A Dissertation

entitled

Investigation of the Effect of Genetic Polymorphisms on the Functional Characteristics of the Human Cytosolic Sulfotransferase 1C4 (hSULT1C4)

by

Saud Abdullah Gohal

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Doctor of Philosophy Degree in

Experimental Therapeutics (Pharmacology/Toxicology)

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July 2021

An Abstract of

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Sulfate conjugation is known to play a crucial role in the metabolism of low-molecular weight xenobiotics, including drugs, as well as endogenous compounds such as catecholamines, thyroid and steroid hormones, and bile acid. The responsible enzymes, called the cytosolic sulfotransferases (SULTs), act as catalysts in mediating the transfer of a sulfonate group from the donor 3-phosphoadenosine-5-phosphosulfate (PAPS) to substrate compounds containing hydroxyl or amino groups. The primary objective of this study is to determine the effect of single nucleotide polymorphisms (SNPs) of the human *SULT1C4* gene on the sulfation of selected endogenous substrates and drug compounds by SULT1C4 allozymes. In preparation for this study, a broad database search for *SULT1C4* single nucleotide polymorphisms (SNPs) was conducted, and ten missense *SULT1C4* SNPs were selected. cDNAs were then generated in correspondence to the selected missense *SULT1C4* SNPs using site-directed mutagenesis.

Thereafter, recombinant SULT1C4 allozymes were bacterially expressed and purified. The purified SULT1C4 allozymes were evaluated for their sulfating activities toward a panel of compounds that act as SULT1C4 substrates, including 4-nitrophenol (4-NP), doxorubicin, acetaminophen, dextrophan, *O*-desmethylquinidine (ODM-quinidine), *O*-desmethylquinine (ODM-quinine), estrone (E1), and estradiol (E2). The results showed clearly that SULT1C4 allozymes exhibited differential sulfating activities towards the different substrates tested. Collectively, the study presented shed substantial light on the potential of *SULT1C4* genetic polymorphisms to affect the sulfating activity of corresponding allozymes. This information may help elucidate a more robust understanding of the therapeutic efficacy and toxicity of drugs and other exogenous compounds in individuals with different *SULT1C4* genotypes. Furthermore, *SULT1C4* SNPs may impact the biotransformation of other substrates, such as E1 and E2 hormones, and may affect disease processes associated with abnormal estrogen levels.

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List of Abbreviations

AAD.....IA antiarrhythmic drug
ADP.....Adenosine-5'-Diphosphate
AML ..... Myeloblastic leukemia
APS .....Adenosine-5'-Phosphosulfate
ATP .....Adenosine-5'-Triphosphate

ccRCC .....Clear cell renal cell carcinoma
cDNA .....Complementary Deoxyribonucleic Acid
CHES .....Sodium acetate, 2- (Cyclohexylamino) Ethanesulfonic Acid
COMT .....Catechol *O*-Methyl Transferase
CYP .....Cytochrome P-450

DHEA .....Dehydroepiandrosterone
DMSO .....Dimethyl Sulfoxide
DNA .....Deoxyribonucleic Acid
DTT .....Dithiothreitol

E1 .....Estrone
E2 .....Estradiol
E. coli .....*Escherichia Coli*
EDTA.....Ethylenediamine tetraacetic acid
ERs.....Estrogen receptors

GIT..... Gastrointestinal Tract
Glu/E .....Glutamic acid
GST.....Glutathione S-Transferases

HEPES .....N-2-hydroxylpiperazine-N′-2-ethanesulfonic acid
HSTs.....Hydroxysteroid sulfotransferases

IPTG.....Isopropyl β-D-1-Thiogalactopyranoside

kDa.....Kilodalton

LB .....Luria Broth

NATs .....N-Acetyltransferases
NASH .....Nonalcoholic steatohepatitis
NCBI .....National Center for Biotechnology Information
4-NP 4-Nitrophenol

ODM ……*O*-Desmethyl
OD600 nm .....Optical Density at 600 nm Wave Length

PAPS .....3'-phosphoadenosine-5'-phosphosulfate
PCR .....Polymerase Chain Reaction
PPi.....Pyrophosphate
PSB ..... 5'-Phosphosulphate-Binding
PSTs .....Phenolic Sulfotransferases

SDS–PAGE .....Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
SNPs .....Single Nucleotide Polymorphisms
SULT1A1 .....Human Cytosolic Sulfotransferases Family 1A Member 1
SULT1A2 .....Human Cytosolic Sulfotransferases Family 1A Member 2
SULT1A3 .....Human Cytosolic Sulfotransferases Family 1A Member 3
SULT1B1 .....Human Cytosolic Sulfotransferases Family 1B Member 1
SULT1C2 .....Human Cytosolic Sulfotransferases Family 1C Member 2
SULT1C3 .....Human Cytosolic Sulfotransferases Family 1C Member 3
SULT1C4 .....Human Cytosolic Sulfotransferases Family 1C Member 4
SULT1E1 .....Human Cytosolic Sulfotransferases Family 1E Member 1
SULT2A1 .....Human Cytosolic Sulfotransferases Family 2A Member 1
SULT2B1a .....Human Cytosolic Sulfotransferases Family 2B Member 1a
SULT2B1b .....Human Cytosolic Sulfotransferases Family 2B Member 1b
SULT4A1 .....Human Cytosolic Sulfotransferases Family 4A Member 1
SULT6B1 .....Human Cytosolic Sulfotransferases Family 6B Member 1
SULTs .....Human Cytosolic Sulfotransferases enzymes

TLC .....Cellulose Thin-Layer Chromatography

UGTs.....Uridine 5'-Diphospho-Glucuronosyltransferases

WT.....Wild-type enzyme

List of Symbols

°C .....Celsius
µl .....Microliter
ml .....Milliliter
L .....Liter
µg .....Mirogram
mg .....Milligram
g.....Gram
µM .....Micromolar
mM .....Millimolar
nmol .....Nanomole
mmol .....Millimole
rpm .....Rate Per Minute
min .....Minute
$SO3^-$.....Sulfonate Group
Km .....Michaelis Constant
Vmax ..... Maximal Velocity
Vmax/*Km*.……Reflects Catalytic Efficiency
β .....Angle of distortion

Chapter 1

Introduction

1.1. Metabolism of Low-Molecular Weight Endogenous Compounds and Xenobiotics

The human body is constantly exposed to a large number of structurally diverse chemicals; ranging from endogenous compounds, including neurotransmitters and hormones, to exogenous chemicals and xenobiotics, such as drugs and toxins. Within the body, metabolizing enzymes biotransform (or metabolize) these chemicals, facilitating their removal and preventing their harmful accumulation in tissues (Parkinson & Ogilvie, 2008). Such enzymes occur at high levels in the gastrointestinal tract (GIT), but are also present at lower levels in most body tissues (Gonzalez & Tukey, 2006). Some metabolizing enzymes have broad specificities, allowing them to biotransform both endogenous molecules and exogenous compounds. Typically, metabolism by these enzymes is a deactivating process. For example, xenobiotics are rendered more hydrophilic forms, allowing them to be more easily removed from the body. In contrast, for some compounds such as procarcinogens and prodrugs, enzymatic processes generate metabolites that have potent biological activities or even toxic properties.

1.2. The Metabolic Pathways Involved in Xenobiotic Biotransformation

With few exceptions, xenobiotic substances are subject to one or more enzymatic reactions within the body; this metabolism eventually results in their elimination through urine and/or bile. During the course of metabolism, lipophilic agents are transformed into more hydrophilic derivatives through two general types of reactions, referred to as Phase I and Phase II reactions (Gonzalez & Tukey, 2006).

1.2.1. Phase I Functionalization Reactions:

Phase I enzymes act to transform lipophilic molecules into more hydrophilic derivatives. These enzymes carry out oxidation, reduction, or hydrolytic reactions that serve to either introduce or expose a polar functional group (e.g. –COOH, -OH ,-NH2, or-SH). The enzymes most commonly implicated in Phase I reactions belong to the cytochrome P450 (CYP) superfamily, which is divided into families and subfamilies (Gonzalez & Tukey, 2006; Parkinson & Ogilvie, 2008). More than 50 of these CYPs have been identified in humans, the majority of which are known contribute to metabolic processes. The CYP2C, CYP2D, and CYP3A subfamilies are among the most active and therefore contribute greatly to metabolism (Danielson, 2002). Of particular note is the liver enzyme CYP3A4, which has been shown to biotransform more than 50% of clinically approved drugs (Danielson, 2002; Gonzalez & Tukey, 2006).

1.2.2. Phase II Conjugation Reactions:

Phase II reactions act to facilitate the elimination of xenobiotics by producing metabolites that are typically highly hydrophilic and, in most cases, biologically inactive. These enzymes carry out conjugation reactions such as glucuronidation, sulfation, and acetylation. Phase II enzymes are classified into main classes: UDP-glucuronosyltransferases (UGTs); cytosolic sulfotransferases (SULTs); catechol O-methyl transferase (COMT); N-acetyltransferases (NATs); methyltransferases; and glutathione S-transferases (GSTs) (Jancova et al., 2010). Most Phase II enzymes localize to the cytosol; the exceptions are UGTs, which localize to the endoplasmic reticulum (Gonzalez & Tukey, 2006). Notably, some Phase II reactions produce metabolites that are pharmacologically active or have increased toxicity (Mulder and Jakoby, 1990). One example is the glutathione conjugation of bromobenzene and hydroquinone, the product of which has enhanced nephrotoxicity (Hinson & Forkert, 1995).

1.3. The role of sulfation in Xenobiotic Biotransformation and Endobiotic Homeostasis

Phase II sulfate conjugation, first discovered by Eugen Baumann in the late 19th century and mediated by SULTs, is a major pathway for the biotransformation of a wide variety of molecules (Baumann, 1876). Generally, sulfation increases the substrate's hydrophilicity and contributes to its inactivation as well as subsequent elimination from the body. Specifically, sulfation reactions involve the transfer of a sulfonate moiety

(SO3-) from 3‘-phosphoadenosine-5‘phosphosulfate (PAPS), the universal sulfate donor, to a nucleophilic moiety in the substrate that contains amino or hydroxyl groups (**Figure 1.1**) (Coughtrie, 2002). This process has been implicated in not only modulating the activity of xenobiotics, but also in the homeostasis of key endobiotics such as catecholamines, cholesterol, neurotransmitters, and hormones (Strott, 2002).

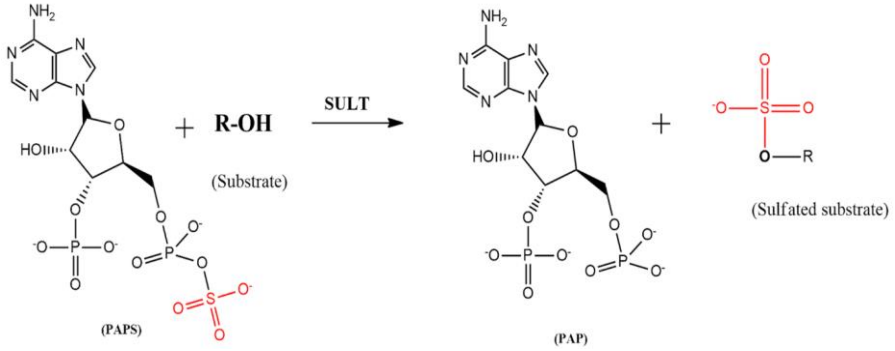

Figure 1.1. Sulfation reaction mediated by human sulfotransferases

The sulfate donor, PAPS, is synthesized in the cytosol by a two-step process (**Figure 1.2**) (Strott, 2002). First, adenosine-5’-phosphosulfate (APS) is produced from adenosine triphosphate by ATP sulfurylase, and then APS is converted into PAPS by APS- kinase.

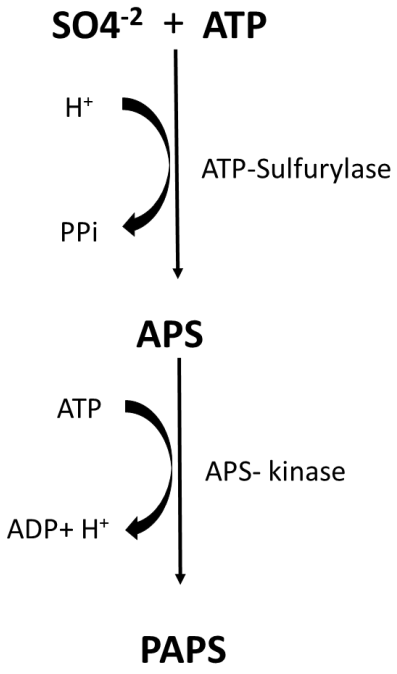

Figure 1.2. Formation of PAPS via two-step process

1.4. Classification of SULTs and Substarte Specificities

Cytosolic SULTs sulfate small molecules such as hormones, drugs, and xenobiotics (**Figure 1.3**) (Weinshilboum & Otterness, 1994)**.** There are a total of 13 cytosolic SULTs, comprising four gene families: SULT1, SULT2, SULT4, and SULT6. Within each family, members share at least 45% amino acid sequence identity; members of sub-families, which are designated by a letter after the family number, share at least 60% sequence identity (Blanchard et al., 2004).

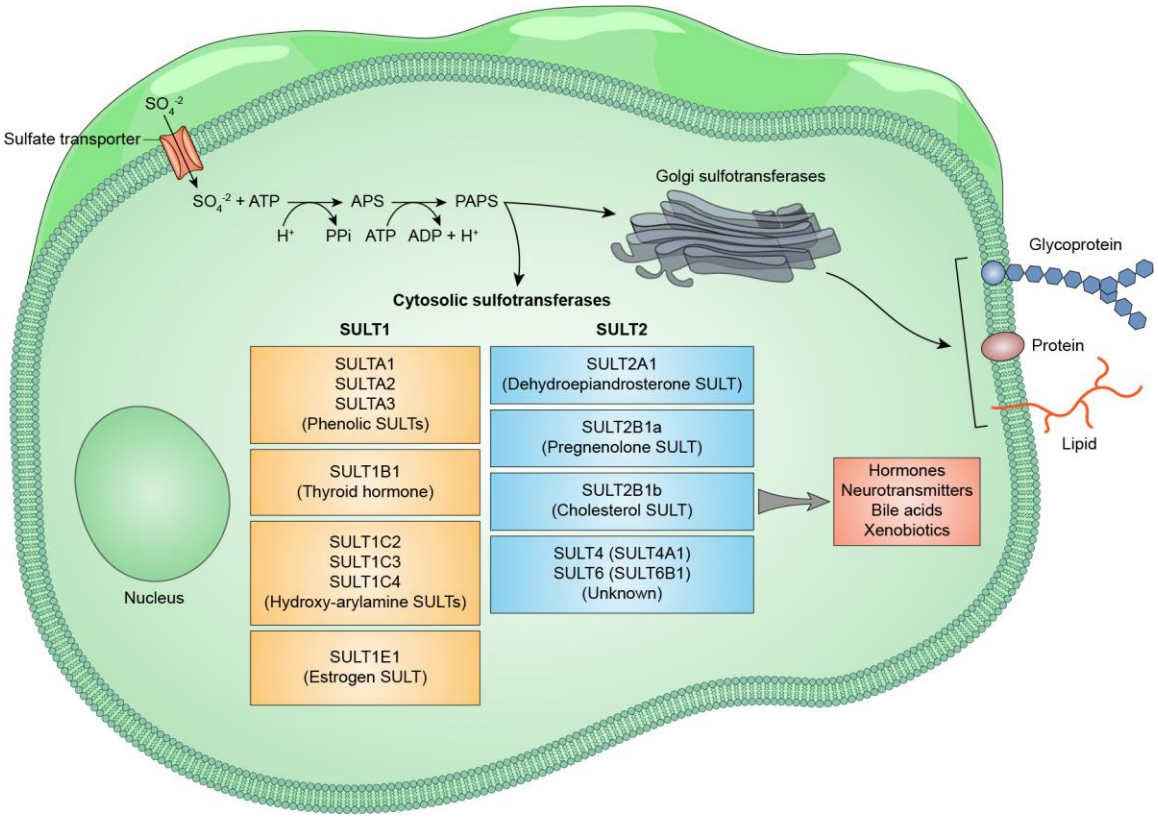

Figure 1.3. The sulfate conjugation pathway.

The SULT1 family, previously referred to as phenolic sulfotransferases (PSTs), have been shown to metabolize phenolic compounds such as estrogens, catecholamines, and thyroid hormones (Lindsay et al., 2008; Tibbs et al., 2015). This family is divided into four sub-families: SULT1A, SULT1B, SULT1C, and SULT1E (Weinshilboum et al., 1997). Genes encoding for the SULT1A family members, SULT1A1, SULT1A2, and SULT1A3, are all located on chromosome 16p11.2-12.1 (Freimuth et al., 2004). SULT1A1 and SULT1A2 are 96% identical and are capable of sulfating the same substrates, which include β-naphthol, 4-nitrophenol, and minoxidil (Gamage et al., 2006;

Ozawa et al., 1995). SULT1A3, also known as catecholamine sulfotransferase, mediates the sulfation of amine neurotransmitters such as dopamine, serotonin, epinephrine, and norepinephrine (Dooley, 1998; Taskinen et al., 2003). The SULT1B subfamily contains only one member, SULT1B1; this enzyme has a high specificity for thyroid hormones, but can also sulfate compounds such as 1-naphthol and 4-nitrophenol (Fujita et al., 1997; Sakakibara et al., 1995; Wang et al., 1998). The SULT1C subfamily, consisting of SULT1C2, SULT1C3, and SULT1C4, sulfates compounds containing a hydroxylamine; these include β-naphthylamine, N-hydroxy-4-aminobiphenyl, and N-hydroxy-2-acetylaminofluorene, as well as endogenous and exogenous estrogenic compounds (Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998). Finally, SULT1E1, also termed the estrogen sulfotransferase, is the most efficient at sulfating estrogenic compounds such as estrone and 17β-estradiol, along with structurally-related xenobiotics (Adjei & Weinshilboum, 2002; Nash et al., 1988).

The SULT2 family, previously termed hydroxysteroid sulfotransferases (HSTs), acts on steroids and hydroxysteroids such as oxysterols, pregnenolone, dehydroepiandrosterone, and cholesterol. Its members are grouped into two subfamilies based on substrate specificity: SULT2A1, the dehydroepiandrosterone sulfotransferase, and SULT2B1, which is further classified into two isoforms, SULT2B1a, pregnenolone sulfotransferase, and SULT2B1b, cholesterol sulfotransferase (Falany & Rohn-Glowacki, 2013; Javitt et al., 2001).

The two remaining families, SULT4 and SULT6, each consist of a single enzyme: SULT4A1 and SULT6B1, respectively (Freimuth et al., 2004). Little is currently known in terms of their tissue distribution and substrate specificity. General information about

all SULT enzymes, such as tissue distribution and representative substrates, is given in **Table 1.1**.

Table 1.1. List of human cytosolic sulfotransferases

Enzyme	No.of Amino Acid	Standard Substrate	Tissue Localization	References
SULT1A1	295	4-nitrophenol	Liver, gastrointestinal tract, brain, platelets, kidney, skin, lung, fetal liver	(Riches et al., 2009; Wilborn et al., 1993)
SULT1A2	295	4-nitrophenol	Liver and stomach	(Her et al., 1996; Teubner et al., 2007)
SULT1A3	295	Dopamine	Gastrointestinal tract, kidney, lung, brain, fetal liver	(Aksoy & Weinshilboum, 1995; Zhu et al., 1993)
SULT1B1	296	Iodothyronines	Liver, stomach colon, rectum, blood leukocytes, kidney	(Fujita et al., 1997)
SULT1C2	296	4-nitrophenol	Fetal liver, fetal kidney, stomach, thyroid, spleen	(Freimuth et al., 2000; Sakakibara et al., 1998)
SULT1C3	304	Thyroid hormones and bile acids	In fetal: liver, spleen, kidney	(Freimuth et al., 2000); (Kurogi et al., 2017)
SULT1C4	302	4-nitrophenol	Breast, ovary, kidney, spinal cord. In fetal: lung, kidney, liver, heart.	(Freimuth et al., 2000; Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998)
SULT1E1	294	17β-estradiol	Reproductive organs, breast, liver, jejunum, skin, brain	(Aksoy et al., 1994)
SULT2A1	285	dehydroepiandrosterone	Liver, brain, adrenal cortex, bone marrow, jejunum	(Kong et al., 1992; Otterness et al., 1992)

Enzyme	No.of Amino Acid	Standard Substrate	Tissue Localization	References
SULT2B1a	350	Pregnenolone	Respiratory system, placenta, skin, prostate	(Falany et al., 2006)
SULT2B1b	365	Cholesterol	Lung, brain placenta, skin, prostate	((Falany et al., 2006; Geese & Raftogianis, 2001)
SULT4A1	284	Unknown	Prostate, cervix, testis, bladder, gastrointestinal tract, trachea, brain	(Falany et al., 2000; Sidharthan et al., 2014)
SULT6B1	265	Unknown	Kidney, testis	(Freimuth et al., 2004)

1.5. Genetic Polymorphisms of Human Cytosolic Sulfotransferase Genes

As biomarkers such as SNPs have been integrated into genetic and epidemiological studies, genetic variation in members of the SULT superfamily has gathered much attention. The integration of genetic and epidemiological information helps provide insight into the relationships between human genotypes and phenotypic variability, as well as improving the prediction of pharmacological responses to drugs, side effects, and toxicity. **Table 1.2** reviews some examples of the human SNPs reported in SULT genes.

Table 1.2. Examples of the reported SNPs in human SULTs

SNPs of SULTs	Effects	Reference
SULT1A1*2	Associated with breast cancer in Asian women	(Wang et al., 2010)
SULT1A1*4	Associated with esophageal squamous cell carcinoma	(Shah et al., 2016)
SULTA2*2	Increase risk of early-onset breast cancer	(Hou et al., 2002)
SULT1A3 *2	Low enzymatic activity toward dopamine	(Ginsberg et al., 2010)
SULT1C2 *3 SULT1C2 *3	Decrease 4-nitrophenol-sulfating activity	(Freimuth et al., 2001)
SULT1E1(Asp22Tyr) *SULT1E1* (Ala32Val)	Decrease 17β-estradiol -sulfating activity	(Adjei et al., 2003)
SULT2A1*3 SULT2A1*4	Decrease dehydroepiandrosterone -sulfating activity	(Glatt et al., 2001)
SULT2B1b (Pro149Leu)	Involved in autosomal recessive congenital ichthyoses	(Heinz et al., 2017)

1.6. Overview of the Human SULT1C4 Gene and Protien

In 1998, Yoshinari et al. were the first to isolate SULT1C4 cDNA from the human fetal liver library (Yoshinari et al., 1998). Human *SULT1C4* gene is approximately 10 kb in length and consist of seven exons. The gene is located in

chromosomal region 2q11.2 (Freimuth et al., 2000). SULT1C4 enzyme (**Figure 1.4**) has been shown to be highly expressed in the lung and kidney during human fetal development, and is present to a much lower extent in the adult ovary, kidney, brain, and spinal cord (Sakakibara et al., 1998). To this effect, SULT1C4 is capable of metabolizing steroid hormones, such as estrogens, that are involved in fetal development (Hui et al., 2008), and the sulfation of exogenous estrogens, such as dietary flavonoids and environmental estrogens (Guidry et al., 2017; Pai et al., 2001). Exposure to exogenous estrogens may inhibit DHEA production by activating a negative feedback mechanism, thereby altering the estrogen levels within the fetus and hindering proper development (Kaludjerovic & Ward, 2012). From another perspective, SULT1C4 may inadvertently catalyze the sulfation of potent pro-carcinogens such as N-hydroxy-2-acetylaminofluorene to highly reactive metabolites that are capable of causing carcinogenicity (Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998). Several studies have also demonstrated the involvement of SULT1C4 in the metabolism of endogenous substrates and therapeutic agents, some of which are listed in **Table 1.3.**

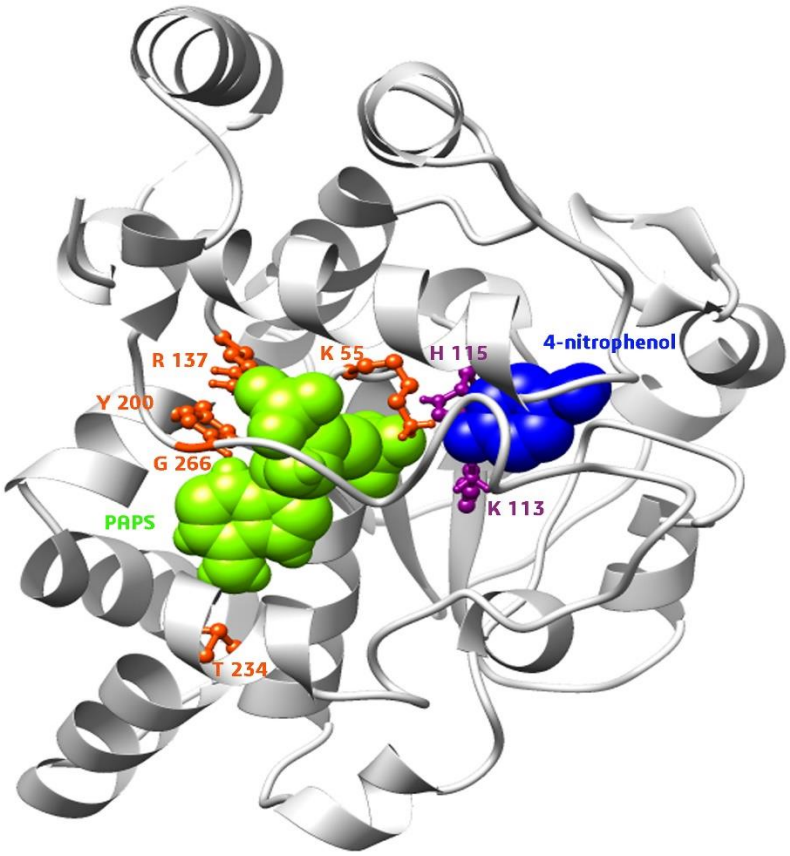

Figure 1.4. SULT1C4 crystal structure complex. The SULT1C4 is shown in light gray color. The PAPS bound to SULT1C4 is represented in chartreuse color. The residues of SULT1C4 which involved in interaction with PAPS are depicted in red orange color. 4-nitrophenol is represented in blue color and the residues of SULT1C4 bound with 4-nitrophenol are illustrated in dark magenta color. The figure was generated using AutoDock Vina software and the reported crystal structure of SULT1C4 (Protein Data Bank code: 2GWH).

Table 1.3. Endogenous substrates and therapeutic agents that act as substrates for SULT1C4.

Substrate	Action	Reference
Estrone (E1)	Steroidal hormone	(Hui et al., 2008)
17 β -Estradiol (E2)	Steroidal hormone	(Hui et al., 2008)
Doxorubicin	Chemotherapeutic agent	(Luo et al., 2016)
Acetaminophen	Analgesic	(Yamamoto et al., 2015)
Dextrophan	Antitussive	(Yamamoto et al., 2016)

1.6.1. Pathophysiological Associations and the Etiological Role of SULT1C4.

The expression of SULT1C4 has also been associated with certain diseases. A study conducted by Hardwick et al. (2013) sought to investigate the expression level of phase II metabolizing enzymes in fatty nonalcoholic steatohepatitis (NASH) and non-fatty/cirrhosis NASH. The study revealed that the expression level of *SULT1C4* was upregulated in both fatty and non-fatty NASH. In view of the significant upregulation of the expression of *SULT1C4* in both stages, the level of SULT1C4 protein was also examined. It was found that the protein expression of SULT1C4 was also significantly increased in both stages of NASH. However, the pathogenic role of SULT1C4 in NASH remains unknown (Hardwick et al., 2013).

With regard to liver disease, it is also worth noting that the expression of SULT1C4 has not been detected in the adult liver (Sakakibara et al., 1998). Given the significance of SULT1C4, it may be of relevance to further investigate the role of SULT1C4 in liver disease. A better understanding of the role of SULT1C4 in liver disease can, in the long run, contribute to the development of better treatment options.

In the case of clear cell renal cell carcinoma (ccRCC), studies have shown that the disease is resistant to chemotherapeutic agents as well as radiation therapy (especially in the case of distant metastasis) (Martinez-Salamanca et al., 2011). A 2017 study, however, revealed that the expression of *SULT1C4* was highly enhanced (up to 2.65 fold) in the ccRCC cell line (Feng et al., 2017). By knowing the expression within ccRCC, the development of agents that are able to treat the disease may become possible.

The expression of *SULT1C4* can also be affected by environmental toxins such as tobacco smoke. Frequent exposure to low-level tobacco smoke has become a significant public health issue. To assess the impact of the exposure, a study was conducted to investigate the genetic alteration, if any, amongst 121 participants who were exposed to low-level tobacco smoke. During the study, small airway epithelium samples were collected and examined. The expression of *SULT1C4* was significantly reduced in individuals who were exposed to tobacco smoke. This finding was also associated with the presence of low nicotine levels in urine samples (Strulovici-Barel et al., 2010). The resultant reduced levels of SULT1C4 are likely due to cigarette smoke toxicants such as N-OH-4-ABP, β-naphthylamine, catechol, and caffeic acid; all strong substrates for SULT1C4 (Yasuda et al., 2007) that potentially may act as inhibitors to SULT1C4.

1.6.2. Genetic Polymorphisms of the Human *SULT1C4* gene.

Single nucleotide polymorphisms (SNPs) have been reported to account for more than 90% of the genetic variation in human DNA (Efferth & Volm, 2005). Although the analysis of *SULT1C4* SNPs is still in the early stages of research, many studies have reported an association between SULT1C4 SNPs and some human phenotypes. One study was conducted to assess the association, if any, between genetic polymorphisms and the relapse rate of patients with acute myeloblastic leukemia (AML). The study involved 110 AML patients, and analyzed the association between the genetic polymorphisms and their relapse rate after treatment with chemotherapeutic agents. The study revealed that the relapse rate was significantly increased in patients whose had a missense mutation (causing the Asp5Glu change) of *SULT1C4*, indicating a positive association between this polymorphic gene and a post-treatment relapse rate in AML. Based on the findings from this study, the *SULT1C4* Asp5Glu polymeric gene can be used as a prognostic biomarker in patients with AML (Monzo et al., 2006).

Another study was focused on the correlation between single polymorphisms from candidate genes and the incidence of uterine leiomyoma in 1,045 premenopausal North American participants. The study revealed that the *SULT1C4* Asp5Glu polymeric change was associated with uterine leiomyoma in European American participants. The research concluded that this *SULT1C4* single nucleotide polymorphism positively impacted the risk and tumor size of patients (Aissani et al., 2015).

In the case of treating castration-resistant prostate cancer, docetaxel is a first-line treatment and thalidomide is a potent anticancer agent that can be used in

combination to treat prostate cancer. However, both drugs show individual variability in response to the treatment. In some cases, docetaxel plasma concentration reaches six-fold differences between patients (Clarke & Rivory, 1999; Hirth et al., 2000). To shed light on this occurrence, a study investigated more than 1200 SNPs in 170 drug disposition genes in patients with castration-resistant prostate cancer who received docetaxel and/or thalidomide. SULT1C4 Asp5Glu was one of the only three polymorphic genes that were associated with a resultant clinical response to the treatment. Interestingly, docetaxel and thalidomide are not known to be metabolized by sulfotransferase enzymes. The SULT1C4 Asp5Glu allozyme, however, may be involved in the disposition of docetaxel and thalidomide by direct metabolism or indirectly metabolizing other agents that influence the activities of these drugs. Another possible explanation is that the SULT1C4 Asp5Glu allozyme may directly affect the disease and may not be involved in either drug activity (Deeken et al., 2010).

1.7. Rationale and Objectives

As previously stated, human SULT1C4 has been shown to be highly expressed in fetal tissues (Sakakibara et al., 1998). Studies have suggested that SULT1C4 may be involved in the sulfation of endogenous compounds, such as E1 and E2 that modulate the development of the fetus (Guidry et al., 2017; Ying Hui et al., 2008; Pai et al., 2001). Several studies have reported an association between *SULT1C4* SNPs and some human phenotypes (Aissani et al., 2015; Deeken et al., 2010; Monzo et al., 2006). Given the

essential role of SULT1C4 in the homeostasis of estrogenic compounds and xenobiotics, the research is driven by **the hypothesis that the missense of single nucleotide polymorphisms (SNPs) of the human *SULT1C4* gene may lead to SULT1C4 allozymes with differential sulfating activities that may affect the sulfation of endogenous substrates including 17β-estradiol, estrone, as well as xenobiotics including drugs.** In order to verify the validity of the above-mentioned hypothesis, the research aims to pursue the following specific objectives:

Specific Aim 1: To search and analyze single nucleotide polymorphisms (SNPs) of human SULT1C4 genes and to collect epidemiological data associated with different SULT1C4 SNPs.

Databases currently available include the National Center for Biotechnology Information (NCBI) and the UniProt Knowledgebase (UniProtKB) were used to search for SULT1C4 SNPs. A total of 2,849 SNPs were identified and classified. These were classified as follows: 4 SNPs in the 3' splice site, 3 SNPs in the 5' splice site, 345 SNPs in the 3' untranslated region, 179 SNPs in the 5' untranslated region, 74 coding synonymous SNPs, 17 frame shift SNPs, 2,004 SNPs in introns, 203 missense SNPs, 10 nonsense SNPs, and 10 stop-gained SNPs. Additionally, information regarding the pathophysiological conditions of different *SULT1C4* SNPs was collected and analyzed from a variety of recent epidemiological studies deposited at PubMed.

Specific Aim 2: To generate cDNAs encoding different human SULT1C4 allozymes and express and purify these SULT1C4 allozymes.

Ten *SULT1C4* cDNAs were generated by using the site-directed mutagenesis technique. The pGEX-2TK prokaryotic plasmid vector that contains wild-type *SULT1C4* cDNA was used as a template, in conjunction with specific mutated primers to PCR-amplify "mutated" *SULTC4* cDNAs. The vector harboring individual "mutated" *SULT1C4* cDNAs was transformed into *E. coli* competent cells for recombinant protein expression. The expressed recombinant SULT1C4 allozyme was purified from the homogenates of the transformed *E. coli* cells and analyzed for purity by SDS-polyacrylamide gel electrophoresis.

Specific Aim 3: To characterize the sulfating activity of recombinants SULT1C4 allozyme toward representative endogenous and exogenous substrates.

This part of the research was accomplished by performing systemic analyses of the sulfating activity of purified human SULT1C4 allozymes toward endogenous compounds (E1, and E2) and xenobiotics (4-nitrophenol, doxorubicin, acetaminophen, dextrophan, *O*-desmethylquinidine, and *O*-desmethylquinine).

Chapter 2

Materials and Methods

2.1. Materials

E1, E2, dextrophan, doxorubicin ODM-quinidine, and ODM-quinine were products of Cayman Chemical Company (Ann Arbor, MI, USA). Acetaminophen, 4-nitrophenol, silica gel thin-layer chromatography (TLC) plates, adenosine-5'-triphosphate (ATP), dithiothreitol (DTT), dimethyl sulfoxide (DMSO), isopropyl-1-thio-β-D-galactopyranoside (IPTG) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Sigma Chemical Company (St. Louis, MO, USA). Polygram® Cellulose 300 TLC plates were from Macherey-Nagel GmbH and Co. KG (Düren, Germany). Cellulose thin-layer chromatography (TLC) plates were from EMD Millipore Corporation (Burlington, MA). QIAprep Spin Miniprep Kit was from QIAGEN (Germantown, MD, USA). PrimeSTAR® Max DNA polymerase was a product of Takara Bio (Mountain View, CA, USA). X-Ray films were from Products International Corporation (Mt Prospect, IL, USA). Carrier-free sodium [^{35}S]sulfate was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA). 3'-Phosphoadenosine-5'-phosphosulfate (PAP[^{35}S]) was synthesized using recombinant human bifunctional PAPS synthase as previously described (Yanagisawa et al., 1998). Ecolume scintillation

cocktail was from MP Biomedicals (Solon, OH). Oligonucleotide primers were synthesized by Eurofins Genomics (Louisville, KY, USA). Protein markers were products of Bioland Scientific LLC. (Paramount, CA, USA). Glutathione Sepharose was from of GE Healthcare Life Sciences (Pittsburgh, PA, USA). All other chemicals used were of the highest grade commercially available.

2.2. Identification and Analysis of the Human *SULT1C4* SNPs.

Databases currently available include the National Center for Biotechnology Information (NCBI) and the UniProt Knowledgebase (UniProtKB) were used to search for SULT1C4 SNPs. A total of 2,849 SNPs were identified and classified. For the purpose of this research, this research focused on SULT1C4 nonsynonymous missense SNPs that are located in the gene coding region, shown in **Table 2.1**, that alter the encoded amino acid to a different amino acid. This alteration may affect protein function and activity, and as a result, the sulfating activity towards endogenous and exogenous substrates of SULT1C4 could be altered. This, in turn, may affect the metabolism of the substrates and increase the risk of diseases

Table 2.1. List of missense coding SNPs in human *SULT1C4* gene

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
1	rs770841161	435	TTA ⇒ TCA	3	L [Leu] ⇒ S [Ser]
2	rs776586800	436	TTA ⇒ TTC	3	L [Leu] ⇒ F [Phe]
3	rs759448103	437	CAC ⇒ TAC	4	H [His] ⇒ Y [Tyr]
4	rs144464562	439	CAC ⇒ CAG	4	H [His] ⇒ Q [Gln]
5	rs923195067	440	GAC ⇒ AAC	5	D [Asp] ⇒ N [Asn]
6	rs1402467 rs52816974 rs61392346	442	GAC ⇒ GAG	5	D [Asp] ⇒ E [Glu]
7	rs769561354	443	ATG ⇒ TTG	6	M [Met] ⇒ L [Leu]
8	rs972556671	444	ATG ⇒ ACG	6	M [Met] ⇒ T [Thr]
9	rs1487958681	445	ATG ⇒ ATA	6	M [Met] ⇒ I [Ile]
10	rs374459677	446	GAG ⇒ CAG	7	E [Glu] ⇒ Q [Gln]
11	rs751025486	448	GAG ⇒ GAC	7	E [Glu] ⇒ D [Asp]
12	rs1309630363	450	GAT ⇒ GTT	8	D [Asp] ⇒ V [Val]
13	rs1206926629	454	TTT ⇒ TTG	9	F [Phe] ⇒ L [Leu]
14	rs756602979	459	TTT ⇒ TAT	11	F [Phe] ⇒ Y [Tyr]
15	rs1482467740	468	ACA ⇒ AAA	14	T [Thr] ⇒ K [Lys]
16	rs1202365786	473	CGC ⇒ AGC CGC ⇒ TGC	16	R [Arg] ⇒ S [Ser] R [Arg] ⇒ C [Cys]
17	rs570353480	474	CGC ⇒ CAC	16	R [Arg] ⇒ H [His]
18	rs368759234	491	GTG ⇒ ATG	22	V [Val] ⇒ M [Met]
19	rs1430067975	496	AAG ⇒ AAC	23	K [Lys] ⇒ N [Asn]
20	rs748381612	498	GGA ⇒ GAA	24	G [Gly] ⇒ E [Glu]
21	rs1483247007	500	ATT ⇒ GTT	25	I [Ile] ⇒ V [Val]
22	rs919454704	510	CCG ⇒ CTG	28	P [Pro] ⇒ L [Leu]
23	rs554156858	515	GAC ⇒ AAC	30	D [Asp] ⇒ N [Asn]
24	rs777726197	516	GAC ⇒ GGC	30	D [Asp] ⇒ G [Gly]
25	rs1364588300	521	TGT ⇒ GGT	32	C [Cys] ⇒ G [Gly]
26	rs1229562613	536	AAG ⇒ GAG	37	K [Lys] ⇒ E [Glu]
27	rs566330437	537	AAG ⇒ ACG AAG ⇒ AGG	37	K [Lys] ⇒ T [Thr] K [Lys] ⇒ R [Arg]
28	rs1051707057	555	GCC ⇒ GAC	43	A [Ala] ⇒ D [Asp]
29	rs536571137	557	AAG ⇒ GAG	44	K [Lys] ⇒ E [Glu]
30	rs1429191256	559	AAG ⇒ AAT	44	K [Lys] ⇒ N [Asn]
31	rs1220815962	563	GAT ⇒ CAT	46	D [Asp] ⇒ H [His]
32	rs1466118688	569	CTG ⇒ CCG	48	L [Leu] ⇒ V [Val]
33	rs775290089	573	CTT ⇒ CCT	49	L [Leu] ⇒ P [Pro]
34	rs1188921693	575	ATT ⇒ TTT	50	I [Ile] ⇒ F [Phe]
35	rs1299640576	588	CCT ⇒ CTT	54	P [Pro] ⇒ L [Leu]
36	rs1165218515	593	GCA⇒ ACA	59	A [Ala] ⇒ T [Thr]
37	rs1032159049	600	ACA ⇒ AAA	58	T [Thr] ⇒ K [Lys]
38	rs368540945	603	ACA ⇒ AGA	59	T [Thr] ⇒ R [Arg]
39	rs1235020291	608	ACT ⇒ TCT	61	T [Thr] ⇒ S [Ser]
40	rs763426887	612	CAG ⇒ CGG	62	Q [Gln] ⇒ R [Arg]
41	rs1369064183	614	GAG ⇒ AAG	63	E [Glu] ⇒ K [Lys]

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
42	rs751879308	618	ATA ⇒ ACA	64	I [Ile] ⇒ T [Thr]
43	rs1326862202	623	GAA ⇒ CAA	66	E [Glu] ⇒ Q [Gln]
44	rs145685280	624	GAA ⇒ GTA	66	E [Glu] ⇒ V [Val]
45	rs1433226851	630	ATA ⇒ ACA	68	I [Ile] ⇒ T [Thr]
46	rs41322445	631	ATA ⇒ ATG	68	I [Ile] ⇒ M [Met]
47	rs965736500	636	AAT ⇒ AGT	70	N [Asn] ⇒ S [Ser]
48	rs756152475	644	GAT ⇒ AAT	73	D [Asp] ⇒ N [Asn]
49	rs749157093	656	AGT ⇒ GGT	77	S [Ser] ⇒ G [Gly]
50	rs1479227868	658	AGT ⇒ AGA	77	S [Ser] ⇒ R [Arg]
51	rs145219467	662	CGG ⇒ TGG	79	R [Arg] ⇒ W [Trp]
52	rs199882807	663	CGG ⇒ CAG	79	R [Arg] ⇒ Q [Gln]
53	rs771649647	666	GCA ⇒ GTA	80	A [Ala] ⇒ V [Val]
54	rs201674274	668	CCG ⇒ ACG	81	P [Pro] ⇒ T [Thr]
55	rs78602560	669	CCG ⇒ CGG	81	P [Pro] ⇒ R [Arg]
56	rs1210703999	672	ACT ⇒ AGT	82	T [Thr] ⇒ S [Ser]
57	rs137946991	681	CGA ⇒ CAA	85	R [Arg] ⇒ Q [Gln]
58	rs764526797	687	CCT ⇒ CTT	87	P [Pro] ⇒ L [Leu]
59	rs1279694759	692	CTC ⇒ TTC	89	L [Leu] ⇒ F [Phe]
60	rs143857621	695	GAA ⇒ AAA	90	E [Glu] ⇒ K [Lys]
61	rs1277013309	696	GAA ⇒ GTA	90	E [Glu] ⇒ V [Val]
62	rs767809500	699	ATG ⇒ ACG	91	M [Met] ⇒ T [Thr]
63	rs917522243	700	ATG ⇒ ATA	91	M [Met] ⇒ I [Ile]
64	rs750644774	701	AAA ⇒ GAA	92	K [Lys] ⇒ E [Glu]
65	rs756309632	702	AAA ⇒ AGA	92	K [Lys] ⇒ R [Arg]
66	rs753879048	717	GGA ⇒ GTA	97	G [Gly] ⇒ V [Val]
67	rs1366207958	734	GCT ⇒ ACT	103	A [Ala] ⇒ T [Thr]
68	rs1434718535	735	GCT ⇒ GTT	103	A [Ala] ⇒ V [Val]
69	rs949770113	738	CAT ⇒ CCT	104	H [His] ⇒ P [Pro]
70	rs1293672567	744	ATG ⇒ AGG	106	M [Met] ⇒ R [Arg]
71	rs1389649938	745	ATG ⇒ ATC	106	M [Met] ⇒ I [Ile]
72	rs1170807657	749	TCA ⇒ GCA	108	S [Ser] ⇒ A [Ala]
73	rs760801583	755	CGG ⇒ TGG	110	R [Arg] ⇒ W [Trp]
74	rs142885329	756	CGG ⇒ CAG	110	P [Pro] ⇒ L [Leu]
75	rs1210969864	767	ACA ⇒ GCA	114	T [Thr] ⇒ A [Ala]
76	rs765115421	770	CAT ⇒ GAT	115	H [His] ⇒ D [Asp]
77	rs752596847	771	CAT ⇒ CGT	115	H [His] ⇒ R [Arg]
78	rs537771943	773	CTT ⇒ TTT	116	L [Leu] ⇒ F [Phe]
79	rs1425074179	777	CCC ⇒ CTC	117	P [Pro] ⇒ L [Leu]
80	rs369096265	779	TTT ⇒ CTT	118	F [Phe] ⇒ L [Leu]
81	rs1459749759	780	TTT ⇒ TCT	118	F [Phe] ⇒ S [Ser]
82	rs777541971	784	CAC ⇒ CAG	119	H [His] ⇒ Q [Gln]
83	rs1456480416	787	TTG ⇒ TTT	120	L [Leu] ⇒ F [Phe]
84	rs1287364327	791	CCA ⇒ TCA	122	P [Pro] ⇒ S [Ser]
85	rs1381550391	794	CCA ⇒ TCA	123	P [Pro] ⇒ S [Ser]
86	rs1437783948	798	TCC ⇒ TGC	124	S [Ser] ⇒ C [Cys]
87	rs746672220	802	TTG ⇒ TTC	125	L [Leu] ⇒ F [Phe]
88	rs780780980	815	TGT ⇒ CGT	130	C [Cys] ⇒ R [Arg]

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
89	rs769317251	819	AAG ⇒ ACG	131	K [Lys] ⇒ T [Thr]
90	rs1427071152	823	ATA ⇒ ATG	132	I [Ile] ⇒ M [Met]
91	rs1433530376	825	ATC ⇒ ACC	133	I [Ile] ⇒ T [Thr]
92	rs750068930	827	TAT ⇒ CAT	134	Y [Tyr] ⇒ H [His]
93	rs1393464165	828	TAT ⇒ TGT	134	Y [Tyr] ⇒ C [Cys]
94	rs779704419	831	GTA ⇒ GAA	135	V [Val] ⇒ E [Glu]
95	rs748668211	833	GCA ⇒ CCA GCA ⇒ ACA	136	A [Ala] ⇒ P [Pro] A [Ala] ⇒ T [Thr]
96	rs145686853	838	CAG ⇒ CAC	137	Q [Gln] ⇒ H [His]
97	rs747292078	863	TAT ⇒ GAT	146	Y [Tyr] ⇒ D [Asp]
98	rs1386522915	866	TAC ⇒ CAC	147	Y [Tyr] ⇒ H [His]
99	rs976487409	867	TAC ⇒ TTC	147	Y [Tyr] ⇒ F [Phe]
100	rs771292711	875	CAA ⇒ GAA	150	Q [Gln] ⇒ E [Glu]
101	rs1229976405	879	AGA ⇒ AAA	151	R [Arg] ⇒ K [Lys]
102	rs776763656	880	AGA ⇒ AGC	151	R [Arg] ⇒ S [Ser]
103	rs759789348	881	ATG ⇒ GTG	152	M [Met] ⇒ V [Val]
104	rs1202408514	882	ATG ⇒ ACG	152	M [Met] ⇒ T [Thr]
105	rs1200526721	891	GCT ⇒ GTT	155	A [Ala] ⇒ V [Val]
106	rs1476651073	893	CTT ⇒ TTT	156	L [Leu] ⇒ F [Phe]
107	rs769869249	894	CTT ⇒ CCT	156	L [Leu] ⇒ P [Pro]
108	rs1451490598	906	GGA ⇒ GAA	160	G [Gly] ⇒ E [Glu]
109	rs1389693027	914	GAA ⇒ CAA	163	E [Glu] ⇒ Q [Gln]
110	rs879242115	917	GAG ⇒ AAG	164	E [Glu] ⇒ K [Lys]
111	rs932471098	920	TAT ⇒ CAT	165	Y [Tyr] ⇒ H [His]
112	rs763979300	924	TTT ⇒ TCT TTT ⇒ TGT	166	F [Phe] ⇒ S [Ser] F [Phe] ⇒ C [Cys]
113	rs372792270	926	GAG ⇒ CAG	167	E [Glu] ⇒ Q [Gln]
114	rs1436221418	929	ACT ⇒ GCT	168	T [Thr] ⇒ A [Ala]
115	rs767872321	936	CTG ⇒ CCG	170	L [Leu] ⇒ P [Pro]
116	rs1324224743	939	GCT ⇒ GTT	171	A [Ala] ⇒ V [Val]
117	rs532365323	941	GGG ⇒ AGG	172	G [Gly] ⇒ R [Arg]
118	rs761566136	947	GTG ⇒ ATG	174	V [Val] ⇒ M [Met]
119	rs769956256	950	TGC ⇒ CGC	175	C [Cys] ⇒ R [Arg]
120	rs775578071	951	TGC ⇒ TCC TGC ⇒ TTC	175	C [Cys] ⇒ S [Ser] C [Cys] ⇒ F [Phe]
121	rs367696220	954	GGC ⇒ GAC GGC ⇒ GTC	177	G [Gly] ⇒ D [Asp] G [Gly] ⇒ V [Val]
122	rs1331001180	965	CAT ⇒ AAT	180	H [His] ⇒ N [Asn]
123	rs749413902	966	CAT ⇒ CGT	180	H [His] ⇒ R [Arg]
124	rs185799497	971	CAT ⇒ TAT	182	H [His] ⇒ Y [Tyr]
125	rs1212505448	972	CAT ⇒ CCT	182	H [His] ⇒ P [Pro]
126	rs182424713	974	GTG ⇒ ATG	183	V [Val] ⇒ M [Met]
127	rs1186470972	983	TGG ⇒ CGG	186	W [Trp] ⇒ R [Arg]
128	rs1414778388	986	TGG ⇒ CGG	187	W [Trp] ⇒ R [Arg]
129	rs767218158	989	GAA ⇒ AAA	188	E [Glu] ⇒ K [Lys]
130	rs554095641	997	AAA ⇒ AAC	190	K [Lys] ⇒ N [Asn]
131	rs1383802633	1007	CGT ⇒ TGT	194	R [Arg] ⇒ C [Cys]

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
132	rs201183375	1008	CGT ⇒ CAT	194	R [Arg] ⇒ H [His]
133	rs772999744	1017	TAT ⇒ TGT TAT ⇒ TCT	197	Y [Tyr] ⇒ C [Cys] Y [Tyr] ⇒ S [Ser]
134	rs1470689410	1019	CTC ⇒ TTC	198	L [Leu] ⇒ F [Phe]
135	rs1335766834	1028	GAG ⇒ AAG	201	E [Glu] ⇒ K [Lys]
136	rs878945162	1029	GAG ⇒ GGG	201	E [Glu] ⇒ G [Gly]
137	rs868757135	1031	GAC ⇒ TAC	202	D [Asp] ⇒ Y [Tyr]
138	rs542725288	1032	GAC ⇒ GTC	202	D [Asp] ⇒ V [Val]
139	rs760421899	1034	ATG ⇒ GTG	203	M [Met] ⇒ V [Val]
140	rs1413599656	1035	ATG ⇒ ACG	203	M [Met] ⇒ T [Thr]
141	rs891790431	1040	AAG ⇒ GAG	205	K [Lys] ⇒ E [Glu]
142	rs1333802131	1052	CAT ⇒ TAT	209	H [His] ⇒ Y [Tyr]
143	rs771993707	1060	ATT ⇒ ATG	211	I [Ile] ⇒ M [Met]
144	rs1347358178	1074	GAA ⇒ GCA	216	E [Glu] ⇒ A [Ala]
145	rs1435244240	1080	ATT ⇒ ACT	218	I [Ile] ⇒ T [Thr]
146	rs760587079	1082	GGG ⇒ AGG	219	G [Gly] ⇒ R [Arg]
147	rs377017374	1088	AAA ⇒ GAA	221	K [Lys] ⇒ E [Glu]
148	rs1342949928	1094	GAT ⇒ CAT	223	D [Asp] ⇒ H [His]
149	rs1195780389	1098	GAC ⇒ GGC	224	D [Asp] ⇒ G [Gly]
150	rs1055170402	1100	AAA ⇒ GAA	225	K [Lys] ⇒ E [Glu]
151	rs776483720	1101	AAA ⇒ ACA AAA ⇒ AGA	225	K [Lys] ⇒ T [Thr] K [Lys] ⇒ R [Arg]
152	rs1395790941	1103	GTT ⇒ TTT	226	V [Val] ⇒ F [Phe]
153	rs1199953483	1104	GTT ⇒ GCT	226	V [Val] ⇒ A [Ala]
154	rs142311160	1109	GAT ⇒ AAT	228	D [Asp] ⇒ N [Asn]
155	rs752203823	1110	GAT ⇒ GGT	228	D [Asp] ⇒ G [Gly]
156	rs1462019630	1111	GAT ⇒ GAA	228	D [Asp] ⇒ E [Glu]
157	rs1169268939	1114	AAA ⇒ AAC	229	K [Lys] ⇒ N [Asn]
158	rs1457343652	1115	ATT ⇒ CTT	230	I [Ile] ⇒ L [Leu]
159	rs1167293080	1116	ATT ⇒ AAT	230	I [Ile] ⇒ N [Asn]
160	rs757736443	1118	GTC ⇒ ATC	231	V [Val] ⇒ I [Ile]
161	rs750911106	1131	TCG ⇒ TTG TCG ⇒ TGG	235	S [Ser] ⇒ L [Leu] S [Ser] ⇒ W [Trp]
162	rs749518195	1133	TTT ⇒ CTT	236	F [Phe] ⇒ L [Leu]
163	rs1300068311	1134	TTT ⇒ TCT	236	F [Phe] ⇒ S [Ser]
164	rs372071845	1136	GAT ⇒ AAT	237	D [Asp] ⇒ N [Asn]
165	rs1327142510	1139	GTC ⇒ ATC	238	V [Val] ⇒ I [Ile]
166	rs1226734105	1146	AAA ⇒ AGA	240	K [Lys] ⇒ R [Arg]
167	rs1202065262	1149	CAG ⇒ CCG	241	Q [Gln] ⇒ P [Pro]
168	rs372131884	1157	ATG ⇒ GTG	244	M [Met] ⇒ V [Val]
169	rs748215141	1164	AAC ⇒ AGC	246	N [Asn] ⇒ S [Ser]
170	rs1312383762	1172	TCG ⇒ CCG	249	S [Ser] ⇒ P [Pro]
171	rs1037911381	1173	TCG ⇒ TTG	249	S [Ser] ⇒ L [Leu]
172	rs746928906	1179	CCT ⇒ CGT	251	P [Pro] ⇒ R [Arg]
173	rs770915181	1182	GCT ⇒ GTT	252	A [Ala] ⇒ V [Val]
174	rs1309232256	1200	TCC ⇒ TTC	258	S [Ser] ⇒ F [Phe]
175	rs1431644337	1203	ATT ⇒ ACT	259	I [Ile] ⇒ T [Thr]

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
176	rs759286821	1204	ATT ⇒ ATG	259	I [Ile] ⇒ M [Met]
177	rs769449918	1213	TTC ⇒ TTG	262	F [Phe] ⇒ L [Leu]
178	rs774975544	1214	ATG ⇒ CTG	263	M [Met] ⇒ L [Leu]
179	rs1393525394	1215	ATG ⇒ ACG	263	M [Met] ⇒ T [Thr]
180	rs762573156	1218	AGA ⇒ AAA AGA ⇒ ACA	264	R [Arg] ⇒ K [Lys] R [Arg] ⇒ T [Thr]
181	rs1362579458	1235	GAC ⇒ TAC	270	D [Asp] ⇒ Y [Tyr]
182	rs946761823	1244	AAA ⇒ GAA	273	K [Lys] ⇒ E [Glu]
183	rs199794832	1245	AAA ⇒ AGA	273	K [Lys] ⇒ R [Arg]
184	rs1308247155	1253	ACC ⇒ GCC	276	T [Thr] ⇒ A [Ala]
185	rs141659142	1256	GTG ⇒ ATG	277	V [Val] ⇒ M [Met]
186	rs372835682	1260	GCT ⇒ GTT	278	A [Ala] ⇒ V [Val]
187	rs1258555343	1262	CAG ⇒ AAG	279	Q [Gln] ⇒ K [Lys]
188	rs761189477	1264	CAG ⇒ CAC	279	Q [Gln] ⇒ H [His]
189	rs1253216712	1275	TTT ⇒ TGT	283	F [Phe] ⇒ C [Cys]
190	rs1210919887	1286	TAC ⇒ CAC	287	Y [Tyr] ⇒ H [His]
191	rs1484688015	1289	AAG ⇒ CAG	288	K [Lys] ⇒ Q [Gln]
192	rs766828224	1290	AAG ⇒ AGG	288	K [Lys] ⇒ R [Arg]
193	rs201728224	1295	AAA ⇒ GAA	290	K [Lys] ⇒ E [Glu]
194	rs1040102631	1301	ACT ⇒ CCT	292	T [Thr] ⇒ P [Pro]
195	rs34501366	1307	ACC ⇒ CCC	294	T [Thr] ⇒ P [Pro]
196	rs765505365	1308	ACC ⇒ ATC	294	T [Thr] ⇒ I [Ile]
197	rs1340301899	1312	AGA ⇒ AGT	295	R [Arg] ⇒ S [Ser]
198	rs373046061	1316	ACT ⇒ TCT	287	T [Thr] ⇒ S [Ser]
199	rs1373708481	1323	CAC ⇒ CGC	299	H [His] ⇒ R [Arg]
200	rs181231789	1325	TTC ⇒ CTC	300	F [Phe] ⇒ L [Leu]
201	rs1299225312	1332	TTC ⇒ TCC	302	F [Phe] ⇒ S [Ser]

2.3. Selection of *SULT1C4* SNPs

A total of ten SULT1C4 missense allozymes were selected, shown in **Table 2.2,** for the purpose of this study on the basis that the selected allozymes showed the most significant alteration in the encoded amino acid and their locations (dimerization motif, PAPS-binding sites, or catalysis) as previously reported in crystal structure of the enzyme

(Allali-Hassani et al., 2007). The selected SULT1C4 missense allozymes are predicted to alter the function of the protein. **Figure 2.1** shows the location of the selected SNPs and the altered amino acid residues in the structure of the SULT1C4 enzyme.

Table 2.2. The selected SULT1C4 allozymes

No.	SNP code no.	Position of nucleotide change	Nucleotide change	Amino acid position	Amino acid change
1	rs368540945	603	A**C**A ⇒ A**G**A	59	T [Thr] ⇒ R [Arg]
2	rs752596847	771	C**A**T ⇒ C**G**T	115	H [His] ⇒ R [Arg]
3	rs748668211	833	**G**CA ⇒ **C**CA	136	A [Ala] ⇒ P [Pro]
4	rs769869249	894	C**T**T ⇒ C**C**T	156	L [Leu] ⇒ P [Pro]
5	rs750911106	1131	T**C**G ⇒ T**T**G	235	S [Ser] ⇒ L [Leu]
6	rs749518195	1133	**T**TT ⇒ **C**TT	236	F [Phe] ⇒ L [Leu]
7	rs372071845	1136	**G**AT ⇒ **A**AT	237	D [Asp] ⇒ N [Asn]
8	rs774975544	1214	**A**TG ⇒ **C**TG	263	M [Met] ⇒ L [Leu]
9	rs762573156	1218	A**G**A ⇒ A**A**A	264	R [Arg] ⇒ K [Lys]
10	rs141659142	1256	**G**TG ⇒ **A**TG	277	V [Val] ⇒ M [Met]

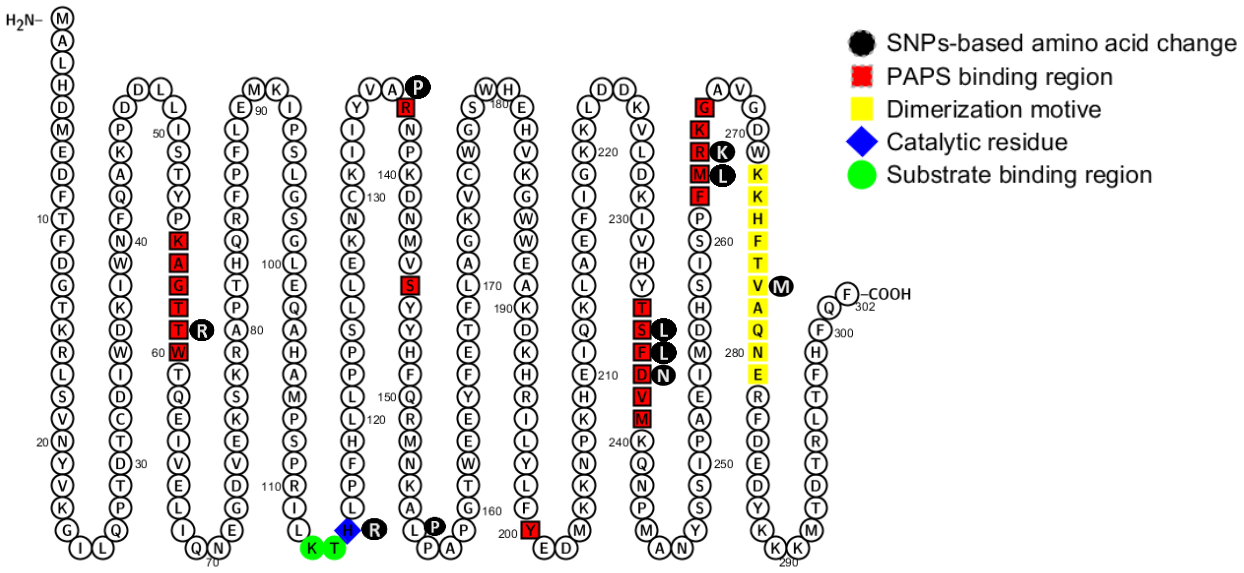

Figure 2.1. The amino acid sequence of the human SULT1C4 demonstrating residues reported to be involved in PAPS-binding, substrate-binding, dimerization, and/or catalysis. Residues in red are involved in PAPS-binding. Residue in blue involved in catalysis. Residues in yellow refer to the dimerization-motif. Residues in green are involved in substrate-binding. Residues circled with black background refer to the substituting amino acids. The figure was generated using Protter, a web tool for interactive protein feature visualization.

2.4. Generation of SULTC4 Allozymes cDNAs

Mutations in *SULT1C4* cDNA corresponding to selected *SULT1C4* polymorphisms were generated through site-directed mutagenesis. Briefly, a sense and antisense mutagenic primer for each selected SULT1C4 allozyme was designed to span

18 nucleotides on either side of each SNP (total length 37 bases; see **Table 2.3**), then applied to wild-type *SULT1C4* cDNA template that had been ligated into the pGEX-2TK prokaryotic expression vector.

Table 2.3. List of human *SULT1C4* cSNPs, their minor allele frequencies, and mutagenic primer sets designed for the PCR-amplification of the corresponding cDNAs. The bold underlined letters indicate altered/mutated nucleotides (SNPs).

hSULT1C4 Allozyme	Mutagenic primer	Minor Allele Frequency
hSULT1C4-Thr59Arg	5'-ATCCTAAAGCAGGAACAA**C**ATGGACTCAGGAGATAT3' 5‘- ACTATCTCCTGAGTCCATGTTGTTCCTGCTTTAGGAT-3'	(0.00002-0.0001)
hSULT1C4-His115Arg	5'-CACGGATCCTGAAAACAC**A**TCTTCCCTTTCACTTGCT-3' 5'-AGCAAGTGAAAGGGAAGATGTGTTTTCAGGATCCGTG-3'	0.00001
hSULT1C4-Ala136Pro	5'-TGTAAGATAATCTATGTA**G**CAAGAAATCCCAAGGACA-3' 5'-TGTCCTTGGGATTTCTTGCTACATAGATTATCTTACA-3'	0.00002
hSULT1C4-Leu156Pro	5'-AAAGAATGAATAAAGCTC**T**TCCTGCTCCAGGAACATG-3' 5'-CATGTTCCTGGAGCAGGAAGAGCTTTATTCATTCTTT-3'	0.00002
hSULT1C4-Ser235Leu	5'-AAATTGTCCATTACACTT**C**GTTTGATGTCATGAAACA-3' 5'-TGTTTCATGACATCAAACGAAGTGTAATGGACAATTT-3'	0.00002
hSULT1C4-Phe236Leu	5'-ATTGTCCATTACACTTCG**T**TTGATGTCATGAAACAGA-3' 5'-TCTGTTTCATGACATCAAACGAAGTGTAATGGACAAT-3'	0.00001
hSULT1C4-Asp237Asn	5'-GTCCATTACACTTCGTTT**G**ATGTCATGAAACAGAATC-3' 5'-GATTCTGTTTCATGACATCAAACGAAGTGTAATGGAC-3'	0.0001
hSULT1C4-Met263Leu	5'-CACTCCATTTCTCCATTC**A**TGAGAAAAGGGGCAGTGG-3' 5'-CCACTGCCCCTTTTCTCATGAATGGAGAAATGGAGTG-3'	0.00003
hSULT1C4-Arg264Lys	5'-CCATTTCTCCATTCATGA**G**AAAAGGGGCAGTGGGAGA-3' 5'-TCTCCCACTGCCCCTTTTCTCATGAATGGAGAAATGG-3'	0.00002

hSULT1C4 Allozyme	Mutagenic primer	Minor Allele Frequency
hSULT1C4-Val277Met	5'-TGGAAGAAACACTTCACC**G**TGGCTCAGAATGAGAGAT-3' 5'-ATCTCTCATTCTGAGCCACGGTGAAGTGTTTCTTCCA-3'	(0.0001-0.0022)

PCR was conducted using mutagenic primers, 1 μL template, and PrimeSTAR® Max DNA polymerase. The thermocycler program featured initial denaturation for 30 seconds at 94 °C, then 12 cycles consisting of denaturation, 0.5 min at 94 °C; annealing, 1 min at 55 °C; and extension, 15 min at 72 °C. The program ended with a final extension step of 7 min at 72 °C. The amplified reactions were incubated for 1 h at 37 °C with the methylation-sensitive restriction enzyme *Dpn* I to digest the methylated, non-mutated template. Subsequently, individual plasmids carrying mutated *SULT1C4* cDNAs were transformed into competent NEB 5-α *E. coli* cells. Briefly, the competent cells were first fully thawed on ice, after which 1 μL of PCR product was added with careful mixing. The cells were left on ice for 30 min, then subjected to heat shock at 42 °C for exactly 40 sec, and again allowed to rest on ice for 5 min. Subsequently, super optimal broth with catabolite (SOC) medium (150 μL) was added to the mixture and incubated for 10 min at 37 °C, followed by further incubation for 30-60 min with continuous shaking (250 rpm). Finally, the mixture was thoroughly mixed by flicking and inversion, then spread onto an agar plate pretreated with ampicillin followed by an overnight incubation at 37°C overnight. Colony PCR followed by agarose gel electrophoresis was performed to verify the presence of (mutated) SULT1C4-pGEX-2TK plasmid. The thermocycler program for colony PCR consisted of an initial denaturation for 2 min at 94 °C, then 35 cycles as follows: denaturing, 30 s at 94 °C; annealing, 30 s at 55 °C; and

extension, 90 s at 72 °C. It concluded with an additional extension step of 5 min at 72 °C. The PCR products were visualized by ethidium bromide staining and agarose gel (1%) electrophoresis (**Figure 2.2**).

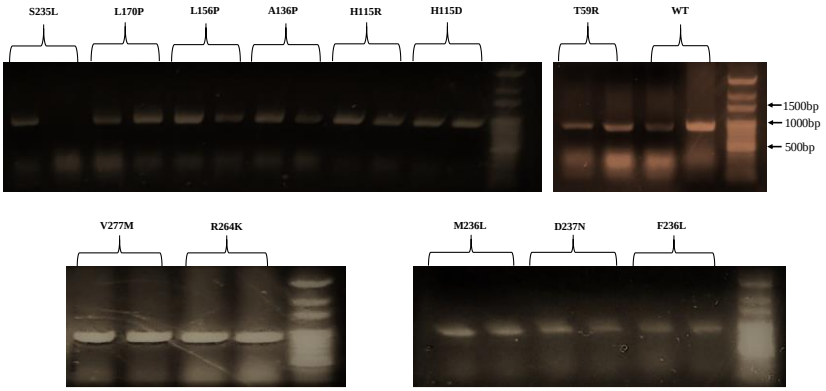

Figure 2.2. Confirmation of wild-type and mutated *SULT1C4* inserts by colony PCR (in NEB 5-α *E. coli* competent cells). DNA ladder band sizes are indicated on the right.

The positive colonies containing *SULT1C4* inserts were grown to amplify the plasmid. Briefly, each chosen colony was cultured overnight in 10 ml LB medium. Then, plasmids were extracted and purified using a QIAprep® Spin Miniprep Kit according to the manufacturer's instructions. The purified plasmids were sequenced (Sanger, Nicklen, & Coulson, 1977), and sequences analyzed with Clustal Omega software to verify each mutation.

2.5. Expression and Purification of Recombinant SULT1C4 Allozymes

Individual vectors confirmed to harbor cDNAs encoding mutated SULT1C4 allozymes were transformed into competent cells (*E. coli* BL21) as described above. Transformations were confirmed by colony PCR and agarose gel electrophoresis as described above (**Figure 2.3**).

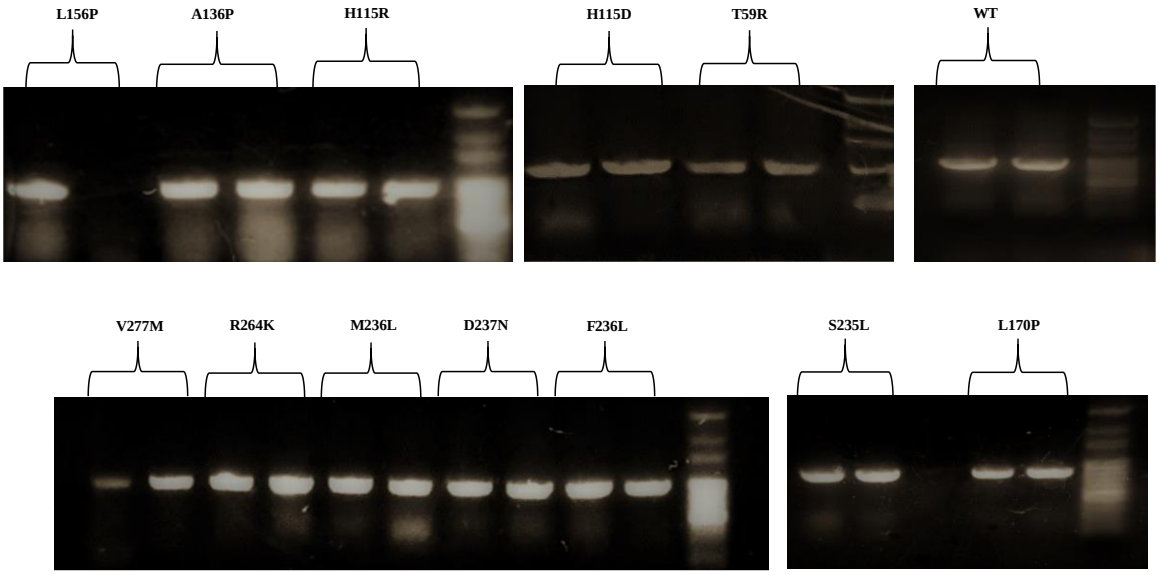

Figure 2.3. Confirmation of wild-type and mutated *SULT1C4* inserts by colony PCR (in BL21 *E. coli* competent cells). DNA ladder band sizes are indicated on the right.

A pilot study was performed to evaluate the expression of recombinant SULT1C4 protein in BL21 competent cells, along with an estimate of the necessary induction time. Subsequently, cells confirmed to be correctly transformed were suspended in a sterile 10 mL of LB medium (allowing for growth) containing 100 μg/ml ampicillin overnight at 25

°C in a shaker. The next morning, cultured cells were added to 1 L of LB medium, again containing ampicillin at a final concentration of 100 μg/ml; these were incubated in a shaker incubator at 37 °C until an OD600nm value of ~0.3 was attained. At that time, 0.1 mM of the protein transcription inducer IPTG was added to induce expression of the transformed protein, and the cells incubated at 25 °C with shaking for 12 hours. Afterwards, the cells were collected by centrifugation and resuspended in 20 ml of ice-cold lysis buffer (containing 4 M NaCl, 1 M Tris-HCl, pH 8. 0, and 0.4 M EDTA). An Aminco French press was used to homogenize the cell suspension. Supernatants were collected by centrifuging the crude homogenates at 10,000 × g for 20 min at 4 °C, then individually fractionated using 1 ml of glutathione-sepharose resin. Unbound proteins were removed from the resin by washing it several times with lysis buffer. The resin bound with GST fusion protein was treated with 3.5 unit/ml of bovine thrombin in a thrombin digestion buffer (containing 1 M Tris-HCl, pH 8.0, 4 M NaCl, and 0.25 M $CaCl_2$) and to cleave off the recombinant SULT1C4 allozyme. The suspension was incubated for 15 min at room temperature with constant agitation. Afterwards, supernatants containing the recombinant SULT1C4 allozymes were collected via centrifugation and evaluated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in order to assess the purity of the recombinant SULLT1C4 allozyme (**Figure 2.4**) (Laemmli, 1970; Shapiro et al., 1967). Protein concentrations were determined using the Bradford method (Bradford, 1976).

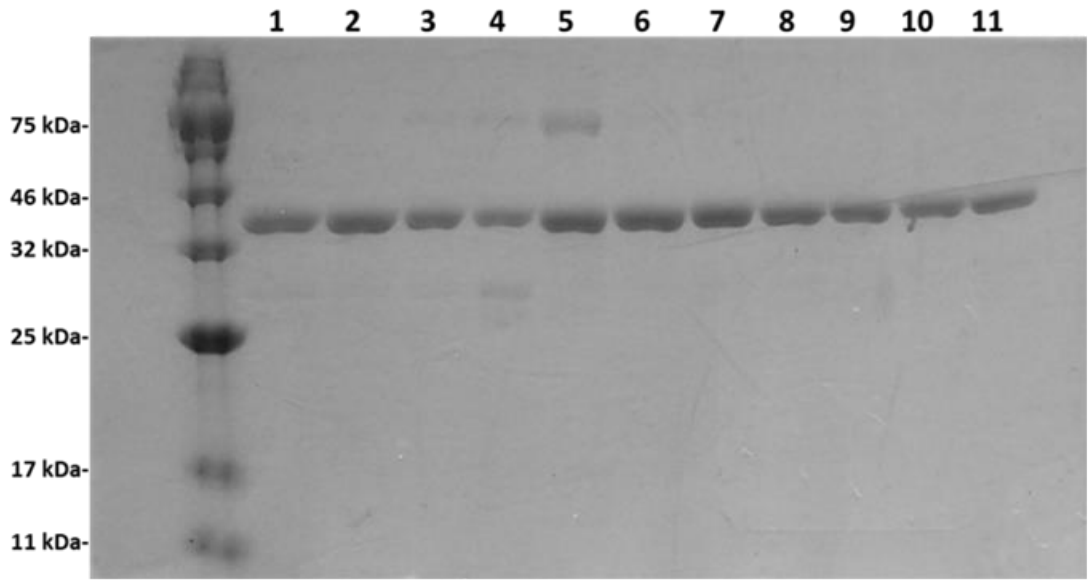

Figure 2.4. SDS gel electrophoretic pattern of the purified human SULT1C4 allozymes. SDS-PAGE was performed on a 12% gel, followed by Coomassie Blue staining. Samples analyzed in lanes 1 through 11 correspond to SULT1C4-WT (wild-type), SULT1C4-T59R, SULT1C4-H115R, SULT1C4-A136P, SULT1C4-L156P, SULT1C4-S235L, SULT1C4-F236L, SULT1C4-D237N, SULT1C4-M263L, SULT1C4-R264K, and SULT1C4-V277M. Positions of protein molecular weight markers are indicated on the left.

2.6. Enzymatic Assay for the Sulfating Activity of SULT1C4 Allozymes

Sulfating activities were determined for the purified recombinant SULT1C4 allozymes and the wild-type enzyme using an established assay procedure, in which

PAP[^{35}S] is employed as a sulfate donor. Activities were determined against a number of endogenous and exogenous substrates at three different concentrations: one well below the reported K_m, one close to the K_m, and one above the K_m; the tested substrates included 4-nitrophenol, doxorubicin, acetaminophen, dextrophan, *O*-desmethylquinidine, *O*-desmethylquinine, E1, and E2. Assays were performed in a 20 μL reaction mixture (pH 7.4) containing 14 μM radiolabeled PAPS (PAP[^{35}S]), purified SULT1C4 (wild-type or allozyme), 1 mM DTT, 50 mM HEPES buffer, and a substrate; negative controls were performed in parallel. The reaction was carried out by incubating at 37 C^{o} for 10 min, then terminated by heating at 100 °C for 3 min, after which precipitates were cleared by centrifugation at 13,000 rpm for 3 min. The radiolabeled sulfated product was evaluated by TLC using 1-2 μl of the cleared mixture. Silica TLC plate was used for doxorubicin, ODM-quinidine, ODM-quinine, E1, and E2. Cellulose TLC plate was used for 4-NP, acetaminophen, and dextrophan. For sulfated acetaminophen and 4-NP, the solvent system consisted of n-butanol/isopropanol/formic acid/water in a ratio of 3:1:1:1 (by volume). For sulfated dextrorphan, the ratio was 2:1:1:2 (by volume). For sulfated doxurubicin, the ratio was 8:2:1:1 (by volume). For sulfated ODM-quinidine and ODM-quinine, the solvent system consisted of n-butanol/acetonitrile at a ratio of 4:2 (by volume). For sulfated E1 and E2, the solvent system consisted of acetic acid/n-butanol in a ratio of 2:1 (by volume). Autoradiography using an X-ray film was performed on the separated mixture to reveal the position of the sulfated product, which was then cut out and the sulfated product eluted by placing it in a vial with 0.5 ml water. The eluted product was mixed thoroughly with 2 ml of Ecolume scintillation liquid and its [^{35}S] radioactivity quantified with a liquid scintillation counter as counts per minute (cpm).

The specific activity was then determined from the radioactivity in terms of nmol sulfated product per minute per mg enzyme.

2.7. Kinetic Studies

The kinetic constants, *Km* and *Vmax*, were determined for wild-type SULT1C4 on a range of substrate concentrations according to the procedure described above. The catalytic efficiency of the wild-type enzyme was then determined as *Vmax*/*Km*.

2.8. Statistical Analysis

Kinetic constants were calculated from data obtained in the kinetic experiments based on non-linear regression of the Michaelis-Menten equation using GraphPad Prism®7. Inter-group comparisons were performed using one-way ANOVA in conjunction with Dunnett's multiple comparison test. Mean values were considered significantly different from that of the wild-type SULT1C4 enzyme at $p<0.05$.

Chapter 3

Effects of the Human SULT1C4 Polymorphisms on the Sulfation of Doxorubicin

3.1. Introduction

Doxorubicin is an anthracycline anticancer agent commonly used to treat a wide variety of cancers including Hodgkin's and non-Hodgkin's lymphomas, sarcoma, ovarian, breast, gastric, lung, and pediatric cancers (Gewirtz, 1999; Thorn et al., 2011). Despite being recognized as one of the most effective chemotherapeutic agents, the use of doxorubicin is complicated by cardiomyopathy and heart failure, which develop in a dose-dependent manner for reasons not fully understood (Lefrak, Piťha, Rosenheim, & Gottlieb, 1973; Von Hoff et al., 1979). It is greatly important to prevent such anthracycline-related cardiac damage, especially as the majority of patients treated with doxorubicin will live on with that damage (van den Anker, 2015). However, the dosage of doxorubicin that results in toxic responses is highly variable between patients. For instance, a dosage of 1000 mg/m^2 may be tolerated by some, while a dosage of merely

200 mg/m^2 may cause acute cardiotoxicity in others (Chang & Wang, 2018). These findings indicate that no "safe" doxorubicin dose is likely to exist where cardiac damage will not occur, and inter-individual variation must be accounted for in treatment. To better understand both the adverse and therapeutic effects of doxorubicin in different individuals, it is essential to elucidate the metabolism and deactivation mechanisms the drug is subject to. Some light has been shed on this subject by previous studies, which have shown that doxorubicin undergoes glucuronidation, sulfation, and demethylation (Lal, Mahajan, Chen, & Chowbay, 2010; Takanashi & Bachur, 1976).

Sulfation is a key process for the biotransformation and excretion of xenobiotics, including drugs; this process is catalyzed by cytosolic sulfotransferases (SULTs) (Coughtrie, 2002; Falany, 1997). These enzymes mediate the transfer of a sulfonate group from the donor co-substrate, 3´-phosphoadenosine-5´phosphosulfate (PAPS), to the hydroxyl or amino group of an acceptor (i.e. the drug) (Falany, 1997). Sulfation increases the substrate's hydrophilicity, and thus can be more readily eliminated from the body (Mulder & Jakoby, 1990; Weinshilboum et al., 1997). Most 'drug metabolizing enzymes,' including the cytochrome P450 (CYP) family and UDP-glucuronosyltransferases (UGTs), are not expressed at significant levels until after birth (Hakkola, Pelkonen, Pasanen, & Raunio, 1998). In contrast, SULTs are highly expressed in the human fetus (Stanley, Hume, & Coughtrie, 2005), suggesting that these enzymes may provide a key chemical defense during fetal development (Coughtrie, 2002). Humans have thirteen distinct SULT enzymes, which are classified into four families: SULT1, SULT2, SULT4, and SULT6 (Freimuth, Wiepert, Chute, Wieben, &

Weinshilboum, 2004). However, out of all human SULTs, it has been reported that SULT1C4 is the only one capable of sulfating doxorubicin (Luo et al., 2016). In the human fetus, SULT1C4 is highly expressed in the kidney, lung, and liver; it is also expressed at lower levels in the fetal heart, infant liver, and in the adult liver, kidney, ovary, spinal cord, and brain (Dubaisi et al., 2018; Dubaisi et al., 2019; Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998). In terms of interindividual variation, it is conceivable that coding SNPs that produce amino acid changes in SULT1C4 may influence its ability to sulfate doxorubicin, thereby impacting the efficacy of the drug and personal sensitivity to adverse effects.

This study carried out a comprehensive database search for nonsynonymous SNPs in human *SULT1C4*. Ten missense SNPs were selected and the corresponding cDNAs were generated. The resultant allozymes were expressed in bacteria. After affinity purification, the allozymes and wild-type enzyme were evaluated for differential sulfating activity toward doxorubicin and the prototype substrate 4-nitrophenol (4-NP).

3.2. Materials and Methods

Materials and Methods were previously described in chapter 2.

3.3. Results

3.3.1. Identification and Analysis of Different Human *SULT1C4* SNPs

SULT1C4 genotypes were systematically searched in two focal SNP databases: the U.S. National Center for Biotechnology Information (NCBI) and the Universal Protein Resource (UniProt). In total, 2,849 *SULT1C4* SNPs were identified and categorized as either coding or non-coding. A SNP was considered coding if annotated as synonymous, non-synonymous (missense), or nonsense, and non-coding if it was located in an intron, 3'-untranslated region (3'UTR), or 5'-untranslated region (5'UTR). Only missense SNPs were considered for downstream analysis, of which 203 were identified and ten were selected for further investigation. This selection was based on SNP location (e.g. proximity to substrate- and PAPS-binding sites) and differences in the physiochemical properties of amino acids between the wild-type and mutant proteins (e.g. acidic to/from basic, polar to/from non-polar, turn inducing to/from non-turn inducing). Details concerning these selected SNPs are provided in **Table 2.2**.

3.3.2. Preparation of Recombinant Human SULT1C4 Allozymes

The ten selected recombinant SULT1C4 allozymes were expressed in bacteria and purified by means of glutathione-Sepharose affinity chromatography. The results from SDS-gel electrophoresis of the selected allozymes and the wild-type enzyme are given in **Figure 2.4**. All enzymes appeared highly homogeneous, and all had an apparent size of ~35.5 kD, consistent with predictions.

3.3.3. Characterization of the Sulfating Activity of Human SULT1C4 Allozymes Toward 4-NP and Doxorubicin

First, concentration dependence of the sulfation of 4-NP (prototypic substrate) and doxorubicin by wild-type SULT1C4 was evaluated (**Figure 3.1**). The sulfation of 4-NP and doxorubicin by SULT1C4 appeared consistent with Michaelis-Menten kinetics until reaching respective substrate concentrations of 10 μM and 2000 μM. After those points, significant substrate inhibition was observed. Kinetic constants (K_m, V_{max}, and V_{max} / K_m) were determined from the sulfation reactions using GraphPad Prism 7 and the Michaelis-Menten equation with non-linear regression (**Table 3.1**). Three substrate concentrations were selected to examine the sulfating activity of SULT1C4 allozymes in relation to the K_m value determined for wild-type SULT1C4: one well below, one near the K_m, and one well above.

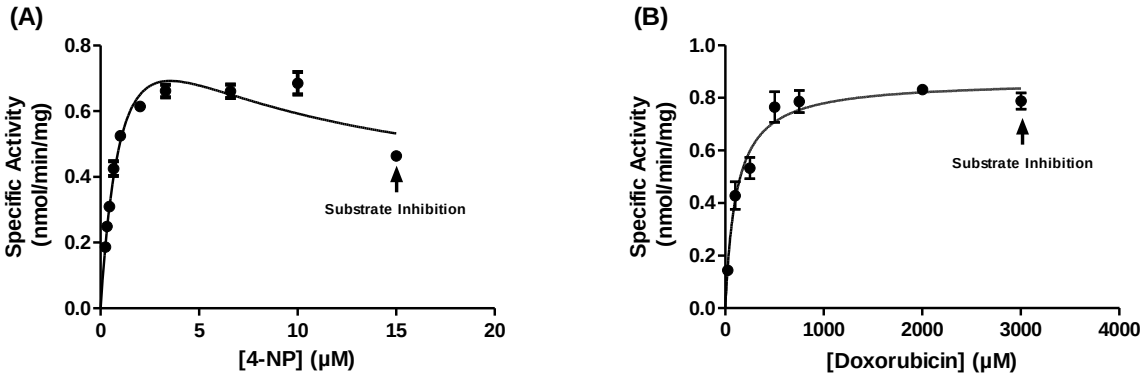

Figure 3.1. Kinetic analysis for the sulfation of the tested substrates by wild-type human SULT1C4. (A) and (B) plots represent the nonlinear Michaelis–Menten enzyme kinetics for 4-NP and doxorubicin, respectively. Data shown represent calculated mean ± standard deviation derived from three experiments.

Table 3.1. Kinetic parameters of the human SULT1C4 wild type with 4-NP and doxorubicin as substrates

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max} /K_m(ml/min/mg)
4-NP	0.570± 0.056	0.751± 0.025	1.320
Doxorubicin	250± 32.277	1.076± 0.054	0.004

With 4-NP as the substrate

At the tested concentrations of 0.1, 0.5, and 2 μM 4-NP, all tested allozymes showed roughly similar patterns of sulfating activity (**Figure 3.2**). Three allozymes (SULT1C4-T59R, SULT1C4-H115R, and SULT1C4-L156P) displayed no activity, while two allozymes (SULT1C4-F236L and SULT1C4-R264k) showed barely noticeable activity. Meanwhile, the sulfating activities of SULT1C4-D237N and SULT1C4-V277M were consistently lower than that of the wild-type enzyme, being reduced by at least by 41% and 19%, respectively. Of the remaining three allozymes, SULT1C4-M263L and SULT1C4-S235L displayed sulfating activities comparable to wild-type at low (0.1 μM) and high (2 μM) substrate concentrations, with notably lower activity (25% and 27%, respectively) at the mid (0.5 μM) substrate concentration. Only one allozyme (SULT1C4-A136P) displayed a sulfating activity higher than the wild-type enzyme at all three substrate concentrations, with an approximate 30% increase at the low (0.1 μM) concentration.

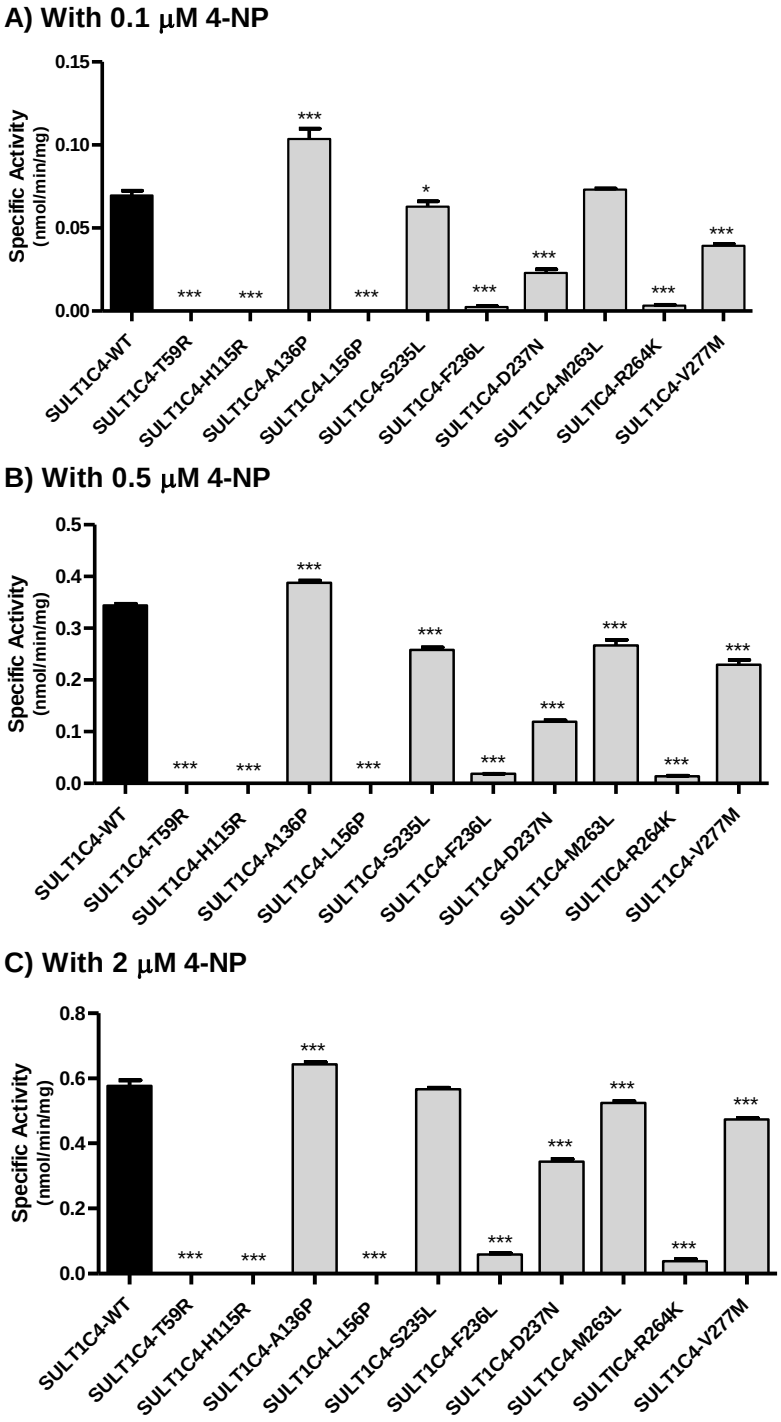

Figure 3.2. Specific activities of the sulfation of 4-NP human SULT1C4 allozymes. Concentrations of 4-NP used in the enzymatic assays were 0.1 μM (A), 0.5 μM (B), and 2 μM (C). Specific activity refers to nmol 4-NP sulfated/min/mg of purified allozyme.

Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

With doxorubicin as the substrate

At the tested concentrations of 50, 250, and 1000 µM doxorubicin, all allozymes tested showed differential activity from the wild-type enzyme (**Figure 3.3**). Five allozymes (SULT1C4-T59R, SULT1C4-H115R, SULT1C4-L156P, SULT1C4-F236L, and SULT1C4-R264k) showed no sulfating activity toward doxorubicin, with the exception of a drastically reduced activity for SULT1C4-F236L at the mid (250 µM) substrate concentration. Meanwhile, two allozymes (SULT1C4-M263L and SULT1C4-S235L) showed respective reductions in sulfating activity of approximately 72% and 21% at the low substrate concentration (50 µM), 38% for both at the mid concentration (250 µM), and 70% and 62% at the high substrate concentration (1000 µM). The remaining three allozymes (SULT1C4-A136P, SULT1C4-D237N, and SULT1C4-V277M) consistently showed higher sulfating activities than the wild-type enzyme, with SULT1C4-D237N exhibiting the strongest activity at approximately 66%, 52%, and 26% increase for respective substrate concentrations of 50, 250, and 1000 µM.

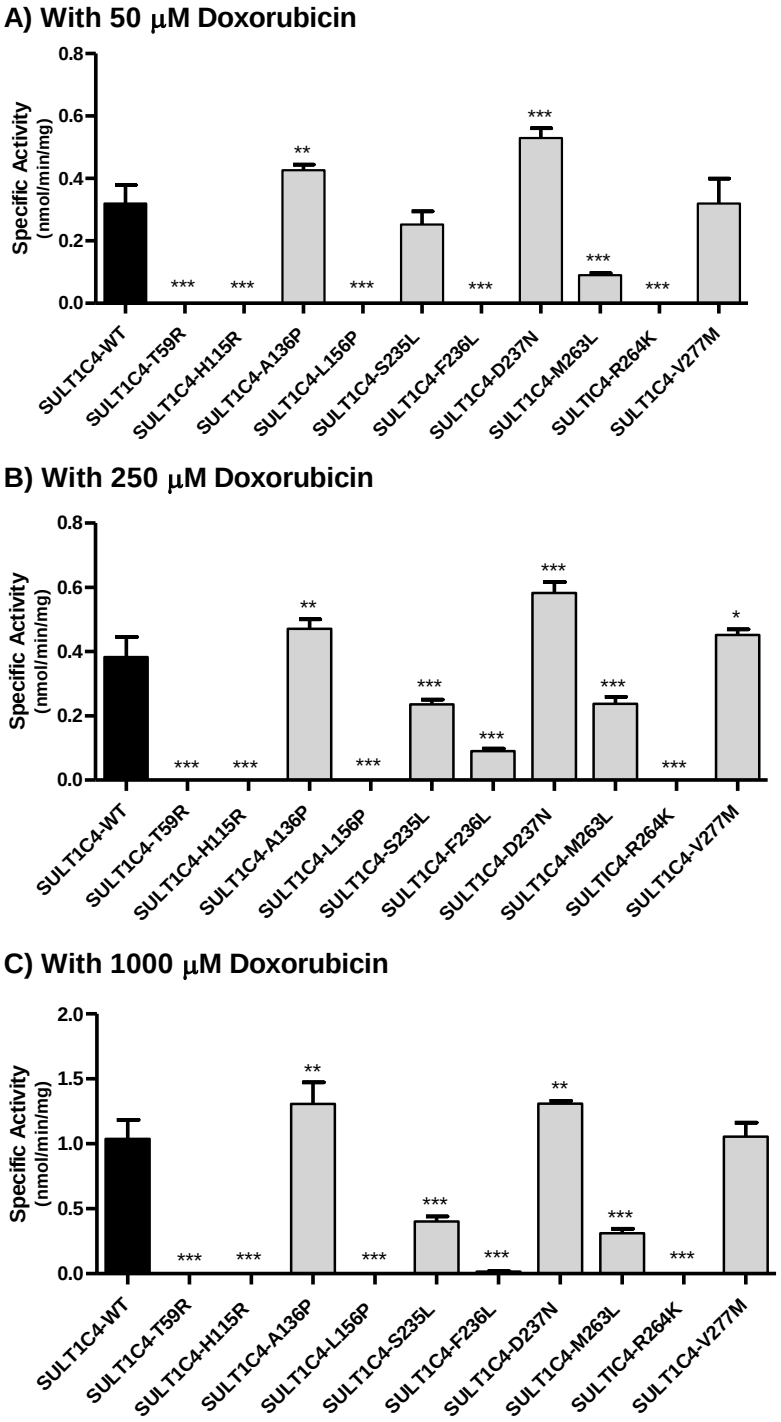

Figure 3.3. Specific activities of the sulfation of doxorubicin human SULT1C4 allozymes. Concentrations of doxorubicin used in the enzymatic assays were 50 μM (A), 250 μM (B), and 1000 μM (C). Specific activity refers to nmol doxorubicin

sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

3.4. Discussion

The chemotherapeutic drug doxorubicin is widely prescribed and used in the treatment of numerous cancers, including prostate cancer, breast cancer, and multiple myeloma (Gewirtz, 1999; Thorn et al., 2011). However, the pharmacokinetic parameters of doxorubicin are subject to considerable variation from patient to patient (Jacquet et al., 1990). How genetic variation affects doxorubicin response has only recently begun to be studied (Thorn et al., 2011). Therefore, remains essential to investigate the possible impact of genetic polymorphisms on doxorubicin metabolism, and therefore the drug's potential efficacy and risk of toxicity for a given patient.

A recent study has shown that in humans, SULT1C4 is the only enzyme that mediates the sulfation of doxorubicin (Luo et al., 2016). The current study identified missense SNPs in this through a systematic database search. Ten of these SNPs were selected for experimental analysis of the encoded proteins. The corresponding cDNAs were produced through site-directed mutagenesis. The resultant recombinant SULT1C4 allozymes were expressed in bacteria and purified. Concentrations of 4-NP (prototypic substrate) and doxorubicin for use in activity assays were determined from a preliminary

study using wild-type SULT1C4, with three values selected for downstream assays (one well below the K_m, one near the K_m, and one well above the K_m; cf. **Table 3.1**). Subsequently, the ten tested SULT1C4 allozymes demonstrated distinct differential sulfating activities towards 4-NP and doxorubicin (cf. **Figures 3.2** and **3.3**).

A crystal structure has been reported for the human SULT1C4 enzyme (Allali-Hassani et al., 2007). A number of residues integral to enzyme function have been identified, including: the 5'-phosphosulphate-binding (PSB) loop (52TYPKAGT58), the PAPS/PAP-binding regions (55KAGTTW60, 234TSFDVM239, 262FMRKG266, Arg137, Ser145, and Tyr200), the catalytic histidine His115, the substrate binding residues (Lys113 and Thr114) (Allali-Hassani et al., 2007), the C-terminal dimerization motif region (272KKHFTVAQNE281) (Petrotchenko, Pedersen, Borchers, Tomer, & Negishi, 2001), and the β-sheet in the N-terminal region, which is important for proper folding of the protein (Allali-Hassani et al., 2007). SNPs that affect these residues or regions are the most likely to alter protein function.

Five of the ten allozymes tested showed a drastic decrease, if not a total loss, of their catalytic activities toward 4-NP and doxorubicin: SULT1C4-T59R, SULT1C4-H115R, SULTL156P, SULT1C4-F236L, and SULT1C4-R264k. No sulfating activity was observed for SULT1C4-T59R possibly on account of substituting a polar uncharged threonine with a positively-charged arginine. This likely disrupted the hydrogen bonding of the enzyme to PAPS. The SULT1C4-H115R allozyme also displayed a complete loss of sulfating activity. Replacement of the catalytic histidine with arginine likely affected

deprotonation of the substrate, thereby suppressing the dissociation of the sulfuryl group from PAPS. Based on work done in human SULT1E, it has been proposed that the catalytic histidine accepts a proton from the phenyl group of 17β-estradiol and thereby facilitates nucleophilic attack on the sulfur atom of PAPS (Negishi et al., 2001; Pedersen, Petrotchenko, Shevtsov, & Negishi, 2002). The lack of sulfating activity observed for SULT1C4-H115R in the current study is consistent with prior reports on other SULTs in which the catalytic histidine is replaced (Chen, 2004; Kakuta, Petrotchenko, Pedersen, & Negishi, 1998; Liu, Suiko, & Sakakibara, 2000; Pedersen et al., 2002). Another allozyme that demonstrated no activity, SULT1C4-L156P, did not contain any substitutions in regions known to be key for enzyme function. However, the mutation in this allozyme introduced a proline residue, which is turn-inducing, and therefore may affect the overall conformation of the protein and its function (Betts & Russell, 2003).

The last two allozymes with minimal activity (SULT1C4-F236L and SULT1C4-R264K) displayed barely-detectable activities on 4-NP at all tested concentrations, with no activity seen for SULT1C4-R264K on doxorubicin. The SULT1C4-F236L allozyme involves the replacement of a residue with an aromatic side-chain to one with an aliphatic side chain. While both are non-polar, the side-chain difference may explain the dramatic decrease in enzyme activity. Meanwhile, the residue affected in SULT1C4-R264K, Arg264, is a conserved residue located within the PAPS binding region (Dong, Ako, & Wu, 2012). In SULT2B1b, this residue is known to forms a hydrogen bond with the negatively-charged O3P phosphate oxygen of PAPS (Lee et al., 2003). Therefore, the

substitution of this arginine with lysine in SULT1C4-R264K can be expected to impair PAPS binding.

Two other allozymes exhibited marked reductions in sulfating activity. In SULT1C4-S235L, the affected serine is conserved in all SULT family members. Research on SULT2B1b suggests that the oxygen in this serine's carbonyl group forms a hydrogen bond with the adenine group of PAPS (Lee et al., 2003). In the allozyme, this polar serine was replaced with the non-polar amino acid leucine, which unsurprisingly had a negative effect on activity toward 4-NP and doxorubicin. Meanwhile, the residue affected in SULT1C4-M263L was located in a PAPS/PAP binding region. The substitution of this methionine with leucine may similarly disrupt hydrogen bonding of the enzyme with PAPS.

Three allozymes demonstrated increased, rather than reduced, sulfating activity toward doxorubicin: SULT1C4-A136P, SULT1C4-D237N, and SULT1C4-V277M. In SULT1C4-A1365P, the replacement of alanine (a non-turn-inducing residue) with proline (a turn-inducing residue) might produce a kink in the peptide chain that acted to enhance the allozyme's activity. Meanwhile, the residues affected in SULT1C4-D237N and SULT1C4-V277M share key characteristics. In SULT1C4-D237N, both the aspartate and the asparagine that replaced it are polar amino acids, while in SULT1C4-V277M, both the valine and methionine are non-polar. The similarity rather than contrast in these two substitutions may explain the enhanced activity observed towards doxorubicin for these allozymes.

In summary, the current study made a systematic evaluation of how genetic polymorphisms in SULT1C4 allozymes affect the enzyme's sulfating activity towards 4-NP and doxorubicin. Activity assays revealed significant differences in the catalytic activities of these allozymes. These results provide crucial insights into the functional effects of SULT polymorphisms. Pending further studies, such information may aid in the individual-level interpretation and prediction of pharmacokinetic profiles and toxicity risk for drugs, such as doxorubicin, catalyzed by SULT1C4 in particular.

Chapter 4

Impact of Genetic Polymorphisms on the Sulfation of Acetaminophen and Dextrophan by Human Cytosolic Sulfotransferase SULT1C4

4.1. Introduction

Often used in combination as a treatment for the common cold and flu, acetaminophen and dextromethorphan are both widely used over-the-counter medications. While acetaminophen is frequently prescribed as a safe and popular analgesic and antipyretic (Bertolini et al., 2006), dextromethorphan is prescribed as an antitussive often used to treat cough associated with upper respiratory tract infections (Bem & Peck, 1992). In adults, acetaminophen has been reported to undergo glucuronidation and sulfation reactions (Steventon, Mitchell, & Waring, 1996). Because of the low expression levels of UDP-glucuronosyltransferases enzymes (UGTs) in the fetus, neonates, and post neonatal development stages, sulfation remains the main metabolic pathway of acetaminophen during these stages (Adjei, Gaedigk, Simon, Weinshilboum, & Leeder, 2008; Bertolini et al., 2006). Dextromethorphan mainly

undergoes O-demethylation by cytochrome P450 2D6 to dextrorphan followed by glucuronidation and sulfoconjugation (Fossati, Vimercati, Caputo, & Valenti, 1995; Ramachander, Williams, & Emele, 1978; Schadel, Wu, Otton, Kalow, & Sellers, 1995). Because dextrorphan is more potent than dextromethorphan (Church, Lodge, & Berry, 1985; Franklin & Murray, 1992), the effect of dextromethorphan as a drug has been attributed to its conversion to dextrorphan (Pechnick & Poland, 2004). The co-administration of dextromethorphan with salicylamide and acetaminophen, analgesic and antipyretic agents, has been shown to improve the antitussive function of dextromethorphan while also extending its duration of action. This suggests that the competitive inhibitory effect of salicylamide and acetaminophen on dextrorphan conjugation via sulfation and/or glucuronidation conjugations may be the cause behind the enhanced activity of the antitussive (Ramachander et al., 1978).

Sulfate conjugation is essential to the metabolism of xenobiotics, (such as drugs), thyroid and steroid hormones, bile acid, and endogenous compounds like catecholamines (Kauffman, 2004; Weinshilboum et al., 1997). Cytosolic sulfotransferases (SULTs) are enzymes that act as a catalyst that enable the transmission of a sulfonate group to hydroxyl or amino group containing substrate compounds from donor 3-phosphoadenosine-5-phosphosulfate (PAPS) (Lipmann, 1958). SULT1, SULT2, SULT4, and SULT6 make up four SULT gene families that categorize thirteen different human SULTs (Freimuth, Wiepert, Chute, Wieben, & Weinshilboum, 2004; Gamage et al., 2006). Specifically, SULT1C4 has been identified as an enzyme integral to the sulfation of acetaminophen (Yamamoto et al., 2015), and one of the primary SULTs responsible

for the sulfation of dextrorphan (Yamamoto et al., 2016). Of importance are the findings of previous studies that have observed high expressions of SULT1C4 in the fetal kidney and lungs while lower expressions of the enzyme were observed in the adult brain, spinal cord, kidney, ovary, and the infant liver, and fetal heart (Dubaisi et al., 2019; Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998). Moreover, multiple SULT genes have been noted to manifest genetic polymorphisms (Bairam et al., 2018; Rasool et al., 2019). Based on these observations, it is likely that *SULT1C4* coding SNPs, responsible for amino acid changes in coded protein products, may affect SULT1C4 allozymes' sulfating activity towards acetaminophen and dextrophan. In doing so, this will also influence the pharmacokinetics of acetaminophen and dextrophan and impact their effectiveness on people with different *SULT1C4* genotypes.

This investigation embarked on a wide-ranging systemic database search for SNPS of the human *SULT1C4* gene, and identified ten missense SNPs that were coded for ten polymorphic SULT1C4 allozymes. The allozymes that were identified were generated, expressed, and purified. The sulfating activity of the resulting purified allozymes towards acetaminophen and dextrophan were then assessed.

4.2. Materials and Methods

Materials and Methods were previously described in chapter 2.

4.3. Results

4.3.1. Identification and Analysis of Different Human *SULT1C4* SNPs

Using the three aforementioned SNP databases, the systemic search for *SULT1C4* genotypes yielded a result of 2,849 identified *SULT1C4* SNPs. The identified *SULT1C4* genotypes were assessed and classified into either coding or non-coding SNP groups. Where the coding SNPs featured nonsense SNPs, non-synonymous (missense) SNPs, and synonymous SNPs. The non-coding SNPs included the 3'-untranslated region (3'UTR), introns, and the 5'-untranslated region (5'UTR) SNPs. 203 *SULT1C4* missense coding SNPs were identified, and of them, ten were selected for further examination. The selection criteria for the specified ten missense SNPs were based on the locations (e.g., proximity to substrate-binding and PAPS-binding site) and changes in the physicochemical properties (e.g., turn inducing to/from non-turn inducing residues, polar to/from non-polar, acidic to/from basic,) of the altered amino acid residues. Accordingly, **Table 2.3** features the sense and antisense primers developed for PCR-amplification, the amino acid locations and variations, and the documented allelic frequency of the selected *SULT1C4* coding SNPs.

4.3.2. Preparation of Recombinant Human SULT1C4 Allozymes

The bacterial expression vector (pGEX-2TK), harboring individual cDNA encoding differing SULT1C4 allozymes, was transferred into BL21 *E. coli* cells. IPTG was then used to initiate the induction of recombinant protein expression. Thereafter, glutathione-Sepharose affinity chromatography was used to fractionate the recombinant

SULT1C4 allozymes from the homogenates of *E. coli* cells. The recombinant SULT1C4 allozymes were then treated with bovine thrombin, thereby causing the allozymes to be released from the bound GST fusion proteins. As illustrated in **Figure 2.4**, the SDS-polyacrylamide gel electrophoretic pattern affirms that the evident molecular weights of the purified SULT1C4 allozymes are consistent with the reported molecular weight (35,520kDa) of the wild-type SULT1C4.

4.3.3. Characterization of the Sulfating Activity of the Wild-Type Human SULT1C4 and Allozymes Toward Acetaminophen and Dextrophan

An analysis of the concentration-dependent sulfation towards acetaminophen and dextrophan by the purified wild-type SULT1C4 was conducted. The analysis found that the sulfation of the two observed substrates adhered to atypical Michaelis-Menten kinetics.; this is illustrated in **Figure 4.1.** A compilation of the determined kinetic parameters for the wild-type SULT1C4 in facilitating the sulfation of the two tested substrates is provided in **Table 4.1.** In accordance with the resulting data, three varying concentrations were identified for each substrate in assessing the sulfating activities of the SULT1C4 allozymes.

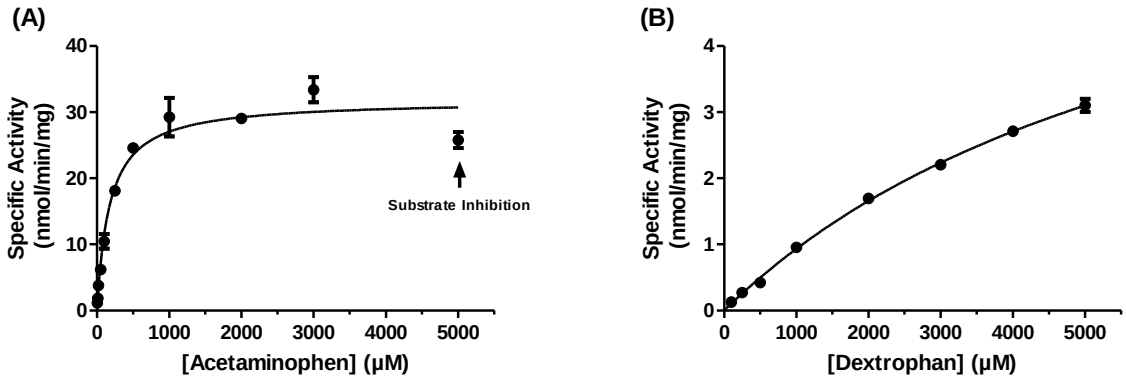

Figure 4.4. Kinetic analysis for the sulfation of the tested substrates by wild-type human SULT1C4. (A) and (B) plots represent the nonlinear Michaelis–Menten enzyme kinetics for acetaminophen and dextrophan, respectively. Data shown represent calculated mean ± standard deviation derived from three experiments.

Table 4.1. Kinetic parameters of the human SULT1C4 wild type with acetaminophen and dextrophan as substrates.

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max} /K_m(ml/min/mg)
Acetaminophen	176.90 ± 14.42	31.77 ± 0.09	0.18
Dextrophan	6944 ± 648.4	7.409± 0.466	0.001

Acetaminophen as a substrate

Using 10 μM acetaminophen as a substrate (**Figure 4.2**), four specific allozymes exhibited sulfating activities that were significantly higher than that of the wild-type enzyme. Those allozymes were SULT1C4-S235L, SULT1C4-D237N, SULT1C4-M263L, and SULT1C4-V277M. Specifically, the sulfating activity of SULT1C4-V277M

was 2.6-fold greater than the wild-type, and the sulfating activities of the other three (SULT1C4-S235L, SULT1C4-D237N, and SULT1C4-M263L) were approximately 14%, 29%, and 41% greater than that of the wild-type enzyme, respectively. Moreover, SULT1C4-V277M was the only allozyme that displayed significantly greater sulfating activities than that of the wild-type enzyme at the three substrate concentrations.

On the other hand, SULT1C4-S235L and SULT1C4-M263L showed sulfating activities similar to that of the wild-type enzyme at the mid-substrate concentration (150 μM), while SULT1C4-S235L and SULT1C4-D237N displayed comparable activity to activity of the wild-type enzyme at a higher substrate concentration (1000 μM). Three of the other SULT1C4 allozymes (SULT1C4-A136P, SULT1C4-F236L, and SULTIC4-R264K) regularly displayed lower sulfating activities than the wild-type enzyme; lower by at least 34%, 56% and 38%, respectively. Of them, SULTIC4-R264K showed the lowest sulfating activity, showing only 8% of the wild-type enzyme's sulfating activity at the middle substrate concentration (150 μM). For the following three allozymes, SULT1C4-T59R, SULT1C4-H115R, and SULT1C4-L156P, no sulfating activity was detected at all three substrate concentrations.

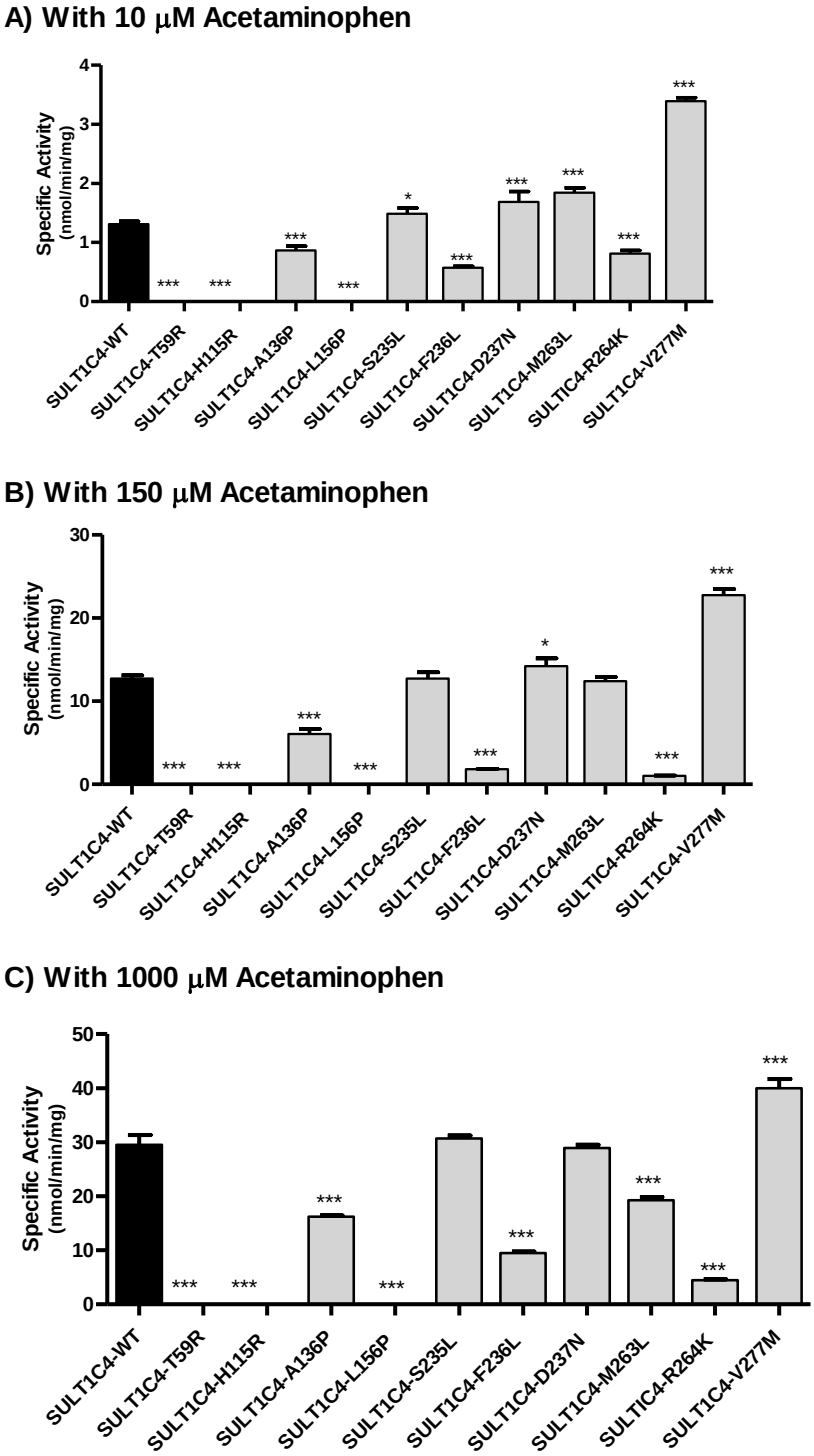

Figure 4.5. Specific activities of the sulfation of acetaminophen human SULT1C4 allozymes. Concentrations of acetaminophen used in the enzymatic assays were 10 μM

(A), 150 μM (B), and 1000 μM (C). Specific activity refers to nmol acetaminophen sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

Dextrorphan as a substrate

Using 500 μM dextrorphan as a substrate (**Figure 4.3**), one allozyme, SULT1C4-V277M, exhibited a greater sulfating activity than the activity of the wild type, whereas SULT1C4-A136P showed sulfating activities similar to that of the wild-type enzyme. Three allozymes, SULT1C4-S235L, SULT1C4-F236L, and SULT1C4-D237N, displayed significantly lower sulfating activities when related to the wild-type enzyme. Specifically, SULT1C4-F236L exhibited the least sulfating activity at 18% of the activity of the wild-type enzyme, while the sulfating activities of the other two, SULT1C4-S235L, and SULT1C4-D237N, were approximately 40% and 67% of the activity of the wild-type enzyme, respectively. For the following five allozymes, no activity was detected at the lower concentration level (500 μM): SULT1C4-T59R, SULT1C4-H115R, SULT1C4-L156P, SULT1C4-M263L, and SULT1C4-R264k, and three of them, (SULT1C4-T59R, SULT1C4-H115R, and SULT1C4-L156P) displayed similar patterns in the mid (2000 μM) and higher (5000 μM) substrate concentrations.

In the mid concentration (2000 μM), the sulfating activities of SULT1C4-A136P and SULT1C4-D237N were similar to the activity of the wild-type enzyme, while the sulfating activity of SULT1C4-A136P was slightly higher (7%), and lower in SULT1C4-

D237N (12%) than the activity of the wild-type enzyme at the higher substrate concentration (5000 μM). Furthermore, SULT1C4-V277M displayed the highest sulfating activity in all three substrate concentrations, reaching up to 29% greater than that of the wild-type enzyme. In contrast, SULT1C4-R264k displayed the least considerable sulfating activity at around 10% of that determined for the wild-type at medium (2000 μM) and high (5000 μM) substrates concentrations.

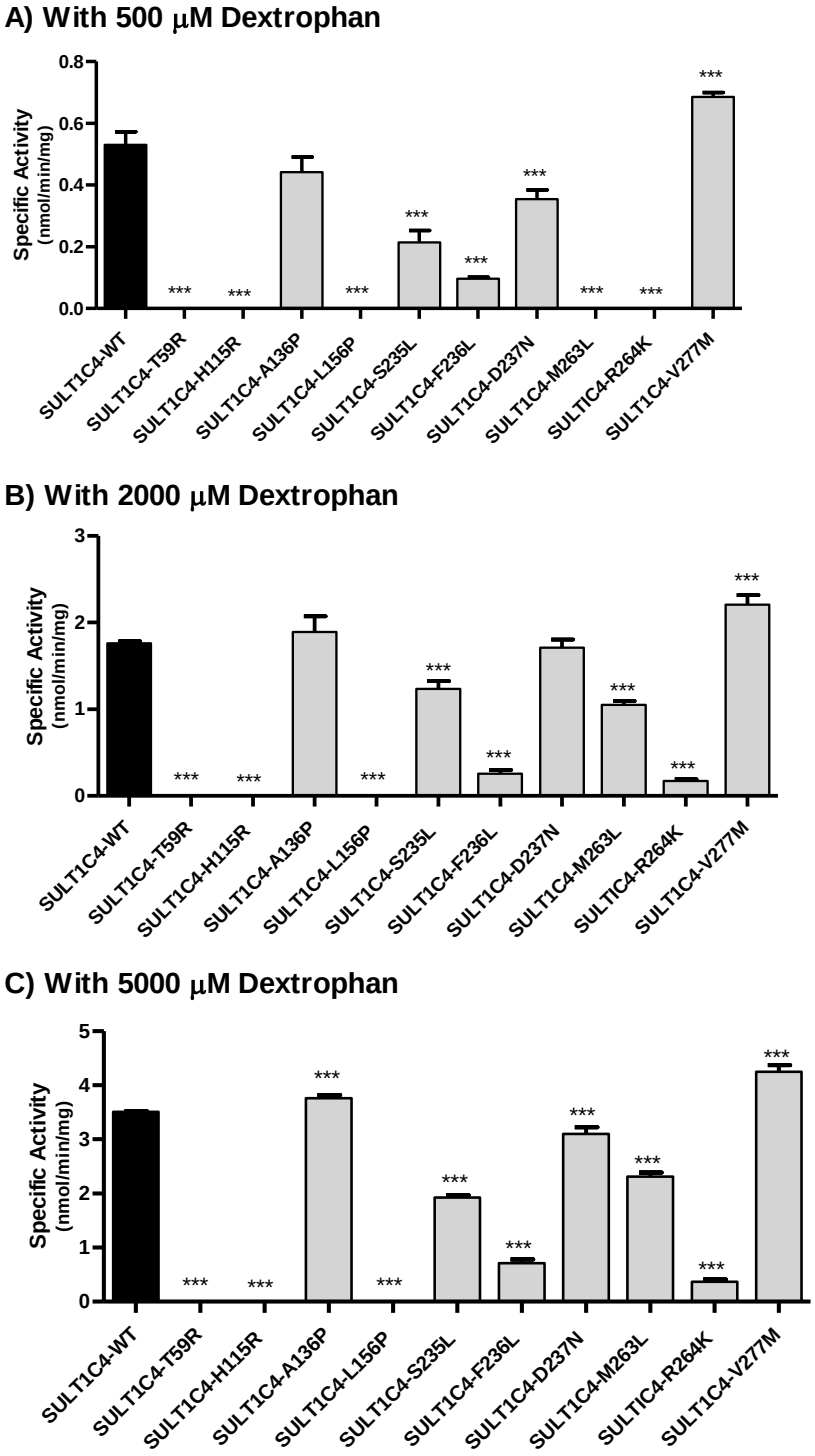

Figure 4.6. Specific activities of the sulfation of dextrophan human SULT1C4 allozymes. Concentrations of dextrophan used in the enzymatic assays were 500 μM (A),

2000 µM (B), and 5000 µM (C). Specific activity refers to nmol dextrophan sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

4.4. Discussion

Acetaminophen and dextromethorphan are two the most commonly used non-narcotic analgesic and antitussive agents, respectively, and are often administered in combination. Given that they are both so widely used, and that they each have a potential for adverse side effects, it is important to fully understand the metabolism of acetaminophen and dextrophan, an active metabolite of dextromethorphan, in individuals with differing metabolic activities. Of particular relevance, several studies have cited that acetaminophen is detoxified primarily through a sulfation reaction in the human fetus (and early on in life) (Adjei et al., 2008; Bertolini et al., 2006). Studies have also indicated that the sulfation pathway is measurably more vital in the pre-natal and post-natal stages of human development, as opposed to adulthood (Adjei et al., 2008; Besunder, Reed, & Blumer, 1988). Because of the SULT1C4's wide substrate range and vast dissemination throughout the human body, SULT1C4 is identified, amongst the known human SULT enzymes, to play an integral role in xenobiotic metabolism and notably maintains a significantly higher expression in the human fetus (Runge-Morris &

Kocarek, 2013; Sakakibara et al., 1998). Current studies have indicated that SULT1C4 is directly involved in the sulfation of acetaminophen (Yamamoto et al., 2015) as well as dextrophan (Yamamoto et al., 2016). Variations in the individual metabolism of acetaminophen and dextrophan have been reported (Peter et al., 2014; Pfaff, Briegel, & Lamprecht, 1983). More importantly, previous research has found that differences in the genetic encoding of enzymes that are responsible for the metabolism of acetaminophen, such as SULTs, may very well be associated with the risk of toxicity induced by acetaminophen in certain individuals (Patel, Tang, & Kalow, 1992; Peter et al., 2014; Zhao & Pickering, 2011). More specifically, a recent study has shown that SNPs in *SULT1A3* affected the sulfating activity of the coded allozymes toward acetaminophen (Bairam et al., 2018). Therefore, the purpose of this study is to explore the influence that single nucleotide polymorphisms (SNPs) of the human *SULT1C4* gene have on the sulfation of acetaminophen and dextrorphan.

Throughout the scope of this study, ten SULT1C4 missense coding SNPs were identified and carefully chosen, and the agreeing SULT1C4 allozymes were expressed, purified. The SULT1C4 allozymes were analyzed with regards to their sulfating activity towards acetaminophen and dextrophan. Based on the kinetic studies completed on the wild-type enzyme, three substrate concentrations were selected for the investigation of the sulfating activity of SULT1C4 allozymes toward acetaminophen and dextrophan. The findings of this study clearly demonstrate that alterations in the amino acid of the SULT1C4 enzyme directly resulted in recorded differential sulfating activities of the allozymes towards the two drugs (cf. **Figure 4.2** and **4.3**). Moreover, the findings of the

study firmly support the conclusion that *SULT1C4* missense coding SNPs affect the sulfation of acetaminophen and dextrophan via coded SULT1C4 allozymes.

The human SULT1C4 enzyme has been noted to possess essential residues that are vital to the function of the enzyme, as reported in its crystal structure (Allali-Hassani et al., 2007). Those residues include: the PAPS/PAP binding regions (55KAGTTW60, 234TSFDVM239, 262FMRKG266, Arg137, Ser145, and Tyr200), the C-terminal dimerization motif region (272KKHFTVAQNE281) (Petrotchenko, Pedersen, Borchers, Tomer, & Negishi, 2001), the residue involved in the catalysis His115, the β-sheet in the N-terminal region (vital to protein folding), the 5'-phosphosulphate-binding (PSB) loop (52TYPKAGT58), and the substrate binding residues (Lys113 and Thr114) (Allali-Hassani et al., 2007). Six of the ten considered SULT1C4 allozymes contained amino acid variations that reside within the PAPS/PAP binding regions: SULT1C4-R264k, SULT1C4-T59R, SULT1C4-M236L, SULT1C4-S235L, and SULT1C4-F236L. One allozyme was noted to feature an amino acid change at the catalytic residue (SULT1C4-H115R). Five of the 10 allozymes exhibited a remarkable decrease in, if not a complete loss of, their catalytic efficiency towards acetaminophen and dextrophan. Those five allozymes are: SULT1C4-R264k, SULT1C4-H115R, SULT1C4-T59R, SULTL156P, and SULT1C4-F236L. Histidine, a catalytic base, is one of the most significant conserved residues residing in all SULTs. Available research suggests that histidine facilitates the nucleophilic attack of the sulfuric atom in PAPs by accepting a proton from the substrate phenyl group (Negishi et al., 2001). In replacing histidine with arginine in SULT1C4-H115R, it was predicted that the deprotonation from the substrates would be affected,

resulting in the suppression of the dissociation of the sulfuryl group from PAPS. The substitution, as predicted, resulted in a total loss of sulfating activity for SULT1C4-H115R. Similarly, previous studies have found that the mutation of the catalytic histidine residue in other cytosolic sulfotransferases caused an entire loss of sulfating activity (Kakuta, Petrotchenko, Pedersen, & Negishi, 1998; Liu, Suiko, & Sakakibara, 2000). Accordingly, the findings of this study are consistent with the results of those previously conducted (Chen, 2004; Kakuta, Petrotchenko, Pedersen, & Negishi, 1998; Liu, Suiko, & Sakakibara, 2000; Pedersen et al., 2002).

No sulfating activity was observed for SULT1C4-T59R. This study proposes the explanation that the substitution of threonine, (a polar uncharged amino acid), with arginine, (a positively charged amino acid), in SULT1C4-T59R resulted in the disruption of the hydrogen bonding between threonine and PAPS. Thereafter, the disruption then destabilized the binding of PAPS to the allozyme. Similarly, the allozyme SULT1C4-L156P, showed a total loss in sulfating activity with regards to both substrates. An explanation of the allozyme's loss of catalytic activity is related to the replacement of non-turn-inducing residues (like leucine) in SULT1C4-L156P with proline (turn-inducing residue), causing an unfavorable alteration in the enzyme structure. A distinct amino acid with its' aliphatic side chain bonded to both the backbone carbon and nitrogen, (resulting in a non-reactive side chain), proline is unable to adopt main chain conformations, thereby resulting in a sharp turn in the polypeptides (Betts & Russell, 2003).

With regards to the SULT1C4-F236L allozyme, which indicated a major reduction in sulfating activity, a change in the side chain from being aromatic to aliphatic, (even though both residues are non-polar), is considered a strong explanation for the weakness of the enzyme activity, given the location of the F236 residue in the PAPs binding region. Concerning the allozyme SULT1C4-R264k, the replacement of arginine with lysine is expected to be the cause behind the reduction of the allozyme's enzymatic activity towards acetaminophen and dextrophan at all of the substrate concentration levels. In particular, arg264 is a conserved residue located at the PAPS binding site (Allali-Hassani et al., 2007). Because preceding studies indicate that arg274 residue in SULT2B1b forms a hydrogen with the negatively charged O3P phosphate oxygen of PAPS (Lee et al., 2003), it follows that the replacement of arginine with lysine in the allozyme subsequently reduced its enzymatic activity towards the substrates.

Throughout the study, SULT1C4-V277M was the only allozyme to show a significantly greater sulfating activity than the activity of the wild-type enzyme for both substrates at all concentration levels. Located in the C-terminal dimerization motif region, Valine277 is involved in mediating the dimerization of two SULT1C4 monomers (Petrotchenko et al., 2001). This is made possible through an amino acid, like methionine, that shares similar amino acid properties with valine, (non-polar, aliphatic side chain). Accordingly, the replacement of valine with methionine in SULT1C4-V277M may have led to the reinforcement of the dimerization capability between two subunits of the enzyme, which may explain the increase in the sulfating activity of SULT1C4-V277M towards acetaminophen and dextrophan.

In summary**,** this exploratory study addresses a specific research gap in gathering, and examining, information regarding the influence of genetic polymorphisms on the sulfating activity of human SULT1C4 towards acetaminophen and dextrophan. The results and findings of the study clearly illustrate that human SULT1C4 allozymes show differential sulfating activities in relation to both substrates. As such, the study strongly supports the conclusion that individuals with different *SULT1C4* genotypes may exhibit differential metabolism of acetaminophen and dextrophan. These findings may contribute significantly to future efforts to develop individualized regimens of both drugs, therein minimizing adverse side effects and improving the drugs' therapeutic efficacy.

Chapter 5

Sulfation of ODM-quinidine and ODM-quinine by the Human Cytosolic Sulfotransferases: A Systematic Analysis

5.1. Introduction

The quinolone alkaloid quinidine and its levorotatory diastereomer quinine are primarily extracted from cinchona bark (Weinreb, 2001). In many respects, their pharmacological activities are similar; however, their clinical applications differ. Since the early 1900s, quinidine has been used for the treatment of arrhythmias; it is considered a class IA antiarrhythmic drug (AAD) (Higgins, Waks, & Josephson, 2015). Meanwhile, quinine is used to treat malaria and to prevent nocturnal leg cramps (Achan et al., 2011; Man-Son-Hing, Wells, & Lau, 1998). Despite their different applications, both drugs share a serious adverse effect, cardiotoxicity (Bailey, 1960; Bonington, Davidson, Winstanley, Pasvol, & Hygiene, 1996). In man, quinidine and quinine are metabolized in a similar fashion. Metabolites of quinidine and quinine are produced by oxidation

reactions that involve either the quinoline or quinuclidine moieties (Bannon, Yu, Cook, Roy, & Villeneuve, 1998). Quinidine is primarily metabolized by the liver, but is also detected unchanged in the urine (13-27%). Several quinidine metabolites have been identified, including *O*-desmethylquinidine (ODM-quinidine), 3-hydroxyquinidine, and quinidine-N-oxide (Drayer et al., 1978; Hoyer, Clawson, Brookshier, Nolan, & Marcus, 1991; Nielsen, Rosholm, & Brøsen, 1995). ODM-quinidine is an active metabolite that exhibits antiarrhythmic activity (Hoyer et al., 1991) as well as inhibitory effects against CYP2D6 (Ching et al., 1995). Meanwhile, *O*-desmethylquinine (ODM-quinine) is an active metabolite of quinine found in human urine (Bannon et al., 1998). Compared to quinine, ODM-quinine has been reported to have lower blood toxicity when used to treat cramps in rats with spinal cord injury (Adnyana, Sukandar, Setiawan, & Christanti, 2013).

Sulfate conjugation is key for metabolizing xenobiotics, such as drugs, and for the biotransformation of endogenous substrates including catecholamines, bile acid, and steroid hormones (Falany, 1997; Weinshilboum & Otterness, 1994). When conjugated to a molecule, the sulfonate group confers a negative charge upon it, thereby enhancing its elimination from the body. Transfer of a sulfonate group from the donor molecule (3-phosphoadenosine-5-phosphosulphate, PAPS) to a substrate containing hydroxyl or amino groups is catalyzed by cytosolic sulfotransferases (SULTs) (Lipmann, 1958). There are four SULT gene superfamilies (SULT1, SULT2, SULT4, and SULT6), which contain distinct subfamilies. Eight SULTs belong to the SULT1 gene family: *SULT1A1* and *SULT1A2* (general detoxification SULTs), *SULTA3* (catecholamine SULT),

SULT1B1 (thyroid hormone SULT), *SULT1C2*, *SULT1C3*, and *SULT1C4* (hydroxyarylamine SULTs), and *SULT1E1* (estrogen SULT). The SULT2 family consists of two genes, *SULT2A1* and *SULT2B1* (hydroxysteroid SULTs). There is one member of the SULT4 family, *SULT4A1*. The last family is SULT6, which contains *SULT6B1* (Blanchard, Freimuth, Buck, Weinshilboum, & Coughtrie, 2004). UDP glucuronosyltransferases and SULTs have been reported to catalyze approximately 40% of drug conjugation reactions; more broadly, a large portion of Phase II conjugation reactions are carried out by these enzymes (Evans & Relling, 1999). This study aimed to identify those human SULTs that can specifically sulfate ODM-quinidine and ODM-quinine. Moreover, reports of single nucleotide polymorphisms (SNPs) in SULT genes (Abunnaja et al., 2018; Bairam et al., 2018; Rasool et al., 2019) raise the question of whether these polymorphisms affect the sulfation of ODM-quinidine and ODM-quinine, thereby impacting their metabolism on the level of individuals.

In this study, we investigated the capability of human SULT enzymes to sulfate ODM-quinidine and ODM-quinine. A kinetic study was conducted to the enzyme that showed the most sulfating activity. Subsequently, the potential effect of polymorphisms was evaluated by comparing the activities of purified SULT1C4 allozymes on both drugs with that of the wild-type.

5.2. Materials and Methods

Materials and Methods were previously described in chapter 2.

5.3. Results

5.3.1. Identification of Human SULT(s) Capable of Sulfating ODM-Quinidine and ODM-Quinine

Thirteen purified human SULTs were screened for sulfating activity toward ODM-quinidine and ODM-quinine (**Table 5.1**). Of these, two (SULT1B1 and SULT1C4) showed sulfating activity toward ODM-quinidine, with SULT1C4 having the higher activity. Meanwhile, SULT1C4 was the only enzyme capable of sulfating ODM-quinine. It therefore appears that SULT1C4 is the main enzyme responsible for sulfating both ODM-quinidine and ODM-quinine.

The sulfating activity of SULT1C4 toward ODM-quinidine and ODM-quinine was further evaluated using a concentration-dependent assay. A range of concentrations were tested for ODM-quinidine (0, 0.05, 0.1, 0.25. 0.5, 0.75, 1, 2, 3, 4, and 5 mM) and ODM-quinine (0, 0.025, 0.05, 0.1, 0.25. 0.5, 0.75, 1, 2, 3, 4, and 5 mM). For both substrates, the sulfating activity of SULT1C4 continuously increased as substrate concentration increased (**Figure 5.1**). No inhibitory concentrations were identified for either drug.

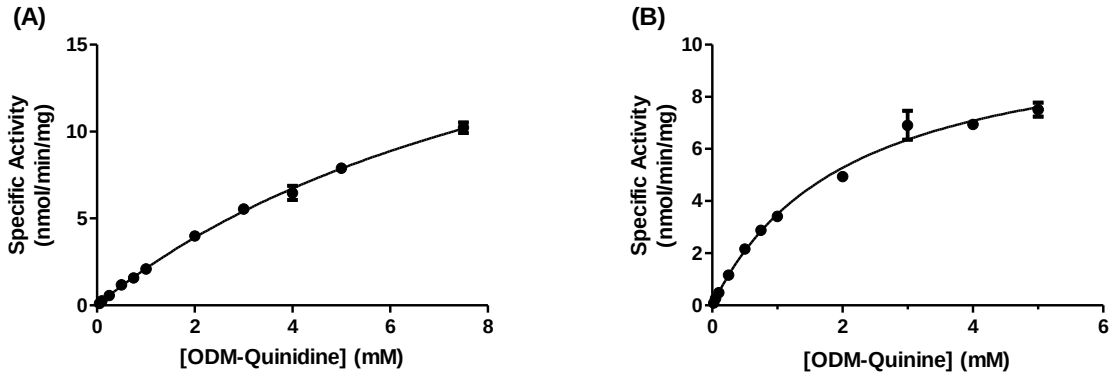

Figure 5.7. Kinetic analysis for the sulfation of the tested substrates by wild-type human SULT1C4. (A) and (B) plots represent the nonlinear Michaelis–Menten enzyme kinetics for ODM-quinidine and ODM-quinine, respectively. Data shown represent calculated mean ± standard deviation derived from three experiments.

Table 5.2. Specific activity of human SULTs with ODM-quinidine and ODM-quinine as substrates.[a]

	Specific activity (nmol/min/mg)	
Substrate	SULT1B1	SULT1C4
ODM-quinidine	0.014 ± 0.002	0.423 ± 0.027
ODM-quinine	N.D.[b]	1.70 ± 0.020

[a]Specific activity refers to nmol substrate sulfated/min/mg purified enzyme. Data shown represent the mean ± standard deviation derived from three experiments. Substrate concentrations were 100 µM for ODM-quinidine and 250 µM for ODM-quinine. [b]N.D. refers to activity not detected

5.3.2. Characterization of the Sulfating Activity of Human SULT1C4 Allozymes Toward ODM-Quinidine and ODM-Quinine

The sulfating activities of SULT1C4 allozymes toward ODM-quinidine and ODM-quinine were compared against wild-type at three substrate concentrations (0.25, 2, and 5 mM).

With ODM-quinidine as substrate

The sulfating activities of SULT1C4 allozymes toward ODM-quinidine differed from those of the wild-type enzyme (**Figure 5.2**). Three allozymes (SULT1C4-T59R, SULT1C4-H115R, and SULT1C4-L156P) displayed complete loss of activity at all substrate concentrations, while two (SULT1C4-F236L and SULT1C4-R264K) showed barely recognizable activity. In contrast, SULT1C4-S235L and SULT1C4-M263L exhibited higher activities (by 26% and 6%, respectively) than wild-type at low (0.25 mM) substrate concentration, then declined in activity (by 11% and 12%, respectively) at the highest substrate concentration (5 mM). The last three allozymes (SULT1C4-A136P, SULT1C4-D237N, and SULT1C4-V277M) displayed increased activity at all substrate concentrations. Among them, SULT1C4-D237N showed the highest activity, being more than two times that of the wild-type. The remaining two (SULT1C4-A136P and SULT1C4-V277M) achieved respective activities of 53% and 72% greater than the wild-type enzyme.

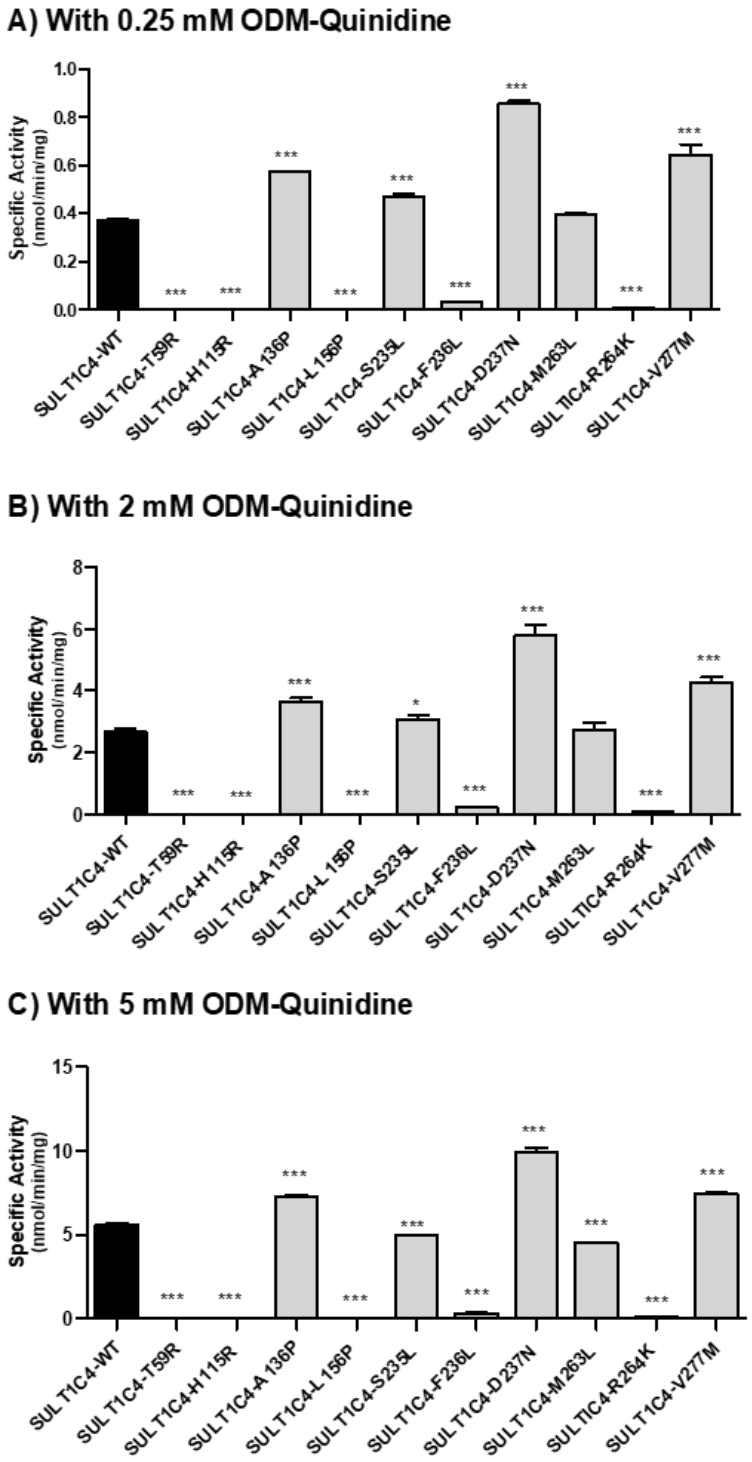

Figure 5.8. Specific activities of the sulfation of ODM-quinidine human SULT1C4 allozymes. Concentrations of ODM-quinidine used in the enzymatic assays were 0.25

mM (A), 2 mM (B), and 5 mM (C). Specific activity refers to nmol ODM-quinidine sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

With ODM-quinine as substrate

Different SULT1C4 allozymes displayed different specific activities against ODM-quinine (**Figure 5.3**). Similar to ODM-quinidine, no sulfating activity was detected from SULT1C4-T59R, SULT1C4-H115R, or SULT1C4-L156P at any concentration of ODM-quinidine (0.25, 2, and 5 mM). Also like ODM-quinidine, SULT1C4-F236L and SULT1C4-R264K had barely noticeable sulfating activity. Meanwhile, SULT1C4-S235L and SULT1C4-M263L exhibited activity consistently lower than the wild-type enzyme; specifically, SULT1C4-S235L respectively demonstrated around 94%, 85%, and 85% of the wild–type activity at low (0.25 mM), mid (2 mM), and high (5 mM) substrate concentrations, while SULT1C4-M263L respectively had around 84%, 76%, and 61% of wild-type activity at the same concentrations. Higher sulfating activity was displayed by the remaining three allozymes (SULT1C4-A136P, SULT1C4-D237N, and SULT1C4-V277M). Of those, SULT1C4-D237N displayed the highest activity, with increases of around 76%, 60%, and 34% at concentrations of 0.25, 2 and 5 mM, respectively. The other two allozymes (SULT1C4-A136P and SULT1C4-V277M) showed increases of up to 19% and 32% compared to the wild-type enzyme.

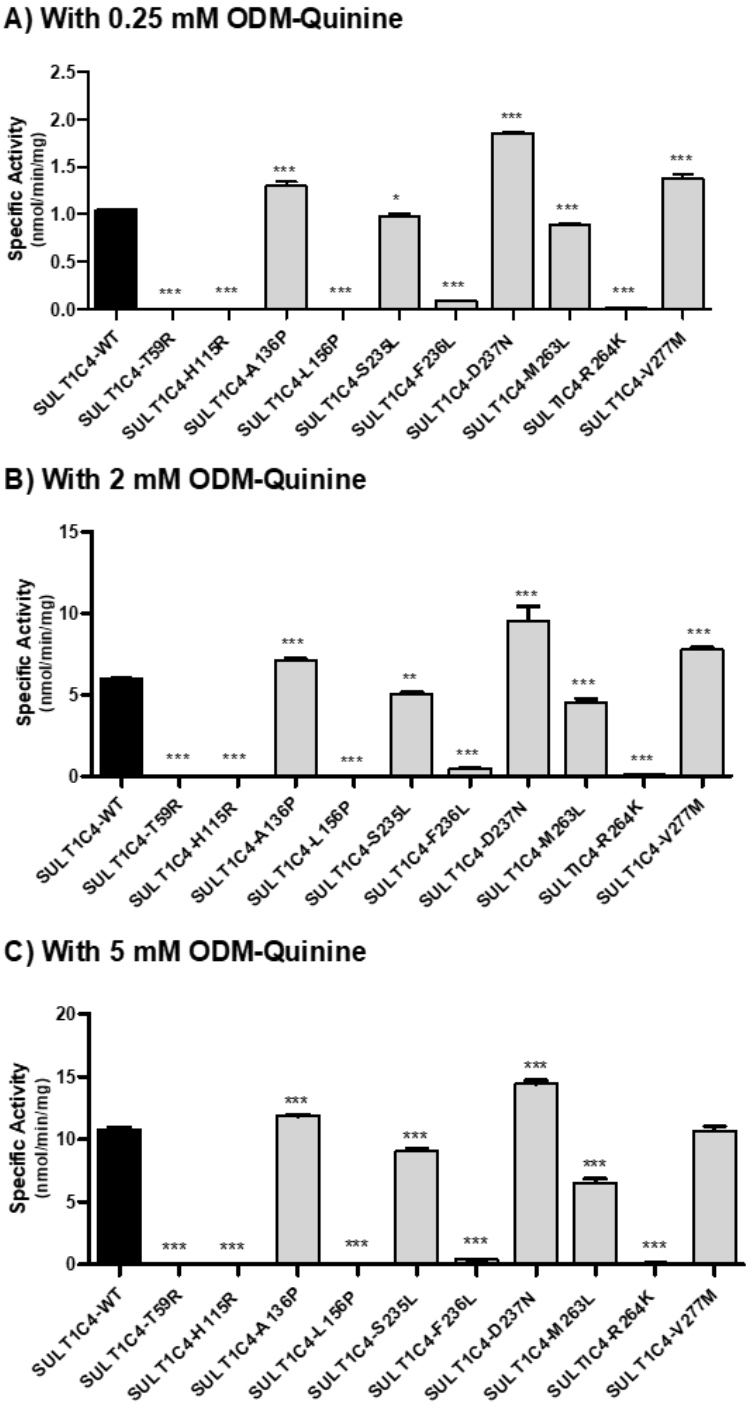

Figure 5.3. Specific activities of the sulfation of ODM-quinine human SULT1C4 allozymes. Concentrations of ODM-quinine used in the enzymatic assays were 0.25 mM (A), 2 mM (B), and 5 mM (C). Specific activity refers to nmol ODM-quinine

sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

5.4. Discussion

ODM-quinidine and ODM-quinine are metabolites of quinidine and quinine, respectively, that possess activity against various diseases. The aim here was to investigate whether human cytosolic sulfotransferases are capable of sulfating these molecules. Thirteen purified SULTs were screened for activity on both substrates. SULT1C4 exhibited the highest sulfating activity toward ODM-quinidine, followed by SULT1B1, and was the only enzyme capable of sulfating ODM-quinine. Accordingly, SULT1C4 is likely to be the primary enzyme mediating sulfation of both molecules in the human body. Given that sulfation reaction can vary between individuals and affect their responses to treatment, it is worth investigating the impact of *SULT1C4* missense SNPs on its activity toward ODM-quinidine and ODM-quinine. Accordantly, ten missense SNPs in *SULT1C4* were selected and site-directed mutagenesis used to generate the corresponding cDNAs. The resulting recombinant SULT1C4 allozymes were expressed, purified, and characterized in terms of their activities toward ODM-quinidine and ODM-quinine.

Concentration-dependent assays of the wild-type SULT1C4 were used to select three substrate concentrations for the profiling of allozyme activities. The results clearly showed that alteration of the amino acid sequence of SULT1C4 affected its ability to sulfate ODM-quinidine and ODM-quinine. In particular, these results strongly indicate that missense SNPs in *SULT1C4* affect the sulfation of ODM-quinidine and ODM-quinine, suggesting potential functional effects for some polymorphisms.

Certain regions of human SULT1C4, as demonstrated in its crystal structure (Allali-Hassani et al., 2007), are essential to the enzyme's function. These regions include the PAPS binding regions (55KAGTTW60, 234TSFDVM239, 262FMRKG266), the PAPS binding residues (Arg137, Ser145, and Tyr200), the Lys113 and Thr114 residues involved in substrate binding, the critical catalytic residue (His115) (Allali-Hassani et al., 2007), and the dimerization motif (272KKHFTVAQNE281) (Petrotchenko, Pedersen, Borchers, Tomer, & Negishi, 2001). In all SULTs, one of the most important and highly conserved residues is the catalytic histidine. This histidine is proposed to accept a proton from a phenyl group on the substrate and subsequently to facilitate a nucleophilic attack on the sulfuric atom in the sulfuryl donor PAPs (Chen, 2004; Negishi et al., 2001). In the SULT1C4-H115R allozyme, this residue is replaced by arginine, which may affect deprotonation and suppress dissociation of the sulfuryl group from PAPS. This is reflected in the complete loss of sulfating activity observed here for SULT1C4-H115R, and in existing research on mutation of the catalytic histidine in other cytosolic sulfotransferases (Kakuta, Petrotchenko, Pedersen, & Negishi, 1998; Liu, Suiko, & Sakakibara, 2000). The SULT1C4-T59R allozyme also demonstrated no

sulfating activity. In this allozyme, the polar uncharged threonine was substituted with positively-charged arginine. The threonine in question normally forms a hydrogen bond with PAPS. Loss of this threonine residue may therefore have destabilized the enzyme's interaction with PAPS. Likewise, the allozyme SULT1C4-L156P displayed no sulfating activity toward both substrates. The side chain of proline is non-reactive due to being uniquely bonded with both the backbone carbon and the nitrogen. This feature renders the amino acid unable to adopt main chain conformations, and in polypeptides produces a sharp turn (Betts & Russell, 2003). When proline replaces a non-turn-inducing residue, including the leucine in SULT1C4-L156P, it may have unfavorable effects on the enzyme's structure and therefore its catalytic activity.

Meanwhile, the allozyme SULT1C4-F236L and SULT1C4-R264K exhibited barely detectible activities. While both the wild-type and variant residues of SULT1C4-F236L allozyme are non-polar, the drop in enzyme activity may be explained by the shift from aromatic to aliphatic side chain combined with the position of F236 in the PAPS binding region. In case of SULT1C4-R264K, the affected residue is both conserved and located in the PAPS binding site (Allali-Hassani et al., 2007). Previous research has indicated its counterpart in SULT2B1b to form a hydrogen bond with the negatively-charged O3P phosphate oxygen in PAPS (Lee et al., 2003). It is therefore reasonable for the substitution of arginine by lysine in SULT1C4-R264K to abolish its enzymatic activity, as observed at all tested concentrations.

On the other hand, two allozymes (SULT1C4-S235L and SULT1C4-M263L) were less affected by amino acid substitutions. This is because the substituted residues share similar amino acid properties. Specifically, both serine and leucine in SULT1C4-S235L are uncharged residues, while methionine and leucine residues in SULT1C4-M263L are nonpolar. As predicted, the substitution of Ser235 with leucine in SULT1C4-S235L, and the substitution of Met263 with leucine in SULT1C4-M263L, did not drastically affect the activity of the two allozymes.

Only three allozymes (SULT1C4-A1365P, SULT1C4-D237N, and SULT1C4-V277M) showed higher activities toward both substrates. Of these, SULT1C4-D237N demonstrated the highest activity. The replacement of Ala136 (non-turning residue) with proline (turn-inducing residue) in SULT1C4-A1365P may produce a favorable turn in the enzyme structure, which may enhance the enzyme activity. On the other hand, the two residues substituted in SULT1C4-D237N, and SULT1C4-V277M share similar properties. In SULT1C4-D237N, the replacement of aspartate 237 with asparagine, which are both polar with aliphatic side chains, may enhance its binding affinity for PAPS, thereby increasing the sulfating activity. The affected residue in SULT1C4-V277M, a valine, mediates enzyme dimerization (Petrotchenko et al., 2001). Its replacement with methionine, which possesses similar properties (non-polar, aliphatic side chain), may have acted to reinforce dimerization of two SULT1C4 subunites, thereby increasing the allozyme's sulfating activity towards ODM-quinidine and ODM-quinine.

In summary, the current study demonstrated that SULT1C4 is the main enzyme capable of mediating the sulfation of ODM-quinidine and ODM-quinine, at least among known human SULTs. Additionally, genetic polymorphisms in *SULT1C4* were shown to affect the activity of the encoded allozymes toward both substrates. These findings suggest the potential for individuals with different *SULT1C4* genotypes to exhibit differential sulfation of ODM-quinidine and ODM-quinine.

Chapter 6

Effects of the Human SULT1C4 Polymorphisms on the Sulfation of Estrone and Estradiol

6.1. Introduction

Estrone (E1) and estradiol (E2) are steroid hormones that play an integral role in modulating the physiological functions during pregnancy in both the mother and the fetus. They are potent forms of endogenous estrogens that exert their biological effects by binding to estrogen receptors (ERs) (Raftogianis, Creveling, Weinshilboum, & Weisz, 2000). Estrogens affect the growth of uterine, vagina, and breast, the timing of parturition and lactation, as well as the regulation of fetal neuro-endocrine maturation and organogenesis (Kaludjerovic & Ward, 2012; Price & Harvey, 1947; Wood, 2014). It is thus important to maintain the homeostasis of estrogens *in vivo*. In this regard, estrogens have been shown to be subjected to oxidation and conjugation reactions (Guengerich, 1990; Zhu & Conney, 1998). Studies have demonstrated the involvement of conjugation reactions, particularly sulfation, in the metabolism of estrogens (Raftogianis et al., 2000).

Conjugated estrogens have been shown to be the most abundant circulating estrogens in fetal and maternal circulation (Wood, 2014). Sulfated estrogens, E1S and E2S, are notably more abundant than unsulfated E1 and E2 in both the pregnant mother and the fetus (Carnegie & Robertson, 1978; Wood, 2014). E1, E2, and their sulfated conjugates, E1S and E2S, have been detected in fetal plasma, as well as various fetal organs, including brain, liver, heart, and kidney and adrenal cortex (Milewich, MacDonald, & Carr, 1989). It is worth noting that while sulfated estrogens are unable to interact with ERs, they have been shown to be capable of modulating fetal neural functions such as the activation of neuroprotective pathways and an overall increase in neural activity (Rabaglino, Richards, Denslow, Keller-Wood, & Wood, 2012; Schumacher et al., 2008).

Sulfate conjugation is known to play a crucial role in the metabolism of xenobiotics, including drugs, as well as endogenous compounds such as catecholamines, thyroid and steroid hormones, and bile acid (Falany, 1997; Kauffman, 2004; Weinshilboum et al., 1997). The responsible enzymes, called the cytosolic sulfotransferases (SULTs), act as catalysts in mediating the transfer of a sulfonate group from the donor 3-phosphoadenosine-5-phosphosulfate (PAPS) to substrate compounds containing hydroxyl or amino groups (Falany, 1997). Since the expression levels of other metabolizing enzymes, such as cytochromes P450 and UDP-glucuronosyltransferases enzymes, have been shown to be low during human fetal development, it has been suggested that SULT-mediated sulfation may play a primary role in the metabolism of above-mentioned endogenous compounds and xenobiotics in the fetus (Guidry, Tibbs, Runge-Morris, & Falany, 2017; Stanley, Hume, & Coughtrie, 2005). In humans, there

are thirteen distinct SULTs that are classified into four SULT gene families, designated SULT1, SULT2, SULT4, and SULT6 (Blanchard, Freimuth, Buck, Weinshilboum, & Coughtrie, 2004). SULT1C4 and other SULTs, such as SULT1A1, SULT1A2, SULT1A3, SULT1E1, and SULT2A1, have been found capable of sulfating E1 and E2 (Hui, Yasuda, Liu, Wu, & Liu, 2008). Studies have shown that SULT1C4 is highly expressed in fetal lungs and kidney, and at lower levels in the fetal heart, infant liver, adult ovary, kidney, brain, and spinal cord (Dubaisi et al., 2019; Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998). It has been proposed that SULT1C4 may be involved in the sulfation of endogenous compounds, such as E1 and E2, that modulate the development of the fetus (Guidry et al., 2017; Hui et al., 2008; Pai, Suiko, Sakakibara, & Liu, 2001). Many *SULT* genes are known to manifest genetic polymorphisms (Bairam et al., 2018; Rasool et al., 2019). It is conceivable that *SULT1C4* coding SNPs, leading to amino acid changes in coded protein products, may influence the sulfating activity of SULT1C4 allozymes toward substrates, including E1 and E2, thereby altering the physiological response to these steroid hormones in the developing fetus and possibly underscore the disease phenotypes associated with specific *SULT1C4* genotypes.

In this study, a comprehensive database search for the human *SULT1C4* SNPs was performed. cDNAs corresponding to ten selected missense *SULT1C4* SNPs were generated. Recombinant SULT1C4 allozymes were bacterially expressed and affinity-purified. Purified SULT1C4 allozymes, in comparison with the wild-type enzyme, were analyzed for their differential sulfating activity toward E1 and E2.

6.2. Materials and Methods

Materials and Methods were previously described in chapter 2.

6.3. Results

6.3.1. Identification and Analysis of Different Human *SULT1C4* SNPs

The National Center for Biotechnology Information (NCBI) and the UniProt Knowledgebase (UniProtKB) databases were searched for *SULT1C4* SNPs. A total of 2,849 SNPs were identified and classified as the following: 4 in the 3'-spliced region, 3 in the 5'-spliced region, 336 in the 3'-untranslated region, 184 in the 5'-untranslated region, 1,975 in introns, 74 synonymous coding SNPs (cSNPs), 203 nonsynonymous cSNPs, 17 frame shift SNPs, 10 nonsense SNPs, and 9 stop-gained SNPs. The scope of the current study was limited to the nonsynonymous cSNPs. Ten SNPs were selected for further investigation on the basis of the location of the SNP (within or in proximity to the dimerization motif, PAPS-binding site, active site and/or substrate binding site) and the chemical nature of the altered amino acid residues (polar to (or from) nonpolar, acidic to (or from) basic, and turn-inducing to (or from) non-turn inducing residues), which may potentially affect protein conformation and function. **Table 2.3** shows the selected *SULT1C4* cSNPs and the mutagenic primers designed for the PCR-amplification of the cDNAs encoding the corresponding SULT1C4 allozymes.

6.3.2. Preparation of Recombinant Human SULT1C4 Allozymes

Recombinant SULT1C4 allozymes were bacterially expressed and purified using glutathione-Sepharose affinity chromatography. **Figure 2.4** shows the SDS-gel electrophoretic pattern of the ten purified SULT1C4 allozymes together with the wild-type enzyme. All ten SULT1C4 allozymes, as well as the wild-type enzyme, appeared highly homogeneous and all migrated at ~35.5 kD position, which is in accordance with their predicted molecular weights.

6.3.3. Kinetic Analysis of the Sulfation of E1 and E2 by Wild-Type SULT1C4.

Enzymatic assays were performed using varying concentrations of E1 or E2 as substrates in order to determine the kinetic constants of the sulfation of E1 and E2 by wild-type SULT1C4. **Figure 6.1** shows the concentration-dependent curves for the sulfation of E1 and E2 by SULT1C4. The SULT1C4-mediated sulfation of E1 or E2 appeared to follow Michaelis-Menten kinetics up to the substrate concentrations of 100 μM and 250 μM, respectively. Significant substrate inhibition was observed at higher concentrations. Data obtained were processed using GraphPad Prism 7 based on Michaelis-Menten equation with non-linear regression. Kinetic constants (K_m, V_{max}, and V_{max} / K_m) obtained are compiled in **Table 6.1**. Wild-type SULT1C4 displayed similar K_m values, 49.63 and 49.34 nmol/min/mg, respectively, for E1 and E2. In regard to V_{max}, SULT1C4 displayed a value (8.75 ± 0.18 nmol/min/mg) with E1 as substrate, which is approximately 28% higher than that (6. 26 ± 0. 04 nmol/min/mg) with E2 as substrate.

Based on calculated V_{max}/K_m, it therefore appears that SULT1C4 has a slightly higher catalytic efficiency with E1 vs. E2 as substrate.

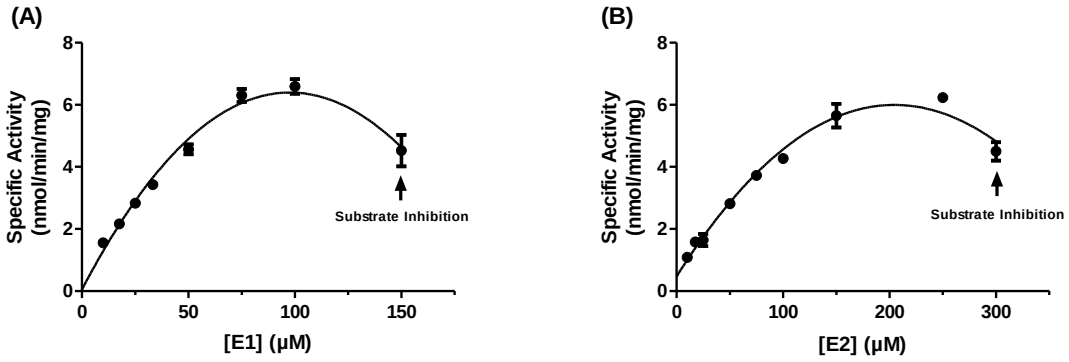

Figure 6.1. Kinetic analysis for the sulfation of the tested substrates by wild-type human SULT1C4. (A) and (B) plots represent the nonlinear Michaelis–Menten enzyme kinetics for E1 and E2, respectively. Data shown represent calculated mean ± standard deviation derived from three experiments.

Table 6.1. Kinetic parameters of the human SULT1C4 wild type with E1 and E2 as substrates.

Substrate	K_m (μM)	V_{max} (nmol/min/mg)	V_{max} /K_m(ml/min/mg)
E1	49.625± 3.152	8.747± 0.179	0.176
E2	49.340± 2.204	6.256± 0.036	0.127

6.3.4. Enzymatic Characterization of SULT1C4 Allozymes.

The sulfating activity of SULT1C4 allozymes, in comparison with the wild-type enzyme, toward E1 and E2 was analyzed. Based on the K_m values determined for wild-type SULT1C4 (cf. **Table 6.1**, three substrate concentrations (one well below the K_m, one around the K_m, and one well above the K_m) were used to assay for the sulfating activity of SULT1C4 allozymes as well as the wild-type enzyme.

With E1 as the substrate.

Activity data shown in **Figure 6.2** indicated that the SULT1C4 allozymes displayed differential sulfating activities toward E1 at each of the three concentrations (5, 50, and 100 µM) tested. Of the ten human SULT1C4 allozymes, two (SULT1C4-T59R and SULT1C4-H115R) showed no detectable sulfating activities, while three (SULT1C4-L156P, SULT1C4-F236L, and SULT1C4-R264k) displayed barely detectable activities at all three substrate concentrations. Of the remaining five, three allozymes (SULT1C4-A136P, SULT1C4-S235L and SULT1C4- M236L) consistently showed lower sulfating activities than the wild-type enzyme at all three substrate concentrations. Among these three, the sulfating activity of SULT1C4-S235L was greater than 60% lower compared with the wild-type enzyme, while SULT1C4-A136P showed approximately 51%, 58%, and 34% lower sulfating activities at, respectively, 5, 50, and 100 µM substrate concentrations. On the other hand, SULT1C4-M236L exhibited 10% lower activity at 5 µM substrate concentration, and a ~34% lower activity at 100 µM substrate concentration. For the two remaining allozymes, SULT1C4-V277M exhibited a decrease, compared with the wild-type, in the sulfating activity by approximately 34%

and 19% at low (5 μM) and high (100 μM) substrate concentrations, respectively, while showing no significant difference at the mid (50 μM) substrate concentration. Whereas SULT1C4-D237N displayed comparable sulfating activity at mid (50 μM) and high (100 μM) substrate concentrations, with a lower (by ~14%) sulfating activity at low (5 μM) substrate concertation.

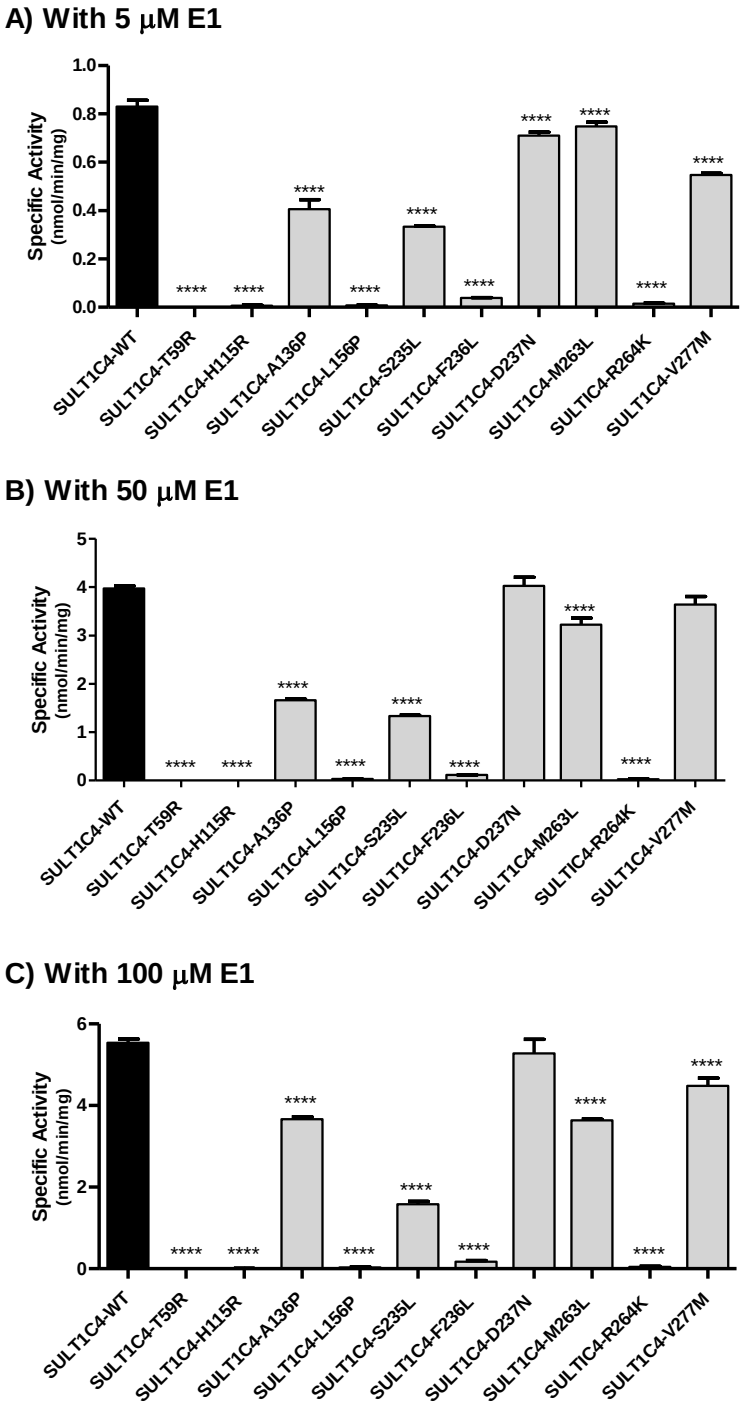

Figure 6.2. Specific activities of the sulfation of E1 human SULT1C4 allozymes. Concentrations of E1 used in the enzymatic assays were 5 μM (A), 50 μM (B), and 100

μM (C). Specific activity refers to nmol E1 sulfated/min/mg of purified allozyme. Data shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

With E2 as the substrate.

Like with E1, differential E2-sulfating activities, shown in **Figure 6.3**, were found for the SULT1C4 allozymes tested compared with the wild-type enzyme. Among the ten human SULT1C4 allozymes, two allozymes (SULT1C4-T59R and SULT1C4-H115R) showed no activities at all three substrate concentrations. No considerable activity was seen among these three allozymes: SULT1C4-L156P, SULT1C4-F236L, and SULT1C4-R264k. On the other hand, two allozymes (SULT1C4-R S235L and SULT1C4-A136P) exhibited lower sulfating activity than the wild type enzyme at the three substrate concentrations (5, 50, and 100 μM), being reduced by at least 31% and 29%, respectively. Meanwhile, the remaining three allozymes (SULT1C4-D237N, SULT1C4-M236L, and SULT1C4-V277M) showed comparative sulfating activity with the wild-type enzyme at 5 and 50 μM substrate concentrations. At 100 μM of E2, the activity of SULT1C4-V277M was approximately 18% lower than the wild-type SULT1C4, while SULT1C4-M236L showed comparative sulfating activity with the wild-type enzyme. Among the ten allozymes, SULT1C4-D237N was the only enzyme that showed a significantly higher sulfating activity than the wild type enzyme, with an approximate 18% increase at higher substrate concentration.

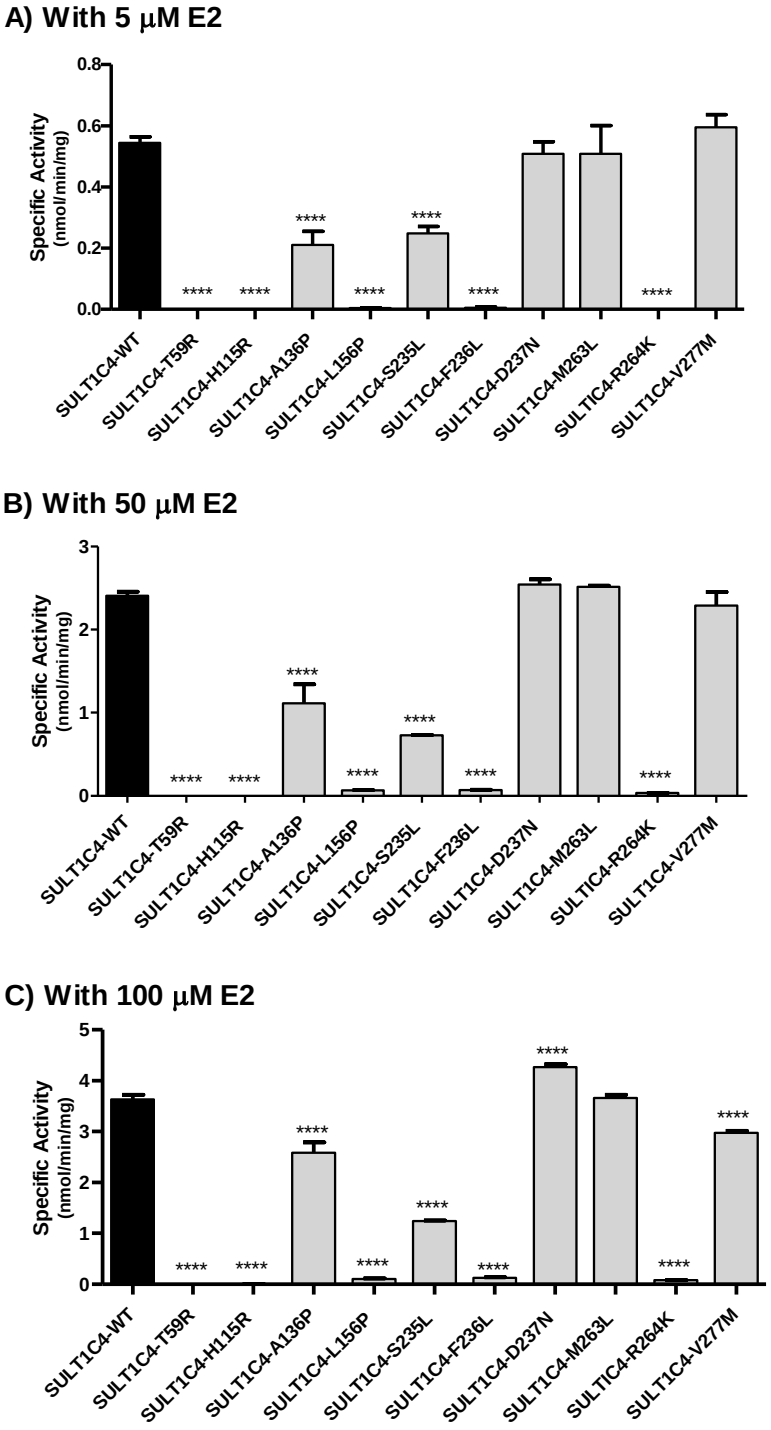

Figure 6.3. Specific activities of the sulfation of E2 human SULT1C4 allozymes. Concentrations of E2 used in the enzymatic assays were 5 μM (A), 50 μM (B), and 100 μM (C). Specific activity refers to nmol E2 sulfated/min/mg of purified allozyme. Data

shown represent mean ± standard deviation derived from three determinations. WT refers to wild-type SULT1C4. Data that was statistically significant with regards to the SULT1C4-wild-type is: *p<0. 05.

6.4. Discussion

Estrogens play a crucial role in human fetal development (Kaludjerovic & Ward, 2012). It has been reported that during pregnancy elevated levels of E1 and E2 are present in both fetal and maternal circulations (Carnegie & Robertson, 1978). In regard to estrogen homeostasis, several human SULTs, including SULT1C4, have been shown to mediate the sulfation of E1 and E2 (Hui et al., 2008). It is noted that compared with other SULTs, SULT1C4 has been shown to be expressed at higher levels in fetal tissues (Dubaisi et al., 2019; Runge-Morris & Kocarek, 2013; Sakakibara et al., 1998), implying that SULT1C4 may have a larger role in estrogen hemostasis during fetal development (Guidry et al., 2017; Paakki et al., 2000). Interestingly, previous studies have demonstrated that genetic polymorphisms of human *SULT1A1 and SULT1E1* genes may affect the enzymatic activity of the resulting SULT1A1 and SULT1E1 allozymes towards estrogens (Adjei et al., 2003; Nagar, Walther, & Blanchard, 2006). In view of the potentially important role of SULT1C4 in the homeostasis of estrogens in fetal tissues, we were interested in investigating how single nucleotide polymorphisms of the *SULT1C4* gene may impact on the sulfating activity of SULT1C4 allozymes toward E1 and E2.

The current study embarked with a systematic database search in order to identify the missense cSNPs of human *SULT1C4* gene, from which ten missense cSNPs were selected. Thereafter, site-directed mutagenesis was performed to generate corresponding cDNAs, followed by the expression and purification of recombinant SULT1C4 allozymes. In a preliminary study, kinetic experiments on the sulfation of E1 or E2 by wild-type SULT1C4 were carried out. The sulfating activities of SULT1C4 allozymes towards E1 and E2 were then analyzed using three concentrations (one well below the K_m, one around K_m, and one well above the K_m of the wild-type enzyme; cf. **Table 2**) of E1 or E2. Activity data obtained indicated clearly that different SULT1C4 allozymes displayed differential sulfating activities towards E1 and E2 (cf. **Figures 3** and **4**).

The crystal structure of human SULT1C4 enzyme has been solved (Allali-Hassani et al., 2007). Key residues that are integral to the functioning of the enzyme include the 5'-phosphosulphate-binding (PSB) loop (52TYPKAGT58), the PAPS/PAP-binding regions (55KAGTTW60, 234TSFDVM239, 262FMRKG266, Arg137, Ser145, and Tyr200), the residue involved in catalysis His115, the substrate binding residues (Lys113 and Thr114) (Allali-Hassani et al., 2007), the C-terminal dimerization motif region (272KKHFTVAQNE281) (Petrotchenko, Pedersen, Borchers, Tomer, & Negishi, 2001) as well as the β-sheet in the N-terminal region, which is important in protein folding (Allali-Hassani et al., 2007). Of the ten SULT1C4 allozymes, six (SULT1C4-T59R, SULT1C4-S235L, SULT1C4-F236L, SULT1C4-M236L, SULT1C4-D237N, and SULT1C4-R264K) contain amino acid variations within the PAPS/PAP binding regions.

Of the six allozymes, one allozyme (SULT1C4-T59R) showed no sulfating activity, possibly due to the substitution of polar uncharged threonine with positively-charged arginine which disrupted hydrogen bonding between PAPS and the enzyme, thereby destabilizing the interaction. Two allozymes (SULT1C4-F236L and SULT1C4-R264K) displayed barely detectable activities on E1 and E2 at all tested concentrations. Although both phenylalanine and leucine are non-polar residues, the change from an aromatic to an aliphatic side chain in SULT1C4-F236L may underscore the dramatic decrease in enzyme activity. In the case of SULT1C4-R264K, Arg264 has been shown to be a conserved residue in the PAPS binding region (Dong, Ako, & Wu, 2012). Notably, previous studies have shown that Arg274 in SULT2B1b forms a hydrogen bond with the negatively-charged O3P phosphate oxygen of PAPS (Lee et al., 2003). It appears therefore that the substitution of arginine with lysine in SULT1C4-R264K is likely similarly detrimental to the PAPS binding. In SULT1C4-S235L, a polar serine residue that is conserved in all SULT enzymes and involved in PAPS binding was replaced with the non-polar leucine, resulting in a significant decrease of E1 and E2 sulfating activities. It has been suggested that in SULT2B1b, the oxygen in the carbonyl group of this serine forms a hydrogen bond with the adenine group of PAPS (Lee et al., 2003). The replacement of Ser235 with leucine in SULT1C4-S235L therefore may disrupt the hydrogen bonding with PAPS. For SULT1C4-D237N and SULT1C4-M263L which exhibited only slight changes in sulfating activity, the amino acid substitutions also occur in PAPS/PAP binding regions. It is noted that the amino acid residues involved in these two allozymes share key characteristics. In SULT1C4-D237N, aspartate and asparagine are both polar, while in SULT1C4-M263L, methionine and leucine are both non-polar,

with aliphatic side chains. These respective similarities may explain the minimal effects on sulfating activity observed for SULT1C4-D237N and SULT1C4-M263L.

Other than the PAPS/PAP binding regions, an important residue conserved in all SULTs, which affects the catalytic activity, is histidine115 (Allali-Hassani et al., 2007). This histidine residue in human SULT1E has been proposed to accept a proton from the phenyl group of the E2 molecule and thereby facilitate the nucleophilic attack of the sulfuric atom in PAPS (Negishi et al., 2001; Pedersen, Petrotchenko, Shevtsov, & Negishi, 2002). It is therefore not surprising that SULT1C4-H115R displayed a complete loss of sulfating activity. The replacement of the catalytic histidine with arginine likely affects the deprotonation of the substrate, thereby suppressing the dissociation of the sulfuryl group from PAPS. It is noted that the lack of sulfating activity for SULT1C4-H115R is consistent with previous studies showing the loss of sulfating activity due to the change of the catalytic histidine for other SULTs (Chen, 2004; Kakuta, Petrotchenko, Pedersen, & Negishi, 1998; Liu, Suiko, & Sakakibara, 2000; Pedersen et al., 2002).

One allozyme analyzed, SULT1C4-V277M, involves amino acid substitution present in the C-terminal dimerization motif (spanning 272KKHFTVAQNE281). SULT1C4-V277M, however, demonstrated sulfating activity comparable to that of the wild-type enzyme. The substitution of Val227 with methionine, therefore, likely did not affect the dimerization of the two subunits of the enzyme.

Two allozymes analyzed, SULT1C4-A1365P and SULT1C4-L156P, did not carry substitutions in regions critically important for the functioning of the enzyme. However, both SULT1C4-A1365P and SULT1C4-L156P involved amino acid substitutions with a proline residue. Proline is known to be a turn-inducing residue and its presence may generate a sharp turn in the polypeptide, thereby affecting the overall conformation (Betts & Russell, 2003). It is therefore not surprising that SULT1C4-L156P displayed barely detectable sulfating activity, and SULT1C4-A1365P showed a significant decrease in sulfating activity for both E1 and E2. The substitution of proline with non-turn-inducing residues, as in the case of SULT1C4-A1365P and SULT1C4-L156P, may similarly underscore the loss of sulfating activity in these allozymes.

In conclusion, the current study was designed to examine the impact of single nucleotide polymorphisms of the *SULT1C4* gene on the sulfating activity of SULT1C4 allozymes towards E1 and E2. Differential sulfating activities were detected for different SULT1C4 allozymes. The results obtained may have implications in the differential metabolism of estrogens in different individuals, as well as fetus vs. mother, with different *SULTC4* genotypes. Further studies are warranted in order to clarify the effects of the *SULT1C4* genotype on the homeostasis of estrogens particularly during fetal development.

Chapter 8

Summary and Conclusions

This study aimed to investigate the effects of missense polymorphisms in human *SULT1C4* on the enzyme's sulfating activity toward the experimental substrate (4-NP) and several therapeutic agents (doxorubicin, acetaminophen, dextrophan, *O*-desmethylquinine, and *O*-desmethylquinidine). First, a database search was used to identify human *SULT1C4* polymorphisms. Ten missense SNPs were selected for activity assay of the encoded allozymes based on the predicted functional and locational importance of the amino acid substitutions. Mutagenized cDNAs corresponding to the selected SNPs were generated and their proteins expressed and purified. Activity assays using the purified SULT1C4 allozymes revealed dramatic differences in specific activities toward all tested compounds. Three allozymes (SULT1C4-T59R, SULT1C4-H115R, and SULT1C4-L156P) displayed no sulfating activities, while two allozymes (SULT1C4-F236L and SULT1C4-R264k) showed barely noticeable activities. The remaining five allozymes (SULT1C4-A136P, SULT1C4-S235L, SULT1C4-D237N, SULT1C4-M263L, and SULT1C4-V277M) all exhibited sulfating activities that deviated

from that of wild-type SULT1C4. The results from these assays might yield insights into the functional effects of SULT polymorphisms that are crucial to anticipating individualized pharmacokinetic and toxicity profiles based on a patient's *SULT1C4* genotype. Such information may also be used to better elucidate the specific role of SULT1C4 in the metabolism of a given drug and provide a means for explaining therapeutic failures.

The current study also endeavored to elucidate the effects of missense cSNPs on the capability of SULT1C4 to sulfate two endogenous substrates, E1 and E2. Clearly differential sulfating activities were observed for the ten SULT1C4 allozymes against these compounds. Specifically, two allozymes (SULT1C4-T59R and SULT1C4-H115R) showed no detectible activities, three had barely detectible activities (SULT1C4-L156P, SULT1C4-F236L, and SULT1C4-R264K), and the remaining allozymes showed differential sulfating activities. These findings suggest that individuals with distinct *SULT1C4* genotypes may have differential efficacies at biotransforming these compounds, which are the most potent of endogenous estrogens. With the addition of epidemiological studies, these results may help clarify the effects of *SULT1C4* genotypes on the homeostasis of estrogens, particularly during fetal development.

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Chapter 1

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