), 6.98 (d, J = 8.5 Hz, 2H), 7.08 to 7.12 (m, 3H), 7.16 to 7.20 (m, 2H), 9.41 (s, 1H, NH). 1H NMR (DMSO-d6, 500 MHz, cis-isomer) δ 0.85 (t, J = 7.5 Hz, 3H), 2.40 (q, J = 7.5 Hz, 2H), 2.34 (s, 3H), 2.97 (t, J = 5.5 Hz, 2H), 4.08 (t, J = 5.5 Hz, 2H), 6.40 (d, J = 8.5 Hz, 2H), 6.60 (d, J = 8.5 Hz, 2H), 6.95 (d, J = 8.5 Hz, 2H), 7.09 to 7.12 (m, 5H), 7.17 to 7.20 (m, 2H), 9.17 (s, 1H, NH).

Separation and Collection of 4-OH-TAM and Endoxifen Isomers. trans- and cis-4-OH-TAM, and trans- and cis-endoxifen, were separated from trans-cis-4-OH-TAM (70:30) and trans-cis-endoxifen (60:40) mixtures, respectively, by HPLC using a Waters 510 system (Waters, Milford, MA) equipped with an autosampler (model 717) and a UV detector operated at 254 nm (A-Max Model 480). The substrates were separated on a 5-μm Luna C18 analytical column (4.6 × 250 mm Phenomenex, Torrance, CA) in series with a 5-μm C18 guard column (4.0 mm in length × 3.0 mm i.d.; Phenomenex). For separation of trans- and cis-4-OH-TAM, the isocratic elution conditions were 20% buffer A (0.1% triethylamine; pH 7.4) and 80% acetonitrile at a 1 ml/min flow rate at room temperature. For separation of trans- and cis-endoxifen, the following isocratic conditions were 5% buffer A (0.25% triethylamine; pH 7.4) and 95% acetonitrile at a 1 ml/min flow rate with the HPLC column kept at 4°C. The collected pure trans- and cis-isomers of 4-OH-TAM and endoxifen were dried, and then they were resuspended in 100% ethanol and stored at −20°C.

Glucuronidation Assays. Glucuronidation activity of homogenates from human UGT1A- and UGT2B-overexpressing cells toward trans- and cis-4-OH-TAM and trans- and cis-endoxifen were performed after an initial incubation of homogenate protein (100 μg–1 mg) with α-methilcin (50 μg/mg protein) for 15 min in an ice bath. Glucuronidation reactions were then performed in a final reaction volume of 100 μl at 37°C in 50 mM Tris-HCl, pH 7.4, 10 mM MgCl2, 4 mM UDPGA, and 1 μM to 1.4 mM TAM metabolite as indicated in the text. The concentration of substrate concentrations used for kinetic analysis was dependent on the Ks values obtained for each active UGT. Reactions were terminated after 60 min by the addition of 100 μl of cold methanol on ice. Mixtures were centrifuged for 10 min at 4°C at 16,100g, and the supernatants were collected and loaded onto the HPLC column. Samples were analyzed for glucuronidated 4-OH-TAM and endoxifen metabolites by HPLC using a Gold 126 Solvent Module HPLC system (Beckman Coulter, Fullerton, CA) equipped with an automatic injector (model 508) and a UV detector operated at 254 nm (model 168). HPLC was performed using a 3-μm Luna C18(2) analytical column (4.6 × 150 mm Phenomenex) in series with a 5-μm Aquasil C18 guard column (10 μm in length × 3.0 mm i.d.; Phenomenex). The gradient elution conditions for kinetic assays with trans- or cis-4-OH-TAM was as follows: starting with 75% buffer A (100 mM ammonium acetate; pH 5.0) and 25% acetonitrile, the flow rate was maintained at 0.5 ml/min over 5 min; a subsequent linear gradient to 75% acetonitrile/25% buffer A over 25 min was performed and then maintained at 75% acetonitrile for 10 min. trans-4-OH-TAM-O-glucuronide, trans-4-OH-TAM-O-glucuronide, cis-4-OH-TAM-O-glucuronide, cis-4-OH-TAM-N-glucuronide, and cis-4-OH-TAM-O-glucuronide were confirmed by 1 M NaOH hydrolysis, and sensitivity to β-glucuronidase as described previously (Sun et al., 2006). For characterization of endoxifen glucuronides, the gradient elution conditions were the same as the gradient programs described above except that the pH of buffer A was maintained at 0.5 ml/min over 5 min; a subsequent linear gradient to 75% acetonitrile/25% buffer A over 25 min was performed and then maintained at 75% acetonitrile for 10 min. trans-4-OH-TAM-O-glucuronide, trans-4-OH-TAM-O-glucuronide, cis-4-OH-TAM-O-glucuronide, cis-4-OH-TAM-N-glucuronide, and cis-4-OH-TAM-O-glucuronide were confirmed by 1 M NaOH hydrolysis, and sensitivity to β-glucuronidase as described previously (Sun et al., 2006). For characterization of endoxifen glucuronides, the gradient elution conditions were the same as the gradient programs described above except that the pH of buffer A was maintained at 0.5 ml/min over 5 min; a subsequent linear gradient to 75% acetonitrile/25% buffer A over 25 min was performed and then maintained at 75% acetonitrile for 10 min.
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 linear 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.

**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 below. 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:peak4 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-glulo...
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. 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. 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.
HLMs (results not shown), suggesting that these glucuronides were the O-glucuronides of 4-OH-TAM isomers. The products corresponding to the predicted trans- and cis-4-OH-TAM-O-glucuronides were collected and analyzed using liquid chromatography with electrospray MS detection. The mass spectrum for the trans-4-OH-TAM-O-glucuronide (with a calculated molecular weight of 563.29) showed a clear [M⁺] ion at m/z 563.75 (Fig. 3E). A virtually identical pattern was observed for cis-4-OH-TAM-O-glucuronide with a clear [M⁺] ion at m/z 563.80 (data not shown). These data suggest that the glucuronides derived from both isomers were in fact monoglucuronides.

UGT1A3 was the only UGT tested in this analysis to form two glucuronide peaks (peaks 1 and 2) against 4-OH-TAM, and this was specific for the trans-isomer (data not shown). Like the peak 2 formed by HLMs (Fig. 3A), UGT1A3-catalyzed peak 2 was sensitive to both 1 M NaOH and β-glucuronidase treatment (data not shown). Similar to that reported previously (Sun et al., 2006), UGT1A4 only formed N-glucuronides against both the trans- and cis-isomers of 4-OH-TAM; no O-glucuronides were observed against 4-OH-TAM isomers. Of the UGTs tested, only 1A6, 2B4, 2B10, and 2B11 did not exhibit detectable glucuronidation activity against both isomers of 4-OH-TAM.

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 UGT1A10-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-endoxifen-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⁺] 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 glucuronide acid moiety (molecular weight = 176 g/mol; Fig. 4E). A virtually identical pattern was observed for cis-endoxifen-O-glucuronide with a clear [M⁺] 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-
glucuronidases 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 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 1A1, 1A3, 1A7, 1A8, 1A9, 1A10, 2B7, 2B15, and 2B17 exhibited detectable levels of O-glucuronidating activity against trans- and cis-4-OH-TAM (Table 1). UGT1A4 selectively exhibited trans-4-OH-TAM (Table 1). UGT1A4 selectively exhibited trans-4-OH-TAM (Table 1). UGT1A4 selectively exhibited trans-4-OH-TAM (Table 1). UGT1A4 selectively exhibited trans-4-OH-TAM (Table 1). UGT1A4 selectively exhibited trans-4-OH-TAM (Table 1). UGT1A4 selectively exhibited N-glucuronidation activity against both 4-OH-TAM isomers in this 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. After normalizing for UGT1A protein expression 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 Vₘₐₓ/Kₐ₅ 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 Vₘₐₓ/Kₐ₅ was 2B7 ≥ 2B17 ≥ 2B4 ≥ 2B5 ≥ 2B6 ≥ 2B16 ≥ 2B13 ≥ 2B12 ≥ 2B11 ≥ 2B10 ≥ 2B9 ≥ 2B8 ≥ 2B3 ≥ 2B2 ≥ 2B1 ≥ 2B0. The 2B1 isomer was selected for the trans-4-OH-TAM, the order of O-glucuronidation activity based upon Vₘₐₓ/Kₐ₅ was 1A10 > 1A8 > 1A7 > 1A6 > 1A5 > 1A4 > 1A3 > 1A2 > 1A1 > 1A0 > 1A9 ≥ 1A8 > 1A7 ≥ 1A6 ≥ 1A5 ≥ 1A4 ≥ 1A3 ≥ 1A2 ≥ 1A1 ≥ 1A0 for the UGT1A family and 2B7 > 2B15 > 2B17 for the UGT2B family. Overall, the UGT2B family members exhibited the lowest Km 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 Km (22.7 μM) against trans-4-OH-TAM and 1A7 exhibited the lowest (7.4 μM) against cis-4-OH-TAM.

Of the UGTs tested in this study, UGT1A3 was the only enzyme exhibiting both O- and N-glucuronidation activity against 4-OH-TAM. The Km and Vₘₐₓ/Kₐ₅ of UGT1A3 for trans-4-OH-TAM-N-glucuronide formation were 193 μM and 0.005 μl·min⁻¹·μg⁻¹; no 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 kinetic analysis (Vₘₐₓ/Kₐ₅) were 1A10 > 1A8 > 1A7 > 1A6 > 1A5 > 1A4 > 1A3 > 1A2 > 1A1 for UGTs 1.2B7, 2B15, and 2B17 protein levels were calculated against known amounts of UGT2B7 standard protein (from Gentest) with quantification made relative to the levels of β-actin observed in each lane. UGT2B7 Std 1, 200 ng of UGT2B7 protein; UGT2B7 Std 2, 250 ng of UGT2B7 protein. Twenty micrograms of homogenate protein was loaded in each lane for UGTs 2B7, 2B15, and 2B17-overexpressing cells. The negative control HEK293 (20 μg of homogenate protein) lane shows no UGT2B expression in the parental HEK293 cells.

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
isomers of 4-OH-TAM (Sun et al., 2006). The N1-glucuronidation activity exhibited by UGT1A4 to form a quaternary ammonium-linked glucuronide with the N,N-dimethyamino alky 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 it is 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 denethylation 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_max/K_M and low apparent K_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_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_max/K_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_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_max/K_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 extrahaepatic 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 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-glucuronidating 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


Coughtrie MW, Burchell B, and Bend JR (1986) A general assay for UDPglucuronosyltrans-


Address correspondence to: Dr. Philip Lazarus, Penn State Cancer Institute, Penn State University College of Medicine, Rm. C379RD, MC-H069, 500 University Dr., Hershey, PA 17033. E-mail: plazarus@psu.edu