The Effects of Endoxifen and Other Major Metabolites of Tamoxifen on the Sulfation of Estradiol Catalyzed by Human Cytosolic Sulfotransferases hSULT1E1 and hSULT1A1*1

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ABSTRACT
Tamoxifen is successfully used for both treatment and prevention of estrogen-dependent breast cancer, yet side effects and development of resistance remain problematic. Endoxifen is a major active metabolite of tamoxifen that is being investigated for clinical use. We hypothesized that endoxifen and perhaps other major metabolites of tamoxifen may affect the ability of human estrogen sulfotransferase 1E1 (hSULT1E1) and human phenol sulfotransferase 1A1 isoform 1 (hSULT1A1*1) to catalyze the sulfation of estradiol, an important mechanism in termination of estrogen signaling through loss of activity at estrogen receptors. Our results indicated that endoxifen, N-desmethyltamoxifen (N-desTAM), 4-hydroxytamoxifen (4-OHTAM), and tamoxifen-N-oxide were weak inhibitors of hSULT1E1 with $K_i$ values ranging from 10 $\mu$M to 38 $\mu$M (i.e., over 1000 times higher than the 8.1 nM $K_m$ value for estradiol as substrate for the enzyme). In contrast to the results with hSULT1E1, endoxifen and 4-OHTAM were significant inhibitors of the sulfation of 2.0 $\mu$M estradiol catalyzed by hSULT1A1*1, with $IC_{50}$ values (9.9 $\mu$M and 1.6 $\mu$M, respectively) that were similar to the $K_m$ value (1.5 $\mu$M) for estradiol as substrate for this enzyme. Additional investigation of the interaction of these metabolites with the two sulfotransferases revealed that endoxifen, 4-OHTAM, and N-desTAM were substrates for hSULT1E1 and hSULT1A1*1, although the relative catalytic efficiencies varied with both the substrate and the enzyme. These results may assist in future elucidation of cell- and tissue-specific effects of tamoxifen and its metabolites.

Introduction
Tamoxifen has been successfully used in the treatment of estrogen-dependent breast cancer for decades; however, its use is limited by a low incidence of endometrial cancer in some patient populations (van Leeuwen et al., 1994; Bernstein et al., 1999). Drug resistance and disease recurrence also occur with tamoxifen therapy. Tamoxifen functions as an anti-estrogen through the formation of 4-hydroxytamoxifen (4-OHTAM) and endoxifen-N-oxide were weak inhibitors of hSULT1E1 with $K_i$ values ranging from 10 $\mu$M to 38 $\mu$M (i.e., over 1000 times higher than the 8.1 nM $K_m$ value for estradiol as substrate for the enzyme). In contrast to the results with hSULT1E1, endoxifen and 4-OHTAM were significant inhibitors of the sulfation of 2.0 $\mu$M estradiol catalyzed by hSULT1A1*1, with $IC_{50}$ values (9.9 $\mu$M and 1.6 $\mu$M, respectively) that were similar to the $K_m$ value (1.5 $\mu$M) for estradiol as substrate for this enzyme. Additional investigation of the interaction of these metabolites with the two sulfotransferases revealed that endoxifen, 4-OHTAM, and N-desTAM were substrates for hSULT1E1 and hSULT1A1*1, although the relative catalytic efficiencies varied with both the substrate and the enzyme. These results may assist in future elucidation of cell- and tissue-specific effects of tamoxifen and its metabolites.

This work was supported by the National Institutes of Health National Cancer Institute [Grant R01 CA038683].

dx.doi.org/10.1124/dmd.115.063206

This article has supplemental material available at dmd.aspetjournals.org.

ABBRIVATIONS: DTT, dithiothreitol; ESI-MS, electrospray ionization-mass spectrometry; hSULT2A1, human hydroxysteroid sulfotransferase 2A1; hSULT1A1, human phenol sulfotransferase 1A1; hSULT1A1*1, human phenol sulfotransferase 1A1 isoform 1; hSULT1E1, human estrogen sulfotransferase 1E1; LC-MS, liquid chromatography-mass spectrometry; N-desTAM, N-desmethyltamoxifen; N-desTAM-S, N-desTAM sulfamate; 4-OHTAM, 4-hydroxytamoxifen; PAPS, adenosine 3′-phosphate 5′-phosphosulfate lithium salt hydrate; TAM-NO, tamoxifen-N-oxide; 4-TAM-SO₄, 4-hydroxytamoxifen sulfate; TLC, thin-layer chromatography.
tamoxifen metabolites were also inhibitors of estradiol sulfation catalyzed by hSULT1E1 and hSULT1A1*. Decreases in the catalytic activity of either enzyme may increase the physiologic concentrations of unconjugated (active) estradiol as a mechanism of clinical resistance. Moreover, the involvement of estrogen in endometrial carcinogenesis (Rižner, 2013; Hernandez-Ramon et al., 2014) may relate to the endometrial cancer side effect of tamoxifen.

**Materials and Methods**

**Chemicals and Instruments.** Expression plasmids (pReceiver-B02) for hSULT1E1 and hSULT1A1* were obtained from GeneCopoeia (Rockville, MD). The Pure Yield Plasmid Mini-Prep System was obtained from Promega (Madison, WI). Antisense (5'-CAG CCT AGG AAC GCC CAA CTT-3') and sense (5'-GGC TAG AGG ATC GAG ATC GAT-3') primers for sequencing were obtained from Integrated DNA Technologies (Coralville, IA). Escherichia coli BL21 (DE3) cells were obtained from Life Technologies (Grand Island, NY). DNA grade Hydroxyapatite was purchased from Bio-Rad (Hercules, CA). Bacto tryptone and yeast extract were purchased from BD Biosciences (Sparks, MD). Ampicillin, dithiothreitol (DTT), and granulated LB broth (Miller’s LB Broth) were obtained from Research Products International (Mount Pleasant, IL). Thin-layer chromatography (TLC) sheets (60 Å silica gel without indicator) were obtained from EMD Millipore (Billerica, MA). Adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate (PAPS) was obtained from Sigma-Aldrich (St. Louis, MO) and purified upon arrival using a previously described protocol (Sekura, 1981) to a purity greater than 99% as determined by high-pressure liquid chromatography (Sheng and Duffel, 2001). 2-Mercaptoethanol, estradiol, estradiol-sulfate, potassium phosphate, (Z)-tamoxifen, (Z)-N-desmethyltamoxifen HCl, (Z)-4-hydroxytamoxifen, and (E/Z)-4-hydroxy-N-desmethyl-tamoxifen hydrochloride (endoxifen) were purchased from Sigma-Aldrich at the highest available purity (>98%). Synthesis of tamoxifen-N-oxide (TAM-NO) was performed, as described elsewhere (Foster et al., 1980; Mani and Kuperf, 1991). N-desethyltamoxifen-sulfamate (N-desTAM-S) and 4-hydroxytamoxifen-sulfate (4-TAM-SO4) were synthesized from sulfuryl chloride, according to our previously published method (Squirewell et al., 2014). [3H]-Estradiol (81.0 Ci/mmol) was obtained from Perkin Elmer (Waltham, MA). Radioactive samples were analyzed with a Tri-Carb 2900TR liquid scintillation counter using EcoNo-Safe liquid scintillation cocktail (Research Products International, Mount Prospect, IL). Data were analyzed using the Enzyme Kinetics Module (version 1.3) of Sigma Plot 11.0 (Systat Software, San Jose, CA).

**Expression and Purification of Recombinant hSULT1E1.** A pReceiver-B02 expression clone (0.6 µl, 114 ng) harboring the gene encoding the native form of hSULT1E1 was transformed into E. coli BL21 (DE3) cells (50 µl). The expression and extraction of hSULT1E1 from these cells were carried out utilizing a procedure similar to that described above for hSULT1E1. The cell extract (300 mg protein) was applied to a DE-52 anion exchange column (2.5 × 20 cm) equilibrated with buffer B [50 mM Tris-HCl, pH 7.5, containing 0.25 M NaCl, 1 mM DTT, 10% (v/v) glycerol, and 0.05% (v/v) Tween 20] and then washed with approximately 1 L buffer B to remove proteins that did not bind to this column. Once the absorbance of the eluate at 280 nm had reached a baseline value, the hSULT1E1 was eluted with a linear gradient formed between 200 ml buffer B and 200 ml buffer C containing 0.1 M KCl. The fractions containing hSULT1E1 were then combined and concentrated by ultrafiltration using an Amicon membrane. Approximately 10 mg purified hSULT1E1 was recovered from the hydroxyapatite column. The subunit molecular mass of hSULT1E1 was found to be approximately 35 kDa by SDS-PAGE, which is consistent with previously reported data for this enzyme (Aksoy et al., 1994). The purity of hSULT1E1 was greater than 94% when analyzed by densitometry on SDS-PAGE. At each purification step, hSULT1E1-containing fractions were identified and quantitated with a previously described methylene blue assay (Duffel et al., 1989) using 25 µM estradiol as substrate. Protein concentration was determined using a standard Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. The complete DNA coding sequence of hSULT1E1 was verified using the sense and antisense sequencing primers described above.

**Expression and Purification of Recombinant hSULT1A1*.** A pReceiver-B02 expression clone (0.6 µl, 108 ng) harboring the gene encoding the native form of hSULT1A1* was transformed into E. coli BL21 (DE3) cells (50 µl). The expression and extraction of hSULT1A1* from these cells were carried out utilizing a procedure similar to that described above for hSULT1E1. The cell extract (300 mg protein) was applied to a DE-52 anion exchange column (2.5 × 15 cm) equilibrated with buffer B and washed with approximately 1 L buffer B to elute those proteins that did not bind to the column. Once the absorbance of the eluate at 280 nm had reached a baseline value, the hSULT1A1* was eluted with a linear gradient formed between 200 ml buffer B and 200 ml buffer C containing 0.1 M KCl. The fractions containing hSULT1A1* were then combined, and the concentration of potassium chloride was reduced through successive dialution and concentration by ultrafiltration with the dilutions carried out using the same buffer to be employed for the subsequent hydroxyapatite chromatography step [i.e., buffer C: 10 mM potassium phosphate, pH 6.8, 0.25 M sucrose, 1 mM DTT, and 0.05% (v/v) Tween 20]. The resulting protein (26 mg) was applied to a column of hydroxyapatite (2.5 × 3.0 cm) that had been equilibrated with 80 ml buffer B and 80 ml buffer C containing 0.4 M potassium phosphate. The fractions containing hSULT1A1* activity were pooled and concentrated by ultrafiltration using an Amicon membrane. Approximately 10 mg purified hSULT1A1* was recovered from the hydroxyapatite column. The subunit molecular mass of hSULT1A1* was found to be approximately 35 kDa by SDS-PAGE, which is consistent with previously reported data for this enzyme (Aksoy et al., 1994). The purity of hSULT1A1* was greater than 94% when analyzed by densitometry on SDS-PAGE. At each purification step, hSULT1A1*-containing fractions were identified and quantitated with a previously described methylene blue assay (Duffel et al., 1989) using 25 µM estradiol as substrate. Protein concentration was determined using a standard Bradford assay (Bradford, 1976) with bovine serum albumin as a standard. The complete DNA coding sequence of hSULT1A1* was verified using the sense and antisense sequencing primers described above.

**Expression and Purification of Recombinant hSULT1A1**. A pReceiver-B02 expression clone (0.6 µl, 114 ng) harboring the gene encoding the native form of hSULT1A1* was transformed into E. coli BL21 (DE3) cells (50 µl). The expression and extraction of hSULT1A1* from these cells were carried out utilizing a procedure similar to that described above for hSULT1E1. The cell extract (300 mg protein) was applied to a DE-52 anion exchange column (2.5 × 15 cm) equilibrated with buffer B and washed with approximately 1 L buffer B to elute those proteins that did not bind to the column. Once the absorbance of the eluate at 280 nm had reached a baseline value, the hSULT1A1* was eluted with a linear gradient formed between 200 ml buffer B and 200 ml buffer C containing 0.1 M KCl. The fractions containing hSULT1A1* were then combined, and the concentration of potassium chloride was reduced through successive dialution and concentration by ultrafiltration with buffer C. The protein obtained from the DE-52 anion exchange column (40 mg protein) was applied to a hydroxyapatite column (2.5 × 5.0 cm) that had been equilibrated with buffer C, and the column was then washed with buffer C to remove all nonbinding proteins. Once the absorbance of the eluate at 280 nm returned to baseline, separation was achieved with a linear gradient formed between 100 ml buffer C and 100 ml buffer C containing 0.4 M potassium phosphate. Analysis by SDS-PAGE revealed minor impurities after the column of hydroxyapatite. Thus, to prepare this mixture for the next step in purification, the fractions containing hSULT1A1* activity were combined and the concentration of potassium phosphate reduced through successive dialution and concentration by ultrafiltration with buffer C. The protein obtained from the DE-52 anion exchange column (25 mg protein) was applied to a hydroxyapatite column (2.5 × 10 cm) that had been equilibrated with buffer C and the column was then washed with buffer C to remove all nonbinding proteins. Once the absorbance of the eluate at 280 nm returned to baseline, separation was achieved with a linear gradient formed between 100 ml buffer C and 100 ml buffer C containing 0.4 M potassium phosphate. The fractions containing hSULT1A1* with the highest activity were combined and concentrated. Approximately 8 mg purified hSULT1A1* was recovered from the second column of hydroxyapatite. The subunit molecular mass of the hSULT1A1* was found to be approximately 844 Squirewell and Duffel
Inhibition of hSULT1E1-Catalyzed Sulfation of Estradiol. Assays for estradiol sulfation catalyzed by hSULT1E1 were performed utilizing the following procedure. Each 200 μl reaction was performed at pH 7.4 and contained 0.25 M potassium phosphate, 50 μM PAPS, and 8.3 mM 2-mercaptoethanol. [3H]-Estradiol and tamoxifen metabolites were dissolved in absolute ethanol, and they were added to the reaction mixture in volumes such that the final concentration of ethanol in each assay was 2% (v/v). The reactions were initiated by the addition of 1.0 μl purified hSULT1E1 (3.0 ng) and incubated for 4 minutes at 37°C. The reactions were then terminated by the addition of 800 μl 0.25 M Tris-HCl, pH 8.7 (Nishiyama et al., 2002), and 4.0 ml chloroform. Samples were vortexed vigorously for 20 seconds and subjected to centrifugation at 1500 rpm for 5 minutes to separate the phases. A 500 μl aliquot of the upper aqueous phase containing [3H]-estradiol-sulfate was added to 10 ml liquid scintillation cocktail, and the radioactivity was determined using a Perkin Elmer Tri-Carb 2900TR liquid scintillation analyzer.

Tamoxifen Metabolites as Substrates for hSULT1E1 and hSULT1A1*1. Tamoxifen metabolites were investigated as substrates for the enzymes using a previously described protocol that determines the incorporation of a radiolabeled sulfuryl moiety from [35S]-PAPS into products of the reaction (Lyon et al., 1981). Each 50 μl reaction was performed at pH 7.4 and contained 0.25 M potassium phosphate, 50 μM PAPS, and 8.3 mM 2-mercaptoethanol. [3H]-Estradiol and tamoxifen metabolites were dissolved in absolute ethanol, and they were added to the reaction mixture in volumes such that the final concentration of ethanol in each assay was 2% (v/v). The reactions were initiated by the addition of 1.0 μl purified hSULT1A1*1 (0.74 μg) and incubated for 10 minutes at 37°C. The reactions were then terminated by the addition of 800 μl 0.25 M Tris-HCl, pH 8.7 (Nishiyama et al., 2002), and 4.0 ml chloroform. Samples were vortexed vigorously for 20 seconds and subjected to centrifugation at 1500 rpm for 5 minutes to separate the phases. A 500 μl aliquot of the upper aqueous phase containing [3H]-estradiol-sulfate was added to 10 ml liquid scintillation cocktail, and the radioactivity was determined as described above.

Results

Metabolites of Tamoxifen Are Weak Inhibitors of the Sulfation of Estradiol Catalyzed by hSULT1E1. Endoxifen, 4-OHTAM, TAM-NO, and N-desTAM were investigated as inhibitors of hSULT1E1 using estradiol as substrate. The sulfation of estradiol was initially examined with 200 μM PAPS and a substrate concentration range between 5.0 and 200 nM to determine the concentrations of estradiol where minimal substrate inhibition occurred (Fig. 1). The sulfation of estradiol could not be described using a simple substrate inhibition model, nor could the data be described using an equation that assumes partial substrate inhibition as noted in previous studies with hSULT1E1 (Zhang et al., 1998). Due to variations in the methodology and reaction conditions used to determine the sulfation of estradiol, it is possible that changes in the enzyme environment (i.e., pH 7.4 in the current study versus pH 6.3 at pH 7.4) and used 50 μM substrate with 50 μM PAPS in the presence of the 0.25 M potassium phosphate, 8.3 mM 2-mercaptoethanol, and 2% ethanol (v/v). The reactions were initiated with the addition of either hSULT1E1 (4.6 μg) or hSULT1A1*1 (3.7 μg) at 37°C for 60 minutes and terminated with 50 μl methanol. A 10 μl aliquot of each sample was analyzed using a Waters Aquity UPLC (BEH C18 column (2.1 mm × 100 mm; 1.7 μm) operated at a flow rate of 0.25 ml/min and a UV wavelength of 213 nm. A linear gradient system was programmed to 40% acetonitrile with 0.1% (v/v) formic acid for 15 minutes and 40–70% (v/v) acetonitrile with 0.1% (v/v) formic acid for 5 minutes, and then sustained at 70% acetonitrile with 0.1% formic acid for 10 minutes. The liquid chromatography–eluate was subjected to mass spectral analysis through interface with an electrospray ionization source operated in negative ion mode.

Identification of Enzyme Reaction Products by Liquid Chromatography and Mass Spectrometry. Products of sulfation catalyzed by hSULT1E1 and hSULT1A1*1 were identified by liquid chromatography–mass spectrometry (LC-MS) analysis on a Waters Q-TOF Premieri mass spectrometer, as described previously (Squirewell et al., 2014). Briefly, each 50 μl reaction was performed at pH 7.4 and used 50 μM substrate with 50 μM PAPS in the presence of the 0.25 M potassium phosphate, 8.3 mM 2-mercaptoethanol, and 2% ethanol (v/v). The reactions were initiated with the addition of either hSULT1E1 (4.6 μg) or hSULT1A1*1 (3.7 μg) at 37°C for 60 minutes and terminated with 50 μl methanol. A 10 μl aliquot of each sample was analyzed using a Waters Aquity UPLC (BEH C18 column (2.1 mm × 100 mm; 1.7 μm) operated at a flow rate of 0.25 ml/min and a UV wavelength of 213 nm. A linear gradient system was programmed to 40% acetonitrile with 0.1% (v/v) formic acid for 15 minutes and 40–70% (v/v) acetonitrile with 0.1% (v/v) formic acid for 5 minutes, and then sustained at 70% acetonitrile with 0.1% formic acid for 10 minutes. The liquid chromatography–eluate was subjected to mass spectral analysis through interface with an electrospray ionization source operated in negative ion mode.
Sulfation of estradiol for uninhibited controls were 62, 67, 58, and 69 nmol/min/mg protein for studies with endoxifen, N-desTAM, 4-OHTAM, and TAM-NO, respectively. Data are the means ± S.E. from triplicate determinations.

In previous work) could contribute to the differences in substrate inhibition that were observed. Thus, an equation that accurately represents substrate inhibition during the hSULT1E1-catalyzed sulfation of estradiol at pH 7.4 may be more complex than previously assumed. In efforts to determine the kinetic constants for estradiol sulfation and to verify the kinetic mechanism of hSULT1E1, estradiol (50 μM) was examined with varied PAPS concentrations (50 nM–100 μM) to determine those concentrations of PAPS where minimal substrate inhibition occurred. Substrate inhibition was not observed with PAPS at pH 7.4 (Supplemental Fig. 1), and this has been previously reported elsewhere (Falany et al., 1995). Sulfation rates were then examined with varied concentrations of estradiol (4 nM–40 nM) and varied concentrations of PAPS (0.2 μM–10.0 μM). The data from this study were best described with a sequential rate equation (Fig. 2), which is in agreement with the kinetic mechanism of hSULT1E1 previously determined by Zhang et al. (1998). PAPS displayed a K_m value of 1.2 ± 0.3 μM, and the K_m, K_i, V_max, and k_cat/K_m derived from the hSULT1E1-catalyzed sulfation of estradiol was determined to be 8.1 ± 1.6 nM, 56 ± 4 nM, 179 ± 9 nmol/min/mg, and 1.6 ± 0.3 minute⁻¹nM⁻¹, respectively.

Endoxifen, 4-OHTAM, N-desTAM, and TAM-NO were all weak inhibitors of estradiol sulfation catalyzed by hSULT1E1 (Fig. 3). Tamoxifen did not exhibit significant inhibition of hSULT1E1 up to the limits of its solubility in the assay (data not shown). Endoxifen, 4-OHTAM, TAM-NO, and N-desTAM displayed greater than 95% inhibition of the enzyme within their solubility limits. The calculated IC_{50} values ranged from 7.0 μM to 21.0 μM for the inhibition of the sulfation of 7.0 nM estradiol, with 4-OHTAM being the most potent inhibitor. The kinetic mechanism of inhibition, apparent (app) maximum velocity (V_max), Michaelis-Menten constant (K_m), inhibitor dissociation constant (K_i), and catalytic efficiency constant (k_cat/K_m) for inhibitors of the hSULT1E1-catalyzed sulfation of estradiol are reported in Table 1, with the initial velocity data in Supplemental Fig. 2. N-desTAM was a mixed inhibitor of hSULT1E1 with a K_i value of 10 μM, whereas endoxifen and 4-OHTAM were noncompetitive inhibitors with K_i values of 30 μM and 38 μM, respectively. Initial velocity data for TAM-NO showed a significant deviation from a noncompetitive inhibition model at 80 μM inhibitor concentration (Supplemental Fig. 2D). Other standard inhibition models (e.g., competitive and mixed inhibition) also failed to describe this behavior at higher inhibitor concentration. This observation was reproducible in later studies with TAM-NO, and its cause remains unclear. Also of note, the estradiol concentrations used to determine the inhibitor dissociation constant for each metabolite (0.5–1.3 × K_m) were lower than the estradiol concentrations used in initial velocity studies with hSULT1E1 (0.5–5 × K_m). Thus, V_max and K_m values for the hSULT1E1-catalyzed sulfation of estradiol in the presence of metabolites (Table 1) are higher because they do not account for the substrate inhibition reflected in the V_max and K_m values (179 nmol/min/mg and 8.1 nM, respectively) for estradiol when determined at higher substrate concentrations in the absence of tamoxifen metabolites.

**Endoxifen and 4-OHTAM Are Potent Inhibitors of the Sulfation of Estradiol Catalyzed by hSULT1A1*1.** Endoxifen, 4-OHTAM, TAM-NO, and N-desTAM were investigated as inhibitors of hSULT1A1*1 at pH 7.4 using estradiol as substrate. The sulfation of estradiol was initially examined with PAPS (50 μM) and varied concentrations of estradiol (0.1–25.0 μM) to determine the concentrations of estradiol where minimal substrate inhibition occurred (Fig. 4). The K_m, K_i, V_max, and k_cat/K_m derived from the hSULT1A1*1-catalyzed sulfation of estradiol were 1.5 ± 0.2 μM, 14 ± 2 μM, 11 ± 1 nmol/min/mg, and 0.5 ± 0.1 minute⁻¹μM⁻¹, respectively. Estradiol sulfation was later examined using a single concentration of estradiol (5 μM) with varied concentrations of PAPS (1.0–100 μM) to determine the concentrations of PAPS that were saturating for the enzyme (Supplemental Fig. 3). Subsequent inhibition studies used 50 μM PAPS as cosubstrate. Of the metabolites studied, only endoxifen and 4-OHTAM were significant inhibitors of estradiol sulfation catalyzed by hSULT1A1*1 (Fig. 5). These metabolites displayed greater than 95% inhibition of the enzyme within their solubility limits, with IC_{50} values of 1.6 ± 0.9 μM for 4-OHTAM and 9.9 ± 0.9 μM for endoxifen. TAM-NO was also an inhibitor of hSULT1A1*1; however, the calculated IC_{50} value for this metabolite was greater than 100 μM when examined with 2 μM estradiol as substrate. N-desTAM and tamoxifen (data not shown) were not significant inhibitors of estradiol sulfation within their solubility limits. Initial velocity data on the inhibition of hSULT1A1*1 are shown in Supplemental Fig. 4. At estradiol concentrations of 0.5–2.5 μM, the data for 4-OHTAM-mediated inhibition of the enzyme were described well by

![Fig. 3. Inhibition of the hSULT1E1-catalyzed sulfation of 7.0 nM estradiol by major metabolites of tamoxifen. Rates of sulfation of estradiol for uninhibited controls were 62, 67, 58, and 69 nmol/min/mg protein for studies with endoxifen, N-desTAM, 4-OHTAM, and TAM-NO, respectively. Data are the means ± S.E. from triplicate determinations.](image-url)
a competitive inhibition model with a $K_i$ value of 1.6 ± 0.1 μM (apparent $K_m$ and $V_{max}$ values under these reaction conditions were 3.9 ± 0.6 μM and 18 ± 2 nmol/min/mg, respectively). Although the data fit a competitive inhibition model for 4-OHTAM as inhibitor at these low concentrations of estradiol, there was a significant deviation of endoxifen from any simple inhibition models, as was especially apparent at 40 μM endoxifen (Supplemental Fig. 4A). This observation was reproducible in later studies with endoxifen, and its cause remains unclear.

**Characterization of 4-OHTAM, N-desTAM, and Endoxifen as Substrates for hSULT1E1.** Previous studies have shown that 4-OHTAM is a substrate for hSULT1E1 (Falany et al., 2006). However, the sulfation kinetics of either endoxifen or N-desTAM with hSULT1E1 have never been fully examined. In efforts to ascertain the metabolic fate of these metabolites, endoxifen, 4-OHTAM, and TAM-NO, respectively. The calculated IC50 uninhibited controls were 5.1, 5.8, 5.7, and 5.7 nmol/min/mg protein for studies with endoxifen, N-desTAM, and TAM-NO, respectively. The calculated IC50 values under these reaction conditions were 3.9 ± 0.6 μM and 18 ± 2 nmol/min/mg, respectively. Although the data fit a competitive inhibition model for 4-OHTAM as inhibitor at these low concentrations of estradiol, there was a significant deviation of endoxifen from any simple inhibition models, as was especially apparent at 40 μM endoxifen (Supplemental Fig. 4A). This observation was reproducible in later studies with endoxifen, and its cause remains unclear. The calculated IC50 values under these reaction conditions were 3.9 ± 0.6 μM and 18 ± 2 nmol/min/mg, respectively. Although the data fit a competitive inhibition model for 4-OHTAM as inhibitor at these low concentrations of estradiol, there was a significant deviation of endoxifen from any simple inhibition models, as was especially apparent at 40 μM endoxifen (Supplemental Fig. 4A). This observation was reproducible in later studies with endoxifen, and its cause remains unclear.

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The sulfation of each metabolite was determined using 0.86 μg purified enzyme in the presence of the indicated concentrations of inhibitor. Rates of sulfation of estradiol for uninhibited controls were 5.1, 5.8, 5.7, and 5.7 nmol/min/mg protein for studies with endoxifen, N-desTAM, 4-OHTAM, and TAM-NO, respectively. The calculated IC50 values for 4-OHTAM and endoxifen were 1.6 ± 0.9 μM and 9.9 ± 0.9 μM, respectively. Data points are the means ± S.E. from triplicate determinations.

**TABLE 2.**

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
<th>$k_{cat}/K_m$ (min$^{-1}$ μM$^{-1}$)</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OHTAM</td>
<td>24 ± 5</td>
<td>12 ± 1</td>
<td>0.036 ± 0.008</td>
<td>387 ± 133</td>
</tr>
<tr>
<td>Endoxifen</td>
<td>24 ± 5</td>
<td>19 ± 2</td>
<td>0.057 ± 0.013</td>
<td>283 ± 86</td>
</tr>
<tr>
<td>N-desTAM</td>
<td>96 ± 52</td>
<td>26 ± 11</td>
<td>0.019 ± 0.013</td>
<td>144 ± 105</td>
</tr>
</tbody>
</table>
Human SULT1E1 catalyzes the sulfation of estrogens and various endogenous and exogenous molecules that contain phenol functional groups. Although known to catalyze the sulfation of hydroxysteroids such as dehydroepiandrosterone and pregnenolone, hSULT1E1 functions primarily in the sulfation of estradiol. Estradiol promotes cellular growth and proliferation when bound to the estrogen receptor (Clemons and Goss, 2001), and it is also conjugated in a reaction catalyzed by hSULT1E1 as one mechanism for inactivation of its role in cell signaling via the estrogen receptor. Although hSULT1E1 is the principal enzyme responsible for the sulfation of estradiol at physiologic substrate concentrations (Zhang et al., 1998), hSULT1A1*1 is also capable of catalyzing the sulfation of estradiol, albeit at micromolar concentrations (Falany, 1997; Shatalova et al., 2005).

Due to the roles of hSULT1E1 and hSULT1A1*1 in estrogen metabolism, we were interested in determining the interactions of tamoxifen and its major metabolites with these enzymes. We hypothesized that major metabolites of tamoxifen could inhibit the catalytic function of hSULT1E1 and/or hSULT1A1*1 and thus potentially serve as modulators of estrogen metabolism. Changes in the concentration of hormonally active estradiol might then play a role in the endometrial effects of tamoxifen as well as in the observed differential responses to tamoxifen therapy. Each metabolite was a weak inhibitor of hSULT1E1 when examined with estradiol (7.0 nM) as substrate, with IC50 values ranging from 7.0 μM to 21.0 μM (Table 1). Furthermore, the inhibition constant (Ki) for inhibitors of hSULT1E1 ranged from 10 μM to 38 μM (Table 1), and these values were orders of magnitude higher than the Km value (8.1 nM) determined for estradiol sulfation. The weak inhibition of hSULT1E1 by 4-OHTAM, N-desTAM, and endoxifen suggests that these metabolites are unlikely to interfere in the inactivation of estradiol in tissues that express hSULT1E1. Also of note in this regard, previous studies have shown that hSULT1E1 is poorly expressed in breast cancer tissues (Falany and Falany, 1996; Suzuki et al., 2003). Nonetheless, even if other tumor tissues were to express hSULT1E1, the weak interactions with this isoform of sulfotransferase relative to those of estradiol suggest that inhibition of hSULT1E1 by endoxifen and the other tamoxifen metabolites examined is unlikely to play a role in altering estradiol concentrations within tumor tissues.

Endoxifen was shown to be a relatively good substrate for hSULT1E1 with a calculated kcat/Km of 0.057 ± 0.013 minute⁻¹ μM⁻¹, which suggests that hSULT1E1 may contribute to the in vivo formation of endoxifen-O-sulfate (Supplemental Fig. 5). This information may be useful when evaluating the pharmacokinetic properties of endoxifen. The properties of sulfated metabolites of tamoxifen have been largely overlooked, although our recent findings show that the product sulfamate of N-desTAM, N-desTAM-S, is a potent inhibitor of the sulfation of endogenous steroid substrates catalyzed by hSULT2A1 (Squirewell et al., 2014). Thus, the pharmacokinetic properties of sulfated tamoxifen metabolites as well as their effects on surrounding tissues are subjects for future investigations.

### Table 3.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Km (μM)</th>
<th>Vmax (nmmol/min/mg)</th>
<th>kcat/Km (μM⁻¹ min⁻¹)</th>
<th>KI (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-OHTAM</td>
<td>26 ± 5</td>
<td>20 ± 3</td>
<td>0.050 ± 0.012</td>
<td>84 ± 19</td>
</tr>
<tr>
<td>Endoxifen</td>
<td>118 ± 82</td>
<td>35 ± 20</td>
<td>0.020 ± 0.018</td>
<td>26 ± 18</td>
</tr>
<tr>
<td>N-desTAM</td>
<td>44 ± 14</td>
<td>19 ± 2.0</td>
<td>0.003 ± 0.001</td>
<td></td>
</tr>
</tbody>
</table>

Figures:

**Fig. 7.** Sulfation of 4-OHTAM, N-desTAM, and endoxifen catalyzed by hSULT1A1*1. Data are the means ± S.E. from triplicate determinations. Curves represent fit of the data to a simple Michaelis Menten equation (for 4-OHTAM) and to an equation for uncompetitive substrate inhibition (for 4-OHTAM and endoxifen).

**Fig. 8.** (A) Inhibition of the hSULT1E1-catalyzed sulfation of 7.0 nM estradiol by N-desTAM-S with a calculated IC50 value of 5.6 ± 0.9 μM, and inhibition by 4-TAM-SO4 with an IC50 value greater than 100 μM. (B) Inhibition of the hSULT1A1*1-catalyzed sulfation of 2.0 μM estradiol by N-desTAM-S with a calculated IC50 value of 14 ± 1 μM, and inhibition by 4-TAM-SO4 with an IC50 value greater than 70 μM.
N-desTAM was a notably good substrate for hSULT1E1 with a calculated $K_{cat}/K_m$ higher than the catalytic efficiency constant determined for the sulfation of this metabolite either by hSULT1A1*1 or hSULT2A1 in breast cancer (Falany et al., 1996) as well as the abundance of the metabolites in relation to thermally labile and low-activity hSULT1A1*2 allele, as the formation of 4-OHTAM on breast cancer might reduce and follow endoxifen or 4-OHTAM might increase from its direct use. Thus, changes in the catalytic activity or expression of hSULT1A1*1 could significantly alter the efficacy of tamoxifen (Mercer et al., 2010). Thus, changes in breast cancer cells and potential role(s) in the therapeutic efficacy of tamoxifen as one mechanism of clinical resistance. This may have implications in the ongoing clinical trials of endoxifen (NCT01327781 and NCT01273168; ClinicalTrials.gov), because the steady-state tissue concentrations of endoxifen might increase from its direct use.

Of the metabolites studied, 4-OHTAM was the best substrate for the hSULT1A1*1 (Table 3). Moreover, the rate of sulfation for 4-OHTAM was higher with hSULT1A1*1 than for either hSULT1E1 (Table 2) or hSULT2A1 (Squirewell et al., 2014). These studies suggest that hSULT1A1*1 could be important for the in vivo formation of 4-OHTAM, the rate of sulfation for concentrations of endoxifen might increase from its direct use. Such inhibition might increase the localized concentrations of hormonally active estradiol, thus decreasing the therapeutic efficacy of tamoxifen metabolism in tissues reported in the nanomolar range (Brauch et al., 2009). The pharmacokinetics and pharmacodynamics of endoxifen and its metabolites in breast cancer cells significantly increases 4-hydroxytamoxifen-induced apoptosis. However, because 4-OHTAM and endoxifen were relatively potent inhibitors of estradiol sulfation catalyzed by hSULT1A1*1, there is a potential for these metabolites to alter the concentrations of hormonally active estrogen in tissues where hSULT1A1*1 is expressed and hSULT1E1 is not. Additional roles of hSULT1E1 and hSULT1A1*1 in the variable response to tamoxifen therapy will be the subject of future investigations.

Acknowledgments

The authors thank Dr. Duncan I. Mackie for helpful advice and assistance in the bacterial expression of hSULT1E1 and hSULT1A1*1.

Authorship Contributions

Participated in research design: Squirewell, Duffel.
Conducted experiments: Squirewell, Duffel.
Performed data analysis: Squirewell, Duffel.
Wrote or contributed to writing of the manuscript: Squirewell, Duffel.

References

Kadlubar FF, and Kadlubar SA (2010) Expression of sulfotransferase isoform 1A1 (SULT1A1) in breast cancer is associated with an increased patient survival catalyzed by this particular isoform of sulfotransferase. However, it is important to note that 4-TAM-SO$_4^-$ is also a product of sulfation by hSULT1A1*1, which suggests that these metabolites of tamoxifen are unlikely to interfere with estrogen inactivation catalyzed by this particular isoform of sulfotransferase. However, because 4-OHTAM and endoxifen were relatively potent inhibitors of estradiol sulfation catalyzed by hSULT1A1*1, there is a potential for these metabolites to alter the concentrations of hormonally active estrogen in tissues where hSULT1A1*1 is expressed and hSULT1E1 is not.


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