

# Endoxifen and Other Metabolites of Tamoxifen Inhibit Human Hydroxysteroid Sulfotransferase 2A1 (hSULT2A1)<sup>§</sup>

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## ABSTRACT

Although tamoxifen is a successful agent for treatment and prevention of estrogen-dependent breast cancer, its use has been limited by the low incidence of endometrial cancer. Human hydroxysteroid sulfotransferase 2A1 (hSULT2A1) catalyzes the formation of an  $\alpha$ -sulfoxy metabolite of tamoxifen that is reactive toward DNA, and this has been implicated in its carcinogenicity. Also, hSULT2A1 functions in the metabolism of steroid hormones such as dehydroepiandrosterone (DHEA) and pregnenolone (PREG). These roles of hSULT2A1 in steroid hormone metabolism and in generating a reactive metabolite of tamoxifen led us to examine its interactions with tamoxifen and several of its major metabolites. We hypothesized that metabolites of tamoxifen may regulate the catalytic activity of hSULT2A1, either through direct inhibition or through serving as alternate substrates for the enzyme. We found that

4-hydroxy-*N*-desmethyltamoxifen (endoxifen) is a potent inhibitor of hSULT2A1-catalyzed sulfation of PREG and DHEA, with  $K_i$  values of 3.5 and 2.8  $\mu\text{M}$ , respectively. In the hSULT2A1-catalyzed sulfation of PREG, 4-hydroxytamoxifen (4-OHTAM) and *N*-desmethyltamoxifen (*N*-desTAM) exhibited  $K_i$  values of 12.7 and 9.8  $\mu\text{M}$ , respectively, whereas corresponding  $K_i$  values of 19.4 and 17.2  $\mu\text{M}$  were observed with DHEA as substrate. A  $K_i$  value of 9.1  $\mu\text{M}$  was observed for tamoxifen-*N*-oxide with DHEA as substrate, and this increased to 16.9  $\mu\text{M}$  for the hSULT2A1-catalyzed sulfation of PREG. Three metabolites were substrates for hSULT2A1, with relative sulfation rates of 4-OHTAM > *N*-desTAM > endoxifen. These results may be useful in interpreting ongoing clinical trials of endoxifen and in improving the design of related molecules.

## Introduction

Human hydroxysteroid sulfotransferase (hSULT2A1) is a cytosolic enzyme that catalyzes the sulfation of various endogenous and exogenous molecules (Gamage et al., 2006; Duffel, 2010; James and Ambadapadi, 2013). It is highly expressed in the liver and adrenal gland, and present in other tissues as well (Rainey and Nakamura, 2008; Riches et al., 2009). Moreover, it catalyzes the sulfation of dehydroepiandrosterone (DHEA) and pregnenolone (PREG) (Falany et al., 1989), two of the most abundant circulating steroid hormones in humans. Although the detoxication of many hydrophobic xenobiotics that contain alcohol functional groups is one of the important roles of SULT2A1, the sulfation of benzylic and allylic alcohols catalyzed by this enzyme can sometimes generate bioactive electrophilic products that are reactive toward DNA and proteins (Phillips et al., 1994; Surh and Miller, 1994; Shibutani et al., 1998b; Duffel, 2010).

Tamoxifen is a selective estrogen receptor modulator that has been successfully used for decades in the treatment and more recently the prevention of estrogen-dependent breast cancer (Fisher et al., 1998; Jordan, 2003, 2007). However, there is also an increased risk for the

development of endometrial cancer as a side effect of tamoxifen (Bernstein et al., 1999). The carcinogenic effects of tamoxifen are complex and may include a combination of estrogen-receptor mediated hormonal effects and metabolic activation of tamoxifen metabolites to electrophiles that are genotoxic (Dowers et al., 2006). One of the major potential genotoxic species derived from tamoxifen is its  $\alpha$ -sulfoxy metabolite, a product derived from the cytochrome P450 (P450)-catalyzed oxidation of tamoxifen to an allylic  $\alpha$ -hydroxy derivative that is then a substrate for hSULT2A1 (Shibutani et al., 1998a). The resulting sulfuric acid ester is a good leaving group and forms an electrophilic carbocation intermediate that reacts with nucleophilic sites on DNA, thereby forming covalent tamoxifen-DNA adducts (Shibutani et al., 1998a).

Tamoxifen is initially metabolized through oxidative reactions catalyzed by several forms of P450. The most abundant of these initial metabolites in humans is *N*-desmethyltamoxifen (*N*-desTAM), which is formed via an oxidative demethylation reaction catalyzed by CYP2D6, 3A4, 1A1, and/or 1A2 (Crewe et al., 2002; Desta et al., 2004). *N*-desTAM is further metabolized in a reaction catalyzed by CYP2D6 to form 4-hydroxy-*N*-desmethyltamoxifen (endoxifen) (Desta et al., 2004). Endoxifen is 100 times more potent than tamoxifen as an antiestrogen and equipotent with another P450-mediated oxidative metabolite, 4-hydroxytamoxifen (4-OHTAM) (Lim et al., 2005, 2006). Although now known to be present at lower serum concentrations than

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**ABBREVIATIONS:** DHEA, dehydroepiandrosterone; DMSO, dimethylsulfoxide; ESI, electrospray ionization; hSULT2A1, human hydroxysteroid sulfotransferase 2A1; LC, liquid chromatography; MS, mass spectrometry; *N*-desTAM, *N*-desmethyltamoxifen; *N*-desTAM-S, *N*-desmethyltamoxifen sulfamate; 4-OHTAM, 4-hydroxytamoxifen;  $\alpha$ -OHTAM,  $\alpha$ -hydroxytamoxifen; P450, cytochrome P450; PAPS, adenosine 3'-phosphate 5'-phosphosulfate; PREG, pregnenolone; SULT, sulfotransferase; 4-TAM-SO<sub>4</sub>, 4-hydroxytamoxifen sulfate; TAM, tamoxifen; TAM-NO, tamoxifen *N*-oxide; TLC, thin-layer chromatography.

endoxifen (Gjerde et al., 2012), 4-OHTAM was the first metabolite of tamoxifen (TAM) identified with a high affinity for estrogen receptors (Jordan et al., 1977). The microsomal flavin-containing monooxygenases catalyze oxidation of the tertiary amine of tamoxifen to form tamoxifen-*N*-oxide (TAM-NO) (Mani et al., 1993), a metabolite that is also of recent interest due to its potential role(s) in the activity of tamoxifen (Gjerde et al., 2012). As reviewed by Brauch et al. (2009), other metabolic products of tamoxifen include sulfate and glucuronide conjugates as well as various minor metabolites.

Pharmacogenetic differences in responses to tamoxifen have been of much interest in understanding individual variability in its clinical effectiveness (Brauch et al., 2009; Ruddy et al., 2013). While such differences may be of use in individualizing dosages of tamoxifen, another strategy has been to investigate the administration of endoxifen as an antitumor drug through clinical trials (NCT01327781 and NCT01273168; ClinicalTrials.gov). Due to the involvement of hSULT2A1 in the metabolism and transport of steroid hormones and in the genotoxicity of the  $\alpha$ -hydroxy metabolite of TAM, we have explored the potential for metabolites of TAM to inhibit the catalytic activity of hSULT2A1. Such inhibition might prevent formation of the genotoxic  $\alpha$ -hydroxy metabolite in some tissues or it might alter steroid hormone homeostasis by interfering with the inactivation of steroid substrates for the enzyme. Moreover, because inhibitors of an enzyme could be exerting their effect through serving as alternate substrates, we have also examined this possibility. Thus, our study examines the interactions of endoxifen, 4-OHTAM, N-desTAM, and TAM-NO with the human hydroxysteroid sulfotransferase hSULT2A1.

## Materials and Methods

**Chemicals and Instruments.** Thin-layer chromatography (TLC) sheets (60 Å, Silica Gel F<sub>254</sub>, and 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 et al., 2001). We purchased 2-mercaptoethanol, DHEA, PREG, potassium phosphate, (*Z*)-tamoxifen, (*Z*)-*N*-desmethyltamoxifen HCl, (*Z*)-4-hydroxytamoxifen, and (*E/Z*)-4-hydroxy-*N*-desmethyltamoxifen hydrochloride hydrate (endoxifen) from Sigma-Aldrich at the highest available purity ( $\geq 98\%$ ). [<sup>3</sup>H]DHEA (70.5 Ci/mmol), [<sup>3</sup>H]DHEA-sulfate (63.0 Ci/mmol), and [<sup>3</sup>H]pregnenolone (22.9 Ci/mmol) were obtained from Perkin Elmer (Waltham, MA). [<sup>3</sup>H]pregnenolone sulfate (0.20 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). All radioactive samples were analyzed in Econo-Safe liquid scintillation cocktail (Research Products International, Mount Prospect, IL) using a Perkin-Elmer Tri-Carb 2900TR Liquid Scintillation Counter.

**Expression and Purification of Recombinant Human SULT2A1.** SULT2A1 was expressed and extracted from BL21 (DE3) *Escherichia coli* as previously described elsewhere (Liu et al., 2006; Gulcan et al., 2008). The enzyme was purified using DE-52 (Sigma-Aldrich) anion exchange chromatography followed by two hydroxyapatite (Bio-Rad; Hercules, CA) columns to homogeneity as determined by SDS-PAGE. Protein concentration was determined at each step of the purification process with a modified Lowry method using bovine serum albumin as a standard (Bensadoun and Weinstein, 1976). Chromatography fractions were analyzed for enzyme activity using a previously reported methylene blue assay (Nose and Lipmann, 1958; Duffel et al., 1989).

**Inhibition of hSULT2A1-Catalyzed Sulfation of DHEA.** Assays for the sulfation of DHEA were performed under first-order reaction kinetics as previously described elsewhere (Gulcan and Duffel, 2011). Each 200- $\mu$ l reaction was performed at pH 7.4 and contained 0.25 M potassium phosphate, 0.20 mM PAPS, and 8.3 mM 2-mercaptoethanol. [<sup>3</sup>H]DHEA 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 purified hSULT2A1 (0.03  $\mu$ g) and incubated for 4 minutes at 37°C. The reactions were

then terminated by the addition of 800  $\mu$ l of 50 mM potassium hydroxide and 500  $\mu$ l of chloroform. Samples were vortexed vigorously for 20 seconds and subjected to centrifugation at 3500 rpm for 5 minutes to separate the phases. A 100- $\mu$ l aliquot of the upper aqueous phase containing [<sup>3</sup>H]DHEA-sulfate was added to 10 ml of liquid scintillation cocktail, and the radioactivity was determined by liquid scintillation analysis.

**Inhibition of hSULT2A1-Catalyzed Sulfation of Pregnenolone.** Assays for the sulfation of pregnenolone were performed using the following general procedure. Each 100- $\mu$ l assay was performed at pH 7.4 and contained 0.25 M potassium phosphate, 0.20 mM PAPS, and 8.3 mM 2-mercaptoethanol. [<sup>3</sup>H]pregnenolone 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 purified hSULT2A1 (0.03  $\mu$ g), incubated at 37°C for 4 minutes, and terminated with an equal reaction volume of methanol. A 10- $\mu$ l aliquot of the resulting mixture was applied to Silica Gel 60 TLC sheets (without indicator) and developed in chloroform/methanol/acetone/acetic acid/water (80:20:40:20:10) (Fuda et al., 2002) until the solvent moved approximately 8 cm from the origin. The area of the TLC sheet encompassing 2–5 cm from the origin contained [<sup>3</sup>H]pregnenolone sulfate, and this section was excised and placed in 10 ml of scintillation cocktail supplemented with 500  $\mu$ l of methanol, and the radioactivity was determined as described earlier. The location of [<sup>3</sup>H]pregnenolone sulfate on TLC sheets was previously determined with unlabeled pregnenolone sulfate. Maximum recovery of pregnenolone sulfate under these conditions was 75%, and assay results were corrected for this extraction efficiency.

**Analysis of Inhibition Data.** Endoxifen, 4-OHTAM, N-desTAM, and TAM-NO were used as inhibitors of the sulfation of DHEA and PREG at the indicated concentrations. 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 (Systat Software, San Jose, CA), and the model with the highest value for the coefficient of determination,  $r^2$ , was selected. In cases where  $r^2$  was not statistically significantly different, the model with the lowest corrected Akaike information criterion was selected.

**Tamoxifen Metabolites as Substrates for hSULT2A1.** Tamoxifen metabolites were investigated as substrates for hSULT2A1 using a previously described protocol that determines the incorporation of a radiolabeled sulfonyl 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 0.25 M potassium phosphate, 0.20 mM [<sup>35</sup>S]PAPS, 8.3 mM 2-mercaptoethanol, and the indicated concentrations of tamoxifen metabolite dissolved in dimethylsulfoxide (DMSO), with a final DMSO concentration of 2% (v/v). The reactions were initiated by the addition of purified hSULT2A1 (0.52  $\mu$ g), incubated for 20 minutes at 37°C, and terminated with 50  $\mu$ l of methanol. A 10- $\mu$ l aliquot of the resulting mixture was applied to Silica Gel 60 TLC sheets (without 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 of scintillation cocktail for determination of radioactivity. The location of the sulfated products on TLC was determined before the radiolabeled assay using synthesized standards for 4-TAM-SO<sub>4</sub> and *N*-desmethyltamoxifen sulfamate (N-desTAM-S).

**Identification of Enzyme Reaction Products by Mass Spectrometry.** Products of the hSULT2A1-catalyzed sulfation of 4-OHTAM, N-desTAM, and endoxifen were identified using liquid chromatography with mass spectrometry (LC-MS) analysis performed on a Waters Q-TOF Premiere mass spectrometer (Waters Corporation, Milford, MA). Each 50- $\mu$ l reaction was performed at pH 7.4 and used 50  $\mu$ M substrate in the presence of 0.25 M potassium phosphate, 0.20 mM PAPS, 8.3 mM 2-mercaptoethanol, and 2% ethanol (v/v). The reactions were initiated with the addition of hSULT2A1 (2.6  $\mu$ g) at 37°C for 60 minutes and terminated with 50  $\mu$ l of methanol. A 10- $\mu$ l aliquot of each sample was analyzed using a Waters Aquity BEH C18 column (2.1 mm  $\times$  100 mm; 1.7  $\mu$ m) using a flow rate of 0.25 ml/min and UV analysis at 213 nm. A linear gradient system was programmed to 40% acetonitrile with 0.1% (v/v) formic acid for 15 minutes, 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

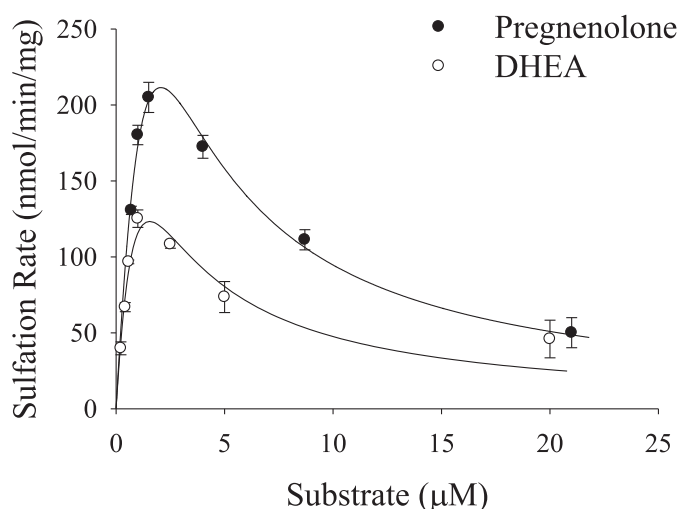
analysis (LC-MS) through an interface with an electrospray ionization (ESI) source operated in negative ion mode.

**Synthesis of TAM-NO.** The *N*-oxide of tamoxifen was synthesized using a procedure previously described elsewhere (Foster et al., 1980). Aqueous hydrogen peroxide (1 ml, 30% w/w) was added to a solution of tamoxifen (15 mg, 40  $\mu$ mol) dissolved in methanol (3.0 ml). The reaction was stirred at room temperature for 10 hours in the dark. The resulting product was examined for homogeneity on Silica Gel 60 F<sub>254</sub> analytical thin layer chromatography sheets developed in chloroform/methanol/ammonium hydroxide (8:2:0.05), which revealed tamoxifen *N*-oxide ( $R_f = 0.40$ ), which was well separated from any residual tamoxifen ( $R_f = 0.69$ ). Excess hydrogen peroxide and water were removed by concentration of the sample under nitrogen, addition of 1.0 ml of ethanol, repeating this procedure two more times, and then performing the concentration and solvent addition procedure three times using 1.0-ml aliquots of dry benzene. Afterward, the sample was dried under nitrogen and stored overnight in a vacuum desiccator over phosphorus pentoxide to afford a white solid (15.3 mg, 97% yield). Negative ion ESI-MS  $m/z = 388.20$  [ $M-H$ ]<sup>-</sup> (calculated  $m/z = 388.2277$ ); [<sup>1</sup>H NMR: (300 MHz, CDCl<sub>3</sub>) ppm ( $\delta$ ) 0.94 (t, 3H, CH<sub>2</sub>CH<sub>3</sub>); 2.48 (q, 2H, CH<sub>2</sub>CH<sub>3</sub>); 3.29 [s, 6H, N(CH<sub>3</sub>)<sub>2</sub>]; 3.65 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 4.69 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 6.57 (d, 2H, Ph); 6.79 (d, 2H, Ph); 7.11–7.39 (m, Ph).] The melting point was 136–138°C.

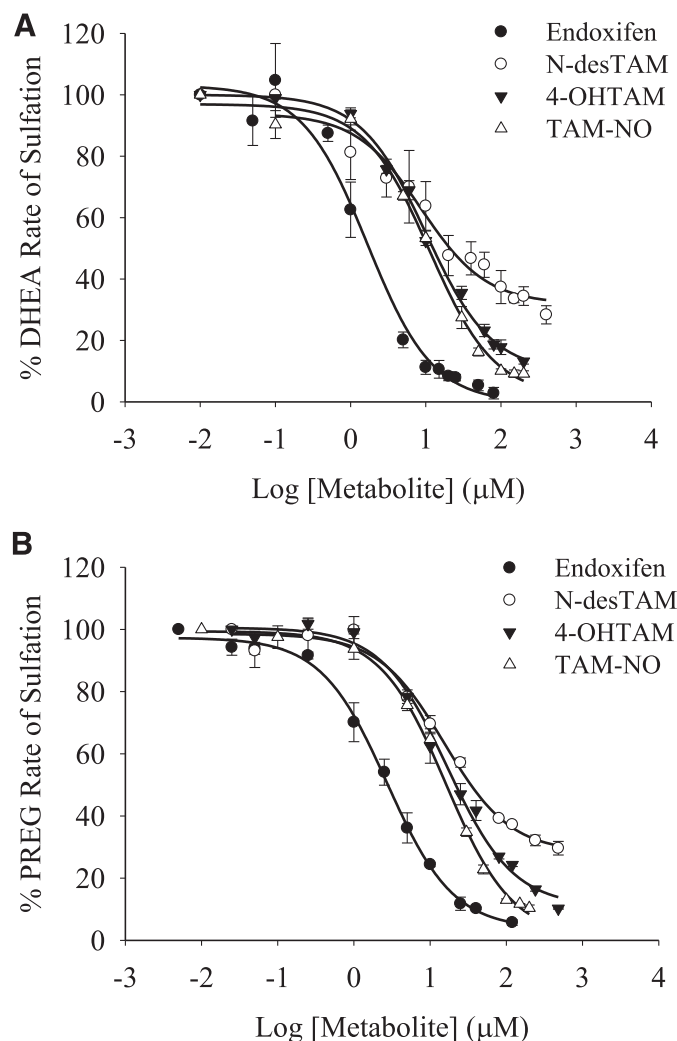
**Synthesis of N-desTAM-S Ammonium Salt.** The sulfamate of N-desTAM was prepared using sulfuryl imidazolium triflate (2,2,2-trichloroethoxysulfuryl-(2-methyl)-*N*-methyl-imidazolium triflate) as the sulfating reagent, and the synthesis of this reagent has been previously reported elsewhere (Ingram and Taylor, 2006; Desoky et al., 2011). We added 1,2-dimethylimidazole (14  $\mu$ l, 157  $\mu$ mol) to a solution of N-desTAM (28 mg, 69  $\mu$ mol) and sulfuryl imidazolium triflate (94 mg, 207  $\mu$ mol) dissolved in dichloromethane (5 ml). The mixture was stirred at 0°C for 1 hour and gradually warmed to room temperature for a 12-hour reaction, then purified on a Silica Gel 60 flash column (1 cm  $\times$  10 cm) using ethyl acetate/hexanes (33:67) as mobile phase. The eluate was concentrated, dissolved in DMSO (2 ml), and then added to a solution of ammonium formate (52 mg, 828  $\mu$ mol) and zinc dust (27 mg, 414  $\mu$ mol) (Liu et al., 2004) in methanol (1 ml). The reaction was stirred at room temperature for 2 hours and filtered through celite under vacuum. Methanol was removed by rotary evaporation, and DMSO was removed by freeze-drying. The resulting mixture was purified on a Silica Gel 60 flash column (1 cm  $\times$  10 cm) using dichloromethane/methanol/ammonium hydroxide (20:4:1) as mobile phase. The sample was concentrated and the solvents removed to afford a yellow, flaky solid (28 mg, 90% yield). Negative ion ESI-MS  $m/z = 436.1586$  [ $M-H$ ]<sup>-</sup> (calculated  $m/z = 436.1588$ ); [<sup>1</sup>H NMR:

(600 MHz, methanol-*d*<sub>4</sub>) ppm ( $\delta$ ) 0.92 (t, 3H, CH<sub>2</sub>CH<sub>3</sub>); 2.47 (q, 2H, CH<sub>2</sub>CH<sub>3</sub>); 2.75 [s, 6H, N(CH<sub>3</sub>)<sub>2</sub>]; 3.29 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 4.04 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 6.57 (d, 2H, Ph); 6.76 (d, 2H, Ph); 7.10–7.26 (m, Ph).] The melting point was 132–138°C.

**Synthesis of 4-Hydroxytamoxifen Sulfate Ammonium Salt.** The sulfuric acid ester of 4-OHTAM, 4-hydroxytamoxifen sulfate (4-TAM-SO<sub>4</sub>), was prepared similarly to the synthesis of N-desTAM-S with slight modifications. We added 1,2-dimethylimidazole (2  $\mu$ l, 23  $\mu$ mol) to a solution of (*Z*)-4-OHTAM (4 mg, 10  $\mu$ mol) and sulfuryl imidazolium triflate (14 mg, 30  $\mu$ mol) dissolved in dichloromethane (5 ml). The mixture was stirred at 0°C for 1 hour and gradually warmed to room temperature for a 24-hour reaction. The product was then purified on a Silica Gel 60 flash column (1 cm  $\times$  10 cm) using chloroform/methanol (80:20) as the mobile phase. The resulting reaction product was concentrated and then added to a solution of ammonium formate (8 mg, 120  $\mu$ mol) and zinc (4 mg, 60  $\mu$ mol) dissolved in methanol (3 ml). The reaction was stirred at room temperature for 30 minutes and vacuum-filtered through celite. Afterward, the mixture was purified on a Silica Gel 60 flash column (1 cm  $\times$  10 cm) using dichloromethane/methanol/ammonium hydroxide (20: 4: 1) as the mobile phase, and the eluate was concentrated and then lyophilized to yield a white powder (5 mg; 36% yield). Positive ion ESI-MS  $m/z = 468.1838$  [ $M + H$ ]<sup>+</sup> (calculated  $m/z = 468.1852$ ); [<sup>1</sup>H NMR (600 MHz, dimethylsulfoxide-*d*<sub>6</sub>)



**Fig. 1.** Initial velocities of hSULT2A1-catalyzed sulfation of DHEA and pregnenolone in the presence of 200  $\mu$ M PAPS. The  $K_m$  and  $V_{max}$  for DHEA were 1.7  $\mu$ M and 398 nmol/min per mg, respectively, and these values increase to 4.4  $\mu$ M and 1112 nmol/min per mg for pregnenolone sulfation. Data are mean  $\pm$  S.E. from triplicate determinations and were obtained using the same preparation of purified enzyme for both substrates.



**Fig. 2.** Inhibition of the hSULT2A1-catalyzed sulfation of (A) 1.0  $\mu$ M DHEA and (B) 0.4  $\mu$ M pregnenolone by major metabolites of tamoxifen. Sulfation rates of uninhibited controls for endoxifen, N-desTAM, 4-OHTAM, and TAM-NO were 87, 98, 97, and 111 nmol/min per mg, respectively, for DHEA sulfation, whereas the uninhibited rates for these metabolites were 63, 92, 68, and 90 nmol/min per mg, respectively, when determining the sulfation of pregnenolone. Data are mean  $\pm$  S.E. from triplicate determinations.

TABLE 1

Inhibition of hSULT2A1-catalyzed sulfation of DHEA by metabolites of tamoxifen

The sulfation of DHEA was determined using 0.03  $\mu\text{g}$  of purified hSULT2A1 in the presence of varied concentrations of inhibitor and either 1  $\mu\text{M}$  DHEA (for  $\text{IC}_{50}$  values) or 0.22–1.0  $\mu\text{M}$  DHEA for determination of the mechanism of inhibition and related kinetic constants. The data are expressed as mean  $\pm$  S.E. from three independent experiments. Calculation of  $k_{\text{cat}}$  values was based on 33,678 as the subunit molecular mass of hSULT2A1.

Metabolite	$\text{IC}_{50}$	Type of Inhibition	$V_{\text{max}}$	$K_{\text{m}}$	$k_{\text{cat}}/K_{\text{m}}$	$K_{\text{i}}$
			nmol/min per mg	$\mu\text{M}$	$\text{min}^{-1}\mu\text{M}^{-1}$	$\mu\text{M}$
Endoxifen	1.7 $\pm$ 0.4	Noncompetitive	243 $\pm$ 17	0.7 $\pm$ 0.1	10.9	2.8 $\pm$ 0.2
N-desTAM	8.3 $\pm$ 2.6	Competitive	260 $\pm$ 24	0.8 $\pm$ 0.1	11.0	17.2 $\pm$ 2.0
4-OHTAM	10.0 $\pm$ 1.0	Noncompetitive	178 $\pm$ 7	0.5 $\pm$ 0.1	11.8	19.4 $\pm$ 1.6
TAM-NO	11.1 $\pm$ 1.1	Noncompetitive	592 $\pm$ 75	3.5 $\pm$ 0.5	5.7	9.6 $\pm$ 0.2

revealed an isomeric mixture of 4-TAM-SO<sub>4</sub>. [Major isomer of 4-TAM-SO<sub>4</sub>-ppm ( $\delta$ ) 0.86 (t, 3H, CH<sub>2</sub>CH<sub>3</sub>); 2.40 (q, 2H, CH<sub>2</sub>CH<sub>3</sub>); 2.69 [s, 6H, N(CH<sub>3</sub>)<sub>2</sub>]; 3.26 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 4.11 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 6.66 (d, 2H, Ph); 6.78 (d, 2H, Ph); 7.08–7.26 (m, Ph).] [Minor isomer of 4-TAM-SO<sub>4</sub>-ppm ( $\delta$ ) 0.86 (t, 3H, CH<sub>2</sub>CH<sub>3</sub>); 2.40 (q, 2H, CH<sub>2</sub>CH<sub>3</sub>); 2.75 [s, 6H, N(CH<sub>3</sub>)<sub>2</sub>]; 3.36 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 4.28 (t, 2H, OCH<sub>2</sub>CH<sub>2</sub>N); 6.71 (d, 2H, Ph); 6.82 (d, 2H, Ph); 7.00 (d, 2H, Ph); 7.08–7.26 (m, Ph).] Melting point: 270–275°C

## Results

**Metabolites of Tamoxifen as Inhibitors of the Sulfation of DHEA and Pregnenolone Catalyzed by hSULT2A1.** Tamoxifen metabolites were investigated as inhibitors of hSULT2A1 using DHEA and pregnenolone as substrates. The sulfation of either DHEA or pregnenolone was initially examined using a concentration range between 0.2 and 20.0  $\mu\text{M}$  for DHEA, and 0.5 and 22  $\mu\text{M}$  for pregnenolone to determine the substrate concentrations where minimal substrate inhibition occurred with each (Fig. 1). Under the assay conditions described, we determined that endoxifen, 4-OHTAM, N-desTAM, and TAM-NO were all inhibitors of DHEA sulfation (Fig. 2A). Tamoxifen did not exhibit significant inhibition of hSULT2A1 up to the limits of its solubility in the assay (data not shown). Endoxifen, 4-OHTAM, and TAM-NO displayed greater than 95% inhibition of the enzyme within their solubility limits, whereas N-desTAM reached only approximately 70% inhibition at its limit of solubility. As seen in Table 1, the calculated  $\text{IC}_{50}$  (half-maximal inhibitory concentration) values ranged from 1.7 to 11.1  $\mu\text{M}$  for the inhibition of the sulfation of 1.0  $\mu\text{M}$  DHEA, with endoxifen being the most potent inhibitor. The kinetic mechanism of inhibition, inhibitor dissociation constant ( $K_{\text{i}}$ ), catalytic efficiency constant ( $k_{\text{cat}}/K_{\text{m}}$ ), Michaelis-Menten constant ( $K_{\text{m}}$ ), and maximal velocity ( $V_{\text{max}}$ ) for inhibitors of the hSULT2A1-catalyzed sulfation of DHEA are shown in Table 1, with initial velocity data in Supplemental Fig. 1. Endoxifen, 4-OHTAM, and TAM-NO were noncompetitive inhibitors with  $K_{\text{i}}$  values of 2.8, 19.4, and 9.6  $\mu\text{M}$ ,

respectively, whereas N-desTAM was a competitive inhibitor of DHEA sulfation with a  $K_{\text{i}}$  value of 17.2  $\mu\text{M}$ .

After our findings with DHEA, we used pregnenolone to determine whether the inhibition observed was affected by the substrate used. We found that endoxifen, 4-OHTAM, N-desTAM, TAM-NO were inhibitors of pregnenolone sulfation, and the inhibition by each metabolite was similar to our results with DHEA (Fig. 2A, 2B). The calculated  $\text{IC}_{50}$  values ranged from 2.7 to 16.7  $\mu\text{M}$  for the inhibition of 0.4  $\mu\text{M}$  pregnenolone, with endoxifen being the most potent inhibitor. As with DHEA as substrate, we determined the kinetic mechanism of inhibition and the kinetic parameters  $V_{\text{max}}$ ,  $K_{\text{m}}$ ,  $K_{\text{i}}$ , and  $k_{\text{cat}}/K_{\text{m}}$  for inhibitors of the hSULT2A1-catalyzed sulfation of pregnenolone. We report these values in Table 2, with initial velocity data provided in Supplemental Fig. 2. Endoxifen, 4-OHTAM, and TAM-NO were either mixed or noncompetitive inhibitors with  $K_{\text{i}}$  values of 3.5, 12.7, and 16.9  $\mu\text{M}$ , respectively, whereas the effect of N-desTAM was best described as a competitive inhibitor of pregnenolone sulfation with a  $K_{\text{i}}$  value of 9.8  $\mu\text{M}$ .

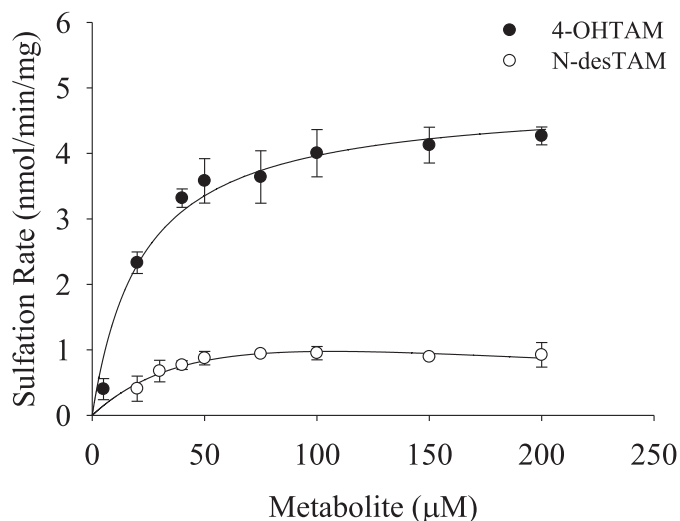
**Characterization of 4-OHTAM, N-desTAM, and Endoxifen as Substrates for hSULT2A1.** Previous studies have shown that hSULT2A1 is capable of catalyzing the sulfation of 4-OHTAM (Falany et al., 2006). Because the *N*-sulfoconjugation of aliphatic secondary amines is catalyzed by this enzyme (Senggunprai et al., 2009), we examined the ability of hSULT2A1 to catalyze the sulfation of N-desTAM. Moreover, because endoxifen has both a phenolic group in the 4-position as well as the secondary aliphatic amine, we also characterized the kinetics of sulfation of endoxifen. Our results showed that 4-OHTAM and N-desTAM were substrates for the enzyme (Fig. 3). The kinetics of 4-OHTAM sulfation was best described using a Michaelis-Menten equation (i.e., no substrate inhibition was evident) whereas the data for the sulfation of N-desTAM was best described using a substrate inhibition model (Fig. 3). The  $K_{\text{m}}$ ,  $V_{\text{max}}$ , and  $k_{\text{cat}}/K_{\text{m}}$  values for the sulfation of 4-OHTAM were determined to be 22.3  $\pm$  3.4  $\mu\text{M}$ , 4.9  $\pm$  0.1 nmol/min per mg, and 0.015  $\text{min}^{-1}\mu\text{M}^{-1}$ , respectively. The  $K_{\text{m}}$ ,  $K_{\text{i}}$ ,  $V_{\text{max}}$ , and  $k_{\text{cat}}/K_{\text{m}}$  values for N-desTAM sulfation were

TABLE 2

Inhibition of hSULT2A1-catalyzed sulfation of pregnenolone by metabolites of tamoxifen

The sulfation of pregnenolone was determined using 0.03  $\mu\text{g}$  of purified hSULT2A1 in the presence of varied concentrations of inhibitor and either 0.4  $\mu\text{M}$  pregnenolone (for  $\text{IC}_{50}$  values) or 0.2–1.0  $\mu\text{M}$  DHEA for determination of the mechanism of inhibition and related kinetic constants. The data are expressed as mean  $\pm$  S.E. from three independent experiments. Calculation of  $k_{\text{cat}}$  values was based on 33,678 as the subunit molecular mass of hSULT2A1.

Metabolite	$\text{IC}_{50}$	Type of Inhibition	$V_{\text{max}}$	$K_{\text{m}}$	$k_{\text{cat}}/K_{\text{m}}$	$K_{\text{i}}$
			nmol/min per mg	$\mu\text{M}$	$\text{min}^{-1}\mu\text{M}^{-1}$	$\mu\text{M}$
Endoxifen	2.7 $\pm$ 1.1	Mixed	332 $\pm$ 54	1.2 $\pm$ 0.3	9.3	3.5 $\pm$ 0.7
N-desTAM	15.0 $\pm$ 1.2	Competitive	630 $\pm$ 127	2.1 $\pm$ 0.5	10.0	9.8 $\pm$ 1.2
4-OHTAM	16.7 $\pm$ 1.1	Mixed	522 $\pm$ 103	2.0 $\pm$ 0.5	8.5	12.7 $\pm$ 2.1
TAM-NO	16.1 $\pm$ 1.1	Noncompetitive	647 $\pm$ 121	2.3 $\pm$ 0.5	9.5	16.9 $\pm$ 0.6



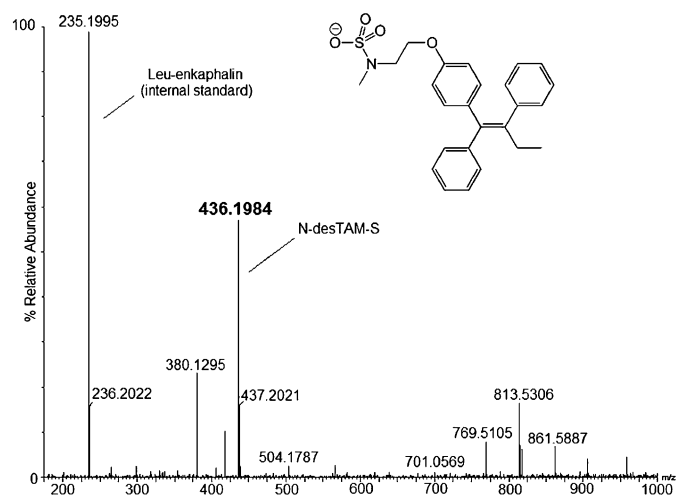
**Fig. 3.** Sulfation of 4-OHTAM and N-desTAM catalyzed by hSULT2A1. Data are mean  $\pm$  S.E. from triplicate determinations. Curves represent fit of the data to a simple Michaelis-Menten equation (for 4-OHTAM) and to an equation describing uncompetitive substrate inhibition (for N-desTAM).

determined to be  $72.7 \pm 49.8 \mu\text{M}$ ,  $156 \pm 135 \mu\text{M}$ ,  $2.3 \pm 4.4 \text{ nmol/min per mg}$ , and  $0.0024 \text{ min}^{-1} \mu\text{M}^{-1}$ , respectively. Thus, the enzyme displayed higher catalytic activity with 4-OHTAM than with N-desTAM as seen by the 6-fold higher  $k_{\text{cat}}/K_m$ .

Because the sulfamate of N-desTAM (i.e., N-desTAM-S) has not been previously reported as a metabolite, we synthesized it as a standard for analysis of N-desTAM-S formed in enzymatic reactions. The synthetic procedure for N-desTAM is outlined in Supplemental Fig. 3. We synthesized 4-hydroxytamoxifen sulfate (4-TAM-SO<sub>4</sub>) from (Z)-4-OHTAM, and the synthetic scheme is outlined in Supplemental Fig. 4. The proton NMR spectrum of 4-TAM-SO<sub>4</sub> revealed evidence of both (Z) and (E) isomers of 4-TAM-SO<sub>4</sub>, and this was observed even though the isomeric purity of (Z)-4-OHTAM was validated by NMR before synthesizing the sulfuric acid ester of 4-OHTAM. The enzymatic reactions were analyzed by LC-MS, and the negative ion ESI-MS of the product formed by the hSULT2A1-catalyzed sulfation of N-desTAM is seen in Fig. 4.

Endoxifen-sulfate was also identified as a product of sulfation catalyzed by hSULT2A1. Analysis of the enzyme-catalyzed reaction revealed a product  $m/z$  of 452.1930 by negative ion ESI-MS (Fig. 5). Endoxifen has two potential sites for sulfation, but we were able to determine that sulfation had occurred at the phenolic hydroxyl group due to the single charge of the parent mass when analyzed in negative ion mode. Sulfation of the aliphatic amino group of endoxifen would result in a doubly charged species with an approximate  $m/z$  of 226, and we saw no evidence of this ion in ESI-MS analysis of our samples. The product of 4-OHTAM sulfation (4-TAM-SO<sub>4</sub>) is shown in Supplemental Fig. 5 with an  $m/z$  of 466.2050, as determined by negative ion ESI-MS. The retention times of 4-TAM-SO<sub>4</sub>, N-desTAM-S, and endoxifen-sulfate from the LC (not shown) were 16.26, 22.38, and 16.05 minutes, respectively.

**N-desTAM-S and 4-TAM-SO<sub>4</sub> as Inhibitors of DHEA Sulfation Catalyzed by hSULT2A1.** As seen in Fig. 6, N-desTAM-S was a potent inhibitor of DHEA sulfation. The calculated values for IC<sub>50</sub> and K<sub>i</sub> for inhibition of the enzyme were 7.7 and 4.8  $\mu\text{M}$ , respectively. We determined 4-TAM-SO<sub>4</sub> to be a very weak inhibitor of the enzyme, with an IC<sub>50</sub> value greater than 70  $\mu\text{M}$  when examined with 1.0  $\mu\text{M}$  DHEA as substrate (data not shown).



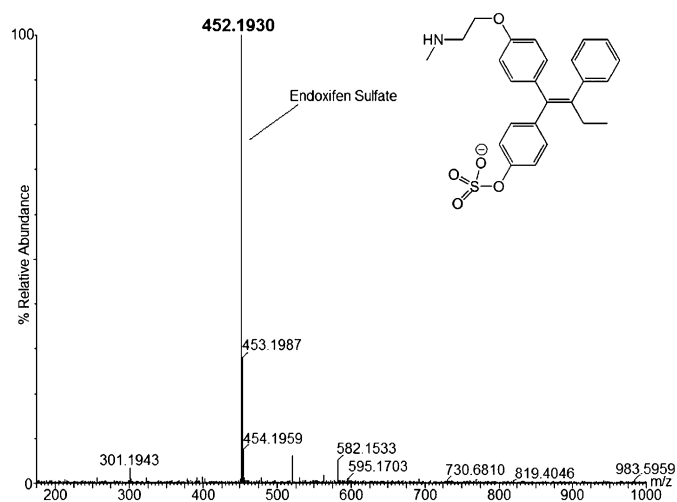
**Fig. 4.** LC-MS analysis of N-desTAM-S formed in a reaction catalyzed by hSULT2A1.

## Discussion

The full benefits of tamoxifen as a breast cancer therapeutic agent are compromised by its low incidence of endometrial cancer (Fornander et al., 1993; Kedar et al., 1994), and the formation of genotoxic tamoxifen-DNA adducts has been proposed to be a key step in this carcinogenic response (Shibutani et al., 1999). A major mechanism for this genotoxicity begins with the cytochrome P450-mediated oxidation of tamoxifen to  $\alpha$ -hydroxytamoxifen ( $\alpha$ -OHTAM), which then undergoes sulfation catalyzed by hSULT2A1 to form an electrophilic  $\alpha$ -sulfoxy intermediate that reacts with DNA to form covalent adducts (Shibutani et al., 1998a). Other mechanisms, such as those involving ortho-quinone and quinone-methide derivatives of 4-OHTAM, have also been explored as potentially responsible for toxicities seen with tamoxifen (Dowers et al., 2006).

An involvement of this hSULT2A1-mediated mechanism for the initiation of endometrial cancer by tamoxifen is consistent with several findings. First, there is evidence of hSULT2A1 mRNA expression in endometrial tissue (Singh et al., 2008), and immunohistochemical localization of the enzyme in specific cell-types within human endometrium has been reported (Andersson et al., 2010). Furthermore, in vitro studies have shown the ability of the enzyme to catalyze formation of  $\alpha$ -tamoxifen sulfate, with subsequent DNA-adduct formation (Shibutani et al., 1998b).

The role of hSULT2A1 in TAM-induced endometrial cancer, however, remains controversial. For example, one study failed to detect catalytic activities of hSULT2A1 in cytosolic preparations from human endometrium (Rubin et al., 1999). There is also a controversy with respect to the formation of tamoxifen-DNA adducts. Although several studies have found tamoxifen-DNA adducts in endometrial tissue from women treated with tamoxifen (Hemminki et al., 1996; Shibutani et al., 2000; Hernandez-Ramon et al., 2014) or from incubation of tamoxifen with human endometrial explants (Andersson et al., 2010), there are other reports that such adducts were not detected in endometrium (Carmichael et al., 1996, 1999; Beland et al., 2004). As recently reviewed, there is clear evidence for involvement of estrogens in endometrial carcinogenesis (Rizner, 2013), and several metabolites of tamoxifen have been shown to have estrogen agonist properties (Jordan and Gosden, 1982; Jordan, 2007). Thus, it has been hypothesized that the combination of genotoxicity due to formation of tamoxifen-DNA adducts, deficiencies in DNA repair, and estrogenic effects all combine to contribute to the risk of endometrial cancer after treatment with tamoxifen (Hernandez-Ramon et al., 2014).

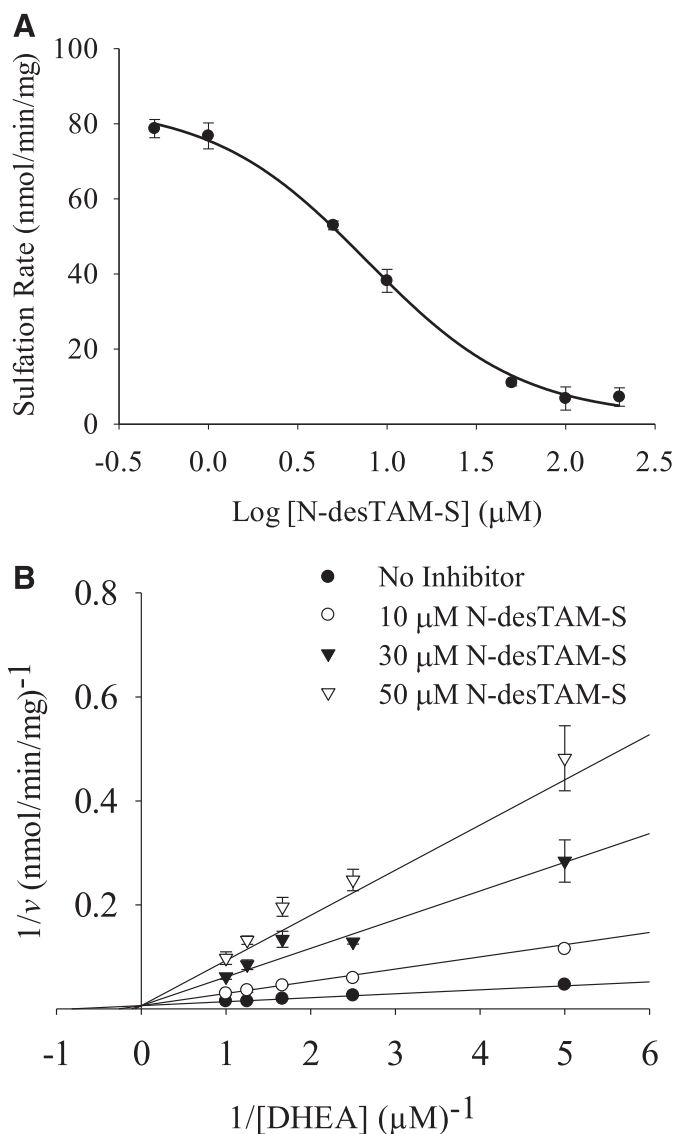


**Fig. 5.** LC-MS analysis of endoxifen-sulfate formed in a reaction catalyzed by hSULT2A1.

In addition to metabolic sulfation of tamoxifen metabolites and other xenobiotics, hSULT2A1 catalyzes the sulfation of DHEA to form DHEA-sulfate, one of the most abundant circulating steroid hormones in humans. DHEA-sulfate is hydrolyzed to the parent alcohol in peripheral tissues where it serves as a precursor for the biosynthesis of both estrogens and androgens. Thus, hSULT2A1 is important in maintaining the homeostasis of these steroid hormones, and inhibition by metabolites of tamoxifen might be expected to affect androgen or estrogen synthesis in a tissue-specific manner. Such alterations in cellular steroid hormones, either systemically or at the level of individual tissues, may have consequences that relate to either therapeutic or toxic effects of tamoxifen and its metabolites.

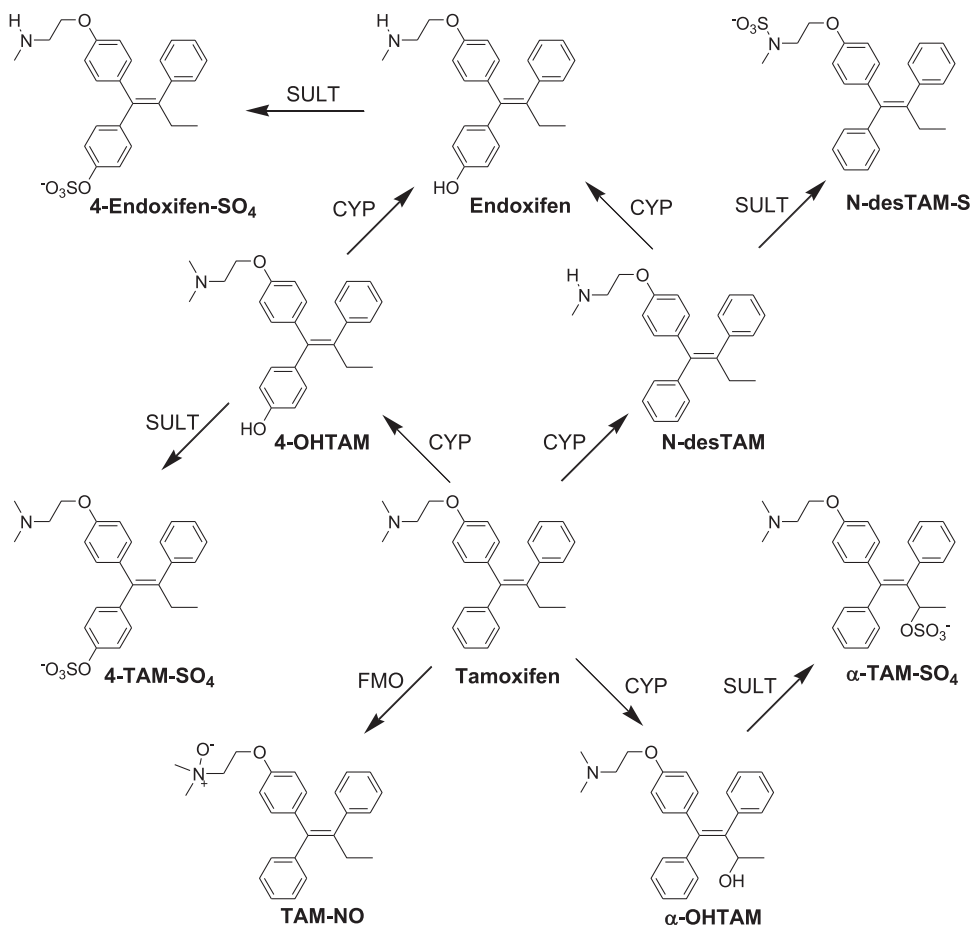
Given the complexity of the carcinogenic response to tamoxifen and the potential role(s) of hSULT2A1, we were interested in the determining the interactions of tamoxifen and its major metabolites with hSULT2A1. We hypothesized that the major metabolites of tamoxifen could inhibit the catalytic activity of hSULT2A1 and thus serve as potential modulators of the function of this enzyme in steroid hormone metabolism and in the genotoxicity of tamoxifen. Of the metabolites tested, endoxifen was the most potent inhibitor of the sulfation of 1.0  $\mu\text{M}$  DHEA and 0.4  $\mu\text{M}$  pregnenolone with  $\text{IC}_{50}$  values of 1.7 and 2.7  $\mu\text{M}$ , respectively (Tables 1 and 2). This was an interesting finding because the inhibition constants for interactions between the hSULT2A1 and endoxifen were of similar magnitude to the  $K_m$  values observed for the sulfation of DHEA and pregnenolone. The range of serum concentrations reported for DHEA and pregnenolone are between 5 and 24 nM, and 1 and 6 nM, respectively (Labrie et al., 1997), whereas the mean plasma concentrations of tamoxifen metabolites have been reported to be in the general range of 14–130 nM for endoxifen, 3–17 nM for 4-OHTAM, 15–24 nM for TAM-NO, and 280–800 nM for N-desTAM (Brauch et al., 2009). If the serum concentrations of DHEA, pregnenolone, and the tamoxifen metabolites are an indication of their concentrations in peripheral tissues, then the metabolites have a potential to alter the homeostasis of steroid hormones by inhibiting their sulfation catalyzed by hSULT2A1. These effects would be amplified if the intracellular concentrations of the metabolites were to exceed those of the hydroxysteroids, and some 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).

Because endoxifen, 4-OHTAM, N-desTAM, and TAM-NO effectively inhibited hSULT2A1, we expect that in some cellular environments where



**Fig. 6.** Inhibition of the hSULT2A1-catalyzed sulfation of (A) 1.0  $\mu\text{M}$  DHEA by N-desTAM-S with an  $\text{IC}_{50}$  value of  $7.7 \pm 1.2 \mu\text{M}$ , and (B) competitive inhibition by N-desTAM-S with values for  $K_i$ ,  $K_m$ , and  $V_{\text{max}}$  determined to be  $4.8 \pm 0.3 \mu\text{M}$ ,  $1.2 \pm 0.2 \mu\text{M}$ , and  $159 \pm 15 \text{ nmol/min per mg}$ , respectively. Data are mean  $\pm$  S.E. from triplicate determinations.

these metabolites are produced sufficient concentrations may be present to inhibit the hSULT2A1-catalyzed sulfation of  $\alpha$ -OHTAM. In fact, hSULT2A1 has a much lower catalytic efficiency with  $\alpha$ -OHTAM than with DHEA (Apak and Duffel, 2004), and  $\alpha$ -OHTAM is reported to have a mean plasma concentration of only 1 nM (Brauch et al., 2009). In the present study, endoxifen was an inhibitor of hSULT2A1 with  $K_i$  values of 2.8 and 3.5  $\mu\text{M}$ , respectively, for DHEA and pregnenolone as substrates. We previously determined a  $K_m$  value of  $136 \pm 7 \mu\text{M}$  for the sulfation of  $E$ -( $\pm$ )- $\alpha$ -OHTAM catalyzed by hSULT2A1 with a  $k_{\text{cat}}/K_m$  value of  $5.1 \pm 0.3$  (Apak and Duffel, 2004). Thus, it would be expected that endoxifen could inhibit the sulfation of  $\alpha$ -OHTAM under the appropriate in vivo conditions. Furthermore, we identified N-desTAM-S as a potent inhibitor of DHEA sulfation with a  $K_i$  value of 4.8  $\mu\text{M}$  (Fig. 6B), which was significantly lower than that seen for the parent metabolite. The combination of N-desTAM serving as a substrate (i.e., binding in a catalytically productive conformation at the active site) and the affinity of the enzyme for the product sulfamate suggest that N-desTAM may also contribute to inhibition of hSULT2A1.



**Fig. 7.** Summary of the roles of sulfotransferases in the metabolism of tamoxifen and endoxifen shown within the context of pathways for formation of those metabolites of tamoxifen examined in the current study. CYP, cytochrome P450 monooxygenase; 4-Endoxifen-SO<sub>4</sub>, endoxifen-4-sulfate; FMO, microsomal flavin-containing monooxygenase; α-TAM-SO<sub>4</sub>, α-tamoxifen sulfate.

The benefits of tamoxifen therapy depend on the *in vivo* formation of its active metabolites 4-OHTAM and endoxifen, which are derived from the CYP2D6-mediated oxidation of tamoxifen (Dehal and Kupfer, 1997) and N-desTAM (Brauch et al., 2009), respectively. Polymorphisms in CYP2D6 have been shown to result in lower plasma levels of endoxifen and to increase the risk of breast cancer mortality in tamoxifen-treated women (Lammers et al., 2010). To overcome the pharmacogenetic variability between tamoxifen users, endoxifen has been proposed as an independent therapeutic agent for the treatment of patients with estrogen receptor-positive breast tumors and hormone receptor-positive solid tumors (NCT01327781 and NCT01273168; ClinicalTrials.gov). Our current results suggest that an additional advantage of the direct use of endoxifen might be the lack of conversion to reactive intermediates analogous to α-tamoxifen sulfate through inhibition of the sulfation of any α-hydroxy metabolite that might be formed. It remains to be determined if this is a beneficial effect of the clinical use of endoxifen, but it also remains to be seen whether the observed inhibition of sulfation of endogenous steroid hormones by endoxifen has any effect on either therapeutics or toxicity.

It must be recognized, however, that the role of sulfation in the endometrium is broader than just those reactions where hSULT2A1 is involved. A brief summary of metabolic pathways involving sulfation of tamoxifen metabolites is seen in Fig. 7. Those metabolic reactions noted in Fig. 7 as being catalyzed by sulfotransferases (SULTs), as also with the reactions catalyzed by P450s, may represent involvement of one or more enzyme isoforms. Indeed, previous studies have indicated the presence of SULT1A1, SULT1A3, and SULT1E1 in normal human endometrium (Rubin et al., 1999). Interactions with

multiple SULTs may be important in regulating active levels of tamoxifen metabolites with either antagonist or agonist properties at estrogen receptors. Although the sulfation of 4-OHTAM is catalyzed by multiple isoforms of sulfotransferase (Falany et al., 2006), a full appreciation of the varied roles of sulfation in the metabolism of tamoxifen will require further studies on the specificities and inhibition of the individual SULTs as they relate to cellular concentrations of pharmacologically/toxicologically active metabolites.

In summary, we determined that 4-OHTAM, TAM-NO, N-desTAM, and endoxifen were inhibitors of the sulfation of DHEA and pregnenolone catalyzed by hSULT2A1. Endoxifen was the most potent inhibitor of the enzyme, which suggests that this metabolite may affect the roles of hSULT2A1 in steroid hormone metabolism and in a metabolic pathway for genotoxicity that involves this enzyme. N-desTAM was a substrate for hSULT2A1, and the product of this reaction, N-desTAM-S, displayed greater inhibition of the enzyme than its unconjugated precursor. Thus, endoxifen, N-desTAM, and N-desTAM-S might serve protective roles in some tissues as they may inhibit the sulfation of α-OHTAM. A more complete understanding of the interactions of tamoxifen metabolites with sulfotransferase-dependent pathways for steroid hormone metabolism and drug toxicities will await additional *in vitro* and *in vivo* studies.

#### Authorship Contributions

*Participated in research design:* Squirewell, Qin, Duffel.

*Conducted experiments:* Squirewell, Qin.

*Performed data analysis:* Squirewell, Duffel.

*Wrote or contributed to the writing of the manuscript:* Squirewell, Qin, Duffel.

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