Potent Inhibition of Human Sulfotransferase 1A1 by 17 α -Ethinylestradiol: Role of 3'-Phosphoadenosine 5'-Phosphosulfate Binding and Structural Rearrangements in Regulating Inhibition and Activity^[S]

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ABSTRACT:

Sulfotransferase (SULT) 1A1 is the major drug/xenobiotic-conjugating SULT isoform in human liver because of its broad substrate reactivity and high expression level. SULT1A1 sulfates estrogens with low micromolar K_m values consistent with its affinity for sulfation of many small phenolic compounds. Binding studies showed the unexpected ability of 17 α -ethinylestradiol (EE2) to bind and inhibit SULT1A1 activity toward *p*-nitrophenol and β -naphthol at low nanomolar concentrations, whereas EE2 was not sulfated until significantly higher concentrations were reached. EE2 had a K_i of 10 nM for inhibiting *p*-nitrophenol and β -naphthol sulfation and inhibited 17 β -estradiol (E2) sulfation in intact human MCF-7 breast cancer cells with a K_i of 19 nM. In contrast, the K_m for EE2 sulfation by SULT1A1 was 700 nM. The K_d for EE2 binding of pure SULT1A1

was $0.5 \pm 0.15 \mu\text{M}$; however, the K_d for EE2 binding to the SULT1A1-PAP complex was >100 -fold lower ($4.3 \pm 1.7 \text{ nM}$). The K_d for E2 binding to SULT1A1 changed from 2.3 ± 0.9 to $1.2 \pm 0.56 \mu\text{M}$ in the presence of PAP. Docking studies with E2 indicate that E2 binds in a competent orientation in the resolved structure of SULT1A1 in the both presence and absence of 3'-phosphoadenosine 5'-phosphosulfate (PAPS). However, EE2 binds in a catalytically competent orientation in the absence of PAPS but in a noncompetent orientation via formation of a charge interaction with Tyr108 if PAPS is bound first. In conclusion, EE2 is a potent inhibitor, but not a substrate, of SULT1A1 at low nanomolar concentrations, indicating the possibility of drug-drug interactions during contraceptive therapy.

Introduction

17 α -Ethinylestradiol (EE2) is a major active component of many oral contraceptives and is extensively metabolized in human tissues. After ingestion, EE2 undergoes intestinal sulfation as well as hepatic oxidation, glucuronidation, and sulfation. The absorption, metabolism, and pharmacokinetic properties of EE2 have been well documented, and sulfation represents a major aspect of its first-pass metabolism. Because of the extensive usage of EE2 in women of child-bearing age and its extensive metabolism, several drug-drug interactions have been described. Many of these interactions involve the cytochromes P450 (P450), resulting in the inhibition or the induction of the P450s,

leading to increased EE2 metabolism and clearance (Barditch-Crovo et al., 1999; Wang et al., 2004; Zhang et al., 2007). Although EE2 is readily sulfated in humans, less is known concerning the interactions and inhibition of the sulfotransferase (SULT) isoforms by EE2 that may result in clinically relevant drug-drug interactions (Sinofsky and Pasquale, 1998; Christensen et al., 2007).

SULTs activate or inactivate compounds by transferring a sulfonate group from 3'-phosphoadenosine 5'-phosphosulfate (PAPS), the obligate sulfonate donor, to a hydroxyl or amine group (Falany, 1997). Conjugation with a charged sulfonate group generally renders the acceptor compound more water-soluble and results in increased excretion in either urine or bile. Sulfation has a major role in regulating the activity and metabolism of hydroxysteroids and estrogens in humans (Falany et al., 1995a, 2002; Raftogianis et al., 2000). The biological activities of 17 β -estradiol (E2) and EE2 are primarily regulated in tissues by sulfation catalyzed by SULT1E1 (Kotov et al., 1999; Raftogianis et al., 2000; Falany et al., 2002). The K_m for E2 and EE2 sulfation by SULT1E1 is approximately 4 nM, and activation of estrogen receptors is regulated by sulfation at low picomolar concentrations in endometrial cells (Zhang et al., 1998; Kotov et al., 1999).

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ABBREVIATIONS: EE2, 17 α -ethinylestradiol; P450, cytochrome P450; SULT, sulfotransferase; PAPS, 3'-phosphoadenosine, 5' phosphosulfate; E2, 17 β -estradiol; GI, gastrointestinal; PAP, 3',5'-phosphoadenosine; TLC, thin-layer chromatography; MBP, maltose binding protein; PNP, *p*-nitrophenol; PDB, Protein Data Bank; MOE, molecular operating environment; BFE, binding free energy; aa, amino acids.

Although SULT1E1 is the major isoform responsible for E2 and EE2 sulfation at physiological concentrations, several other SULT isoforms also conjugate these estrogens. It is well documented that the average serum level of EE2 in women taking oral contraceptives is 61 pM (Klehr-Bathmann and Kuhl, 1995) and the average serum level of E2 for premenopausal women ranges from 160 pM to 1.5 nM (Stricker et al., 2006). However, it is not known what the concentrations are at the tissue level for both hormones. E2 and EE2 are conjugated by SULT1A1, although the K_m for E2 sulfation is approximately 2.4 μ M, which is similar to its affinity for many small phenolic substrates (Falany et al., 1994). EE2 is conjugated by SULT1A1 with an affinity similar to that of E2 (Falany et al., 1994; Kotov et al., 1999). SULT1A1 is one of the major human drug/xenobiotic-metabolizing isoforms and is abundantly expressed in the liver and GI tract as well as being widely expressed at varying levels in many other tissues. One facet of the importance of SULT1A1 is its broad substrate reactivity, including small phenolic drugs and xenobiotics, estrogens, polyphenolic nutraceuticals, thyroid hormones, and *N*-hydroxyaromatic amine promutagens (Glatt et al., 2000; Nishiyama et al., 2002; Nakano et al., 2004; Pacifici, 2005). Understanding the mechanisms for regulating the activity or expression of SULT1A1 via drug-drug interactions would provide valuable insights into the role of sulfation in drug therapy and physiology.

The structure of EE2 is slightly different from that of E2. As well as possessing two hydroxyl groups, a D-ring 17 α -hydroxyl and an A-ring 3 β -hydroxyl group, EE2 has an azido group at the 17-position (Fig. 1). The SULT isoforms responsible for intestinal EE2 sulfation have not been well described; however, SULT1E1 and P450s have been reported to be the major metabolizers of EE2 in human liver (Schrage et al., 2004; Wang et al., 2004). With a relatively high K_m for EE2, SULT1A1 probably has only a small role in EE2 sulfation in liver; however, in tissues such as skin where SULT1A1 is highly expressed, it may have a greater role, especially in situations in which estrogens, including EE2, are applied topically. However, our understanding of the effects of EE2 on SULT activity have not included the possibility of potent inhibition of SULT1A1 in the absence of significant sulfation activity.

The current study focuses on the novel observation that EE2 was able to potently inhibit SULT1A1 at low nanomolar concentrations without being significantly conjugated, whereas it is a substrate for conjugation by SULT1A1 at significantly higher concentrations. Therefore, EE2 may have the unrecognized ability to inhibit SULT1A1 activity *in vivo* at relevant therapeutic concentrations with-

out significant evidence of sulfation. Defining a mechanism for the switch from inhibition to sulfation activity with increasing EE2 concentrations involved the analysis of the structural arrangements in the SULTs elicited by PAPS binding and the effects on EE2 binding. Cook et al. (2010) have recently described the effect of PAPS-elicited structural changes on the binding and substrate selectivity of SULT2A1; however, structural plasticity in the SULTs, including SULT1A1, is not well described partly because of the lack of high-quality resolved structures without PAPS or 5'-phosphoadenosine (PAP) bound (Allali-Hassani et al., 2007). Therefore, a combination of binding studies, kinetic analysis, and homology modeling was used to define a mechanism for the potent inhibition. The high inhibitory potency of EE2 for SULT1A1 suggests a new mechanism for inhibitory drug interactions involving sulfation in women taking EE2 as a contraceptive.

Materials and Methods

Materials. The Molecular Operating Environment license was purchased from Chemical Computing Group (Montréal, QC, Canada). [3 S]PAPS (2.2 Ci/mmol), [3 H]E2 (70.0 Ci/mmol), and [14 C] β -naphthol (4.7 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). PAPS was purchased from Dr. Sanford Singer at the University of Dayton (Dayton, OH). Silica gel thin-layer chromatography (TLC) plates (250 mm) were obtained from Whatman (Clifton, NJ). DEAE-Sepharose CL-6B was purchased from GE Healthcare (Chalfont St. Giles, Buckinghamshire, UK). E2 and EE2 were purchased from Sigma-Aldrich (St. Louis, MO). All other reagents were reagent grade and were purchased from Thermo Fisher Scientific (Waltham, MA).

Activity Assays of E2 and EE2 with SULT1A1. Chloroform extraction assays were used for the determination of E2 sulfation activity using [3 H]E2 as described previously (Falany et al., 1995b). To assay EE2 sulfation, nonradio-labeled EE2 was sulfated using [35 S]PAPS, and the [35 S]EE2 was resolved by TLC and quantified by scintillation counting as described previously (Meloche and Falany, 2001). E2 and EE2 sulfation by SULT1A1 was assayed with final concentrations of 20 and 6 μ M, respectively. Both assay procedures used 0.5 μ g of pure SULT1A1 protein and 10 μ M PAPS. To determine the K_m values of E2 and EE2 sulfation by SULT1A1, estrogen concentrations from 0.1 to 5 μ M were used. K_m and V_{max} were determined by nonlinear regression using the Michaelis-Menten equation (Bell and Bell, 1988).

Expression and Purification of SULT1A1. SULT1A1 was expressed in *Escherichia coli* with a maltose-binding protein (MBP) tag as described previously (Cook et al., 2009). The MBP tag was cleaved by factor Xa digestion to allow purification of the active native SULT1A1 enzyme. In brief, SULT1A1 with an amino-terminal MBP attached was expressed using the pMAL-c2 vector. The MBP moiety was attached so that factor Xa cleavage occurs immediately before the initial methionine in SULT1A1. The MBP-SULT1A1 fusion protein is purified by amylose affinity chromatography, the MBP is removed by factor Xa digestion, and the SULT1A1 is further purified by DEAE-Sepharose chromatography and passage through a second small amylose affinity column. Protein concentrations are estimated using the Bio-Rad protein assay with γ -globulin as a standard.

EE2 Inhibition of SULT1A1. To determine the K_i for EE2 inhibition of SULT1A1 activity, three different substrates [E2, *p*-nitrophenol (PNP), and β -naphthol] were used. The chloroform extraction assay was used with the radiolabeled substrates [3 H]E2 (5 μ M) and [14 C] β -naphthol (1.5 μ M). A barium precipitation assay was used for PNP sulfation assays with [35 S]PAPS (Falany et al., 1990). All reactions were 125 μ l and contained 0.2 μ g of pure SULT1A1 with EE2 concentrations ranging from 0 to 100 nM. At concentrations greater than 100 nM SULT1A1 began to sulfate EE2 and interfere with the determination of the inhibition constants. K_i values were determined using Michaelis-Menten noncompetitive and competitive inhibition kinetics (Bell and Bell, 1988).

Inhibition of SULT1A1 Activity in MCF-7 Cells with EE2. MCF-7 cells were cultured in Dulbecco's modified Eagle's medium with 7.5% fetal bovine serum, passed into 24-well plates, and allowed to grow to 70 to 80% confluence. The cells were then washed twice with PBS and incubated with 250 μ l

FIG. 1. Structures of 17 β -estradiol (E2) and 17 α -ethinylestradiol (EE2).

of serum-free medium containing 5 μM [^3H]E2 and 0, 5, 10, 20, or 50 nM EE2 for 2 h. Immediately after addition of the serum-free medium, a 50- μL aliquot of medium was removed to represent time 0 for each concentration of EE2. After a 2-h incubation, duplicate 100- μL aliquots of medium were removed for determination of [^3H]E2 sulfate formation by chloroform extraction and scintillation counting. After removal of media samples, the cells were washed several times with PBS and extracted with chloroform to determine the levels of [^3H]E2 sulfate retained in the cells.

K_d Determination for E2 and EE2 Binding to SULT1A1. The intrinsic fluorescence of pure SULT1A1 was measured at room temperature using excitation at 280 nm and emission of 340 nm with a PerkinElmer LS-5 fluorescence spectrophotometer. To determine K_d values, the SULT1A1 concentration was maintained below the anticipated K_d values. The SULT1A1 concentration was 0.1 μM for the E2 binding studies and 50 nM for EE2 binding to SULT1A1 in the absence of PAP. To determine EE2 binding to SULT1A1 with bound PAP, a 1 nM enzyme concentration was used. PAP was used in the substrate binding studies because PAPS would allow the sulfation reaction to occur. Increasing concentrations of substrates were added in 2- μL aliquots, mixed, and allowed to equilibrate for 3 min before the fluorescence determination. K_d values were calculated from double-reciprocal plots of fluorescence change versus substrate concentration for which K_d was determined from the x -intercept as $-1/K_d$.

Homology Modeling of SULT1A1. Recent studies have reported that PAPS binding to human SULT2A1 results in a structural rearrangement of the PAPS and substrate binding sites that alters substrate selectivity and reactivity (Cook et al., 2010). The human SULT1A1 structures in the RCSB Protein Data Bank were resolved with bound PAP (closed conformation) and either E2 (PDB 2D06) or *p*-nitrophenol (PDB 1LS6). There are no high-quality structures of SULT1A1 without PAP bound (open conformation). To generate an open model of SULT1A1, MODELLER (Eswar et al., 2007) was used to align the SULT1A1 and SULT2A1 sequences. The MODELLER program creates homology and comparative models by satisfying spatial restraints (Sali and Blundell, 1993) and can model de novo loops of protein structures. The backbone of SULT1A1 was threaded on the open structure of SULT2A1 (PDB 1J99) as a template, and the side chains were added. The system was energy-minimized using the AMBER99 energy field. The predicted models were then submitted to the National Institutes of Health SAVES (Structural Analysis Verification Server, <http://nihserver.mbi.ucla.edu/SAVES/>) for analysis with PROCHECK, VERIFY-3D, and ERRAT to check bond angles, atom distances, and dihedral properties for unallowable structural characteristics in the model. A passing model is considered a 90% or higher score.

Docking Studies of E2 and EE2 to SULT1A1. Molecular docking studies were performed using the integrated set of programs in MOE. The PDB files for the substrates were generated using LigX that can build three-dimensional structures in MOE (Labute, 2005). The PDB files for the two closed structures of SULT1A1 (2D06 and 1LS6) as well as the open model of SULT1A1 were used in the docking studies. The SULT1A1 structures, models, and substrates were protonated using Protonate 3D (Bower et al., 1997) assuming pH 7.0 and energy-minimized using the AMBER99 energy field. Then Triangle Matcher (Wildman and Crippen, 2001) was used to orient the ligands in the active site based on the charge groups and spatial fit. Triangle Matcher performs a random walk with the ligand in the active site to determine optimal binding orientations. The binding orientations are scored by calculation of binding free energy (BFE) using London dG (Wildman and Crippen, 2001) and Affinity dG (Feher and Schmidt, 2000), which are designed to function at physiological pH and salt concentrations. Binding orientations are then refined using FlexX that functions as a Monte Carlo program to generate 10^6 orientations for each initial placement established by Triangle Matcher that fixes a point around which to vary the orientation. The orientations are then scored by London dG and Affinity dG to define the lowest BFE conformation. The London dG scoring function favors hydrophobic interactions increasing the binding of the substrate to the active site, and the Affinity dG scoring function favors electrostatic interactions, which in turn underestimates the hydrophobic bonds in the active site. The program then displays an output of the 10 conformations with the lowest BFE. Acceptable BFEs are considered to have values less than -6.5 .

TABLE 1

Kinetic constants for E2 and EE2 sulfation determined with pure SULT1A1

Reactions were performed as described under *Materials and Methods* with four different concentrations of E2 or EE2 and PAPS as denoted under *Materials and Methods*.

	K_m		$V_{\max}$	$V_{\max}/K_m$
	Substrate	PAPS		
	μM			
E2	2.4 ± 0.9	0.36 ± 0.06	16 ± 2.1	6.7
EE2	0.69 ± 0.13	0.45 ± 0.12	11 ± 0.77	15.9

Results

E2 and EE2 Sulfation by SULT1A1. E2 has been shown to be a substrate for several human SULT isoforms, including SULT1E1 and SULT1A1 (Falany et al., 1994; Raftogianis et al., 2000). SULT1E1 sulfates E2 with a significantly lower K_m (4 nM) than SULT1A1 (2.4 μM); however, the K_m observed with SULT1A1 is typical of its affinity and activity with a variety of small neutral phenolic compounds including PNP, 1-naphthol, and 2-naphthol (Falany et al., 1994; Falany, 1997). We have previously reported that E2 sulfation by SULT1E1 shows a random bi-bi reaction mechanism (Zhang et al., 1998); however, the apparent mechanism for E2 sulfation by SULT1A1 has not been reported. The results of the kinetic analysis of E2 and EE2 sulfation by SULT1A1 using four concentrations of PAPS and either E2 or EE2 are shown in Table 1.

Because E2 and EE2 are both conjugated by SULT1A1 at the phenolic 3-OH position, the kinetics of sulfation of both compounds by pure SULT1A1 were compared. Table 1 shows that the K_m for EE2 sulfation (0.69 μM) was approximately 3-fold lower than that for E2, whereas the K_m for PAPS in both sets of reactions was not significantly different. The K_{cat} for EE2 sulfation by SULT1A1 was 2.4-fold greater than the K_{cat} for E2, indicating slightly better sulfation of EE2 than of E2. Neither E2 nor EE2 showed substrate inhibition with increasing substrate concentrations up to 20 μM after which the steroids were not soluble in the reaction mixture (Fig. 2).

K_d Determinations of E2 and EE2 Binding to SULT1A1. To establish the disassociation constants (K_d) for E2 and EE2 binding to SULT1A1 in the presence and absence of bound PAP, the change in intrinsic fluorescence of the pure enzyme generated by interaction with increasing estrogen concentrations was measured (Cook et al., 2010). Figure 3 shows the decrease in intrinsic fluorescence with increasing concentrations of E2 and EE2 in both the presence and absence of PAP. These results indicate that both estrogens are able to bind SULT1A1 in both the presence and absence of bound PAP. The K_d for E2 binding to SULT1A1 was determined to be $2.3 \pm 0.9 \mu\text{M}$ in the absence of PAP and $1.2 \pm 0.6 \mu\text{M}$ in the presence of PAP, suggesting that PAPS binding slightly increases the affinity for E2. In contrast, the K_d for EE2 binding to SULT1A1 was $0.5 \pm 0.15 \mu\text{M}$; however, the K_d for EE2 was >100 -fold lower ($4.3 \pm 1.7 \text{ nM}$) for binding to the SULT1A1-PAP complex. Whereas the K_d for E2 binding to the SULT1A1-PAP complex was approximately 50% of the K_m for E2 sulfation, the K_d for EE2 binding to the SULT1A1-PAP complex was 175-fold lower than its K_m , indicating that EE2 may be a potent inhibitor of SULT1A1.

Inhibition of SULT1A1 by EE2. Although EE2 is sulfated by SULT1A1 at micromolar concentrations (Fig. 2), the binding results (Fig. 3) suggest that EE2 may be a potent inhibitor at low nanomolar concentrations, especially in the presence of relatively high PAPS concentrations. Figure 4 shows the inhibition of PNP, 2-naphthol, and E2 sulfation by pure SULT1A1 with increasing nanomolar concentrations of EE2. With all three SULT1A1 substrates, EE2 concentrations well below 100 nM showed potent inhibition. As EE2 concen-

FIG. 2. SULT1A1 activity with E2 and EE2. A, reactions with E2 were performed using a chloroform extraction assay with [³H]E2. B, reactions with EE2 were performed using a TLC assay with [³⁵S]PAPS. The reactions above were performed at 10 μ M PAPS, and reactions were also performed at 0.1, 0.5, 1, and 5 μ M PAPS to determine whether there was any change in the K_m of PAPS. The K_m values of E2, EE2, and PAPS and the V_{max} values were determined on the basis of Michaelis-Menten kinetics and are listed in Table 1.

trations increased above 100 nM, the sulfation of EE2 by SULT1A1 began to interfere with the assays. EE2 inhibited the sulfation of three typical SULT1A1 substrates, E2, PNP, and 2-naphthol, with K_i values of 14.9 ± 2.8 , 8.9 ± 5.4 , and 19 ± 9.8 nM, respectively.

Inhibition of SULT1A1 Activity by EE2 in MCF-7 Cells. To further characterize the potent inhibition of SULT1A1, the ability of EE2 to inhibit E2 sulfation in intact MCF-7 breast cancer cells was examined. Intact MCF-7 cells express high levels of SULT1A1 and do not express SULT1E1 (Falany and Falany, 1996; Falany et al., 2002). MCF-7 cells were treated with 5 μ M [³H]E2 and increasing EE2 concentrations; then [³H]E2 sulfate levels in the medium were determined. There was no detectable [³H]E2 sulfate in the cell lysate, indicating that it was rapidly transported. Figure 5 shows that EE2 inhibits E2 sulfation with a calculated IC_{50} of 20 ± 9.8 nM in the intact MCF-7 cells. The MCF-7 cells did not take up either PNP or β -naphthol efficiently enough to allow for inhibition studies.

Homology Modeling of SULT1A1. Two high-quality crystal structures, 2D06 and 1LS6, are available for SULT1A1 and were resolved with bound PAP and E2 or PNP, respectively (Gamage et al., 2003, 2005). Figure 6 shows an alignment of the structures of 2D06 (Gamage et al., 2005) and 1LS6 (Gamage et al., 2003). The only region of difference in the structures is in loop 1 (aa 85–90) where

FIG. 3. K_d determination of E2 and EE2 with SULT1A1. The intrinsic fluorescence of SULT1A1 was measured at an excitation of 280 nm and emission of 340 nm, and the substrate was titrated into the solution to determine the K_d of E2 (A and B) and EE2 (C and D). The titrations were performed to the open enzyme without bound PAP (A and C) and then the closed enzyme with 10 μ M PAP (B and D). The affinity for E2 was determined to be 2.3 ± 0.9 and 1.2 ± 0.56 μ M in the presence of 10 μ M PAP. The affinity for EE2 was 0.5 ± 0.15 and 4.3 ± 1.7 nM in the presence of 10 μ M PAP. The concentration of enzyme was adjusted for K_d determinations, with 0.1 μ M enzyme for E2 titrations and 50 nM enzyme for the without PAP titration with EE2 and 1 nM enzyme for the with PAP titration with E2.

FIG. 4. Inhibition of SULT1A1 activity by EE2. Increasing concentrations of EE2 were used to inhibit the ability of SULT1A1 to sulfate E2 at 5 μ M (A) and two prototypical substrates *p*-nitrophenol at 3 μ M (B) and β -naphthol at 1.5 μ M (C). Reactions also contained 10 μ M PAPS and 0.2 μ g of SULT1A1 in a volume of 125 μ L.

FIG. 5. Inhibition of SULT1A1 activity in MCF-7 cells by EE2. MCF-7 cells were passed in Dulbecco's modified Eagle's medium with 7.5% fetal bovine serum into 24-well plates. Reactions were performed with 750,000 cells per well. The cells were washed with PBS twice and then were incubated with serum-free medium and 5 μ M [3 H]EE2 and increasing concentrations of EE2 (0–50 nM). After 2 h, the medium was removed, and cells were extracted with CHCl_3 and counted by scintillation spectroscopy (a time 0 at each concentration was used for a blank control). The IC_{50} for inhibition of E2 sulfation in the intact cells was 20 ± 9.8 nM.

loop 1 of **1LS6** is displaced slightly more (1.2–1.8 Å) into the substrate binding pocket than in **2D06**. There are currently no high-quality open crystal structures (without PAP/PAPS bound) reported for SULT1A1. Studies of human SULT2A1 have shown that the binding of PAP(S) can have a significant effect on the apparent structure and substrate binding in the active site (Cook et al., 2010). SULT2A1 exists in an open conformation, and the binding of PAPS results in a structural rearrangement that decreases the volume of the substrate binding pocket by approximately 60% and alters the reactivity of SULT2A1 with different substrates (Cook et al., 2010). The closure of loop 3 in SULT2A1 results from a charge interaction between the 3'-phospho group of PAPS and Arg257 in loop 3.

The open model of SULT2A1 was used as a template to generate an open homology model of SULT1A1. The SULT1A1 open homology model shows no difference in the basic structure of the core of the enzyme compared with SULT1A1 resolved in the presence of PAP. However, comparison of the open model with the closed resolved **2D06** structure identified several small structural changes: loop K85–P90 has a 5-Å shift; loop M140–P150 has a 3-Å shift; and loop M237–W261 has a 3-Å shift (Supplemental Fig. 1). The M140–P150 loop contains Glu146 that has been described as the essential amino acid contributing to the substrate selectivity differences between SULT1A1 and SULT1A3 (Dajani et al., 1998). These loops are located adjacent to the substrate binding pocket of SULT1A1, and the combined changes suggest a decrease of approximately 40% in the volume of the substrate binding pocket in the closed conformation elicited by PAPS binding.

Docking Studies of SULT1A1. To validate that the open models generated from the resolved closed structures are accurate, docking of a known substrate, E2, to both the open and closed models was performed. Kinetic and binding studies have previously shown that E2 binds to both conformations of SULT1A1. The docking studies showed that E2 had a BFE of -7.9 kJ/mol in the open conformation and -8.5 kJ/mol in the closed conformation (Fig. 7). A competent orientation for sulfonate transfer occurs when the hydroxyl group of E2 is within 3 Å of the active site His108. The docking studies showed E2 with the 3-hydroxyl in a competent orientation for sulfonate transfer in both the open and closed SULT1A1 models.

Because E2 docked into both the open and closed SULT1A1 structures in a competent orientation, EE2 was docked into both the open SULT1A1 model and closed resolved structure. The open homology model generated with either **2D06** or **1LS6** showed a BFE of -8.5 kJ/mol with the 3'-hydroxyl of EE2 oriented competently to the active site His108 (Fig. 8a). In contrast, the closed resolved structures demonstrated two different options for the docking of EE2 to SULT1A1. The **1LS6** structure predicts a BFE of -10.2 kJ/mol with a hydrophobic interaction with Ile89 orienting EE2 to the active site His108 in a position favorable for sulfation (Fig. 8b). The **2D06** structure also predicts a BFE of -10.2 kJ/mol; however, the hydrophobic interaction with Ile89 orients the 3-hydroxyl to bond with Tyr169 in a position that is not catalytically favorable (Fig. 8c). This indicates that the **2D06** structure resolved with E2 correlates better with the kinetic properties of the pure enzyme. The small change in the position of Ile89 in loop 1 between the two resolved structures appears to be the major factor that defines EE2 as a potent inhibitor at nanomolar concentrations rather a substrate.

Discussion

Drug-drug interactions associated with EE2 therapy have been primarily studied in regard to the cytochromes P450. Few studies investigating interactions with the SULTs or other major conjugating enzyme systems have been reported. The inhibition of SULT1A1 at low nanomolar concentrations of EE2 while allowing for EE2 sulfation at higher concentrations suggests a new paradigm for investigating and understanding drug-drug interactions involving SULT1A1 and possibly other isoforms. SULT1A1 is the major xenobiotic sulfating isoform in liver and is widely expressed throughout the body (Hempel et al., 2007); however, its interactions in EE2 therapy are not well studied. The potent inhibition of SULT1A1 was unexpected and indicates that more attention should be paid to sulfation of both drugs and xenobiotics during concurrent therapy with EE2-containing contraceptives. Lack of sulfation activity at the inhibitory concentrations also suggests a relatively long duration of inhibition. Combined with the high levels of expression of SULT1A1 in the GI tract, brain, and lungs as well as liver, the effects of EE2 inhibition may be tissue-selective. The potent inhibition of SULT1A1 by EE2 needs to be considered as a possible mechanism for large numbers of pharmacokinetic drug-drug interactions involving EE2 (Zhang et al., 2007).

FIG. 6. Comparison of the resolved structures of SULT1A1. The structure of SULT1A1 resolved with PAP and PNP (**1LS6**) is shown in green. The structure resolved with E2 and PAP (**2D06**) is shown in blue. The structures were aligned in MacPyMol. Loop 1 (aa 85–90) is shown in red in **2D06** and in sand in **1LS6**. The position of E2 in **2D06** and PAPS (yellow) in both structures is included for reference.

Fig. 7. Modeling of E2 docking in the open and closed conformations of SULT1A1. E2 was docked in both the open (A) and closed (B) conformations of SULT1A1 using Amber99 and Triangle Matcher in MOE. In the open homology model of SULT1A1, E2 had a BFE of -7.9 kJ/mol and a BFE of -8.5 kJ/mol in the closed resolved structures. In both situations, E2 was positioned with the 3'- β -hydroxyl in a position consistent with sulfonate transfer.

Comparison of E2 and EE2 sulfation by and inhibition of SULT1A1 illustrates the complexity of substrate binding, sulfation, and inhibition in the SULT family. Although E2 and EE2 are structurally very similar, EE2 demonstrates a much greater ability to bind and inhibit SULT1A1 without being conjugated in both in vitro assays and in MCF-7 cells. The observation that EE2 had a K_d of 500 nM for binding pure SULT1A1 in the absence of PAP was consistent with a K_m for EE2 sulfation of 700 nM. However, the determination of a K_d of 4 nM for EE2 binding of SULT1A1 in the presence of PAP was unexpected because EE2 is not a substrate at this low concentration. In addition, EE2 also inhibited the sulfation of several common SULT1A1 substrates with K_i values in the 9 to 20 nM range. The ability of EE2 to inhibit the sulfation of E2 in intact MCF-7 breast cancer cells that express SULT1A1 also showed an IC_{50} of 20 nM (Fig. 5). Therefore, it was of interest to determine how EE2 was a potent inhibitor of SULT1A1 at concentrations approximately 175-fold lower than the concentrations at which it was a substrate for conjugation. The lack of sulfation at the low inhibitory concentrations also suggests that the inhibitory complex may be relatively long-lasting.

Analyzing the possible role of structural rearrangements in SULT1A1 was necessary to understand the potent inhibition of SULT1A1 by EE2. Although closed SULT1A1 structures have been resolved with acceptor substrates including E2 (Gamage et al., 2005) and PNP (Gamage et al., 2003), no high-quality open structures of SULT1A1 have been reported (Allali-Hassani et al., 2007), probably because of the flexibility of the loop 3 region without the stabilizing effect of the Arg257-PAPS interaction. Therefore, models of SULT1A1 in an open conformation were generated using SULT2A1 as a template. The open homology models generated from either 2D06 or 1LS6 displayed very similar binding properties and bound EE2 with similar BFE in a competent orientation for sulfation. Because EE2 is not a substrate at low nanomolar concentrations, the closed 2D06 structure is apparently more predictive of the conformation binding of EE2 with the low K_d than the 1LS6 structure. The major difference in the 2D06 and 1LS6 structures is the position of the K85-P90 loop forming a portion of the substrate binding pocket. Interaction of the ethinyl group in EE2 with ILE89 in this loop appears to be associated with altering the position of the 3-hydroxyl so that instead of interacting with the active site His108 it interacts with Tyr169.

The kinetic data for EE2 inhibition of and sulfation by SULT1A1 are best represented by its interactions with the 2D06 structure of SULT1A1 and the open models generated using SULT2A1 as a template. The binding of PAP(S) to SULT1A1 appears to result in a more compact substrate binding pocket due to the rearrangement of several unstructured loops. In contrast, PAPS binding in SULT2A1 results primarily in a 10-Å shift in loop 3 (aa 228–253), resulting in a decrease in the volume of the substrate binding pocket. A smaller PAPS-elicited shift is observed in loop 3 in SULT1A1; however, the position of loop 1 appears important for orienting EE2 binding. Loop1

in the SULT1 family is not present in the SULT2 family, indicating that even though compounds such as E2 may be sulfated by multiple SULT isoforms (SULT1E1, 1A1 and 2A1), the kinetic and inhibitory properties may be significantly affected by small sequence and structural changes.

EE2 interactions with SULT1A1 also suggest that order of addition of substrates has an effect on the ternary complex. At low nanomolar concentrations, EE2 appears to only bind if PAPS is already bound, and the resulting ternary complex is not catalytically active. As EE2 concentrations increase above the K_d for EE2 binding to the open conformation increases. If EE2 binds before PAPS then sulfation can occur. Complicit in this process is the fact that once EE2 is bound in a competent orientation in SULT1A1, the binding of PAPS does not result in an inactivating change in the position of the EE2 molecule. More detailed kinetic and binding analyses are required to refine the stability and reactivity of the inhibitory complex.

Characterization of reaction mechanisms in the SULTs has not indicated consistent results. Some of the differences may be related to the substrates and SULT isoforms used in the studies. SULT2A1 has recently been reported to show apparently different binding and reaction mechanisms with dehydroepiandrosterone and raloxifene (Cook et al., 2010). Because of the high affinity of EE2 for the SULT1A1-PAPS complex and generation of a nonproductive complex, for efficient sulfation EE2 must apparently bind to the enzyme before PAPS. In contrast to EE2, E2 is not a high-affinity inhibitor of SULT1A1. The K_d for E2 binding to pure SULT1A1 in the absence of PAP is 2.3 μ M, and the K_d decreases to 1.2 μ M if the enzyme is saturated with PAP before addition of E2. This observation is consistent with the docking results indicating that E2 binds in a competent orientation in the open and both closed models of SULT1A1 with only a relatively small change in BFE. The change in affinity combined with the K_i of PAP for the E2-SULT1A1 complex demonstrates that whereas the reaction mechanism for SULT1A1 sulfation of E2 is an apparent random order reaction, there is a preference for the PAPS to bind first and E2 second. The K_i values of the products in the E2 sulfation reaction show that E2 sulfate has a poor affinity for the SULT1A1-PAPS complex, probably because the two sulfates are required to be within 5 Å of each other. In addition, PAP has a poor affinity for the E2-SULT1A1 complex, which would indicate a preference of PAPS for the free enzyme.

The novel observation that EE2 was capable of inhibiting SULT1A1 both in vitro and in intact cells at low nanomolar concentrations without being sulfated provides a new venue for drug-drug interactions. In addition, the mechanism by which increasing EE2 concentrations allowed for EE2 sulfation activity provides insight into the structural rearrangements in the SULTs associated with substrate binding and reactivity. SULT1A1 is highly expressed in liver as well as in the GI tract and is considered the major human SULT isoform involved in xenobiotic sulfation in liver. Therefore, the ability to inhibit SULT1A1 at low EE2 nanomolar concentrations without the EE2 being readily sulfated indicates possible drug-drug interactions

Fig. 8. Modeling of EE2 docking in the open and closed conformations of SULT1A1. Docking of EE2 to the SULT1A1 models was performed using the same programs for E2 docking in Fig. 7. EE2 docked to the open SULT1A1 homology model with a BFE of -8.5 kJ/mol (A) with the 3-OH oriented to the His108 in a position consistent with hydrogen abstraction. Docking of EE2 in the closed resolved structures of SULT1A1 showed two populations of structures with similar BFEs. The first orientation was found with the SULT1A1 structure resolved with bound PNP (1LS6). This structure shows a hydrophobic interaction with Ile89 in loop 1 that orients the EE2 to interact with the His108 with a BFE of -10.2 kJ/mol (B) in an orientation favorable for sulfation. In contrast, EE2 binding to the resolved structure generated with bound E2 (2D06; C) predicted an interaction of EE2 with Ile89 that oriented the 3-OH moiety to hydrogen bond with Tyr169. This results in a noncatalytically competent orientation with a BFE of -10.2 kJ/mol.

with compounds that are extensively sulfated by SULT1A1, especially during first-pass metabolism. Because PAPS levels have been reported to be significantly different between tissues (Klaassen and Boles, 1997), the dependence of EE2 inhibition on PAPS binding suggests that cellular PAPS levels may be a factor in regulating its SULT1A1 inhibitory activity. Overall, our understanding of SULT activity and inhibition will require a better understanding of the interactions of structure, binding, and catalysis, especially for in silico predictions with specific drug sulfation and interactions.

Authorship Contributions

Participated in research design: Rohn, Cook, Leyh, and Falany.

Conducted experiments: Rohn, Cook, Leyh, and Falany.

Performed data analysis: Rohn, Cook, Leyh, and Falany.

Wrote or contributed to the writing of the manuscript: Rohn, Cook, Leyh, Kadlubar, and Falany.

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