Effects of Human *SULT2A1* Genetic Polymorphisms on the Sulfation of Tibolone

Ethan Miller[1,*], **Munaf H. Zalzala**[1,2,*], **Maryam S. Abunnaja**[1], **Katsuhisa Kurogi**[1,3], **Yoichi Sakakibara**[3], **Masahito Suiko**[3], and **Ming-Cheh Liu**[1,**]

[1]Department of Pharmacology, College of Pharmacy and Pharmaceutical Sciences, University of Toledo Health Science Campus, Toledo, OH 43614 USA

[2]Department of Pharmacology and Toxicology, College of Pharmacy, University of Baghdad, Baghdad, Iraq

[3]Department of Biochemistry and Applied Biosciences, University of Miyazaki, Miyazaki 889-2192 Japan

Abstract

Background and Objectives—Previous studies have demonstrated the metabolism of tibolone through sulfation, with the cytosolic sulfotransferase (SULT) SULT2A1 as the major responsible enzyme. The current study aimed to investigate how *SULT2A1* genetic polymorphisms may affect the dehydroepiandrosterone (DHEA)- and tibolone-sulfating activity of SULT2A1.

Methods—Site-directed mutagenesis was employed to generate cDNAs encoding ten different SULT2A1 allozymes. Recombinant SULT2A1 allozymes were expressed in BL21 *E. coli* cells, and purified using glutathionine-Sepharose affinity chromatography. An established sulfotransferase assay was used to analyze DHEA- and tibolone-sulfating activity of the purified SULT2A1 allozymes.

Results—The nine human SULT2A1 allozymes plus the wild-type SULT2A1 were found to display differential sulfating activity toward DHEA and tibolone. Kinetic analysis revealed that different SULT2A1 allozymes exhibited differential substrate affinity and catalytic efficiency toward the two substrates tested.

Conclusion—The results obtained provided useful information concerning the differential metabolism of tibolone through sulfation in individuals with different *SULT2A1* genotypes.

1. Introduction

Tibolone, a synthetic steroid hormone drug, is commonly used in hormone replacement therapy in postmenopausal women as well as for the treatment of endometriosis [1–8]. The

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[**]Corresponding Author: Ming-Cheh Liu, Ph.D., Department of Pharmacology, College of Pharmacy and Pharmaceutical Sciences, University of Toledo Health Science Campus, 3000 Arlington Avenue, Toledo, OH 43614 USA, Tel: (419) 383-1918, Fax: (419) 383-1909, ming.liu@utoledo.edu.
[*]These two authors made equal contributions to the work presented in this paper.

metabolism of tibolone in humans has been shown to involve both Phase I and Phase II metabolic reactions, with sulfation being the most important one in the latter category [9]. Using recombinant human cytosolic sulfotransferases (SULTs), it has been demonstrated that tibolone may be subjected to SULT-mediated sulfation, with SULT2A1 being the major responsible enzyme [10]. In addition to its therapeutic role, there are adverse effects associated with the use of tibolone, with studies showing correlations with breast, kidney, liver and vascular disorders [11]. Further investigation into the metabolism of tibolone, in particular sulfation, may provide insightful information concerning reasons underlying these health risks.

The cytosolic sulfotransferases (SULTs) are a major group of Phase II enzymes involved in the metabolism of key endogenous compounds such as catecholamine neurotransmitters and thyroid/steroid hormones, as well as a variety of xenobiotics including drugs [12–15]. SULT enzymes catalyze the transfer of the sulfonate group from the donor compound, 3′-phosphoadenosine 5′-phosphosulfate (PAPS), to the hydroxyl or amine group of substrate compounds [16]. The addition of the sulfonate group may inactivate and/or increase the water-solubility of the substrate compounds, thereby facilitate their excretion from the body [12–15]. In humans, there are thirteen SULTs that display distinct substrate specificity and/or tissue distribution [17]. As mentioned above, sulfation has been shown to be the most important Phase II reaction in the metabolism of tibolone *in vivo* [9], and that SULT2A1 has been identified as a major enzyme mediating the sulfation of tibolone [10]. While the major enzyme capable of sulfating tibolone has been identified, a lesser understood aspect is how genetic polymorphisms of the responsible enzyme, SULT2A1, may affect the differential metabolism of tibolone in different individuals. Like with many other genes, single nucleotide polymorphisms (SNPs) of SULT genes have been reported [15, 18, 19]. An earlier study sequencing DNA samples from African-American and Caucasian-American subjects revealed three non-synonymous coding SNPs (cSNPs) for the *SULT2A1* gene [20]. Interestingly, the corresponding allozymes expressed in COS-7 cells displayed differential sulfating activity toward dehydroepiandrosterone (DHEA), an endogenous substrate [12]. These findings, therefore, raised an interesting issue whether SULT2A1 genetic polymorphisms may have an impact on the metabolism of tibolone through sulfation, thereby influencing the efficacy of the drug in different individuals.

In this study, we report the generation, expression, and purification of ten SULT2A1 allozymes. The differential sulfating activities of the purified SULT2A1 allozymes toward tibolone and DHEA were examined and their kinetic constants determined.

2. Materials and Methods

2.1. Materials

Dehydroepiandrosterone (DHEA), tibolone, dithiothreitol (DTT), dimethylsulfoxide (DMSO), 3-(N-morpholino) propanesulfonic acid (MOPS), and isopropyl-1-thio-β-D-galactopyranoside (IPTG) were products of Sigma Chemical Company (St. Louis, MO USA). Carrier-free sodium [^{35}S]sulfate was a product of Perkin-Elmer (Waltham, MA USA). Ecolume scintillation cocktail was purchased from MP Biomedical (Solon, OH USA). Cellulose thin-layer chromatography (TLC) plates were from EMD chemicals

(Gibbstown, NJ USA). Recombinant human bifunctional ATP sulfurylase/adenosine 5′-phosphosulfate kinase was prepared as previously described [21]. EX *Taq* DNA polymerase was a product of Takara Bio (Mountain View, CA USA). Protein molecular weight markers were from New England Biolabs, Inc. (Ipswich, MA USA). Oligonucleotide primers were synthesized by MWG Biotech (Louisville, KY USA). All other reagents were of the highest grades commercially available.

2.2. Preparation of SULT2A1 allozymes

The QuikChange site-directed mutagenesis kit from Stratagene (La Jolla, CA USA) was used for the generation of cDNAs encoding SULT2A1 allozymes. Briefly, wild-type SULT2A1 cDNA packaged in pGEX-2TK prokaryotic expression vector was used as the template in conjunction with specific mutagenic primers (see Table 1 for the mutagenic primers used). The amplification conditions were 12 cycles of 30 s at 95°C, 1 min at 55°C, and 6 min at 68°C. The "mutated" SULT2A1 sequences were verified by nucleotide sequencing [22]. pGEX-2TK vector harboring individual mutated SULT2A1 sequence was transformed into competent XL1-Blue *E. coli* cells. The transformed cells, grown to $A_{600\ nm}$ = ~0.5 in 1 liter of Luria broth (LB) medium supplemented with 100 μg/ml ampicillin and induced with 0.1 mM IPTG overnight at room temperature, were collected by centrifugation and homogenized in 20 ml of an ice-cold lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mm NaCl, and 1 mM EDTA) using an Aminco French press. The crude homogenate thus prepared was subjected to centrifugation at $10,000 \times g$ for 30 min at 4°C. The supernatant collected was fractionated using 0.5 ml of glutathione-Sepharose, and the bound fusion protein was treated with 2 ml of a thrombin digestion buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 2.5 mM $CaCl_2$) containing 5 units/ml bovine thrombin. Following a 1-h incubation at room temperature with constant agitation, the preparation was subjected to centrifugation. The recombinant enzyme present in the supernatant collected was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and subjected to the enzymatic characterization as described below.

2.3. Enzymatic assay

The sulfating activity of the recombinant human SULT2A1 allozymes was determined using PAP[^{35}S] as the sulfonate donor. The reaction mixture for the standard enzymatic assay, prepared in a final volume of 20 μl, contained, 50 mM MOPS at pH 7.0, 14 μM of PAP[^{35}S], 1 mM DTT, and 10 μM substrate (DHEA or tibolone). Controls with water or DMSO replacing substrate were also included. The reaction was started by the addition of the enzyme, allowed to continue at 37°C for 10 minutes, and terminated by placing the thin-walled tube containing the reaction mixture on a heating block at 100°C for 2 minute. Heated reaction mixture was subjected to centrifugation to pellet down the precipitates formed. Afterwards, 2 μl of the reaction mixture was spotted on a cellulose TLC plate and the spotted TLC plate was subjected to TLC analysis using a solvent system containing n-butanol, isopropanol, 88% formic acid and H_2O in a ratio of 3:1:1:1 (by volume) [23]. Upon completion of TLC, the TLC plate was air-dried and autoradiographed by using an X-ray film. The radioactive spot corresponding to the sulfated product was located and cut out and eluted in 0.5 ml water in a glass vial. 4.5 ml of Ecolume scintillation liquid was added to each vial, mixed thoroughly, and the radioactivity therein was counted by using a liquid

scintillation counter. The cpm count obtained was used to calculate the specific activity in the unit of nmol of sulfated product/minute/mg enzyme. For the kinetic studies on the sulfation of DHEA and tibolone, different concentrations (1 μM, 2 μM, 5 μM, 10 μM, and 20 μM) of these substrate compounds and 50 mM MOPS buffer at pH 7.0 were used. The specific activity data were analyzed based on the Michaelis-Menten equation to calculate the kinetic constants. GraphPad Prisim6 software was used in data analysis.

2.4. Miscellaneous methods

The sulfonate donor, PAP [^{35}S], was synthesized from ATP and carrier-free [^{35}S]sulfate using the bifunctional human ATP sulfurylase/APS kinase [21]. The synthesized PAP[^{35}S] was adjusted to the desired concentration and specific activity by the addition of nonradioactive (cold) PAPS. SDS-PAGE was performed on a 12% polyacrylamide gel using the method of Laemmli [24]. Protein determination was based on the method of Bradford [25] with bovine serum albumin as a standard.

3. Results

3.1. Preparation of human SULT2A1 allozymes

Based on the procedure described in the "Materials and Methods" section, cDNAs encoding different SULT2A1 allozymes packaged in pGEX-2TK prokaryotic expression vector were individually transformed into BL 21 *E. coli* host cells for expressing the recombinant enzymes. As shown in Figure 1, the recombinant SULT2A1 allozymes fractionated from the homogenates of transformed *E. coli* cells using glutathione-Sepharose and cleaved off the bound fusion proteins by thrombin digestion appeared to be highly homogeneous upon SDS-polyacrylamide gel electrophoresis, with apparent molecular weights similar to predicted molecular weight (33,780) of SULT2A1.

3.2. Sulfation of DHEA and tibolone by human SULT2A1 allozymes

Purified SULT2A1 allozymes were assayed for sulfating activity using DHEA and tibolone as substrates. As shown in Figure 2A, the ten allozymes exhibited specific activities ranging 31.64 – 47.08 nmol/min/mg with DHEA as the substrate. With tibolone as the substrate, the allozymes exhibited specific activities ranging 0.98 – 2.25 nmol/min/mg. It is noted that both substrates were tested at a concentration of 10 μM. While the variation in DHEA-sulfating activity among the ten SULT2A1 allozymes was not as dramatic as that previously reported [20], the differences are significant and may have functional implications *in vivo*. The variation in tibolone-sulfating activity, on the other hand, was considerably bigger (close to 2.3 fold) among the ten SULT2A1 allozymes (Figure 2B).

3.3. Kinetics of the sulfation of DHEA and tibolone by human SULT2A1 allozymes

Enzymatic assays were carried out using varying concentrations of DHEA or tibolone. Data obtained were used to generate Lineweaver-Burke double-reciprocal plots in order to calculate the K_m, V_{max}, and V_{max}/K_m for each of the ten SULT2A1 allozymes in catalyzing the sulfation of DHEA or tibolone. Figure 3 shows the Lineweaver-Burke double reciprocal plots of the sulfation of, respectively, DHEA and tibolone by the wild-type SULT2A1 (SULT2A1*1). The values of K_m, V_{max}, and V_{max}/K_m determined for the ten SULT2A1

allozymes are compiled in Table 2. With DHEA as the substrate, the K_m values determined for the ten SULT2A1 allozymes spanned a range of 8.71 – 11.91 μM, while the V_{max} values spanned a range of 61.41 – 93.64 nmol/min/mg enzyme. Calculated V_{max}/K_m determined for the ten allozymes varied in the range of 6.39 – 8.46, which in general correlate reasonably well with the specific activity data shown in **2A**. With tibolone as the substrate, the K_m values determined for the ten SULT2A1 allozymes spanned a range of 8.69 – 15.98 μM, while the V_{max} values spanned a range of 2.33 – 5.16 nmol/min/mg enzyme. Calculated V_{max}/K_m determined for the ten allozymes varied in the range of 0.18 – 0.47, which also correlate reasonably well with the specific activity data shown in Figure 2B.

4. Discussion

The current study was designed to clarify how genetic polymorphisms may impact the sulfation of tibolone in different individuals with different *SULT2A1* genotypes. Evidence indicating individual variation in the enzymatic activity of SULT2A1, previously named the DHEA sulfotransferase, was first reported in 1993 [26]. Of the 94 human hepatic tissue samples analyzed, a 4.6-fold variation in DHEA-sulfating activity was observed. Moreover, the frequency distribution of DHEA-sulfating activity among the samples tested appeared to be bimodal, with approximately 25% in a high activity subgroup [26]. These findings provided the first indication of underlying genetic variations that may influence the sulfating activity of DHEA. In a later study sequencing DNA samples from 120 human subjects, three non-synonymous coding SNPs for the *SULT2A1* gene were detected [20]. The corresponding SULT2A1 allozymes, expressed in COS-1 cells, displayed differential DHEA-sulfating activity. These data showed clearly a genotype-phenotype relationship for SULT2A1. It is thus important to clarify further how genetic polymorphisms of SULT2A1 may influence the metabolism of not only the endogenous compounds such as DHEA, but also drugs including tibolone, through sulfation. By searching the NCBI databases, we have identified ten non-synonymous coding SNPs for *SULT2A1* (cf. Table 1). To find out how different amino acid changes may affect the sulfating activity of the SULT2A1 protein products, our approach was to prepare recombinant SULT2A1 allozymes for enzymatic characterization with DHEA and tibolone as substrates.

Previous studies have revealed DHEA as a major endogenous substrate for SULT2A1 [27]. For tibolone, SULT2A1 was reported to be the major enzyme responsible for its sulfation [9]. In view of the genetic polymorphisms of SULT2A1, it is an interesting question whether different SULT2A1 allozymes may display differential sulfating activities toward not only the endogenous DHEA, but also drugs such as tibolone. Data from the enzymatic assays showed indeed significant differences in both DHEA- and tibolone-sulfating activities among the ten SULT2A1 allozymes analyzed. To examine further the effects of genetic polymorphisms on the sulfation of DHEA and tibolone by SULT2A1, kinetic experiments were performed on each of the ten SULT2A1 allozymes. Kinetic constants (K_m, V_{max}, and V_{max}/K_m) obtained indicated that the ten SULT2A1 allozymes displayed differential affinities for DHEA or tibolone, as well as intrinsic catalytic activities toward the two substrates. Collectively, the results derived from the above-mentioned studies provide useful information concerning the enzymatic mechanism underlying the differential sulfation of DHEA and tibolone by different SULT2A1 allozymes. It thus appears that *SULT2A1*

polymorphisms may have implications in not only the metabolism of key endogenous compounds such as DHEA, but also drugs like tibolone that may be metabolized through sulfation by SULT2A1.

5. Conclusions

The current study was designed to clarify how *SULT2A1* genetic polymorphisms may affect the DHEA- and tibolone-sulfating activities of SULT2A1 allozymes. Results obtained showed indeed differential DHEA- and tibolone-sulfating activities among the ten SULT2A1 allozymes analyzed. Kinetic analysis revealed further that different SULT2A1 allozymes exhibited differential substrate affinity and catalytic efficiency toward the two substrates tested. Additional studies are warranted in order to clarify how *SULT2A1* polymorphisms may impact on the expression and stability of SULT2A1 allozymes and thus the metabolism and pharmacokinetics of tibolone *in vivo*.

Acknowledgments

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Key Points

- Human SULT2A1 allozymes displayed differential sulfating activities toward DHEA and tibolone.

- Kinetic analysis revealed that different SULT2A1 allozymes exhibited differential substrate affinity and catalytic efficiency toward DHEA and tibolone.
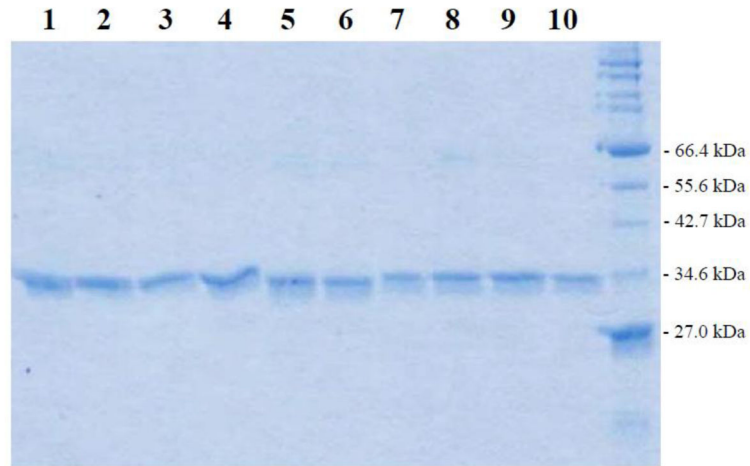
Figure 1.
SDS gel electrophoretic pattern of the purified human SULT2A1 allozymes. SDS-PAGE was performed on a 12% gel, followed by Coomassie blue staining. Samples analyzed in lanes 1 through 1 correspond to SULT2A1 allozymes 1 through 10. Positions of protein molecular weight markers co-electrophoresed are marked on the right.
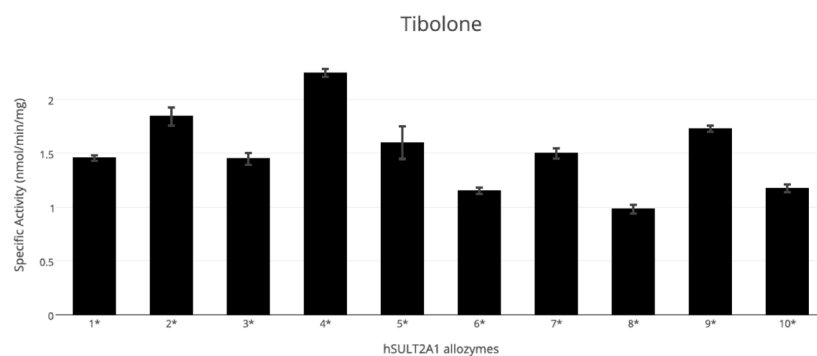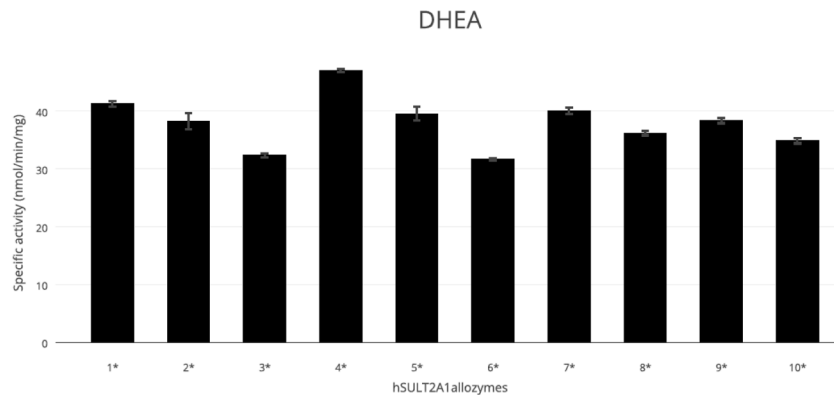
(A)

(B)

Figure 2.
Specific activities of the sulfation of (A) DHEA and (B) tibolone by human SULT2A1 allozymes. Concentration of DHEA or tibolone used in the enzymatic assays was 10 μM. Data shown represent mean ± standard deviation derived from four independent experiments.
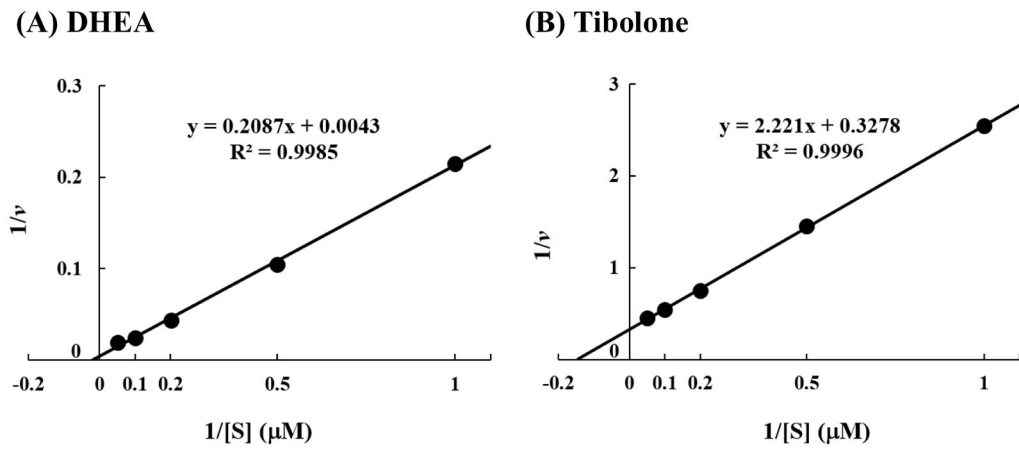
(A) DHEA

(B) Tibolone

Figure 3.
Lineweaver-Burke double-reciprocal plot of the sulfation of (A) DHEA and (B) tibolone by wild-type human SULT2A1 (SULT2A1*1).

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Table 1

Primer sets used in the site-directed mutagenesis of the cDNA encoding human human SULT2A1

SULT2A1 Allozyme	Amino Acid Substitution	Mutagenic Primer Set
SULT2A1*2	Met57Thr	5′-gagattctctgcctgacgcactccaggggggat-3′ 5′-atccccttggagtgcgtcaggcagagaatctc-3′
SULT2A1*3	Thr90Ser	5′-acagcactcagtgaatcggagagtccacgttta-3′ 5′-taaacgtggactctccgattcactgagtgctgt-3′
SULT2A1*4	Leu159Val	5′-tgtcaaggaaccgtggtatatggtcatggttt-3′ 5′-aaacatgacccatataccacagttccttgaca-3′
SULT2A1*5	Glu186Val	5′-ttactgagttatgaggtgctgaaacaggacaca 3′ 5′-tgtgtcctgtttcagcacctcataactcagtaa-3′
SULT2A1*6	Met57Thr/Glu186Val	*2 + *5
SULT2A1*7	Ala63Pro	5′-ccaaggggggatcccaagtggatc-3′ 5′-gatcacttgggatccccttgg-3′
SULT2A1*8	Lys227Glu	5′-tgaaagaaaacgagatgtccaat-3′ 5′-attggacatctcgttttctttca-3′
SULT2A1*9	Ala261Thr	5′-acttcacagtgacccaagctgaa-3′ 5′-ttcagcttgggtcactgtgaagt-3′
SULT2A1*10	Ala63Pro/Ala261Thr	*7 + *9

SULT, cytosolic sulfotransferase.

Table 2

Kinetic constants of the sulfation of DHEA and tibolone by human SULT2A1 allozymes

DHEA as Substrate			
	V_{max} (nmol/min/mg)	K_m (µM)	V_{max}/K_m
SULT2A1*1	83.2 ± 4.6	10.9 ± 1.3	7.63
SULT2A1*2	75.5 ± 4.6	9.96 ± 1.4	7.58
SULT2A1*3	76.8 ± 3.7	11.9 ± 1.2	6.45
SULT2A1*4	93.6 ± 6.7	11.1 ± 1.7	8.46
SULT2A1*5	84.6 ± 5.3	11.8 ± 1.5	7.14
SULT2A1*6	61.4 ± 3.6	9.6 ± 3.6	6.37
SULT2A1*7	81.6 ± 5.0	11.1 ± 1.4	7.38
SULT2A1*8	73.5 ± 3.4	10.8 ± 1.1	6.79
SULT2A1*9	73.3 ± 5.2	10.0 ± 1.5	7.34
SULT2A1*10	62.8 ± 5.3	8.7 ± 1.4	7.22
Tibolone as Substrate			
SULT2A1*1	3.6 ± 0.2	13.0 ± 1.7	0.28
SULT2A1*2	3.6 ± 0.1	8.7 ± 0.8	0.41
SULT2A1*3	2.8 ± 0.2	8.9 ± 1.4	0.32
SULT2A1*4	5.2 ± 0.3	10.9 ± 1.5	0.47
SULT2A1*5	3.4 ± 0.2	10.1 ± 1.4	0.34
SULT2A1*6	2.9 ± 0.2	14.3 ± 2.0	0.20
SULT2A1*7	3.7 ± 0.4	12.6 ± 2.3	0.29
SULT2A1*8	2.8 ± 0.3	16.0 ± 3.0	0.18
SULT2A1*9	3.8 ± 0.4	11.0 ± 2.2	0.34
SULT2A1*10	2.3 ± 0.2	8.9 ± 1.6	0.26

Data shown represent mean ± standard deviation calculated based on the results obtained from four independent kinetic experiments.

SULT, cytosolic sulfotransferase.