16 Sulfotransferases

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16.1 INTRODUCTION TO HUMAN SULFOTRANSFERASES

In the nineteenth century, one of the difficulties associated with surgery was the high postoperative mortality rate that many patients suffered (as high as 50%) most often due to infection [1,2]. Carbolic acid (phenol) was an early antiseptic that was used to reduce postoperative infections and thus reduce mortality [3]. In the late 1870s, a German chemist named Eugen Baumann was studying the elimination of carbolic acid in urine. He was able to isolate and characterize phenol sulfate from the urine of a patient who was administered carbolic acid as an antiseptic [4]. Many years later (1957), the molecule which served as a sulfate donor in this process was characterized [5]. Robbins and Lipman demonstrated that when protein fractions from liver cytosol were separated, one fraction contained the sulfate-activating system and a second fraction contained the transferring enzyme or a "phenol sulfokinase." The identity of the sulfate donor molecule was determined from the sulfate-activating fraction. These investigators were able to isolate and identify what is now known as *3′-phosphoadenosine 5′-phosphosulfate* (PAPS), which is the physiological donor molecule or cofactor in sulfotransferase (SULT)-mediated conjugation reactions [5,6]. Since these early discoveries, from a historical perspective, the SULT field has moved forward relatively rapidly.

Today, SULTs are recognized as an important family of enzymes. They are not only involved in the metabolism of xenobiotics such as carbolic acid but also involved in important processes such as supporting placental estrogen biosynthesis, modulating estrogenic stimulation of the endometrium, and influencing the levels of receptor-active

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thyroid hormone [7–17]. Thus, the SULT field has grown beyond the conjugation of xenobiotics, and it is now recognized that SULTs play an important role in biological processes as well. It should be noted that many insightful and complete reviews of SULT enzymology have preceded this chapter [18–24]. Moreover, the reader is directed to the references in this chapter to expand into additional topics according to his or her level of interest. However, for the purposes of this book, the scope of the current review focuses most directly on the topic of drug metabolism and the role that SULTs play in this area. An emphasis is placed on the practicality of studying SULTs as they apply to drug discovery and drug development. While a great deal of information is available for cytochrome P450 *in vivo–in vitro* correlations, *in vitro* assay conditions, selective substrates and inhibitors, and so on, this information is not always readily available for the SULT family of enzymes. In many cases, selective probe substrates or inhibitors do not exist, and in other cases, the selectivity of certain inhibitors of substrates has not been well characterized. For the drug metabolism scientist determining, for example, the contribution of a specific SULT isoforms(s) to the formation of a sulfate drug metabolite, it is a difficult task; many of the expectations learned from dealing primarily with cytochrome P450 enzymology do not translate to the world of SULTs. It is the authors' intent that the following chapter reviews some topics that may help applied drug metabolism investigators to point themselves in the right direction for solving practical issues in drug discovery/development.

16.1.1 Classification and Nomenclature

There are two classes of mammalian SULTs: SULTs that are membrane bound and those that are found in the cytosol [18]. Membrane-bound SULTs are located in the Golgi apparatus, and these enzymes are involved in the sulfation of endogenous substrates such as protein and carbohydrates. Membrane SULTs do not currently appear to be involved in the sulfation of xenobiotics [25,26]. As a result, focus is directed toward cytosolic SULTs, which are capable of conjugating a variety of small molecules including both exogenous and endogenous substrates. It is only relatively recent that the nomenclature system for cytosolic SULTs has been established [27]. The members of the cytosolic family of SULTs are all assigned to the superfamily "SULT." In addition, based on the genetic sequence identity, the members of this superfamily are categorized and subcategorized. Those SULTs that share at least 45% amino acid sequence identity belong to the same family. Subfamilies are determined by at least 60% amino acid sequence identity. Thus, SULT1A is a designation for the SULT1 family (family designation uses an Arabic numeral after the superfamily name) and SULT1A also specifies the "A" subfamily. SULT1A1 is the name for isoform "1" (Arabic numeral) within the SULT1A subfamily. Gene names have the same designation as the proteins, but the gene names are listed in italics. Alleles are identified with an asterisk and an Arabic numeral directly after the subfamily isoform number (e.g., *SULT1A1*1*). SULT2B1a and SULT2B1b refer to splicing variants encoded by the same gene but with different amino acid sequences [28]. However, new nomenclature has designated these enzymes as SULT2B1_v1 and SULT2B1_v2, respectively.

This last point serves to illustrate a very real and sometimes confounding issue when dealing with the SULT literature. Much of the SULT body of literature consists of papers written before standardized nomenclature. Readers who are unfamiliar with the subject will be confronted with several names designated for the same protein,

which can lead to potential misunderstanding. Table 16.1 has been constructed to aid in the process of reading "older" literature. For example, the following paper published as recently as 2002 was titled "Phenol Sulfotransferase, ST1A3, as the Main Enzyme Catalyzing Sulfation of Troglitazone in Human Liver" [29]. The term ST1A3 is similar to SULT1A3; however, consultation with Table 16.1 will clarify that this paper is actually referring to SULT1A1.

16.1.2 Protein Structure and Function

The first reported SULT crystal structure was a mouse estrogen sulfotransferase (mSult1e1) [30,31]. Since then, three-dimensional (3D) structures have been determined for human SULTs 1A1, 1A3, 1E1, 2A1, 2B1_v1, and 2B1_v2 [22,32]. SULTs are globular proteins with a single α/β domain containing a central five-stranded parallel β-sheet [20]. Although many structural features identified from mSult1e1 are also observed in human SULTs, the mouse enzyme exists as a monomer, whereas human SULTs are generally present as homodimers [20,33]. A conserved sequence consisting of 10 amino acid residues (KXXXTVXXXE; also known as the *KTVE motif*) located near the C-terminus in most human SULTs has been identified as being involved in dimerization [20,33]. Mutation of the valine residue alone (Val270Glu in SULT1A1, Val266Glu in SULT1E1, and Val260Glu in SULT2A1) is sufficient to convert the dimeric enzymes into monomers [33,34]. The monomeric mSult1e1 has Pro269-Glu270 residues in place of Thr and Val and, as anticipated, a double mutation of Pro269Thr and Glu270Val leads to dimerization of the enzyme [34].

One of the conserved motifs identified in mSult1e1 and in various human SULTs is the structural element that contributes to the binding of cofactor PAPS. This motif consists of nine amino acids corresponding to residues 45-TYPKSGTTW-53 in mSult1e1, and is named the 5′-phosphosulfate binding loop (5′-PSB-loop) because of its interaction with the 5′-phosphate group of 3′-phosphoadenosine 5′-phosphate (PAP) or PAPS [31,32]. Several amino acid residues have been determined to constitute the 3′-phosphate binding (3′-PB) site for PAP or PAPS, including the highly conserved residues Arg130, Ser138, and 257-RKG-259 [30]. Site-directed mutagenesis of Arg257 to Ala or Glu in SULT1A1 results in inactive proteins, illustrating the importance of this residue for the catalytic activity of the enzyme [35]. In addition to two binding motifs, aromatic–aromatic interaction (π-stacking) has also been observed for the adenine ring of the PAP molecule with residues Trp53 and Phe229 [36].

The catalytic mechanism for SULTs has been suggested to involve an inline S_N2-type nucleophilic attack on the PAPS sulfonate by a suitable substrate nucleophile. One early study that helped to shed light on this process was a solved crystal structure of mSult1e1 with the vanadate ion (VO_4^{-3}) [31]. Vanadate (oxidation state +5) can readily acquire a stable 5-coordinated trigonal bipyramidal geometry, which mimics the transition state of both phosphate and sulfate ions. Indeed, Kakuta *et al*. were able to show that the vanadate anion adopted this bipyramidal geometry in the active site, with the three bonded oxygen atoms forming the trigonal plane surrounding the vanadium atom and the apical positions occupied by water on one side and by the terminal oxygen of the 5′-phosphate of PAP on the other. In addition, the overall structure of the mSult1e1-PAP-vanadate complex was shown to be virtually the same as the mSult1e1-PAP-β-estradiol complex obtained by the same investigators [30,31]. Subsequent crystal

TABLE 16.1 Naming Conventions and Properties of Human Sulfotransferases

Human SULT	Other Names	Major Expression Sites	Known Drug Substrates	Known Endogenous Substrates
SULT1A1	P-PST, P-PST-1, TS-PST, TS-PST-1, H-PST, HAST-1, ST1A3	Adult: liver, intestine, kidney, lung, platelets, brain, placenta Fetus: liver	Minoxidil, troglitazone, acetaminophen, ethinyl estradiol	β-Estradiol, T_2, T_3, T_4
SULT1A2	P-PST-2, TS-PST-2, ST1A2, HAST4	Adult: liver, intestine	Minoxidil	Not known
SULT1A3	M-PST, TL-PST, HAST3, ST1A5	Adult: intestine, kidney, lung, platelets, brain, placenta Fetus: liver	Salbutamol, terbutaline, minoxidil, ethinyl estradiol	Dopamine, 3-methyl dopamine, norepinephrine
SULT1B1	ST1B2	Adult: liver, intestine, kidney, lung	—	T_2, T_3, T_4
SULT1C2	ST1C2, SULT1C1, HAST5	Adult: stomach Fetus: liver, kidney	—	Not known
SULT1C4	SULT1C2, hSULT1C	Fetus: liver, kidney, lung	—	Not known
SULT1E1	EST, hEST, ST1E4	Adult: liver, intestine, lung, endometrium Fetus: liver, kidney, lung	Ethinyl estradiol, troglitazone	β-Estradiol, estrone, 24OH-cholesterol, T_2, T_3, T_4
SULT2A1	DHEA-ST, HST, ST2A3	Adult: liver, intestine, lung, adrenal Fetus: liver, adrenal	Ethinyl estradiol, minoxidil,	DHEA, 24OH-cholesterol, T_2, T_3, T_4
SULT2B1_v1	SULT2B1a	Adult: placenta	—	DHEA, pregenolone
SULT2B1_v2	SULT2B1b	Adult: placenta, prostate	—	DHEA, pregenolone, cholesterol, 24OH-cholesterol
SULT4A1	BR-STL	Adult: brain	Not known	Not known

structures obtained from human SULT1E1–PAPS complex and the ternary hSULT1E1-PAP-β-estradiol complex provided additional evidence for the catalytic mechanism of SULT [37]. His107 (of hSULT1E1) has been hypothesized to be a catalytic base and to activate the hydroxyl group of the substrate for nucleophilic attack via proton abstraction. Mutation of this residue (His107Asn) leads to an enzymatically inactive protein [37]. Once activated, the oxygen anion attacks the sulfur atom of the PAPS molecule, resulting in the dissociation of the bridging oxygen between the 5′-phosphate and the sulfate leaving group. A conserved lysine residue (Lys47) has been suggested to act as an acid catalyst by interacting with the bridging oxygen and therefore facilitating the dissociation of the sulfate group from PAP. Interestingly, in the absence of substrate, the crystal structure of PAPS-bound human SULT1E1 showed that Lys47 actually interacted with Ser137 via a side chain interaction (hydrogen bonding) and that this interaction prevented the hydrolysis of PAPS. It was proposed that once the substrate was bound in the active site, and the reaction progressed, the bridging oxygen between the 5′-phosphate and the sulfate group then attained a partial negative charge after the attack of the substrate oxyanion (Fig. 16.1). At this point, the side chain of Lys47 switched from the Ser137 interaction to the partial negative charge of the bridging oxygen, stabilizing this intermediate and thus making the phosphate a better leaving group. Taken together, the results from crystallography and site-directed mutagenesis studies have suggested a detailed structural catalytic mechanism. Although there may be refinements to this proposed mechanism, the data indicate that the conserved serine, lysine, and histidine residues work in concert during the sulfonate transfer process [37].

16.1.3 Tissue Distribution

Sulfation has been shown to be a major biotransformation pathway for a number of xenobiotics (e.g., ethinyl estradiol, salbutamol, and troglitazone) [38–41] as well as an inactivation/regulation mechanism for endogenous chemicals such as dopamine [42]. Section 16.1 briefly touched on some of the important endogenous roles for SULTs. Not surprisingly, broad expressions of SULT enzymes have been identified in various tissues as would be expected for a family of enzymes with many different roles. Major expression sites for different SULT enzymes are summarized in Table 16.1. Using quantitative immunoblotting, Riches *et al*. [43] determined the expression levels of five major SULTs (SULT1A1, SULT1A3, SULT1B1, SULT1E1, and SULT2A1) in adult liver, small intestine, kidney, and lung. The overall amount of SULT expressed in liver and intestine was greater than 10-fold of that observed in lung and kidney. In liver, SULT1A1 and SULT2A1 were determined to be the major SULT isoforms, with SULT1B1 and SULT1E1 expressing at relatively lower levels. There was no detectable level of SULT1A3 in liver. The expression pattern of various SULTs in intestine was markedly different from that in liver, with SULT1A3 and SULT1B1 more abundant than SULT1A1, SULT1E1, and SULT2A1. In a similar study conducted by Teubner *et al*. [44], high SULT expression levels were detected in ileum compared to the remaining intestinal sections (jejunum, cecum, colon, and rectum). Subsequent immunohistochemical analyses revealed that SULTs were primarily expressed in mature (differentiated) enterocytes (similar to cytochrome P450 3A), consistent with the anticipated role of xenobiotic detoxification. It is interesting to note that SULT1C2 was detected in stomach, while SULT1A2 was present at very low levels in liver and intestines. In addition, significant interindividual differences in SULT expression levels were observed in the tissues examined.

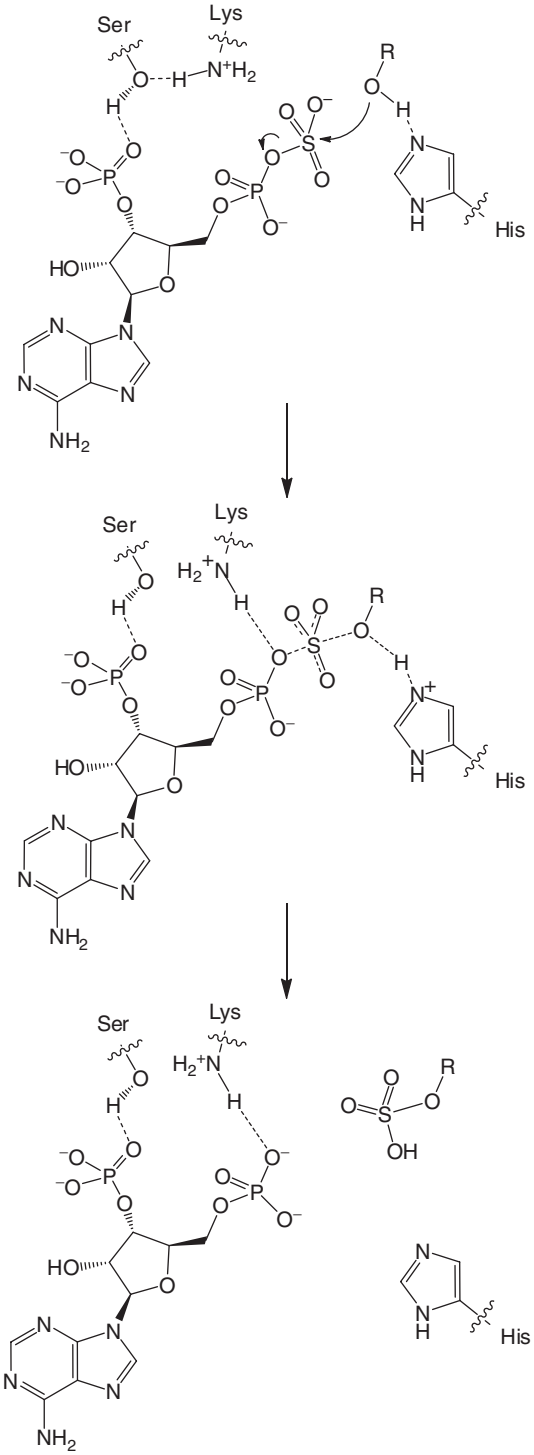

Figure 16.1 Proposed mechanism of sulfotransferase-catalyzed sulfonyl transfer.

Results obtained from reverse transcriptase-polymerase chain reaction (RT-PCR) analyses indicated that SULT1A1, SULT1A3, and SULT4A1 were readily expressed in human brain [45,46]. Immunoblot analyses showed that SULT1A1, SULT1A3, and SULT4A1 proteins were present in all regions of the brain examined; however, the expression patterns for these SULTs were vastly different [47,48]. SULT1A1 was preferentially expressed in cerebellum, occipital, and frontal lobes, whereas SULT1A3 was highly expressed in superior temporal gyrus, hippocampus, and temporal lobe. The expression level of SULT4A1 was relatively consistent among various regions tested. At the cellular level, immunoreactivities for SULTs 1A1 and 1A3 were found in neurons as well as glial cells, but localization of SULT4A1 was mainly associated with neurons. Currently, despite abundance, the role and function of SULT4A1 in human brain is undetermined.

16.1.4 Role in Fetal Development

Similar to adults, the developing fetus is potentially exposed to a number of environmental toxins; it has been hypothesized that SULTs play a protective role during development because a number of SULTs are expressed at high levels during this period [18]. However, it should be added that other xenobiotic-metabolizing enzymes are also present in the fetus, one notable example would be CYP3A7 and other P450 enzymes [49,50]. Several investigators have shown that SULT2A1 [dehydroepiandrosterone (DHEA) SULT] increases during fetal development, and the expression of SULT2A1 is very high in the adrenal from ~12 weeks of gestation onward, which lends credence to the idea that DHEA sulfate is needed during this period to support placental estrogen biosynthesis [51,52]. DHEA is sulfated primarily by SULT2A1 in the adrenal cortex and then desulfated in the placenta to release free DHEA [53]. The DHEA sulfate in placenta is from both maternal and fetal adrenal origin [54,55], and once free DHEA is released, it is then converted into androstenedione by the 3β-hydroxysteroid dehydrogenase type 1 (3β-HSD1), next aromatized to estrone by the cytochrome P450 aromatase and finally reduced by the 17β-HSD type 1 to generate estradiol [56].

Thyroid hormones are critical for development of the fetal and neonatal brain, as well as for many other aspects of fetal growth (e.g., thyroxine). Hypothyroidism in either the mother or fetus often results in fetal disease; in humans, this can include a high incidence of mental retardation [57–59]. The fetal thyroid gland reaches maturity by week 11–12, close to the end of the first trimester, and begins to secrete thyroid hormones by about week 16 [60]. During this period, an adequate supply of maternal thyroid hormones must be sustained to ensure normal neurological development.

The mechanism by which thyroxine (T_4) is converted to the receptor active form 3,3′,5-triiodothyronine (T_3) is a complex interplay of sulfation and deiodination between free T_4 and T_3. When T_4 is secreted by the thyroid gland, it can be activated to form T_3 (outer ring deiodination) or inactivated to reverse T_3 (inner ring deiodination). However, when T_4 is sulfated, it is almost completely converted to receptor inactive reverse T_3. Sulfation of T_3 also accelerates the deiodination of T_3 to T_2 (3,3′-diiodothyronine) [14,15,17,60].

It has been shown that iodothyronine sulfates are present at very high levels in fetal blood and postnatal life, and thus, it has been speculated that sulfation may play part of a protective mechanism that prevents the fetus from excessive T_3 exposure [61,62]. However, other mechanisms are also present and low fetal serum concentrations of

T_3 are due, in part, to the increased activity of the inactivating deiodinase (D_3) and a low expression of the activating deiodinase D_1 (the major activating deiodinase in adults) [63]. Although the major contributing mechanism may be subject to debate, it is interesting that the expression of deiodinase and high sulfate levels of thyroid hormones are all mechanisms that would reduce fetal exposure to T_3.

16.2 SULFATION REACTION

As discussed, since the early work of Eugen Baumann, examples of drug sulfation have grown substantially. Many review papers on this subject have wording such as "Sulfonation of the low molecular weight compounds catalyzed by members of the cytosolic SULT multigene family is an important determinant of the pharmacology and toxicology of a vast array of endogenous and foreign chemicals..." [21]. However, in the pharmaceutical industry as well as in the literature, the overall contribution of sulfation to drug metabolism is often overlooked. For example, in a recent review of cytochrome P450 and other drug-metabolizing enzymes, the author presents a pie schematic outlining the contribution of cytochrome P450s (CYPs or P450s), UDPGA-glucuronosyltransferases (UGTs), esterases, *N*-acetyltransferases (NATs), and monoamine oxidases (MAOs), but no slice of the pie was designated for the contribution from SULTs [64]. In addition, over the course of many collective years of industrial experience (and many hundreds of compounds), we have worked on relatively few drugs that undergo sulfation as the primary means of elimination. However, we have also made the observation that sulfation has grown in importance in the industrial setting in recent years. Several suggestions may be offered to rationalize this undocumented observation. First, in early drug discovery, much attention is focused on P450- and UGT-mediated metabolism. As a consequence, when discovery teams seek to minimize metabolism/clearance caused by these enzymes, the process (likely inadvertently) shifts the chemical space of compounds such that other pathways (e.g., active transport and sulfation) play an increasing role in compound elimination. Second, microsomes-based metabolic stability studies have been utilized as front-line assays for years. Given that drug-metabolizing SULTs are cytosolic, the microsomal system will not yield useful information regarding the potential sulfation of drug candidates. However, because the contemporary quality and availability of cryopreserved hepatocytes has improved, microsomal assays are increasingly complemented by *in vitro* assays using hepatocytes, which generate metabolism information from a whole cell system, and therefore include the contribution to metabolism from many enzymes including SULTs [65]. Regardless of the true reason for the increasing role of sulfation, given the potential for SULTs to conjugate a wide variety of xenobiotic compounds, it would be prudent for drug metabolism scientists (academic or industrial) to be familiar with the basics of SULT conjugation. Accordingly, this section focuses on the basic catalytic mechanism, *in vitro* assays, enzyme kinetics, practical considerations, and examples of conjugation leading to the formation of potential deleterious reactive intermediates.

16.2.1 Catalytic Mechanism

Sulfation (or sulfonation) is the transfer of a sulfonate group ($-SO_3^-$) to an acceptor molecule. A structural mechanism for this reaction was discussed in Section 16.1.2.

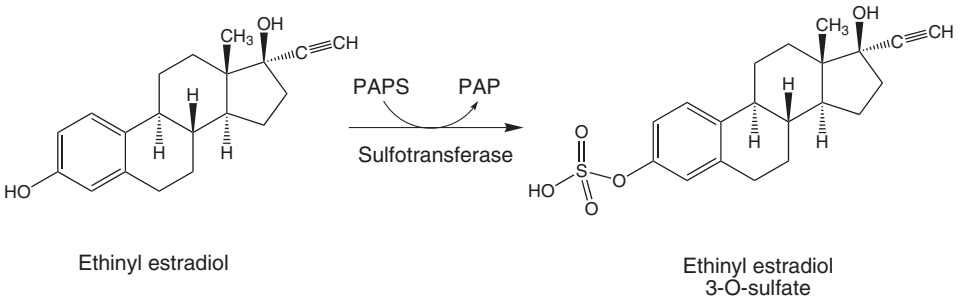

Figure 16.2 Example of a sulfonate conjugation reaction.

The acceptor in this reaction is commonly a hydroxyl group, in which case a sulfate is formed ($RO{-}SO_3H$; Fig. 16.2). It should be noted that some authors prefer to use the term "sulfonation" rather than "sulfation," since technically it is the sulfonate group, not the sulfate group ($-OSO_3^-$), that is being transferred in the reaction. Regardless of terminology, a hydroxyl group can serve as the nucleophile/acceptor and it is also possible for nitrogen or sulfur to act as a nucleophile and form a sulfamate or thiosulfate metabolite, respectively. As discussed in Section 16.1.1, for small molecules, this reaction occurs primarily in the cytosol, catalyzed by cytosolic SULT enzymes. In general, the catalytic turnover of substrates catalyzed by SULT enzymes is relatively low. For example, the k_{cat} for sulfation of β-estradiol by SULT1E1 is ~1.3/s [66]. However, the K_m values for many substrates are low (in the nanomolar range), resulting in high catalytic efficiency (k_{cat}/K_m).

Early crystallography studies were not able to determine a structure with both PAPS and an acceptor molecule bound to SULT simultaneously. Instead, structures of mouse Sult1e1 were solved as mSult1e1-PAP-β-estradiol complex and mSult1e1-PAP-vanadate complex (*vide supra*) [30,31]. Similarly, structures of human SULT1E1-PAPS and human SULT1E1-PAP-β-estradiol complexes were also obtained [37]. Insight into the mechanisms of sulfonate transfer was gained by superimposing the structures of the later two complexes, and the resulting superposition revealed a ternary complex that positioned PAPS with the acceptor substrate in a S_N2-like inline displacement reaction [37]. In Section 16.1.2, a detailed description (hypothesis; Fig. 16.1) of this mechanism was presented. Recently, a group was able to provide a snap shot of the Michaelis–Menten complex with both PAPS and 4-nitrophenol bound to mouse Sult1d1 [67]. The data from this complex also strongly supported the S_N2 inline displacement mechanism as proposed by earlier studies.

16.2.2 Sulfation Assays

For many decades, the barium precipitation assay developed by Foldes and Meek [68] has been commonly adopted for determining SULT activities. This assay takes advantage of the highly effective precipitation process of PAPS in the presence of barium ions. With the use of [^{35}S]-labeled PAPS as the cosubstrate, the unreacted [^{35}S]PAPS can be easily removed from the incubation mixture by precipitation, leaving the soluble, [^{35}S]-labeled sulfate conjugates readily available for quantification by scintillation counting.

However, this simple, quick, and widely applicable [^{35}S]PAPS assay does have some limitations. One major disadvantage is the inability to differentiate what exactly has been sulfated. It has been shown that contaminants of substrate and buffering agent, as well as small peptides present in tissue preparations, can undergo significant sulfation, and as a consequence, there is potential to confound experimental results [69]. The background signal caused by these undesired sulfation products are particularly problematic in determining product formation rates at low substrate concentrations. Many steroids, for example, have K_m values in the low nanomolar region. To overcome this hurdle, thin-layer chromatography (TLC) has been commonly utilized in recent years to replace barium precipitation when [^{35}S]PAPS is used [42,70,71]. The ability to separate various [^{35}S]-labeled sulfate conjugates also enables a more thorough characterization of metabolism and kinetic analysis. For compounds such as raloxifene and 24-hydroxycholesterol, TLC analyses reveal the formation of secondary metabolites (i.e., disulfates) in addition to monosulfates from *in vitro* incubations. In the case of dopamine, sulfation products of dopamine and its metabolite, 3-methyldopamine, have been identified in the incubation of dopamine with human neuroblastoma cells. The usefulness of TLC analysis, however, is limited by resolving power, as illustrated by the inability to separate isomeric monosulfate conjugates [42,70,71].

High performance liquid chromatography (HPLC)-based methods provide much needed resolution for various sulfate conjugates, including isomeric conjugates, leading to more precise measurements of SULT activities and kinetic analyses. Traditional reversed-phase column chromatography using a C18 column is broadly applicable for many endogenous substrates and xenobiotics [70–72]. For highly hydrophilic substrates such as dopamine and 3,4-dihydroxymethamphetamine, separation can now be readily achievable with a fluoro-substituted alkyl column or a hydrophilic interaction chromatography (HILIC) column [73,74]. Depending on the application, column effluent can be analyzed using one or more online detectors, including UV/VIS spectrophotometer, fluorescence spectrophotometer, radiochemical detector, and mass spectrometer. Liquid chromatography-mass spectrometry (LC/MS) appears to be the most versatile and convenient, albeit expensive, method among those listed above. Although the pKa of the sulfate moiety is ~1, a sulfate conjugate can be detected using a positive or negative ionization mode depending on the pH of the mobile phase used [73–75]. Constant neutral loss of 80 Da (sulfonate moiety) is a generic yet selective way for the detection of sulfate conjugates. It should be noted that the loss of sulfonate moiety can readily occur via in-source collision-induced dissociation (i.e., in-source fragmentation). As such, extra care should be taken to optimize the ionization condition for sulfate conjugates. The labile nature of the sulfonate moiety in mass spectrometric analysis makes the identification of the sulfation site very difficult. Additional nuclear magnetic resonance (NMR) analyses and/or preparation of synthetic standards are commonly performed in conjunction with LC/MS analyses [73,74].

16.2.3 Enzyme Kinetics and Some Practical Considerations

16.2.3.1 Kinetics. In Section 16.2.1, the catalytic mechanism of SULTs was presented. As discussed in this section, published data strongly support a ternary S_N2 inline displacement mechanism. One additional question that has been studied is the exact order of binding and the extent to which reaction products (e.g., PAP) are also

bound. In particular, understanding the binding order offers a mechanistic understanding for the phenomena of substrate inhibition (i.e., lower product formation as substrate concentration is increased). Atypical kinetics (e.g., substrate inhibition and autoactivation) displayed by drug-metabolizing enzymes such as P450s and UGTs can also be observed in SULTs [36,66,76–79]. For example, in the case of human, SULT1E1-catalyzed sulfation of β-estradiol, substrate inhibition was so prominent that it was observed even at concentrations below 100 nM [66].

Early studies by Duffel and Jacoby found that the kinetic binding mechanism of aryl SULT (purified from rat liver) was described by a random Bi Bi mechanism with two dead-end product–inhibitor complexes, which was also consistent with a study conducted by Zhang *et al*. using recombinant human SULT1E1 [66,80]. On the other hand, a sequential/ordered mechanism was proposed to describe kinetics determined in human SULT1A3 as well as SULTs obtained from rat and rhesus monkey liver [81–84]. Regardless of the exact order of binding, enzyme kinetic studies have postulated that substrate inhibition can result from noncatalytic dead-end complexes (e.g., enzyme bound to substrate and PAP).

However, formation of dead-end complexes is not the only hypothesis that can rationalize substrate inhibition kinetics. A second mechanism involves more than one substrate bound simultaneously to the active site. This kinetic mechanism was first proposed by Korzekwa *et al*. [76] to rationalize cytochrome P450 3A4 atypical kinetics. Active site occupation by multiple substrates results in a heterologous population of enzyme: Free enzyme (E), enzyme bound with a single substrate (ES), and enzyme bound with multiple substrates (ESS, etc.). The amount of each complex is dependent on the concentration of substrate and the relative substrate binding affinities for each complex. When the catalytic properties of ES differ from those of ESS (e.g., k_{cat} or rate of product release), atypical kinetics can be observed. In the SULT literature, evidence for the plausibility of this mechanism was found when a crystal structure of SULT1A1 was found to contain two molecules of 4-nitrophenol bound in the active site simultaneously [36]. These investigators postulated that overall rate of product formation from ESS was less than ES. Since ESS was formed at higher concentrations of substrate, the product formation curve displayed substrate inhibition as 4-nitrophenol concentration increased.

In addition, when SULT2A1 was cocrystallized with DHEA, two binding orientations were shown to exist for this substrate [85]. One binding orientation was consistent with a catalytic orientation (ES_1), while the other binding site penetrated further into the active site (ES_2); the authors postulated that this orientation might give rise to substrate inhibition [85]. Although different from multiple substrates within one active site, this mechanism would also result in two populations of bound enzyme (ES_1 and ES_2) with different product formation rates. Thus, as the concentration of substrate increases, it is expected that the ES_2 complex (in this case with reduced product formation) would be formed preferentially at higher concentrations of substrate, and consequently, at higher concentrations, substrate inhibition would characterize the product formation curve. On the basis of a kinetic argument, Gamage *et al*. [79] disputed this idea and proposed that the proportion of ES_1 and ES_2 complexes would most likely stay constant as substrate concentration increased and thus would not result in substrate inhibition. Finally, the stoichiometry of β-estradiol binding to human SULT1E1 was found to be 2:1 for each homodimer [66]. In this report, investigators noted that the binding of two molecules of estradiol to each

SULT1E1 subunit suggested that one of the binding sites was a catalytic site, whereas the other site could potentially be allosteric and regulate the turnover of the enzyme.

The allosteric binding site hypothesis was supported by studies with celecoxib, which in *in vitro* could modulate the stereoselectively of ethinyl estradiol (EE) sulfation [86]. Celecoxib was first shown to cause product switching in SULT2A1-catalyzed sulfation of EE and then later with β-estradiol (E2) (from the 3-*O*-position to the 17β-*O*-position) [86,87]. Detailed kinetics studies revealed that increasing concentrations of celecoxib decreased the V_{max} of EE 3-*O*-sulfation by three- to fourfold (without changing the K_m value significantly) and also increased the V_{max} and decreased the K_m for 17β-*O*-sulfate formation by seven- and eightfold, respectively [86]. In addition, the inhibitory effect of celecoxib on EE 3-*O*-sulfation, as indicted by IC_{50} values, was independent of the EE concentrations. Increasing EE concentrations decreased the apparent kinetic constant for celecoxib (as indicated by EE 17β-*O*-sulfation), suggesting that the formation of EE complex does not inhibit the binding of celecoxib. Collectively, these results lead to the conclusion that EE and celecoxib interacted with separate binding sites of SULT2A1 and the site bound by celecoxib could modulate the activity and regioselectivity of EE sulfation.

16.2.3.2 Practical Considerations. In a study conducted by Maiti *et al*. [88], it was shown that human SULT1E1 was inhibited in a time- and concentration-dependent manner by oxidized glutathione (GSSG) and that the redox status of Cys83 was implicated in affecting the enzyme activity. In this study, the presence of PAPS was found to have a protective effect against GSSG oxidative inactivation [88]. Moreover, these investigators also found that SULTs 1A1, 1A3, and 2A1 were not affected by redox conditions. Thus, it would appear that depending on the isoform, and perhaps the substrate, the redox state of key active site cysteine(s) may or may not influence SULT activity. Although the *in vivo* significance of this remains to be determined, the implications for *in vitro* experiments are clear. Investigators performing *in vitro* studies should be cautious to maintain reduced incubation/storage conditions, so as to more closely mimic the standard *in vivo* redox state.

Organic solvents have also been demonstrated to modulate SULT-mediated reactions [72]. In bench-top incubations, the presence of 0.4% (v/v) acetonitrile-activated SULT1E1-catalyzed 1-hydroxypyrene sulfation by ~2.6-fold. When a two-site kinetic model was applied, studies demonstrated that acetonitrile affected V_{max1}, V_{max2}, and the K_i value of 1-hydroxypyrene sulfation, while there was no significant effect on the K_m value (the second binding site was postulated to be inhibitory and the binding constant was termed K_i). This suggests that solvent may potentially alter binding interactions of the second substrate molecule but not the first. Of course, there are other potential interpretations for this data; however, given the potential effects of organic solvents on SULT activities, it was recommended that ethanol should be used as the preferred solvent vehicle [72].

Divalent cations also have been shown to affect the activity of SULT enzymes. SULT enzymatic activity may be stimulated in the presence of both Mn^{2+} and Mg^{2+}. Using point-mutated SULT1A3 enzyme, Pai *et al*. [89] demonstrated that Mn^{2+} may exert a stimulatory effect by interacting with Glu86. Zhang *et al*. [66] found that Mg^{2+} stimulated SULT1E1 in a bell-shaped manner. Unfortunately, these findings were not supplemented with additional experimental work. In addition to SULTs, other drug-metabolizing enzymes are also affected by divalent cations. It has been recommended

that CYP3A4 incubations include Mg^{2+}, and in particular, since the free concentration of Mg^{2+} in liver is ~0.5 mM, it was suggested that incubations with CYP3A4 should include Mg^{2+} at a similar concentration [90]. By analogy, it may be necessary to include divalent cations in SULT incubations. However, the significance of cation inclusion, in terms of improving the ability to extrapolate *in vitro* data to *in vivo*, is not yet known.

Finally, careful attention to the amount of PAPS used in laboratory incubations is important. For example, the inhibition of 1-hydroxypyrene sulfation (using expressed SULT1E1) by mefenamic acid revealed that different inhibitory IC_{50} values could be obtained when different concentrations of PAPS were used in the incubation (data not published). In addition, when Kudlacek *et al*. [91,92] studied the sulfation of minoxidil, they reported a K_m value of 2.9 mM using a PAPS concentration of 0.4 μM. In human platelets, Johnson and Baker [93] studied minoxidil sulfation and reported data using a concentration of 0.86 μM PAPS. The K_m value for PAPS has been reported to range from 0.16 to 0.66 μM. However, the amount of PAPS in liver has been estimated to be ~17–77 nmol/g of tissue, or much higher than the concentrations used in the studies referenced above [33,37,66,94,95]. Saturation conditions for PAPS would be expected to be four to five times the K_m value or ~0.8–3.3 μM. *In vivo*, it would appear that normal PAPS concentration is at a saturating level. Therefore, in laboratory incubations, it would be prudent to also use saturating PAPS concentrations to more closely estimate a relevant *in vivo* concentration and to avoid confounding kinetic results obtained when PAPS concentrations are no longer sufficiently above the K_m value. Moreover, it is important to ensure that sufficient amount of PAPS is used in kinetics experiments such that saturation of product formation observed at high substrate concentrations (e.g., 10 mM) is not an artifact caused by PAPS depletion.

16.2.4 Sulfation of Endogenous Substrates

In Section 16.1.4, the role of SULTs in fetal development was briefly discussed. In this section, other endogenous roles for SULT are explored. One such role is the sulfation of catecholamines. Catecholamines are mediators of physiological stress, and they prepare the body for physical activity. Some physiological responses include increased heart rate, blood pressure, and an increase in blood glucose levels [96]. Circulating levels of catecholamines, such as dopamine, are present primarily in the sulfated form and derive from the sulfation of dietary dopamine or sulfation of dopamine from the myenteric plexus [97]. In fact, the total circulating concentration of free dopamine was found to be less than 4% [98]. This observation is consistent with early experiments by Richter [99], who showed that the majority of orally administered epinephrine was excreted in urine as a sulfate conjugate. Also consistent is the observation that SULT1A3 is expressed at high levels in the intestinal tract (Section 16.1.3), which would contribute significantly to sulfated dopamine derived from the myenteric plexus.

The high level of SULT1A3 expressed in the intestinal tract has been suggested to serve as a "gut–blood" barrier [100]. This hypothesis is supported by the observation that fetal livers contain high levels of SULT1A3 levels, but that after birth (when exogenous dopamine levels will be obtained through the gastrointestinal (GI) tract) the expression of SULT1A3 is "turned off" in the liver [101]. Thus, while fetal livers contain significant SULT1A3 activity, adults do not. The capacity of SULT activity in

the GI tract appears to form a mechanism for detoxifying dietary biogenic amines in postnatal life, whereas the fetal liver takes on this role in prenatal life.

The two most abundant steroid sulfates present in plasma are DHEA and cholesterol. Blood concentrations of both can achieve low micromolar levels; however, unlike DHEA, cholesterol sulfate levels remain relatively constant throughout life [102]. Cholesterol sulfate is perhaps one of the more interesting sulfate conjugates because its physiological role and importance have been extensively studied. Initially, cholesterol sulfate was studied in the context of serving as a precursor for the synthesis of sulfonated steroids. The sulfate conjugate is a direct substrate for the synthesis of other adrenal steroids such as pregnenolone sulfate and DHEA sulfate, without removal of the sulfate moiety [103,104]. Moreover, cholesterol sulfate has also been shown to be a normal constituent of biological membranes (where it acts as a stabilizer) and a regulator of the activity of a number of enzymes (e.g., activating Factor XII, epidermal protein kinase C enzymes, etc.) [97,105,106]. Perhaps the most fascinating role that the sulfate conjugate serves is its involvement in keratinocyte differentiation and development of the epidermal barrier. Cholesterol sulfate conjugate is formed in the basal and spinous layer of the epidermis; these two layers are generally the lowest of the four to five skin layers that compose the epidermis [107,108]. Cholesterol reaches its highest concentration in the granular layer (third layer from the surface) and then concentrations decrease in the stratum corneum (first layer) because of increased sulfatase activity. Thus, sulfated cholesterol begins its journey through the epidermis in the lower layers, and as keratinocytes start to differentiate and migrate out to the surface, cholesterol sulfate is hydrolyzed by steroid sulfatase releasing free cholesterol, which is then available for conjugation in lower layers [107,108]. It has been hypothesized that this cycle plays an important role in normal desquamation (skin peeling or shedding) [109]. This idea is supported by the fact that patients with recessive X-linked ichthyosis, caused by steroid sulfatase deficiency, have difficulty (among other symptoms) with desquamation [110]. Finally, it is interesting that cholesterol is a substrate for SULT2B1_v2 (a splice variant of the *SULT2B1* gene) and that SULT2B1_v2 is significantly expressed in the granular layer, which, noted above, is the layer where cholesterol sulfate reaches the highest concentration [111].

16.2.5 Bioactivation

In the fields of drug metabolism and toxicology, bioactivation of compounds is a crucial topic that has been studied extensively [112,113]. The majority of the bioactivation processes are caused by cytochrome P450-mediated reactions that generate chemically reactive species, potentially leading to various types of toxicity. It has been shown that oxidative metabolites containing electron-deficient groups, such as epoxide and quinone/quinone imine/quinone methide, can form DNA adducts and eventually cause mutagenicity and genotoxicity [114–116]. Sulfation, as it turns out, is another bioactivation mechanism that can generate mutagenic products [117,118]. Sulfate conjugates of *N*-hydroxy aromatic amines (e.g., *N*-hydroxy-acetylaminofluorene) undergo N–O cleavage to form reactive nitrenium ions (Fig. 16.3), resulting in subsequent formation of DNA adducts and gene mutations in bacteria and mammalian cells [119,120]. Similarly, carbocations have been identified to be the reactive species generated from sulfation products of polycyclic benzylic alcohols (e.g., 1-hydroxymethyl pyrene) and allylic alcohols (e.g., 1′-hydroxyestragole) [121,122]. Note that the positive charge of

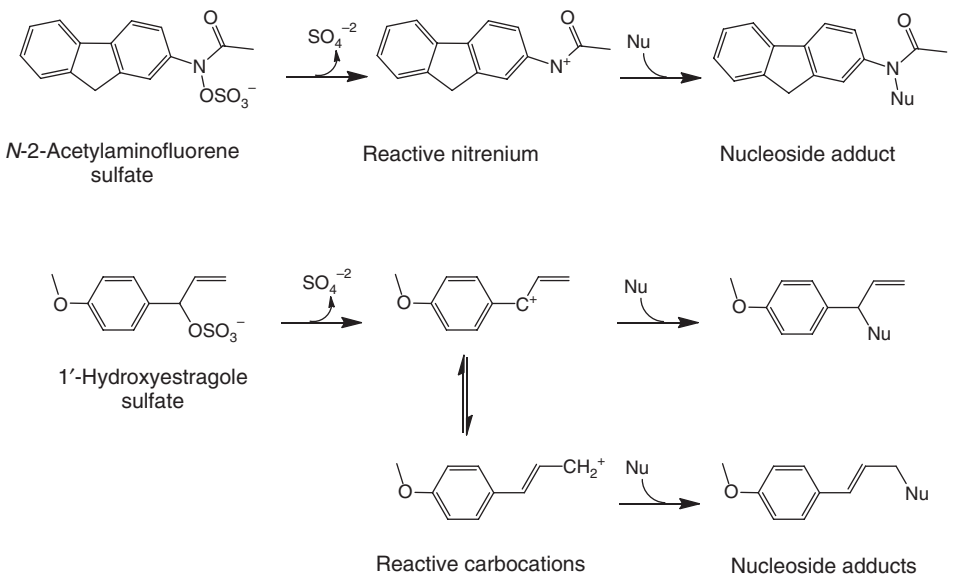

Figure 16.3 Bioactivation of sulfonate conjugates via the formation of reactive nitrenium and carbocation intermediates. Nu, nucleoside.

carbocation/nitrenium is stabilized by the conjugated system as evidenced by DNA adducts with alternative adduction sites arising from resonance structures. Mutagenicity observed from *in vitro* systems has been shown to be compound-dependent and SULT isoform-dependent [117,118]. It has been hypothesized that tissue expression and genetic polymorphism of a particular SULT isoform may influence the genotoxic effects of a given promutagen or procarcinogen. Further evaluations using humanized animal models have been proposed to gain insights on this matter.

16.3 CLINICAL PERSPECTIVES

16.3.1 Polymorphisms

Pioneer studies conducted by Eichelbaum *et al*. and Mahgoub *et al*. in the 1970s illustrated that the reduced metabolism of sparteine/debrisoquine was caused by deficiency of a single gene (now known as *CYP2D6 polymorphism*) [123]. Since then, genetic polymorphisms have been studied for CYPs [124] as well as other drug-metabolizing enzymes such as glutathione *S*-transferases [125], *N*-acetyltransferases [126], UDP-glucuronosyltransferases [127,128], and SULTs [18,129–134].

Polymorphism of SULT1A1 is the most extensively studied among various SULT enzymes. A number of single nucleotide polymorphisms (SNPs) have been identified for *SULT1A1* gene, however, only a few of these SNPs are located at the coding region and affect the amino acid sequence (nonsynonymous cSNPs). *SULT1A1*2* allele, the most common allelic variant other than *SULT1A1*1* allele (wild type), has a G→A mutation at nucleotide 638 in the coding region, resulting in an arginine to histidine substitution at amino acid 213 (Arg213His). By measuring SULT1A1 activity

in human platelet cells with 4-nitrophenol as a probe substrate, individuals homozygous for the *SULT1A1*2* allele were shown to have ~15% of the enzyme activity of *1A1*1/1A1*2* heterozygotes or *1A1*1* homozygotes [132]. The lower enzyme activity of SULT1A1*2 allozyme appears to result from, at least in part, preferential degradation via the proteasome pathway as compared to SULT1A1*1 [133]. The second most common SULT1A1 SNP is A667G, leading to amino acid substitution Met223Val. This variant is termed SULT1A1*3. Kinetics studies showed that SULT1A1*3 allozyme generally exhibits lower K_m and higher V_{max} values for various substrates when compared to the wild-type enzyme. Ethnic variation in allele frequencies for *SULT1A1*2* and *SULT1A1*3* has been reported [130,134]. For *SULT1A1*2*, the frequency was higher in Caucasian (0.254–0.365), African (Nigerian and African-American; 0.269–0.294), and Turkish (0.238) than Japanese (0.168), Chinese (0.045–0.110), Korean (0.116), and Egyptian (0.135) populations. Interestingly, *SULT1A1*3* allele is highly prevalent in African-Americans (frequency 0.229). It is detected in low frequency in Caucasians (0.000–0.029) and is virtually absent in other ethnic groups.

Nonsynonymous SNPs have also been identified for SULT1E1 and SULT2A1 [18,129–131]. Three SNPs (G64T, C95T, and C758A) were observed in *SULT1E1*, resulting in amino acid changes Asp22Tyr, Ala32Val, and Pro253His, respectively. These variants appear to be very rare (allele frequency 0.008) in Caucasians and African-Americans combined. Although the Val32 (SULT1E1*2) allozyme has similar affinities for β-estradiol and cofactor PAPS relative to the wild-type allozyme, the K_m values determined for Tyr22 and His253 allozymes are significantly higher. For SULT2A1, nonsynonymous polymorphisms (A63P, K227D, and A261T) have been identified in African-Americans but not in Caucasian Americans or Japanese. Allozymes expressed in COS cells generally showed decreased enzyme activities and lower immunoreactive protein levels. Surprisingly, the Tyr261 allozyme, resulting from a G→A mutation at nucleotide 781, appears to be a monomer rather than dimer. Despite that difference, the Tyr261 and the wild-type allozymes display comparable affinities for DHEA and PAPS. The allelic frequency for the G781A variant is 0.101–0.144 in different African-Americans populations.

It is important to point out that polymorphisms observed in SULTs have not been reported to significantly alter the elimination of drugs [135,136]. This is likely due to the fact that virtually all drugs undergo sulfation also having alternative elimination pathways such as direct excretion, oxidation, and glucuronidation.

16.3.2 Induction

Many xenobiotics act as perpetrators in pharmacokinetics drug–drug interactions by inducing drug-metabolizing enzymes, which results in an increased elimination of the victim compounds [137,138]. Elevation of drug-metabolizing enzymes usually begins at the transcriptional level when nuclear receptors are activated by the binding of chemical inducers. In the case of CYP enzymes, nuclear receptors such as aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), constitutive androstane receptor (CAR), glucocorticoid receptor (GR), peroxisome proliferator-activated receptor (PPAR), liver X receptor (LXR), and farnesoid X receptor (FXR) have been shown to regulate enzyme expression levels. *In vitro* studies conducted in primary human hepatocytes indicated that activation of AhR, PXR, CAR, LXR, and FXR did not significantly induce mRNA expression of various SULTs [139–141]. In fact, repression of gene expression was

observed for *SULT1E1* and *SULT2A1* in human hepatocytes treated with rifampicin [141,142], a known ligand for human PXR. Such repression was shown to be mediated by hepatocyte nuclear factor 4α. Human hepatocytes treated with dexamethasone and ciprofibrate, prototypical ligands for GR and PPARα, respectively, led to increases in SULT2A1 mRNA and protein levels [140,143]. However, the clinical significance of SULT2A1 induction remains to be determined.

16.3.3 Reaction Phenotyping

Identification of drug-metabolizing enzymes responsible for the biotransformation of a new drug (new chemical entity, NCE) is a necessary and pivotal study in the drug discovery and development process. The results of these studies, usually referred to as *reaction phenotyping studies*, are utilized to evaluate several issues: the potential of the NCE to be involved in a potential drug–drug interaction via inhibition of the enzyme(s) responsible for NCE metabolism; evaluation of the effect of induction on the NCE metabolism; and finally, the possible effects of genetic polymorphism of NCE metabolism. Although the historical focus of reaction phenotyping studies has focused on CYP enzymes, there is an increasing effort in recent years to characterize biotransformations catalyzed by non-CYP enzymes such as SULTs [29,70,75,144,145].

It has been well established for CYP enzymes that the reaction phenotyping studies generally consist of (i) correlation analyses utilizing a bank of individual tissue preparations characterized for enzyme activities; (ii) kinetic determinations with recombinant enzymes and tissue preparations; and (iii) inhibition experiments using isoform-selective chemical inhibitors and/or antibodies [146]. In the authors' experience, the last method is by far the most commonly used in industry. Attempts have been made to adopt these approaches for SULT enzymes; however, the success appears to be handicapped by limited knowledge and/or reagents available.

Correlation analysis involves the comparison of the interindividual variability of the sulfation rate of interest with the previously determined activity or protein level of a given SULT isoform. Hence, in order to determine SULT isoform enzymatic activity, the availability of SULT-isoform-selective probe substrates is vital to the correlation approach. Reasonably good correlation between protein expression and enzyme activity was observed for SULT1A1, SULT1E1, and SULT2A1 (using 4-nitrophenol, β-estradiol, and DHEA as probe substrates, respectively) in human liver cytosol ($r \sim 0.64–0.86$) [43]. However, a selective probe substrate for SULT1B1 has not been identified to-date. Apart from this, the fact that SULTs usually have overlapping substrate specificities [70,145,147] make correlation studies challenging and the results difficult to interpret. As such, the correlation approach has not been widely used for SULT phenotyping.

Screening of sulfate conjugation with recombinant SULT enzymes has been commonly employed as the initial approach, facilitated, at least in part, by the commercially available SULT enzymes (SULT1A1, SULT1A3, SULT1E1, and SULT2A1) representing the majority of isoforms detected in human liver and GI tract [43]. Kinetic studies are then conducted in recombinant enzymes and in tissue preparations to determine the affinity for the substrate (K_m) and the type of kinetics (e.g., hyperbolic vs substrate inhibition). Since the substrate affinity for a single enzyme should be similar regardless of the source/preparation (ideally), comparison of the K_m values determined in recombinant enzymes with that obtained in tissue preparations provide additional evidence

in identifying the SULT enzyme(s) responsible for the sulfation reaction observed in tissue.

Inhibition studies represent a primary methodology for reaction phenotyping. In particular, isoform-selective chemical inhibitors and/or immunoinhibitory antibodies allow direct quantitative assessment of the contribution of individual isoforms at a given drug concentration (often the concentration used is physiologically relevant), as exemplified in a countless number of CYP-mediated biotransformations. Unfortunately, isoform-selective inhibitory antibodies for SULTs are not yet commercially available. 2,6-Dichloro-4-nitrophenol (DCNP) is a potent inhibitor of SULT1A1 (IC_{50} < 0.1 μM) with selectivity of more than 2 orders of magnitude over SULT1A3, SULT1B1, SULT1E1, and SULT2A1 [144,148–150]. The compound is suitable to be utilized as a SULT1A1-selective inhibitor in human liver and GI preparations. Apart from DCNP, there has not been any other isoform-selective inhibitor reported for SULTs. It has been suggested that an ortho-hydroxy-polychlorinated biphenyl (6′-OH-PCB) could potentially serve as a specific inhibitor of SULT1B1 [151]. However, inhibition results in this study may have been confounded by substrate inhibition, poor inhibitor/substrate solubility, as well as the use of a substrate concentration (3-hydroxybenzo[*a*]pyrene) that was ~6-fold greater than the reported K_m value for SULT1A1*1, and ~20-fold lower than the reported K_m for SULT1B1 [152]. Because 6′-OH-PCB may serve as an isoform-selective inhibitor, further experiments to confirm the inhibition of SULT1B1 would be useful. In addition, compounds such as quercetin and estrone could also serve as probe inhibitors but further experiments are needed [75,144]. At 1 μM, quercetin strongly inhibited sulfation activities catalyzed by recombinant SULT1A1 and SULT1E1 but had no effect on SULT1A3 and SULT2A1 activities. Similarly, estrone (at 40 nM) greatly inhibited (>75%) SULT1A3 and SULT1E1 activities but had minimal effect on SULT1A1 and SULT2A1 activities. Caution should be exercised when interpreting inhibition results obtained with quercetin and estrone as their inhibitory effects on SULT1B1 and other SULT enzymes have not been examined. Overall, reaction phenotyping for SULTs can be performed to some extent, and the quality will certainly improve when additional reagents become available in the future.

16.3.4 Drug–Drug Interactions

A large number of *in vitro* studies have been published, which suggest potential *in vivo* SULT drug–drug interactions. However, there are few confirmed *in vivo* drug interactions that are mediated by this class of enzymes. Moreover, as noted above, polymorphisms affecting SULT activity (which can mimic chemical inhibition) have not been clearly associated with significant changes in drug elimination [135,136].

The most commonly published example of a SULT-mediated drug–drug interaction involves an indirect mechanism. In humans, a single dose of acetaminophen (1.5 g) has been shown to cause partial depletion of inorganic sulfate [153]. This observation is relevant to SULT drug–drug interactions because the level of inorganic sulfate affects the level of PAPS [154,155]. The activity of all SULT reactions depend on the availability of PAPS as a cofactor, which in turn is dependent on the rate of PAPS synthesis and degradation [156]. Cellular uptake of inorganic sulfate depends on two uptake mechanisms: one is Na^+ independent and the other is Na^+ dependent [157–159]. Once uptake has occurred within the cell, PAPS is synthesized from inorganic sulfate by what is thought to be a bifunctional protein, associated with ATP-sulfurylase and adenosine

5′-phosphosulfate kinase activities [160]. Thus, indirect drug interactions can occur through a variety of mechanisms. To illustrate one mechanism, the concomitant administration of acetaminophen and a compound that undergoes significant sulfation can potentially lead to a competitive interaction in which a capacity-limited pool of PAPS is depleted by sulfation of one or both drugs [161–163]. In addition, other mechanisms may also include the inhibition of transporters involved in the cellular uptake of inorganic sulfate, or inhibition of PAPS synthase itself [156]. In the case of acetaminophen where doses are high (on a milligram per kilogram basis), competition for and depletion of the PAPS pool has been hypothesized as the mechanism behind interactions with fenoldopam and EE [164,165]. However, a study conducted in healthy male volunteers showed that codosing acetaminophen with troglitazone resulted in no statistically or clinically relevant differences in troglitazone exposure area under the plasma drug concentration-time curve ($AUC_{0-\infty}$) [166]. Troglitazone is metabolized to a sulfate conjugate, a glucuronide conjugate, and an oxidative quinone metabolite. Under steady state conditions, the AUC of the sulfated metabolite was 7–10 times greater than that of parent, whereas quinone exposure was similar to parent. The glucuronide was a minor metabolite (20–40% of parent) [167]. Thus, sulfation represented a major pathway for troglitazone metabolism, yet the coadministration of acetaminophen did not affect exposure. In fact, when troglitazone and acetaminophen were concurrently administered only a slight decrease in the troglitazone sulfate metabolite was observed; the decrease was not judged to be clinically relevant [166].

In addition to an indirect mechanism, SULT-mediated drug–drug interactions may result from a more traditional mechanism where a single SULT serves as the locus of the interaction. One example of this is the interaction of etoricoxib with EE. EE is a primary active component used in many oral contraceptive (OC) formulations; the metabolism of EE is complex, involving cytochrome P450, glucuronosyltransferases and SULT [168]. The absolute oral bioavailability of EE is variable and ranges from 20% to 65% [169]. In urine, the major metabolites excreted are glucuronides (up to 85%) followed by sulfate conjugates (up to 19%) and then low levels of monooxidation products [170]. Because more than one enzymatic pathway is involved in EE metabolism it would expected that EE should be relatively resistant to drug–drug interactions. However, EE is subject to significant gut first-pass metabolism, and it has been shown that up to 38% of the EE dose forms conjugates in human jejunal mucosa and ~90% of the conjugates can be hydrolyzed by sulfatase [171]. These results imply that sulfation of EE is considerable in the GI tract and may be a major factor affecting bioavailability. As a consequence, it has been postulated that inhibition of SULTs in the GI can serve as a locus for drug–drug interactions, affecting the bioavailability of EE [172]. In support of this hypothesis, acetaminophen was orally administered 1 h before OC administration and subsequently, the pharmacokinetics of EE demonstrated that AUC_{0-24} was increased 22% [165]. It was hypothesized that the bioavailability of EE increased in response to depletion of the PAPS pool. Interestingly, although the investigators in this study did not report C_{max} values of EE, the C_{max} appears to have increased 60–70%. This increase in C_{max} was consistent with the hypothesis of lower intestinal SULT activity, which then resulted in increased absorption from the gastrointestinal lumen.

In an example of a direct drug–drug interaction caused by SULT inhibition, etoricoxib has been reported to inhibit EE sulfation activity catalyzed by SULT1E1, the major SULT isoform catalyzing the sulfation of EE [144]. It was further hypothesized

that after an oral dose, the high local intestinal exposures of etoricoxib could interfere directly with first-pass sulfation of EE via inhibition of SULT1E1. Evidence for this inhibition was published later by an *in vivo* drug–drug interaction study involving EE and etoricoxib. Data presented within this report show that coadministration of EE with etoricoxib was associated with a 50–60% increase in EE exposure, and a decrease of EE-sulfate exposure by ~40% [173].

16.4 CONCLUSIONS AND FUTURE PROSPECTS

SULTs are an important class of enzymes and are involved in many endogenous processes such as estrogen biosynthesis, influencing receptor-active thyroid hormone levels and sulfation of catecholamines and steroids (e.g., DHEA or cholesterol). For drug metabolism scientists, sulfation is of interest because it can potentially lead to electron-deficient species, and therefore, it is important to be aware of potential structural alerts that may increase the risk of SULT-mediated reactive intermediates. However, relative to other drug-metabolizing enzymes, SULT-mediated elimination of therapeutic compounds is rather limited; for this reason, and others, the contribution of SULTs to drug metabolism has been underappreciated, particularly in industry. With increased availability and use of high quality hepatocytes, the importance of SULTs to drug metabolism will be become more evident in the future. As a result, drug metabolism scientists will be faced with many challenges. Foremost is the lack of a "chemical toolbox" to study SULT isoform involvement in drug elimination. While reaction phenotyping is readily accomplished with cytochrome P450s, there is no panel of well-characterized SULT-isoform-specific inhibitors. During the course of determining the mechanism for a clinical drug–drug interaction of etoricoxib with ethinyl estradiol, the authors of this chapter screened several hundred compounds in an effort to obtain specific inhibitors for a number of SULT isoforms (unpublished). This effort was largely unsuccessful. Thus, approaches other than screening (such as rational design from crystallography/computational chemistry) may have to be utilized to efficiently generate isoform-specific inhibitors.

The literature contains several examples of isoform-specific or isoform-selective SULT substrates, but the characterization of those substrates against all the relevant SULT isoforms is often lacking. As a consequence, the study of SULT inhibition and/or induction is hampered by the ambiguity surrounding the use of "probe" substrates. In the future, the discovery, development, and characterization of a panel of isoform-selective inhibitors and substrates will greatly increase the success of metabolism studies during drug discovery and development. Increased success in this area will also serve to further highlight the importance of this class of enzymes in drug metabolism [97,98].

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