

DMD # 63206

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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DMD # 63206

Running title:

Interactions of Tamoxifen Metabolites with hSULT1E1 and hSULT1A1\*1

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Number of text pages: 20

Number of tables: 3

Number of figures: 8

Number of references: 49

Number of words in the Abstract: 227

Number of words in Introduction: 750

Number of words in Discussion: 1138

Abbreviations: hSULT1E1, human estrogen sulfotransferase 1E1; hSULT1A1\*1, human phenol sulfotransferase 1A1 isoform 1; hSULT2A1, human hydroxysteroid sulfotransferase 2A1; N-desTAM, *N*-desmethyldamoxifen; N-desTAM-S, *N*-desmethyldamoxifen sulfamate; 4-OHTAM, 4-hydroxydamoxifen; 4-TAM-SO<sub>4</sub>, 4-hydroxydamoxifen sulfate; PAP, adenosine 3',5'-diphosphate; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; TAM-NO, tamoxifen *N*-oxide

## **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 cytosolic sulfotransferases hSULT1E1 and 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 (TAM-NO) were weak inhibitors of hSULT1E1 with  $K_i$  values ranging from 10  $\mu\text{M}$  to 38  $\mu\text{M}$  (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  $\mu\text{M}$  estradiol catalyzed by hSULT1A1\*1, with  $IC_{50}$  values (9.9  $\mu\text{M}$  and 1.6  $\mu\text{M}$ , respectively) that were similar to the  $K_m$  value (1.5  $\mu\text{M}$ ) 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 utilized 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) (Jordan et al., 1977) and endoxifen (Wu et al., 2009; Maximov et al., 2014). However, tamoxifen has estrogenic activity in some tissues (MacGregor and Jordan, 1998), and such effects may be mediated through the estrogen receptor (Thompson et al., 1989). Other studies report estrogenic properties of tamoxifen metabolites (Jordan and Gosden, 1982; Jordan, 2007), and these effects may contribute to differential responses to tamoxifen therapy through estrogen receptor-related hormonal stimulation.

Estrogen is important for normal endocrine function (Pasqualini, 2009). Estradiol binds the estrogen receptor to induce cell growth and proliferation (Clemons and Goss, 2001). As one mechanism of inactivation, estradiol is converted into a sulfuric acid ester (sulfate) in a reaction catalyzed by human estrogen sulfotransferase, hSULT1E1. While hSULT1E1 is the primary sulfotransferase involved in the sulfation of estradiol at physiological substrate concentrations (Zhang et al., 1998), estradiol is also a substrate for human phenol sulfotransferase (hSULT1A1) at higher concentrations (Nagar et al., 2006). Sulfation represents a major route for the hormonal inactivation of estrogens, and this mechanism protects surrounding tissues from excessive estrogenic effects and is associated with tumor regression in estrogen-dependent carcinogenesis (Suzuki et al., 2003).

Polymorphisms within hSULT1A1 may affect individual responses to some therapeutic agents that require metabolism by this enzyme (Raftogianis et al., 1997; Raftogianis et al., 1999). hSULT1A1\*1 is the dominant variant of human phenol sulfotransferase (Coughtrie et al., 1999) and has been extensively studied in relation to the therapeutic outcome and pharmacogenetics of tamoxifen (Nowell et al., 2002; Wegman et al., 2005; Grabinski et al., 2006; Mercer et al., 2010; Serrano et al., 2011). While hSULT1A1\*1 may enhance the therapeutic effects of tamoxifen in breast cancer cells (Mercer et al., 2010), the complete roles of hSULT1A1\*1 or hSULT1E1 in the clinical response to tamoxifen remain to be fully determined.

Major metabolites of tamoxifen including *N*-desmethyldtamoxifen (N-desTAM), tamoxifen-*N*-oxide (TAM-NO), 4-OHTAM, and endoxifen were recently shown to inhibit the sulfation of steroid substrates for human hydroxysteroid sulfotransferase, hSULT2A1 (Squirewell et al., 2014). Thus, we hypothesized that tamoxifen metabolites were also inhibitors of estradiol sulfation catalyzed by hSULT1E1 and hSULT1A1\*1. Decreases in the catalytic activity of either enzyme may increase the physiological concentrations of unconjugated (active) estradiol as a mechanism of clinical resistance. Moreover, the involvement of estrogen in endometrial carcinogenesis (Rizner, 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\*1 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'-GCG 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 Becton Dickinson, Co. (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 angstrom Silica Gel w/o indicator) were obtained from EMD Millipore (Billerica, MA). PAPS (Adenosine 3'-phosphate 5'-phosphosulfate lithium salt hydrate) 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 HPLC (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 hydrate (endoxifen) were purchased from Sigma-Aldrich at the highest available purity ( $\geq 98\%$ ). Synthesis of Tamoxifen-*N*-oxide (TAM-NO) was performed as described elsewhere (Foster et al., 1980; Mani, 1991). *N*-desmethyltamoxifen-sulfamate (N-desTAM-S), and 4-hydroxytamoxifen-sulfate (4-TAM-SO<sub>4</sub>) were synthesized from sulfonyl imidazolium triflate according to our previously published method (Squirewell et al., 2014). [<sup>3</sup>H]-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 Human Estrogen Sulfotransferase 1E1 (hSULT1E1).** A pReceiver-B02 expression clone (2  $\mu$ l, 108 ng) harboring the gene encoding the native form of hSULT1E1 was transformed into *Escherichia coli* BL21 (DE3) cells (50  $\mu$ l). The bacterial cells were incubated on ice for 20 min and then heat-shocked at 42°C for 32 sec. The cells were immediately cooled on ice for 2 min, after which was added 180  $\mu$ l of sterile pre-warmed SOC (Super Optimal broth with Catabolite repression) media. The bacterial cells were grown for 1 hr on a reciprocating shaker (250 rpm) at 37°C. Afterwards, a 35  $\mu$ l aliquot of the cell suspension was added to an LB-ampicillin agar plate containing 100  $\mu$ g/ml ampicillin and incubated at 37°C for 18 hours. A single colony from the LB-ampicillin agar plate was added to 8.0 ml of sterile Luria broth containing 100  $\mu$ g/ml ampicillin and incubated overnight at 37°C on a reciprocating shaker (250 rpm). The bacterial culture was later inoculated into 120 ml of sterile Luria broth containing 100  $\mu$ g/ml ampicillin and incubated overnight at 37°C on a reciprocating shaker (250 rpm). After the incubation, a 20 ml aliquot from the 120 ml bacterial culture was inoculated into 1.0 L of sterile Terrific broth containing 100  $\mu$ g/ml ampicillin and incubated at 37°C on a reciprocating shaker (250 rpm). The bacterial culture was grown to an OD<sub>600</sub> of 1.0 and then induced with 300  $\mu$ M isopropyl-1-thio-D-galactopyranoside. The culture was then incubated on a reciprocating shaker (250 rpm) overnight at 30°C. Cells were subjected to centrifugation at 10,000 x g for 1 hr at 4°C and the supernatant was discarded. The cell pellet was re-suspended in 10 ml of ice-cold bacterial lysis Buffer A [10 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM dithiothreitol, 10 % (v/v) glycerol, 1 mM

phenylmethylsulfonyl fluoride, 1  $\mu$ M pepstatin A, 3.3  $\mu$ M antipain, 10  $\mu$ M trans-epoxysuccinyl-L-leucylamido-(4-guanidino)-butane, and 100  $\mu$ M leupeptin] and snap-frozen in liquid nitrogen. The cells were thawed and supplemented with chicken lysozyme such that the final concentration of lysozyme was 0.5 mg/ml. Cells were gently shaken for 15 min at 4°C and then snap-frozen in liquid nitrogen to complete the cell lysis. The thawed cells were supplemented with DNase (0.5 mg) and gently shaken for 15 min at 4°C. The cytosolic fraction containing hSULT1E1 activity was recovered following centrifugation at 100,000 x g for 1 hr.

The cell extract (440 mg of protein) was applied to a DE-52 anion exchange column (2.5 x 20 cm) equilibrated with Buffer B [50 mM Tris-HCl, pH 7.5, containing 0.25 M sucrose, 1 mM DTT, 10 % (v/v) glycerol, and 0.05 % (v/v) Tween 20] and then washed with approximately 1 L of 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 of Buffer B and 200 ml of buffer B containing 0.1 M KCl. The fractions containing hSULT1E1 were then combined and concentrated by ultrafiltration (Amicon stirred cell with a YM10 membrane, Millipore Corporation, Bedford MA). The concentration of potassium chloride was then reduced through successive dilution 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 column of hydroxyapatite (2.5 x 3.0 cm) that had been equilibrated with Buffer C. Buffer C was used to wash the column and remove all non-binding proteins, and the elution of hSULT1E1 was carried out with a linear gradient formed between 80 ml of Buffer C and 80 ml of Buffer C containing 0.4 M potassium phosphate. The fractions containing hSULT1E1 activity



were pooled and concentrated by ultrafiltration using an Amicon membrane. Approximately 10 mg of 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  $\mu$ M estradiol as substrate. Protein concentration was determined using a standard Bradford assay (Bradford, 1976) with bovine serum albumin (BSA) 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 Human Phenol Sulfotransferase 1A1**

**Variant 1 (hSULT1A1\*1).** A pReceiver-B02 expression clone (0.6  $\mu$ l, 114 ng) harboring the gene encoding the native form of hSULT1A1\*1 was transformed into *Escherichia coli* BL21 (DE3) cells (50  $\mu$ l). The expression and extraction of hSULT1A1\*1 from these cells were carried out utilizing a procedure similar to that described above for hSULT1E1. The cell extract (300 mg of protein) was applied to a DE-52 anion exchange column (2.5 x 15 cm) equilibrated with Buffer B and washed with approximately 1 L of 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\*1 was eluted with a linear gradient formed between 200 ml of Buffer B and 200 ml of Buffer B containing 0.1 M KCl. The fractions containing hSULT1A1\*1 were then combined and the concentration of potassium chloride reduced through successive dilution and concentration by ultrafiltration with buffer C. The protein obtained from the DE-52 anion exchange column (40 mg of protein) was applied to a hydroxyapatite column (2.5 x 5.0 cm) that

had been equilibrated with Buffer C, and the column was then washed with Buffer C to remove all non-binding proteins. Once the absorbance of the eluate at 280 nm returned to baseline, separation was achieved with a linear gradient formed between 100 ml of Buffer C and 100 ml of Buffer C containing 0.4 M potassium phosphate. Analysis by SDS-PAGE revealed minor impurities after the column of hydroxyapatite. Thus, in order to prepare this mixture for the next step in purification, the fractions containing hSULT1A1\*1 activity were combined and the concentration of potassium phosphate reduced through successive dilution and concentration by ultrafiltration with Buffer C. The hSULT1A1\*1 obtained from the hydroxyapatite (16 mg of protein) was loaded onto a second hydroxyapatite column (2.5 x 5.0 cm) equilibrated with Buffer C. Following the initial removal of non-binding proteins, the hSULT1A1\*1 was eluted with a linear gradient formed between 100 ml of Buffer C and 100 ml of Buffer C containing 80 mM potassium phosphate. The fractions containing hSULT1A1\*1 with the highest activity were combined and concentrated. Approximately 8 mg of purified hSULT1A1\*1 was recovered from the second column of hydroxyapatite. The subunit molecular mass of the hSULT1A1\*1 was found to be approximately 34 kDa, which is consistent with previously reported data for this enzyme (Wilborn, 1992). The purity of hSULT1A1\*1 was determined to be greater than 96% when analyzed by densitometry on SDS-PAGE. Chromatography fractions were analyzed for hSULT1A1\*1 activity at pH 7.4 using the methylene blue assay and 25  $\mu$ M 2-naphthol as substrate. Protein concentration was determined at each step of the purification process using the Bradford assay with BSA as a standard. The complete DNA coding sequence of hSULT1A1\*1 was verified using the antisense and sense sequencing primers described above.

**Inhibition of hSULT1E1-catalyzed Sulfation of Estradiol.** Assays for estradiol sulfation catalyzed by hSULT1E1 were performed utilizing the following procedure. Each 200

$\mu$ l reaction was performed at pH 7.4 and contained 0.25 M potassium phosphate, 50  $\mu$ M PAPS, and 8.3 mM 2-mercaptoethanol. [ $^3$ H]-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  $\mu$ l purified hSULT1E1 (3.0 ng) and incubated for 4 min at 37°C. The reactions were then terminated by the addition of 800  $\mu$ l of 0.25 M Tris-HCl, pH 8.7 (Nishiyama et al., 2002) and 4.0 ml of chloroform. Samples were vortexed vigorously for 20 sec and subjected to centrifugation at 1500 rpm for 5 min to separate the phases. A 500  $\mu$ l aliquot of the upper aqueous phase containing [ $^3$ H]-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.

**Inhibition of hSULT1A1\*1-catalyzed Sulfation of Estradiol.** Assays for estradiol sulfation catalyzed by hSULT1A1\*1 were performed as described below. Each 200  $\mu$ l reaction was performed at pH 7.4 and contained 0.25 M potassium phosphate, 50  $\mu$ M PAPS, and 8.3 mM 2-mercaptoethanol. [ $^3$ H]-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  $\mu$ l purified hSULT1A1\*1 (0.74  $\mu$ g) and incubated for 10 min at 37°C. The reactions were then terminated by the addition of 800  $\mu$ l of 0.25 M Tris-HCl, pH 8.7 (Nishiyama et al., 2002) and 4.0 ml of chloroform. Samples were vortexed vigorously for 20 sec and subjected to centrifugation at 1500 rpm for 5 min to separate the phases. A 500  $\mu$ l aliquot of the upper aqueous phase containing [ $^3$ H]-estradiol-sulfate was added to 10 ml liquid scintillation cocktail and the radioactivity was determined as described above.

**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 [<sup>35</sup>S]-PAPS into products of the reaction (Lyon et al., 1981). Each 50  $\mu$ l reaction was performed at pH 7.4 and contained 50  $\mu$ M [<sup>35</sup>S]-PAPS with 0.25 M potassium phosphate, 8.3 mM 2-mercaptoethanol, and the indicated concentrations of tamoxifen metabolites dissolved in DMSO, with a final DMSO concentration of 2 % (v/v). The reactions were initiated by the addition of either purified hSULT1E1 (0.86  $\mu$ g) or hSULT1A1\*1 (0.74  $\mu$ g) at 37°C, incubated for 20 min, and terminated with 50  $\mu$ l methanol. A 10  $\mu$ l aliquot of the resulting mixture was applied to Silica Gel 60 TLC sheets (w/o indicator) and developed in chloroform / methanol (3:7) until the solvent migrated approximately 8 cm from the origin. An area of the TLC sheet 5.5 cm below and including the solvent front (i.e. that contained the section of the radiolabeled sulfated products) was excised and placed in 10 ml scintillation cocktail for determination of radioactivity. The location of the sulfated products on TLC was determined prior to the radiolabeled assay using synthesized standards for 4-TAM-SO<sub>4</sub> and N-desTAM-S.

**Determination of the Kinetic Mechanism of Inhibition.** Endoxifen, 4-OHTAM, N-desTAM, TAM-NO, N-desTAM-S, 4-TAM-SO<sub>4</sub>, and tamoxifen were used as inhibitors of the enzymes. Data were fit to rate equations for competitive, noncompetitive, uncompetitive, or mixed inhibition using a nonlinear least-squares algorithm in the Enzyme Kinetics Module (version 1.3) of Sigma Plot 11.0 and the model with the highest value for the coefficient of determination,  $r^2$ , was selected. In cases where  $r^2$  was not significantly different, the model with the lowest corrected Akaike Information Criterion (AICc) was selected.

**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 analysis on a Waters Q-TOF Premiere mass spectrometer as described previously (Squirewell et al., 2014). Briefly, each 50  $\mu$ l reaction was performed at pH 7.4 and utilized 50  $\mu$ M substrate with 50  $\mu$ M PAPS in the presence of 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  $\mu$ g) or hSULT1A1\*1 (3.7  $\mu$ g) at 37°C for 60 min and terminated with 50  $\mu$ l methanol. A 10  $\mu$ l aliquot of each sample was analyzed using a Waters Aquity (UPLC) BEH C18 column (2.1 mm x 100 mm; 1.7  $\mu$ m) 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 min, 40% - 70% (v/v) acetonitrile with 0.1% (v/v) formic acid for 5 min, and then sustained at 70% acetonitrile with 0.1% formic acid for 10 minutes. The LC-eluate was subjected to mass spectral analysis through interface with an electrospray ionization source operated in negative ion mode.

## **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  $\mu$ M PAPS and a substrate concentration range between 5.0 – 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 vs pH 6.3 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  $\mu$ M) was examined with varied PAPS concentrations (50 nM – 100  $\mu$ M) in order to determine those concentrations of PAPS where minimal substrate inhibition occurred. Substrate inhibition was not observed with PAPS at pH 7.4 (Supplemental Figure 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  $\mu$ M – 10.0  $\mu$ M). The data from this study was 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. (Zhang et al., 1998). PAPS displayed a  $K_m$  value of  $1.2 \pm 0.3 \mu$ M, and the  $K_m$ ,  $K_i$ ,

$V_{\max}$ , and  $k_{\text{cat}}/K_m$  derived from the hSULT1E1-catalyzed sulfation of estradiol was determined to be  $8.1 \pm 1.6$  nM,  $56 \pm 4$  nM,  $179 \pm 9$  nmol/min/mg, and  $1.6 \pm 0.3$  min<sup>-1</sup>nM<sup>-1</sup>, 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  $IC_{50}$  (half-maximal inhibitory concentration) values ranged from 7.0  $\mu$ M to 21.0  $\mu$ M 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 ( $k_{\text{cat}}/K_m$ ) for inhibitors of the hSULT1E1-catalyzed sulfation of estradiol are reported in Table 1, with the initial velocity data in Supplemental Figure 2. N-desTAM was a mixed inhibitor of hSULT1E1 with a  $K_i$  value of 10  $\mu$ M, whereas endoxifen and 4-OHTAM were noncompetitive inhibitors with  $K_i$  values of 30  $\mu$ M, and 38  $\mu$ M, respectively. Initial velocity data for TAM-NO showed a significant deviation from a non-competitive inhibition model at 80  $\mu$ M inhibitor concentration (Supplemental Figure 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 to 1.3  $\times K_m$ ) were lower than the estradiol concentrations used in initial velocity studies with hSULT1E1 (0.5 to 5  $\times K_m$ ). Thus,  $V_{\max}$  and  $K_m$  values for the hSULT1E1-catalyzed sulfation of estradiol in the presence of metabolites (Table 1) are higher since 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  $\mu$ M) and varied concentrations of estradiol (0.1 – 25.0  $\mu$ M) in order 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  $1.5 \pm 0.2 \mu$ M,  $14 \pm 2 \mu$ M,  $11 \pm 1$  nmol/min/mg, and  $0.5 \pm 0.1 \text{ min}^{-1} \mu\text{M}^{-1}$ , respectively. Estradiol sulfation was later examined using a single concentration of estradiol (5  $\mu$ M) with varied concentrations of PAPS (1.0 – 100  $\mu$ M) in order to determine the concentrations of PAPS that were saturating for the enzyme (Supplemental Figure 3). Subsequent inhibition studies utilized 50  $\mu$ M PAPS as co-substrate. 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  $1.6 \pm 0.9 \mu$ M for 4-OHTAM and  $9.9 \pm 0.9 \mu$ M for endoxifen. TAM-NO was also an inhibitor of hSULT1A1\*1, however, the calculated  $IC_{50}$  value for this metabolite was greater than 100  $\mu$ M when examined with 2  $\mu$ M estradiol as substrate. N-desTAM and tamoxifen (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 Figure 4. At estradiol concentrations of 0.5 – 2.5  $\mu$ M, the data for 4-OHTAM-mediated inhibition of the enzyme were described well by a competitive inhibition model with a  $K_i$  value of  $1.6 \pm 0.1 \mu$ M (apparent  $K_m$  and  $V_{max}$  values under these reaction



conditions were  $3.9 \pm 0.6 \mu\text{M}$  and  $18 \pm 2 \text{ nmol/min/mg}$ , respectively). While 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  $40 \mu\text{M}$  endoxifen (Supplemental Figure 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 N-desTAM were examined as substrates for hSULT1E1 (Fig. 6). Sulfation kinetics for endoxifen, 4-OHTAM, and N-desTAM were best described using a substrate inhibition model, and the kinetic constants obtained for the hSULT1E1-catalyzed sulfation of these metabolites are reported in Table 2. Relative sulfation rates for the metabolites were endoxifen > 4-OHTAM > N-desTAM. The enzymatic reactions were analyzed by LC-MS, and the negative ion ESI-MS of products formed by the hSULT1E1-catalyzed sulfation of endoxifen, 4-OHTAM, and N-desTAM (Supplemental Figures 5, 6, and 7, respectively) were similar to those of products catalyzed by hSULT2A1 in our previous study (Squirewell et al., 2014). The retention times of endoxifen-sulfate, 4-TAM-SO<sub>4</sub>, and N-desTAM-S on LC-MS chromatography (not shown) were 16.06, 16.30, and 21.91min, respectively.

**Characterization of 4-OHTAM, N-desTAM, and Endoxifen as Substrates for hSULT1A1\*1.** While 4-OHTAM is a known substrate for hSULT1A1\*1 (Nagar et al., 2006), endoxifen and N-desTAM have never been formally examined as substrates for this enzyme. The results from the current study indicated that 4-OHTAM, N-desTAM, and endoxifen were all

substrates for hSULT1A1\*1. The kinetics of N-desTAM sulfation was best described using a Michaelis-Menten equation whereas the data for the sulfation of 4-OHTAM and endoxifen were best described using a substrate inhibition model (Fig. 7). Kinetic constants obtained from the hSULT1A1\*1-catalyzed sulfation of 4-OHTAM, N-desTAM, and endoxifen are shown in Table 3. Here, hSULT1A1\*1 displayed higher catalytic activity with endoxifen than with N-desTAM as indicated by a 9-fold higher  $k_{cat}/K_m$ . Additionally, the enzyme displayed a much higher catalytic activity with 4-OHTAM than N-desTAM with a 22-fold higher  $k_{cat}/K_m$ . The enzymatic reactions were analyzed by LC-MS, and the negative ion ESI-MS of the products formed by the hSULT1A1\*1-catalyzed sulfation of 4-OHTAM, endoxifen, and N-desTAM (Supplemental Figures 8, 9, and 10, respectively) were similar to previous ESI-MS data from reactions catalyzed by hSULT2A1 (Squirewell et al., 2014). The retention times of 4-TAM-SO<sub>4</sub>, endoxifen-sulfate, and N-desTAM-S on LC-MS chromatography (not shown) were 16.30, 16.07 and 21.81 min, respectively.

**N-desTAMS and 4-TAM-SO<sub>4</sub> are Weak Inhibitors of Estradiol Sulfation Catalyzed by hSULT1E1 and hSULT1A1\*1.** As seen in Fig. 8A, N-desTAM-S was a weak inhibitor of hSULT1E1 with an  $IC_{50}$  value of  $5.6 \pm 0.9 \mu\text{M}$ , and 4-TAM-SO<sub>4</sub> was a very weak inhibitor of the enzyme with  $IC_{50}$  value greater than  $100 \mu\text{M}$ . These metabolites were also weak inhibitors of hSULT1A1\*1 with an  $IC_{50}$  value of  $14 \pm 1 \mu\text{M}$  for N-desTAM-S and an  $IC_{50}$  value greater than  $70 \mu\text{M}$  for 4-TAM-SO<sub>4</sub> (Fig. 8B).

## **Discussion**

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. While hSULT1E1 is the principal enzyme responsible for the sulfation of estradiol at physiological 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  $IC_{50}$  values ranging from 7.0  $\mu$ M – 21.0  $\mu$ M (Table 1). Furthermore, the inhibition constant ( $K_i$ ) for inhibitors of hSULT1E1 ranged from 10  $\mu$ M – 38  $\mu$ M (Table 1), and these values were orders of magnitude higher than the  $K_m$  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 cells (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  $k_{\text{cat}}/K_m$  of  $0.057 \pm 0.013 \text{ min}^{-1}\mu\text{M}^{-1}$ , which suggests that hSULT1E1 may contribute to the in vivo formation of endoxifen-*O*-sulfate (Supplemental Figure 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.

N-desTAM was a notably good substrate for hSULT1E1 with a calculated  $k_{\text{cat}}/K_m$  higher than the catalytic efficiency constant determined for the sulfation of this metabolite either by hSULT1A1\*1 in the current work or by hSULT2A1 in our previous findings (Squirewell et al., 2014). These results suggest that hSULT1E1 might potentially generate sufficient concentrations of N-desTAM-S to inhibit the genotoxic effects of tamoxifen due to the action of hSULT2A1, which is possible given the co-expression of hSULT1E1 and hSULT2A1 in tissues such as the liver (Radomska et al., 1990; Miki et al., 2002) and endometrium (Falany et al., 1998; Rubin et al., 1999; Singh et al., 2007; Andersson et al., 2010).

As with hSULT1E1, we determined that some metabolites of tamoxifen were inhibitors of estradiol sulfation catalyzed by hSULT1A1\*1. This was of particular interest to our studies since the expression of hSULT1A1\*1 in breast cancer is associated with an increased patient survival in tamoxifen-treated women (Nowell et al., 2002; Wegman et al., 2005). Endoxifen and 4-OHTAM were potent inhibitors of the enzyme with  $IC_{50}$  values of 9.9  $\mu$ M and 1.6  $\mu$ M, respectively (Fig. 5). Furthermore, the interactions of either endoxifen or 4-OHTAM with hSULT1A1\*1 were of similar magnitude to the Michaelis constant determined for estradiol sulfation catalyzed by this enzyme ( $K_m = 1.5 \mu$ M). Rizner et al. reports a physiological estradiol concentration in postmenopausal women of only 30 pM, (Rizner, 2013), whereas the mean plasma concentrations of major tamoxifen metabolites are reported to be in the nanomolar range (Brauch et al., 2009). Other studies report that the concentrations of tamoxifen metabolites in tissues are 6- to 60-fold higher than those in serum (Lien et al., 1991; Decensi et al., 2003). Given the high expression of hSULT1A1 in breast cancer cells (Falany and Falany, 1996) as well as the abundance of the metabolites in relation to the physiological estradiol concentrations, endoxifen or 4-OHTAM might potentially inhibit the catalytic function of hSULT1A1 in breast tumor tissue. Such inhibition might increase the localized concentrations of hormonally active estradiol, thus decreasing 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), since 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-TAM-SO<sub>4</sub>, a metabolite of interest due to its reported apoptotic effects in breast cancer cells and potential role(s) in the therapeutic efficacy of tamoxifen (Mercer et al., 2010). Thus, changes in the catalytic activity or expression of hSULT1A1\*1 could significantly alter the formation of 4-TAM-SO<sub>4</sub> in breast cancer tissue. This might be a concern for the population of patients who are homozygous for the thermally labile and low activity hSULT1A1\*2 allele, as the formation of 4-TAM-SO<sub>4</sub> may be limited by this genetic polymorphism. It is important to note that 4-TAM-SO<sub>4</sub> is also a product of sulfation catalyzed by hSULT2A1 and hSULT1E1. However, there is no known evidence that significant expression of either of these enzymes occurs in breast cancer cells.

In summary, this study examined the interactions of major tamoxifen metabolites with purified hSULT1E1 and hSULT1A1\*1. Endoxifen, 4-OHTAM, TAM-NO, and N-desTAM were weak inhibitors of estradiol sulfation catalyzed by hSULT1E1, which suggests that these metabolites of tamoxifen are unlikely to interfere with estrogen inactivation catalyzed by this particular isoform of sulfotransferase. However, since 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.

DMD # 63206

### **Acknowledgments:**

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

DMD # 63206

### **Authorship Contributions:**

*Participated in research design:* Squirewell and Duffel

*Conducted experiments:* Squirewell

*Performed data analysis:* Squirewell and Duffel

*Wrote or contributed to writing of the manuscript:* Squirewell and Duffel



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DMD # 63206

## Footnote

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

## Figure Legends

**Fig 1.** Initial velocities of hSULT1E1-catalyzed sulfation of estradiol in the presence of 50  $\mu$ M PAPS. Data are the means  $\pm$  standard error from triplicate determinations.

**Fig 2.** Initial rates for the sulfation of estradiol catalyzed by hSULT1E1 at low concentrations of estradiol and varied concentrations of PAPS. Data were fit to a sequential rate equation and individual data points are the means  $\pm$  standard error from triplicate determinations.

**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  $\pm$  standard error from triplicate determinations.

**Fig 4.** Initial velocities of hSULT1A1\*1-catalyzed sulfation of estradiol in the presence of 50  $\mu$ M PAPS.

**Fig 5.** Inhibition of the hSULT1A1\*1-catalyzed sulfation of 2.0  $\mu$ M estradiol by major metabolites of tamoxifen. Reactions were conducted with 0.74  $\mu$ g of 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  $IC_{50}$  values for 4-OHTAM and endoxifen were  $1.6 \pm 0.9$   $\mu$ M and  $9.9 \pm 0.9$   $\mu$ M, respectively. Data points are the means  $\pm$  standard error from triplicate determinations.

**Fig 6.** Sulfation of 4-OHTAM, N-desTAM, and endoxifen catalyzed by hSULT1E1. Data are the means  $\pm$  standard error from triplicate determinations. Curves represent the fit of the data to an equation for uncompetitive substrate inhibition.



**Fig 7.** Sulfation of 4-OHTAM, N-desTAM, and endoxifen catalyzed by hSULT1A1\*1. Data are the means  $\pm$  standard error from triplicate determinations. Curves represent fit of the data to a simple Michaelis Menten equation (for N-desTAM) 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  $IC_{50}$  value of  $5.6 \pm 0.9 \mu\text{M}$ , and inhibition by 4-TAM-SO<sub>4</sub> with an  $IC_{50}$  value greater than 100  $\mu\text{M}$ . (B), Inhibition of the hSULT1A1\*1-catalyzed sulfation of 2.0  $\mu\text{M}$  estradiol by N-desTAM-S with a calculated  $IC_{50}$  value of  $14 \pm 1 \mu\text{M}$ , and inhibition by 4-TAM-SO<sub>4</sub> with an  $IC_{50}$  value greater than 70  $\mu\text{M}$ .

DMD # 63206

TABLE 1

*Inhibition of hSULT1E1-catalyzed sulfation of Estradiol by Metabolites of Tamoxifen*

The sulfation of estradiol was determined using 3.0 ng of purified hSULT1E1 in the presence of varied concentrations of inhibitor and either 7.0 nM estradiol (for  $IC_{50}$  values) or 3 nM – 10 nM estradiol for determination of the best-fit to a kinetic model of inhibition and the kinetic constants for that fit to the model. In the case of TAM-NO, the inconclusive fit to a kinetic model is denoted as N.A. The data are expressed as the means  $\pm$  S.E. from three independent experiments. Calculation of  $k_{cat}$  values was based on 70,252 as the dimeric molecular mass of hSULT1E1.

Metabolite	$IC_{50}$	Inhibition Model	$V_{max}(app)$	$K_m(app)$	$k_{cat}/K_m$	$K_i$
	$\mu M$		$nmol/min/mg$	$nM$	$min^{-1}nM^{-1}$	$\mu M$
Endoxifen	$21 \pm 1$	Non-competitive	$680 \pm 237$	$63 \pm 25$	$0.76 \pm 0.4$	$30 \pm 1$
N-desTAM	$8.2 \pm 0.9$	Mixed	$435 \pm 66$	$38 \pm 7$	$0.81 \pm 0.2$	$10 \pm 1$
4-OHTAM	$7.0 \pm 1.1$	Non-competitive	$553 \pm 149$	$57 \pm 17$	$0.68 \pm 0.3$	$38 \pm 1$
TAM-NO	$18 \pm 1$	N.A.				

DMD # 63206

TABLE 2

*Kinetic Constants for the hSULT1E1-catalyzed Sulfation of 4-OHTAM, N-desTAM, and Endoxifen*

The sulfation of each metabolite was determined using 0.86  $\mu\text{g}$  of purified hSULT1E1. Data were fit to a standard uncompetitive substrate inhibition equation and are the means  $\pm$  S.E. from three independent experiments. Calculation of  $k_{\text{cat}}$  values was based on 70,252 as the dimeric molecular mass of hSULT1E1.

Metabolite	$K_m$	$V_{\text{max}}$	$k_{\text{cat}}/K_m$	$K_i$
	$\mu\text{M}$	$\text{nmol}/\text{min}/\text{mg}$	$\text{min}^{-1}\mu\text{M}^{-1}$	$\mu\text{M}$
4-OHTAM	$24 \pm 5$	$12 \pm 1$	$0.036 \pm 0.008$	$387 \pm 133$
Endoxifen	$24 \pm 5$	$19 \pm 2$	$0.057 \pm 0.013$	$283 \pm 86$
N-desTAM	$96 \pm 52$	$26 \pm 11$	$0.019 \pm 0.013$	$144 \pm 105$

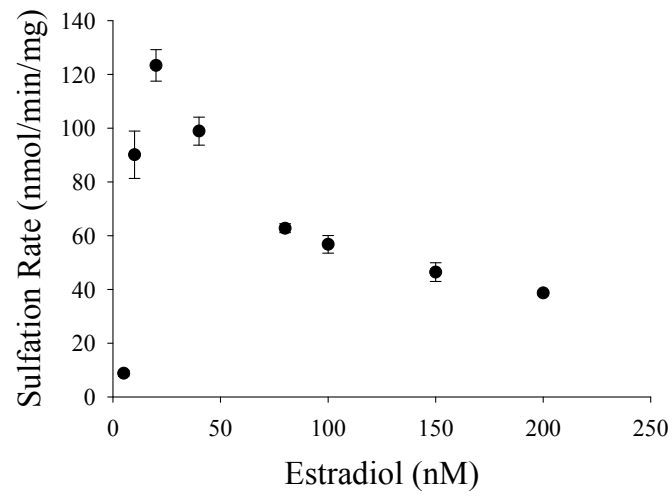
DMD # 63206

TABLE 3

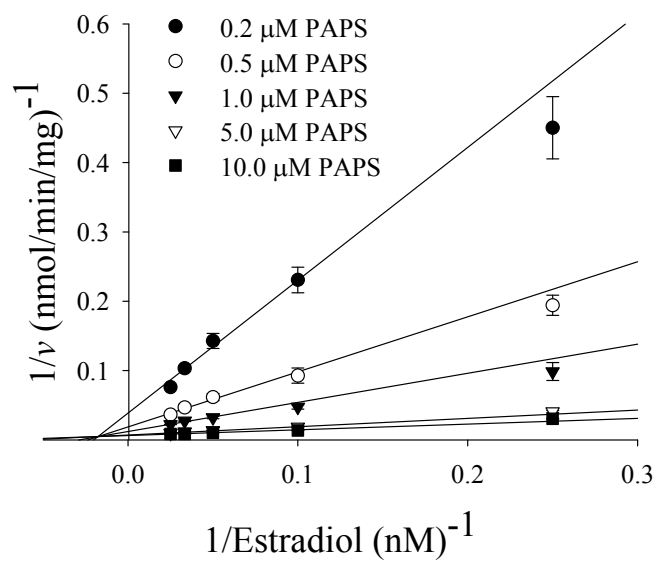
*Kinetic Constants for the hSULT1A1\*1-catalyzed Sulfation of 4-OHTAM, N-desTAM, and Endoxifen*

The sulfation of each metabolite was determined using 0.74  $\mu\text{g}$  of purified hSULT1A1\*1. Data are the means  $\pm$  S.E. from three independent experiments were fit to either a standard uncompetitive substrate inhibition equation (for 4-OHTAM and endoxifen) or a Michaelis Menten equation (for N-desTAM). Calculation of  $k_{\text{cat}}$  values was based on 68,312 as the dimeric molecular mass of hSULT1A1\*1.

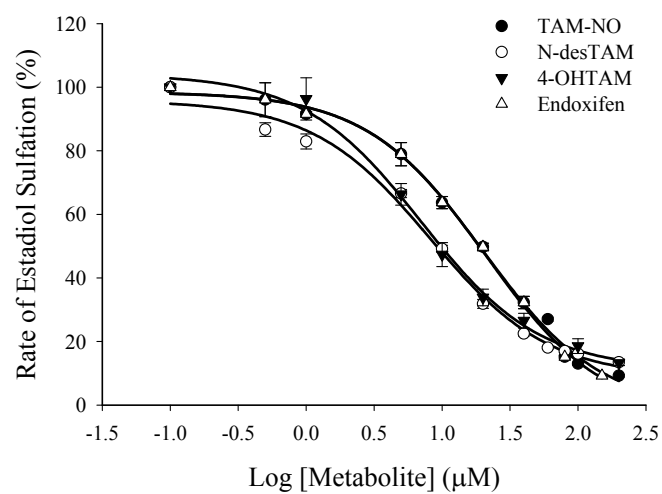
Metabolite	$K_m$	$V_{\text{max}}$	$k_{\text{cat}}/K_m$	$K_i$
	$\mu\text{M}$	$\text{nmol/min/mg}$	$\text{min}^{-1}\mu\text{M}^{-1}$	$\mu\text{M}$
4-OHTAM	$26 \pm 5$	$20 \pm 3$	$0.050 \pm 0.012$	$84 \pm 19$
Endoxifen	$118 \pm 82$	$35 \pm 20$	$0.020 \pm 0.018$	$26 \pm 18$
N-desTAM	$44 \pm 14$	$1.9 \pm 0.2$	$0.003 \pm 0.001$	



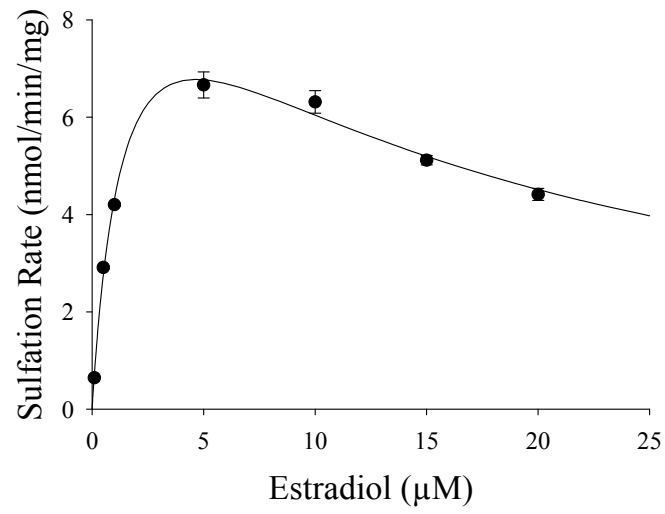
**Fig. 1**



**Fig. 2**

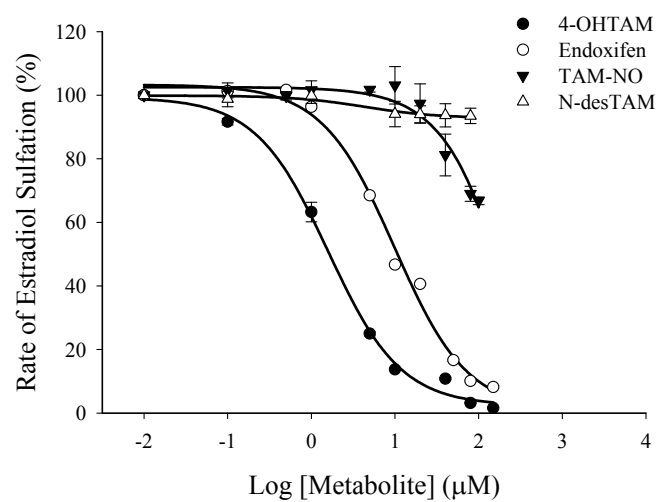


**Fig. 3**

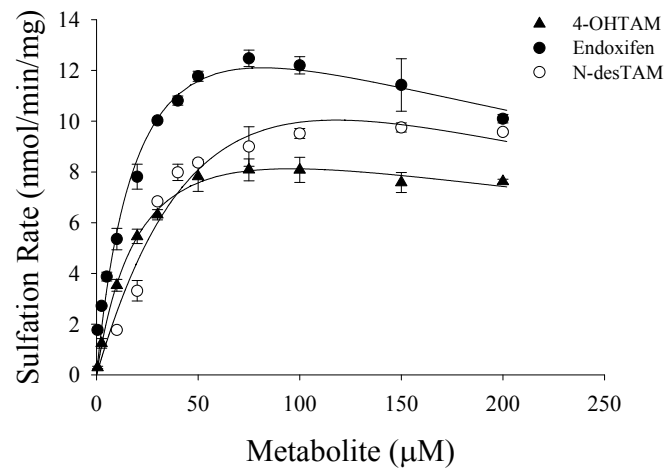


**Fig. 4**

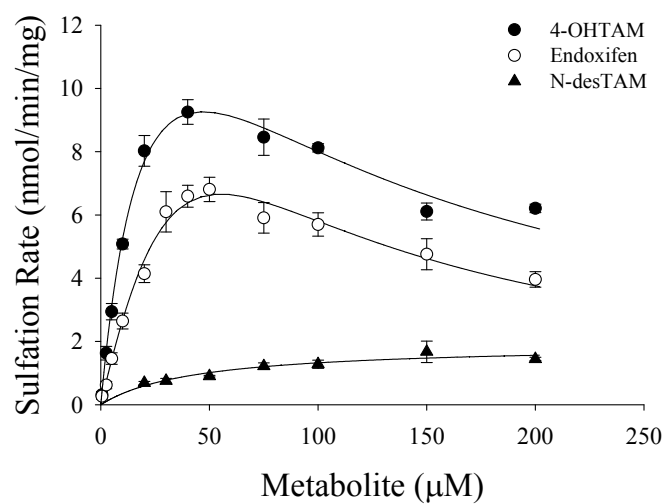




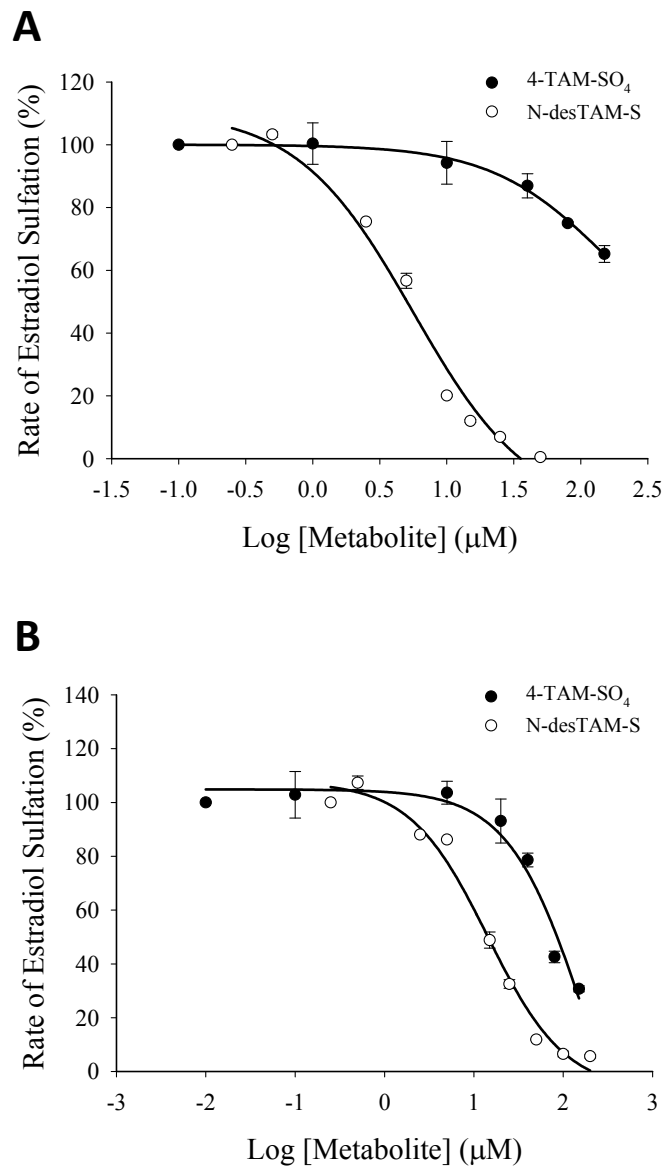
**Fig. 5**



**Fig. 6**



**Fig. 7**



**Fig. 8**