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Molecular cloning, expression, and characterization of a novel zebrafish cytosolic sulfotransferase, SULT5A1

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A Thesis

Entitled

Molecular Cloning, Expression, and Characterization of A Novel Zebrafish

Cytosolic Sulfotransferase, SULT5A1

By

Daniyah Abduljalil Almarghalani

Submitted to the Graduate Faculty as Partial Fulfillment of the Requirements for the

Master of Science Degree in

Pharmaceutical Sciences (Pharmacology/Toxicology)

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The University of Toledo

August 2016

An Abstract of

Molecular Cloning, Expression, and Characterization of A Novel Zebrafish

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Sulfotransferase enzymes (SULTs) are responsible for phase II detoxification of xenobiotics as well as regulation of many endogenous compounds, including thyroid/ steroid hormones, bile acids, and catecholamine neurotransmitters. In recent years, zebrafish is emerging as an important animal model for drug metabolism research. This study is part of an overall effort to establish the zebrafish as a model for studying drug sulfation. By searching the GenBank database, the last remaining zebrafish sequence encoding a putative SULT, designated SULT5A1, was identified. Zebrafish SULT5A1 was subsequently cloned, expressed, purified, and characterized. Substrate specificity of zebrafish SULT5A1 was analyzed using a panel of more than 147 xenobiotics, endogenous compound, bile acids, and commercially available bile alcohols. SULT5A1 showed strong sulfating activity toward bile acid and bile alcohol compounds, including 5α-cyprinol, 5β-cyprinol, 5β- scymnol, 5β-cholestantriol, PZ, and 5α-lithocholic acid. It also exhibited significant activity toward endogenous compound, including DHEA and pregnenolone. However, SULT5A1 showed no activity toward xenobiotics. pH

dependence and kinetic studies were performed using zebrafish SULT5A1 with 5α-cyprinol, 5α-petromyzonol (PZ), DHEA, and pregnenolone as substrates.

Acknowledgements

I would like to express my appreciation and sincere thanks to Dr. Ming-Cheh Liu for being a wonderful advisor, who has always been available for help, excellent guidance, and support. I attribute the level of my master degree to his encouragement, and without him this thesis would not have been completed or written.

I would also like to thank my committee members: Dr. Youssef Sari and Dr. Zahoor Shah for their encouragement and insightful comments. I am also very grateful to Dr. Caren Steinmiller for being my graduate representative.

I owe my deepest gratitude to my father and my mother, who supported my aspirations and dreams. Thanks for all of the prayer and advice that sustained me thus far.

Words cannot express how grateful I am to my sisters, my brothers, and my best friends for their constant inspiration and emotional support.

To all my lab mates, especially, Dr. Katsuhisa Kurogi and my friend Fatemah Alherz, thank you for listening and supporting me through this entire process.

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List of Abbreviations

ACA ....Allocholic Acid

ADP...........................Adenosine-5'-Diphosphate
APS ....Adenosine-5'-Phosphosulfate
ATP...........................Adenosine-5'-Triphosphate

CA..............................Cholic Acid
CDCA ....Chenodeoxycholic Acid

cDNA ....Complementary Deoxyribonucleic Acid

CHES ....Sodium acetate, 2- (Cyclohexylamino) Ethanesulfonic Acid

CYP...........................Cytochrome P-450

DCA ....Deoxycholic Acid

DHEA ....Dehydroepiandrosterone
DHEAS ....Dehydroepiandrosterone Sulfate

DMSO........................Dimethyl Sulfoxide

DNA...........................Deoxyribonucleic Acid

DTT............................Dithiothreitol

E. coli ....*Escherichia Coli*

GCA ....Glycocholic Acid
GCDCA......................Sodium Glycochenodeoxy Cholate
HDCA ....Hyodeoxycholic Acid

HEPES N-2-Hydroxylpiperazine-N2-Ethanesulfonic

HST ....Hydroxysteroid Sulfotransferases

LB ....Lysogeny Broth

MBP ....Maltose-Binding Protein

MES ....2- Morpholinoethanesulfonic Acid
MOPS........................β-naphthol, 3-(N-Morpholino) Propanesulfonic Acid

NCBI ....National Center for Biotechnology Information

$OD_{600\ nm}$ ....Optical Density at 600 nm wavelength

PAPs.........................3'-phosphoadenosine-5'-phosphosulfate

PCR ....Polymerase Chain Reaction

PPi ....Pyrophosphate

PZ ....Petromyzonol

RT-PCR......................Reverse Transcription Polymerase Chain Reaction

S.D ....Standard Deviation

SDS ....Sodium Dodecyl Sulfate
SDS–PAGE................Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

STS...........................Steroid Sulfatase

SULTs........................Human Cytosolic Sulfotransferases Enzymes
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
SULT1C1..................Human Cytosolic Sulfotransferases Family 1C Member 1
SULT1C2..................Human Cytosolic Sulfotransferases Family 1C Member 2
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

List of Symbols

L .... Liter
ml .... Milliliter
µl .... Microliter

g .... Gram
mg .... Milligram
µg .... Mirogram

mM .... Millimolar
µM .... Micromolar

$SO3^-$ .... Sulfonate Group
$SO42^-$ .... Inorganic Sulfate

nmol .... Nanomole
mmol .... Millimole

min .... Minute

^{o}C .... Celsius

Ci .... Curie

V_{Max} .... Maximal Velocity
K_M .... Michaelis Constant
K_{cat} .... Turnover Number
K_{cat}/K_M .... Specificity Constant "catalytic efficiency"

α .... Angle of incidence
β .... Angle of distortion

Chapter 1

1. Introduction

1.1 Overview of Drug Metabolism

Upon their entry into the body, many drugs are subjected to metabolism as do many other xenobiotics. The metabolism of xenobiotics generally involves the same enzymatic pathways and transport systems that are utilized in metabolism of dietary constituents. Over the years, a large number of the so-called drug-metabolizing enzymes have been identified. These drug-metabolizing enzymes are present in nearly all tissues in the body, with the highest levels being present in the gastrointestinal tract and liver. Xenobiotics, as well as endogenous compounds, are extensively metabolized and cleared by the liver, which is considered the major metabolic clearing house for these compounds (Gonzalez FJ 2011).

1.2 Major Phases of Drug Metabolism

The metabolism of xenobiotics including drugs may proceed through two phases, designated Phase I and Phase II. In Phase I, the drug-metabolizing enzymes may mediate oxidation, reduction, or hydrolytic reactions, leading to the introduction of functional groups, such as –OH, –COOH, –O–, –SH, or NH^2. The resulting products will undergo

further metabolism by Phase II drug-metabolizing enzymes, which include several groups of conjugating enzymes. The conjugating enzymes act to increase the water-solubility of drug (xenobiotic) metabolites, thereby facilitate their elimination from the body (Gonzalez FJ 2011).

1.2.1 Phase I Reactions

The most common enzymes involved in the Phase I drug metabolism are cytochrome P -450 (CYP) enzymes (Gonzalez FJ 2011). They constitute a superfamily of heme-containing enzymes (Estabrook 2003). Some of these enzymes are also involved in the synthesis or catabolism of endogenous compounds such as cholesterol, steroids, and bile acids (Wilkinson 2005). More than 50 distinct CYPs have been identified in humans and 75% of them are involved in drug metabolism. CYPs are grouped into families and subfamilies depending on amino acid sequence homology (Blake, Castro et al. 2005). Among them, CYP2C, CYP2D, and CYP3A subfamilies have been shown to be extensively involved in drug metabolism. Particularly, CYP3A4 is considered to be the most abundantly expressed CYP in the liver and is responsible for metabolism of over 50% of clinically used drugs (Gonzalez FJ 2011).

1.2.2 Phase II Reactions (Conjugation Reactions)

Phase II reactions include methylation, sulfonation, acetylation, glucuronidation, and glutathione conjugation reaction. They are considered the true elimination and detoxification pathways for most drugs (Taylor & Francis 2003, Gonzalez FJ 2011).

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Phase II conjugating enzymes mediate the formation of more water-soluble and less biologically active metabolites by endowing them with hydrophilic moieties, thereby facilitating their elimination from the body. In some cases, however, they may convert certain chemical compounds to more highly reactive and carcinogenic metabolites (Goodman, Gilman et al. 2011). All Phase II conjugating enzymes are localized in the cytosol of the cell, with the exception of glucuronidating enzymes that are present in the endoplasmic reticulum (Gonzalez FJ 2011).

1.3 Sulfation and Sulfotransferases

Sulfation is one of the major conjugation reactions that was first discovered in 1876 by Eugen Baumann, who isolated phenyl sulfate from the urine of a patient being treated with phenol as an antiseptic (Baumann 1876). In 1956, Lipmann's group reported the mechanism of action of sulfate conjugation reaction. They identified the sulfate donor, 3'-phosphoadenosine-5'-phosphosulfate (PAPS), used in the sulfation reaction as catalyzed by the cytosolic sulfotransferase (SULT) enzymes (Robbins and Lipmann 1956). SULTs catalyze the transfer of a sulfonate group (SO_3^{-}) from PAPS to a nucleophilic moiety of the substrate compounds (Figure 1-1). Substrates utilized by the SULTs usually contain hydroxyl or amino groups.
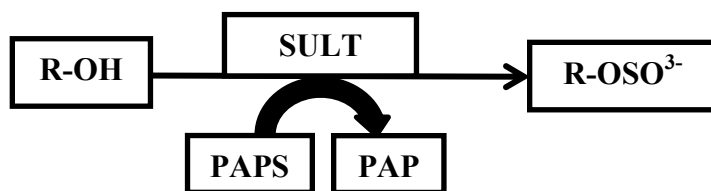
Figure 1-1: General sulfation reaction catalyzed with PAPS as a cofactor

Sulfation reaction may result in the inactivation of the different substrates and/or increase their water solubility, thereby facilitating their excretion from the human body (Liu, Bhuiyan et al. 2010). In some cases, however, sulfation of certain compounds such as N-hydroxy-2-acetylaminofluorene may generate reactive electrophile metabolites that can elicit mutagenic and/or cytotoxic responses (Falany and Kerl 1990, Falany 1991). There are three types of the sulfation reactions, based on the functional groups present in the substrate compounds: *O*-sulfation (ester), *N*-sulfation (amide), and *S*-sulfation (thioester). Generally, *O*-sulfation is considered the most common sulfation reaction involving compounds like steroids and catecholamines (Strott 2002, Chapman, Best et al. 2004). As mentioned above, PAPS is the universal sulfate cofactor for sulfate conjugation reactions. PAPS is synthesized in the cytosol by two consecutive enzymatic reactions (Figure 1-2). In the first step, the enzyme ATP sulfurylase utilizes adenosine-5'-triphosphate (ATP) and inorganic sulfate (SO_4^{2-}) and generate adenosine-5'-phosphosulfate (APS) and pyrophosphate (PPi). In the second step, APS is phosphorylated by the APS kinase using ATP to yield PAPS and adenosine-5'-diphosphate (ADP) (Strott 2002, Cho, Lee et al. 2004).
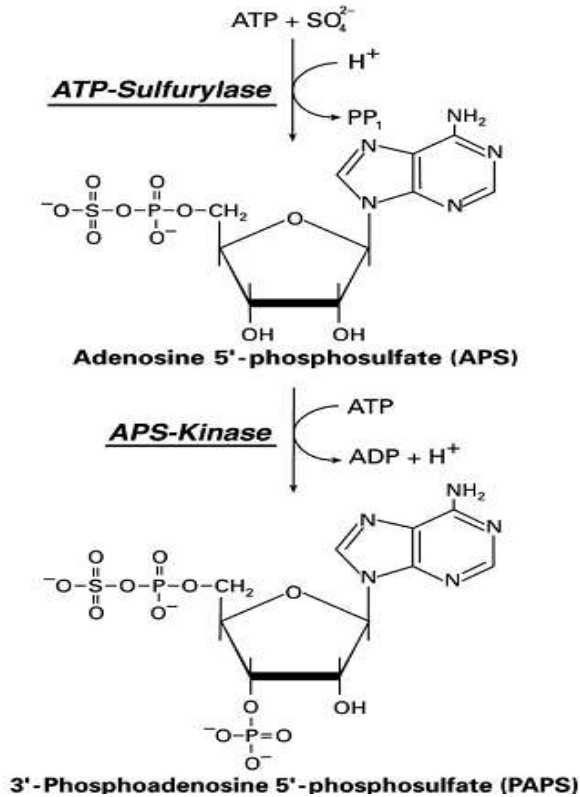
Figure 1-2: Two enzymatic reactions involved in the synthesis of PAPS (Strott 2002)

1.4 Sulfotransferases (SULTs) in Mammals

There are two classes of sulfotransferases in mammals: one is composed of membrane-bound forms located in the trans-Golgi apparatus, and the other comprises the cytosolic sulfotransferases (SULTs). The membrane-bound sulfotransferases are responsible for metabolizing endogenous macromolecules such as glycoproteins, lipids and glycosaminoglycans. The SULTs are responsible for the sulfation of low-molecular weight xenobiotics and endogenous compounds such as bile acids, steroids, and catecholamine neurotransmitters (Falany 1997, Weinshilboum, Otterness et al. 1997, Strott 2002).

In mammalian species, the SULTs comprise a large superfamily of enzymes that are distributed widely throughout the body, including the liver, kidneys, gut, lungs, thyroid glands, adrenal glands, brain, breast tissue, blood, and reproductive systems (Chen, Zhang et al. 2003). Some recent studies have suggested that the SULTs are important for xenobiotic metabolism during fetal development (Wood, Gridley et al. 2003, Duanmu, Weckle et al. 2006). Studies also revealed that these enzymes are also involved in the modulation and homeostasis of thyroid and steroid hormones, catecholamine neurotransmitter, and cholesterol metabolites (Strott 2002, Liu, Bhuiyan et al. 2010).

Based on their amino acid sequences, different SULTs found in vertebrate animals have been categorized into several gene families and subfamilies (Nagata and Yamazoe 2000, Coughtrie 2002, Nimmagadda, Cherala et al. 2006); (Figure 1-3). In humans, thirteen distinct SULTs have been identified and classified into four major SULT families, SULT1, SULT2, SULT4, and SULT6 (Strott 2002, Allali-Hassani, Pan et al. 2007). Studies have shown that members of the SULT1 family are capable of sulfonating phenolic compounds such as catecholamines, while members of the SULT2 family display a higher affinity for hydroxysteroids compounds (Strott 2002, Chapman, Best et al. 2004). The various human SULTs, their tissue/organ expression, and their proposed functional roles are summarized in Table 1.1.

SULT1B1possum
SULT1B1dog
SULT1B1human
SULT1B1rat
SULT1B1mouse
SULT1E1pig
SULT1E1cow
SULT1E1human
SULT1E1guinea-pig
SULT1E1mouse
SULT1E1rat
SULT1E2rat
SULT5A1mouse
SULT2A1rabbit
SULT2A1human
SULT2A1monkey
SULT2A3rat
SULT2A1rat
SULT2A2rat
SULT2A1mouse
SULT2A2mouse
SULT2A2guinea-pig
SULT2A1guinea-pig
SULT2B1mouse
SULT2B1ahuman
SULT2B1bhuman
SULT6A1chicken
SULT3A1mouse
SULT3A1rabbit
SULT4A1human
SULT4A1mouse
SULT4A1rat
SULT1A1platypus
SULT1A1rat
SULT1A1mouse
SULT1A1rabbit
SULT1A3human
SULT1A1monkey
SULT1A1human
SULT1A2human
SULT1A1cow
SULT1A1dog
SULT1D1dog
SULT1D1mouse
SULT1D1rat
SULT1C4human
SULT1Cchicken
SULT1C1rat
SULT1C1mouse
SULT1C2human
SULT1C2rabbit
SULT1C3rat
SULT1C2rat

Figure 1-3: Classification of the SULT gene superfamily (Coughtrie 2002).

Table 1.1: Classification and Function of Human Cytosolic Sulfotransferases:

SULTs Family	SULTs Isoform	Expression	Biological Role	References
SULT1	SULT1A1, SULT1A2, and SULT1A3 (Phenolic SULTs)	SULT1A1 are found in the liver, while high levels of SULT1A3 are identified in the intestine, jejunum, and brain. Low levels of SULT1A2 are detected in the liver.	Both SULT1A1 and SULT1A3 are highly distributed within developing human fetal brain and neurotransmitters. SULT1A3 is highly selective for sulfation of catecholamines. However, no endogenous substrates have been found for SULT1A2.	(Glatt, Boeing et al. 2001, Gamage, Barnett et al. 2006, Yasuda, Yasuda et al. 2009)
	SULT1B1 (Thyroid hormone SULT)	Located in different organs including stomach, intestine, colon, liver, kidney, and thyroids	Highly selective for sulfation of 1-Naphthol , 4 nitrophenol, and iodothyronines	(Freimuth, Wiepert et al. 2004, Gamage, Barnett et al. 2006)

SULTs Family	SULTs Isoform	Expression	Biological Role	References
	SULT1C1, SULT1C2, and SULT1C4 (Hydroxy-arylamine SULTs)	Highly expressed in human fetal tissues, but decline in adults.	In rodents, SULT1C enzymes are able of sulfating the hepatic carcinogen N-hydroxy-2-acetylaminofluorene. However, their role in human is not known.	(Yasuda, Yasuda et al. 2009, Gonzalez FJ 2011)
	SULT1E1 (Estrogen SULT)	Found in liver, hormone-responsive tissues like the breast, testis, adrenal gland, and placenta.	Highly selective for estrogen sulfation (in particular 17β-estradiol)	(Yasuda, Yasuda et al. 2009, Gonzalez FJ 2011)
SULT2	SULT2A1	Highly expressed in liver, small intestine, and adrenal cortex.	SULT2A1 is sulfated (DHEA), SULT2B1a (cholesterol), and SULT2B1b (pregnenolone).	(Thomae, Eckloff et al. 2002, Gamage, Barnett et al. 2006)
	SULT2B1a	Found in prostate and placenta.		
	SULT2B1b	Highly expressed in the skin.		

SULTs Family	SULTs Isoform	Expression	Biological Role	References
SULT4	SULT4A1	Expressed specifically in the brain.	No substrate has yet been found against SULT4.	(FALANY, Xiaowei et al. 2000, Liu, Bhuiyan et al. 2010)
SULT6	SULT6A1	Not fully characterized.		

1.5 Zebrafish as A model for Drug Metabolism Research

Mice and rats have been the most common experimental animal model organisms. Recently, the zebrafish (*Danio rerio*) has emerged as an interestingly popular animal model in different areas of research such as developmental biology, genetics, and pharmacology (Eisen 1996, Liu, Bhuiyan et al. 2010). Compared with other animal models, the zebrafish has numerous advantages that make them valuable as an animal model. The zebrafish has a small size (adult ~ 1- 1.5 inches long) and short generation time (about 3 months). Under optimal conditions, a female zebrafish can produce 100-200 eggs at weekly intervals, which allow for experiments that required a large number of subject material. The zebrafish embryos undergo rapid external developmental within 2-4 days, and their transparency makes them easier to study of developmental stages and observe of morphological changes (Wixon 2000, Langheinrich 2003). Zebrafish can be

easily maintained and breed in a relatively small housing area (Ma, Parng et al. 2003). The zebrafish has a lower husbandry cost compared to other animals like mice and rats (Lieschke and Currie 2007). At early life stages, the zebrafish embryo has been shown to be capable of absorbing tested drugs through the skin (Ma, Parng et al. 2003). Additionally, zebrafish and humans have similar numbers of chromosomes (23 for humans and 25 for zebrafish) which allow for revealing conserved gene functions for studying human genetic diseases and congenital disorders (Postlethwait, Woods et al. 2000). It can therefore be concluded that zebrafish has a great potential to serve as a model for studying infectious diseases, cancers, cardiovascular diseases, genetic disorder, and many other human diseases (Liu, Bhuiyan et al. 2010). Additionally, the unique features of the zebrafish make it an excellent animal model for a systematic research study on the ontogeny of the SULTs and their physiological involvement, including the mitigation of the adverse effects of drugs during the developmental process.

1.6 The SULTs in Zebrafish

In previous studies, nineteen zebrafish SULTs have been cloned, expressed, purified, and characterized. Sequence analysis of these cloned zebrafish SULTs via the BLAST search revealed considerable sequence homology to mammalian SULTs. Of the nineteen zebrafish SULTS that have been cloned, nine of them belong to the SULT1 gene family (ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST8and ST9), three belong to the SULT2 gene family (ST1, ST2, and ST3), five belong to the SULT3 gene family (ST1, ST2, ST3, ST4, and ST5), and two families appear independent from all identified SULTs (SULT4

and SULT6) (Yasuda, Liu et al. 2006, Liu, Bhuiyan et al. 2010, Mohammed, Kurogi et al. 2012). In *in vitro* studies, members of different zebrafish SULTs families displayed differential substrate specificity toward various endogenous and xenobiotic compounds. The zebrafish SULTs appear to play a vital role in the metabolism and detoxification of these compounds (Yasuda, Liu et al. 2006, Liu, Bhuiyan et al. 2010).

1.7 Overview of Bile Acid and Bile Alcohol (Human vs. Zebrafish)

Bile acids and bile alcohols, collectively called the cholanoids, are major metabolic products of cholesterol. Cholanoids display cross-species variation in chemical structure, ranging from C-$_{27}$ bile alcohols in the basal vertebrates (e.g., jawless fishes, cartilaginous fish, and amphibians) to C$_{24}$ bile acids in most reptiles, birds, and mammals (Haslewood 1967, Hofmann, Hagey et al. 2010). C-$_{27}$ bile alcohols appear to be the main cholanoids in all jawless fish and cartilaginous fish as well as cypriniform fish including the zebrafish, *Danio rerio*. Biosynthesis of C-$_{27}$ bile alcohols can occur through intermediate hydroxylation of the side chain of C-$_{27}$ bile alcohols such as 3α, 7α, 12α trihyroxy- 5α/β- cholestane (Kuroki, Shimazu et al. 1985).

The biosynthetic pathway of bile acids in vertebrate is more sophisticated than that of bile alcohols. C$_{24}$ bile acids require a side-chain oxidation by cytochrome P450 (CYP27A1) and subsequent enzymatic reactions to diminish the side-chain of 3 carbon atoms and oxidize the reduced side-chain to produce a bile acid. Usually, primary bile acids are conjugated with the amino acids taurine or glycine (Russell 2003).

In humans, there are two major primary bile acids, chenodeoxycholic acid (CDCA; 3α, 7α-dihydroxy-5β-cholan-24-oic acid) and cholic acid (CA; 3α, 7α, 12α trihydroxy-5β-cholan-24-oic acid). They are biosynthesized in the liver and secreted into the duodenum in the small intestine via the bile duct (Borgstrom, Barrowman et al. 1985). These primary bile acids serve to facilitate digestion and absorption of dietary fat compounds and lipid-soluble vitamins, solubilize and transport of cholesterol and heavy metal cations, stimulate bile flow and biliaryphospholipid secretion, and has bacteriostatic effects by stimulating mucin secretion (Vlahcevic, Heuman et al. 1991). Studies have shown that anaerobic bacteria present in the distal small intestine may deconjugate the taurine and glycine moieties and remove the hydroxyl group at carbon-7 of primary bile acids in order to form secondary bile acids. Secondary bile acids include deoxycholic acid (DCA; 3α, 12α dihydroxy-5β-cholan-24-oic acid) and lithocholic acid (LCA; 3α-hydroxy-5β-cholan-24-oic acid), where LCA is considered to be the most toxic secondary bile acids in mammals. Furthermore, LCA may cause different pathologies such as damaging the intestinal mucosa, formation of gallstones, and hepatotoxicity (Hofmann 1999, Hofmann 2004, Fickert, Fuchsbichler et al. 2006).

A previous study indicated that SULT3 ST2 and SULT3 ST3 were the main bile alcohol/acid-sulfating SULTs in the zebrafish, whereas SULT2A1 was the only SULTs among the thirteen human SULTs that was able to sulfate bile acids and bile alcohol. Additionally, the zebrafish SULTs favored petromyzonol as a bile alcohol substrate than bile acids, while the human SULT2A1 preferred sulfating lithocholic acid over petromyzonol (Kurogi, Krasowski et al. 2011).

1.8 Dehydroepiandrosterone (DHEA) and Pregnenolone: Physiological Role and Metabolism (Human vs. Zebrafish)

In human body, the most abundant steroids are dehydroepiandrosterone (DHEA) and its sulfate ester (DHEAS). DHEA is synthesized from cholesterol mainly in the adrenal glands, and at lower levels in the brain, testis, ovaries, and skin (Zouboulis, Chen et al. 2007, Davis, Panjari et al. 2011). DHEA acts as a metabolic intermediate in the synthesis of the estrogen and androgen sex steroid hormones. The physiological significance of DHEA and its correlation to human health have yet to be fully understood. Several studies have suggested that DHEA may reduce inflammation, modulate endothelial function, improve blood flow, insulin resistance, bone metabolism, cellular immunity, sexual function, cognitive function, memory enhancement, and may also act as neuroprotective agent (Traish, Kang et al. 2011). DHEA is sulfated into its more stable sulfate ester DHEAS in the adrenal zona reticularis, and the responsible enzyme is called the hydroxysteroid sulfotransferases (HST) or DHEA sulfotransferase (SULT2A1). Other SULTs that are capable of sulfating DHEA include SULT1E1, SULT2B1a, and SULT2B1b (Thomae, Eckloff et al. 2002, Gamage, Barnett et al. 2006, Liu, Bhuiyan et al. 2010). DHEAS is converted in peripheral tissues back into DHEA by steroid sulfatase (STS) (Legrain, Massien et al. 2000, Davis, Panjari et al. 2011). In zebrafish, two SULT1s (ST4 and ST9), three SULT2s (ST1, ST2, and ST3), and two SULT3s (ST4 and ST5) have been shown to display significant sulfating activities toward DHEA (Liu, Yang et al. 2005, Yasuda, Liu et al. 2006, Mohammed, Kurogi et al. 2012).

Another steroid that is synthesized from cholesterol in the gonads, adrenal glands, and brain is pregnenolone (Le Goascogne, Robel et al. 1987, Payne and Hales 2004, Vallée 2015). In circulation, the concentration of pregnenolone is low, whereas its level high in the brain (Jo, Abdallah et al. 1989). In humans, pregnenolone has been shown to be sulfated by SULT2A1, SULT2B1a, and SULT2B1b (Strott 2002). Pregnenolone is converted to allopregnenolone by the enzymes 3β- hydroxysteroid dehydrogenase, 3α- hydroxysteroid dehydrogenase, and 5α- reductase type I (Baulieu, Robel et al. 2001). Pregnenolone can also be converted to 7α- hydroxyl-pregnenolone by CYP7B (Rose, Stapleton et al. 1997). The function of pregnenolone has been fairly well characterized during zebrafish embryogenesis (Hsu, Hsu et al. 2006). Studies have suggested that CYP11a1 is highly expressed, as a maternal transcript in early zebrafish embryogenesis. Suppression of the expression of CYP11a1 resulted in a delay of the zebrafish embryonic cell movement (Hsu, Hsiao et al. 2002, Hsu, Liang et al. 2006). Treatment with pregnenolone could help promote the migration of zebrafish embryonic cells (Weng, Liang et al. 2013). Pregnenolone can also enhance cognitive function and prevent negative symptoms in schizophrenia disorders in clinical trials when combined with other anti-psychotic drugs (Marx, Keefe et al. 2009, Ritsner, Gibel et al. 2010). Studies using rats suggested that pregnenolone sulfate could improve learning and memory, and reduce pain (Vallée, Mayo et al. 2001, Chen, Liu et al. 2006). Two zebrafish SULTs, SULT2 ST1 and SULT3 ST4, have been shown to be capable of sulfating pregnenolone (Liu, Yang et al. 2005, Mohammed, Kurogi et al. 2012).

Objectives and Goals

As mentioned previously, nineteen zebrafish SULTs have been identified, purified and characterized. Studies have shown that zebrafish SULTs resemble human SULTs in being able to mediate the sulfation of a variety of xenobiotic and endogenous compounds such as catecholamine hormones, steroid hormones, thyroid hormones, and cholesterol and its metabolites. The aim of this thesis research is to identify and characterize the last remaining zebrafish SULT, designated SULT5A1. In this work, zebrafish SULT5A1 was identified, cloned, purified, and characterized. A panel of more than 147 xenobiotics, endogenous compound, bile acids, and commercially available bile alcohols were tested as substrates for zebrafish SULT5A1 in an effort to unveil its enzymatic characteristics.

Chapter 2

2. Materials and Methods

2.1 Materials.

Dehydroepiandrosterone (DHEA), cholic acid (CA), lithocholic acid (LCA), deoxycholic acid (DCA), sodium chenodeoxy cholate (CDCA), ursodeoxycholic acid (UDCA), hyodeoxycholic acid (HDCA), glycocholic acid (GCA), sodium glycochenodeoxy cholate (GCDCA), taurocholic acid sodium salt hydrate (TCA), sodium taurochenodeoxy cholate (TCDCA), dimethyl sulfoxide (DMSO), sodium dodecyl sulfate (SDS), adenosine 5'-triphosphate (ATP), dithiothreitol (DTT), and sodium acetate, 2-(cyclohexylamino) ethanesulfonic acid (CHES), N-2-hydroxylpiperazine-N2-ethanesulfonic (HEPES), 3-[N-tris-(hydroxymethyl) methylamino[-propanesulfonic acid (TAPS), 3- (cyclohexylamino)-1-propanesulfonic acid (CAPS), β-naphthol, 3-(N-morpholino) propanesulfonic acid (MOPS), 2- morpholinoethanesulfonic acid (MES), were obtained from Sigma Chemical Company (St. Louis, MO). Pregnenolone was from MP Biomedical (Solon, OH). 5α-petromyzonol (PZ) and allocholic acid (ACA) (also known as; 5α-cholic acid) were purchased from Toronto Research Chemical, Inc. (North York, Ontario, Canada). 5α-cyprinol and 5β- cyprinol were kindly provided by Dr. Matthew Krasowski of the University of Iowa. Carrier free sodium [^{35}S]sulfate and Ecolume scintillation cocktail were products of American Radiolabeled Chemicals (St.

Louis, MO). Cellulose thin-layer chromatography (TLC) plates were product of EMD

chemicals (Gibbstown, NJ). Recombinant human bifunctional ATP sulfurylase/adenosine

5'-phosphosulfate kinase used to synthesize PAP[^{35}S] as previously described

(Yanagisawa, *et al*., 1998). TRI reagent was prepared from Molecular Research Center,

Inc. (Cincinnati, OH). *Tag* DNA polymerase was from Promega Corporation (Madison,

WI). Oligonucleotide primers were prepared by MWG Biotech (High Point, NC). All

other chemicals and reagents used were of the highest grade commercially available.

2.2 **Cloning, bacterial expression, and purification of recombinant zebrafish**

SULT5A1.

By searching the GenBank database, a zebrafish sequence (GenBank Accession #

BC124437) encoding a putative SULT (designated SULT5A1) was identified. A

corresponding cDNA (IMAGE Clone ID 8145888) packaged in pME18S-FL3 was

purchased from Open Biosystems. To generate the cDNA for subcloning into the pMAL-

c5x prokaryotic expression vector, sense and antisense oligonucleotide primers designed

based on 5'- and 3'- regions of the coding sequence were synthesized with *Nde* I

restriction site incorporated at the end (Table 2.1). Using these primers, a PCR reaction

was carried out under the action of *EX Taq* DNA polymerase, with the DNA Clone

8145888- pME18S-FL3 as the template. Amplification conditions were 2 min at 94°C

and 20 cycles of 94°C for 35 s, 60°C for 40 s, and 72°C for 1 min. The final reaction

mixture was applied onto a 1% agarose gel, separated by electrophoresis, and visualized

by ethidium bromide staining. The PCR product band detected was excised from the gel,

and the DNA therein was isolated by spin filtration. Purified PCR product was subjected

to *Nde* I restriction and subcloned into *Nde* I -restricted pMAL-c5x vector, and verified

for authenticity by nucleotide sequencing (Sanger, Nicklen et al. 1977). To express the

recombinant zebrafish SULT5A1, competent *Escherichia coli* BL21 (DE3) cells

transformed with pMAL-c5x harboring the cDNA encoding SULT5A1 were grown in 1

L LB medium supplemented with 60 µg/ml ampicillin. After the cell density reached 0.6

$OD_{600\ nm}$, IPTG (0.5 mM final concentration) was added to induce the production of

recombinant maltose-binding protein (MBP)-SULT fusion protein. After a 5-hour

induction at 37°C, the cells were collected by centrifugation and homogenized in 25 ml

ice-cold lysis buffer using an Aminco French Press. Twenty µl of a protease inhibitor

mixture (Roche Diagnostics) were added to the crude homogenate. The crude

homogenate was subjected to centrifugation at 10,000 x g for 15 min at 4°C. The

supernatant collected was fractionated using 2.5 ml of amylose resin. Upon washing with

lysis buffer to remove unbound proteins, the MBP-SULT5A1 fusion protein was eluted

from amylose resin using a stepwise gradient of maltose (1 mM to 10 mM) in 50 mM

Tris-HCl, pH 8.0. The MBP-SULT5A1 fusion protein present in eluted fractions was

analyzed for purity by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and

subjected to enzymatic characterization.

Table 2.1: Sense and Antisense Oligonucleotide Primers used in the cDNA Cloning and RT-PCR Analysis of a New Zebrafish SULT.

Target sequence		Sense and Antisense Oligonucleotide Primers
SULT5A1	Sense:	(5'- GGAAGGATTTCACATATGTCTCGACTAGATGTCACAG AATCTTTTCA-3')
	Antisense:	(5'- GGAAGGATTTCACATATGTTATGTGAGGAGAAGGAT CTCTCCGGATGC-3')

2.3 Sulfotransferase (SULT) Assay.

The sulfating assay of the zebrafish SULT enzyme was performed using radioactive PAP[^{35}S] as the sulfate donor. All substrates tested were dissolved in DMSO, and diluted to a final concentration 50 µM in the final reaction mixture. A control with DMSO replacing substrate was also prepared. The protein concentration of the purified SULT5A1 (in protein fusion form) used in the assays was 0.32 mg/ml. The standard reaction mixture for the enzymatic assay, prepared in a final volume of 20 µl, contained, 50 mM HEPES at pH 7.0, 14 µM of PAP[^{35}S], 1 mM DTT, and 50 µM substrate. The reaction was started by the addition of 2 µl of the enzyme, allowed to continue to proceed at 28°C for 20 minutes, and terminated by heating the tube containing the reaction mixture on a heating block at 100°C for 3 minutes. The precipitates in the heated reaction

mixture were cleared by centrifugation at 13,000×g for 3 minutes. Subsequently, 1 µl of the supernatant reaction mixture was spotted on a cellulose TLC plate, and the plate was subjected to TLC analysis using a solvent system containing nbutanol/isopropanol/formic acid/water in a ratio of (3:1:1:1; by volume). Afterwards, the TLC plate was air-dried and autoradiographed by using a classic X-ray film. The autoradiograph taken from the TLC plate was used to locate the radioactive spot corresponding to the sulfated product, and the spot was cut from the TLC plate, eluted in 0.5 ml water in a glass vial, and mixed thoroughly with 2.5 ml of Ecolume scintillation liquid. The radioactivity was counted by using a liquid scintillation counter. The results obtained were used to calculate the specific activity in the unit of sulfated product formed/minute/mg of SULT5A1.

2.4 pH-dependence Studies.

Endogenous compounds including pregnenolone, dehydroepiandrosterone (DHEA), 5α-petromyzonol (PZ), and 5α-cyprinol were tested as substrates to examine the pH profile of zebrafish SULT5A1. To examine the pH-dependence of the sulfation of these endogenous substrates by zebrafish SULT5A1, different buffers were used (50 mM sodium acetate at 4.5 or 5; MES at 5.5 or 6; HEPES at 7 or 7.5; TAPS at 8 or 8.5; CHES at 9 or 9.5; CAPS at 10, 10.5, 11, or 11.5). The experimental procedure for pH-dependence studies was the same as mentioned previously, except for the buffer used.

2.5 Kinetics Studies.

In the kinetic studies, varying concentrations of the substrates (pregnenolone, dehydroepiandrosterone (DHEA), 5α-petromyzonol (PZ), and 5α-cyprinol) were tested as

substrates for zebrafish SULT5A1. The reaction conditions followed the same procedure as that previously illustrated in the SULT assay. Furthermore, the results obtained were analyzed based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software program.

2.6 Miscellaneous Methods.

The sulfate donor, PAP[^{35}S], was prepared from ATP and carrier-free [^{35}S]sulfate using the recombinant human bifunctional ATP sulfulyase/adenosine 5'-phosphosulfate kinase. Its purity was verified as described earlier (Yanagisawa, Sakakibara et al. 1998). Afterwards, the synthesized PAP[^{35}S] was adjusted to the required concentration and a specific activity of 15 Ci/mmol at 1.4 mM by the addition of non-radioactive (cold) PAPS. Protein was determined based on the Bradford method with bovine serum albumin as a regular standard (Bradford 1976).

2.7 Statistical Analysis.

The data obtained from kinetics studies were analyzed based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software program.

3. Results and Discussions

3.1 Results

3.1.1 Molecular Cloning, Expression, and Purification of the Zebrafish SULT5A1.

By searching the GenBank database, a sequence encoding a zebrafish putative SULT (designated SULT5A1) (GenBank Accession # BC124437) was identified. Based on the sequence information, we designed sense and antisense oligonucleotide primers (Table 2.1.) corresponding to on 5`- and 3`-coding regions of the sequence were synthesized with *Nde* I restriction site incorporated at the end in order to generate the cDNA for subcloning into the pMAL-c5x prokaryotic expression vector. Using these primers and the DNA Clone 8145888- pME18S-FL3 purchased from Open Biosystems as a template, cDNA encoding the zebrafish SULT5A1 was PCR-amplified under the action of *EX Taq* DNA polymerase. Purified PCR product was subjected to *Nde* I restriction and subcloned into *Nde* I -restricted pMAL-c5x vector, and verified for authenticity by nucleotide sequencing (Sanger, Nicklen et al. 1977) pMAL-c5x harboring the cDNA encoding SULT5A1 was transformed into competent *Escherichia coli* BL21 (DE3) cells for expression of recombinant enzyme. The resulting PCR product then was subject to nucleotide sequencing for verifying its authenticity. Figure 3-1 shows the nucleotide and deduced amino acid sequences of the newly cloned zebrafish SULT5A1.

Recombinant zebrafish SULT5A1 was purified from the *E. coli* cell extract as MBP-

fusion protein. Figure 3-2 shows the SDS gel electrophoretic pattern of purified

recombinant zebrafish SULT5A1. Compared with the molecular weight markers co-

electrophoresed, zebrafish SULT5A1 migrated at approximately 78.3 kDa positions.

Taking into consideration of the 42.51 kDa molecular weight of the MBP portion in the

MBP fusion proteins, this result is in agreement with the predicted molecular weight of

35,790.86 of SULT5A1 based on its deduced amino acid sequences. Sequence analysis

based on the BLAST pair wise search showed that the deduced amino acid sequence of

the zebrafish SULT5A1 displays 47% amino acid sequence identity to mouse musculus

SULT5A1, 49% to rat SULT5A1, 59% to xenopus laevis SULT5A1, 60% to xenopus

tropicalis SULT2B1, 66% to the Atlantic salmon SULT2B1, and 78% to

Sinocyclocheilus graham fish SULT2B1.

```
              10           20           30           40           50           60
ATGTCTCGACTAGATGTCACAGAATCTTTTCAGGGCATCAAGTTTCCTGGGCACATGCAT
   M  S  R  L  D  V  T  E  S  F  Q  G  I  K  F  P  G  H  M  H
              70           80           90          100          110          120
ACTGAAGAATCCCTGAATTTTGCAACTGACTTCAAGTTTGAAGAGACAGATACTCTGATA
   T  E  E  S  L  N  F  A  T  D  F  K  F  E  E  T  D  T  L  I
             130          140          150          160          170          180
GTCACCTACCCCAAATCAGGAACCACATGGATGCAGGAAATCCTCACTCTGGTTTTGAGT
   V  T  Y  P  K  S  G  T  T  W  M  Q  E  I  L  T  L  V  L  S
             190          200          210          220          230          240
AAAGGGGACCCGACTATTGCCCAAAGTCAACCTAACTGGGCTCGTGCTCCATGGCTTGAG
   K  G  D  P  T  I  A  Q  S  Q  P  N  W  A  R  A  P  W  L  E
             250          260          270          280          290          300
CAGTACTACTGCCCTGAAGTTCTGAAAGCATCTTCTGGACCTCGCATTATTACTACACAT
   Q  Y  Y  C  P  E  V  L  K  A  S  S  G  P  R  I  I  T  T  H
             310          320          330          340          350          360
CTGCCTTACCATCTGCTGGCACCAGCCCTGCAACACTGCAAAGCAAAGGTGATATATGTG
   L  P  Y  H  L  L  A  P  A  L  Q  H  C  K  A  K  V  I  Y  V
             370          380          390          400          410          420
GCAAGAAATGTCAAAGATGTGTGTGTTTCATATTACCACTTCCACAAGATGGCAAATTTT
   A  R  N  V  K  D  V  C  V  S  Y  Y  H  F  H  K  M  A  N  F
             430          440          450          460          470          480
CTCCCTGACCCAGGAACGTTCCCTGAGTTTCTGAAGAATTTCCTAAAGGGTGACGTGCAT
   L  P  D  P  G  T  F  P  E  F  L  K  N  F  L  K  G  D  V  H
             490          500          510          520          530          540
TATGGATCTTGGTTCGATCATGTCAACGGCTGGACCGGTCATGCTAAAAACTTTCAAAAC
   Y  G  S  W  F  D  H  V  N  G  W  T  G  H  A  K  N  F  Q  N
             550          560          570          580          590          600
TTTTTTTACATCACTTACGAGGAAATGTTTCAGGATCTTCGTGGGTCTCTTCAGAAAGTG
   F  F  Y  I  T  Y  E  E  M  F  Q  D  L  R  G  S  L  Q  K  V
             610          620          630          640          650          660
AGTCATTTCCTGCAGTGCACATTATGCAAAGAAGAGCTGAATCTTGCTGAGAAGCACTGT
   S  H  F  L  Q  C  T  L  C  K  E  E  L  N  L  A  E  K  H  C
             670          680          690          700          710          720
AGCTTCAATAGCATGAAGGAAAATGCCATGGTCAACTATACCCTCATCCCCAAGGAGATC
   S  F  N  S  M  K  E  N  A  M  V  N  Y  T  L  I  P  K  E  I
             730          740          750          760          770          780
ATCGACCACAGCAAGGGAAAGTTCATGAGAAAAGGTAAAGTGGGAGACTGGAAGAACATG
   I  D  H  S  K  G  K  F  M  R  K  G  K  V  G  D  W  K  N  M
             790          800          810          820          830          840
TTTACAGAGGAGCAGAGTGGTCTCGTCGATGAGGTTTACTTTTCACAGATGGCAGATTCC
   F  T  E  E  Q  S  G  L  V  D  E  V  Y  F  S  Q  M  A  D  S
             850          860          870          880          890          900
TCACTGCTGTTTCAATGGGATCAGCCCGGGGAGACTCTAGTGCCCCTGATAAACATTAAT
   S  L  L  F  Q  W  D  Q  P  G  E  T  L  V  P  L  I  N  I  N
             910          920          930          940
AATTCTGCATCCGGAGAGATCCTTCTCCTCACATAA
   N  S  A  S  G  E  I  L  L  L  T  *
```

Figure 3-1: The nucleotide and deduced amino acid sequences of the zebrafish
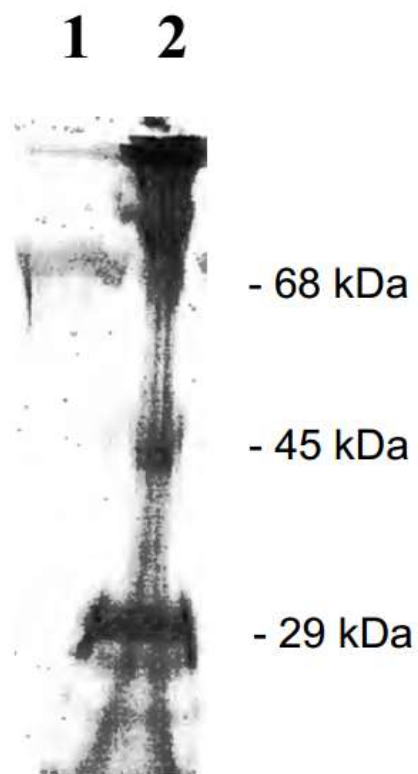
SULT5A1.

Figure 3-2: SDS gel electrophoretic pattern of the purified recombinant zebrafish SULT5A1. SDS-PAGE was performed on a 12% gel, followed by Coomassie blue staining. Positions of protein molecular weight markers co-electrophoresed are marked on the right, β-lactoglobulin (Mr = 18,400), carbonic anhydrase (Mr = 29,000), ovalbumin (Mr = 43,000), bovine serum albumin (Mr = 68,000), and β-galactosidase (Mr = 116,000).

3.1.2 Substrate Specificity of SULT5A1.

A number of endogenous and xenobiotic compounds were selected and tested as substrates for purified SULT5A1 under standard assay conditions described in the **Materials and Methods** section. SULT5A1 displayed differential sulfating activities

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towards different substrates tested. Specifically, SULT5A1 exhibited strong sulfating activities towards bile alcohols (5α-cyprinol, 5β-cyprinol, 5β- scymnol, 5β-cholestantriol, and 5α-petromyzonol (PZ)), bile acid (5α-lithocholic acid), and endogenous compounds (DHEA and pregnenolone). In addition, SULT5A1 displayed weaker activities towards the bile alcohol (5α-mixynol), bile acids (isochenodeoxycholic acid, isodeoxycholic acid, and isocholic acid,), primary bile acid (allocholic acid (ACA)), and Transdehydroandesterone. In contrast, SULT5A1 showed no detectable activities towards other compounds including 25-Hydroxy- Cholesterol, bile acids (3α, 7α, 12α- trihydroxy-5α- cholestan-26-oic acid, and norlithocholic acid), primary bile acids (cholic acid (CA), chenodeoxycholic acid (CDCA), urosodeoxycholic acid (UDCA)), conjugated bile acids (glycocholic acid (GCA), glcochenodeoxycholic acid (GCDCA), taurocholic acid (TCA), and taurochenodeoxycholic acid (TCDCA)), and secondary bile acids (deoxycholic acid (DCA), lithocholic acid (LCA), and hyodeoxycholic acid (HDCA)) (cf. Table 3.1).

Table 3.1: Specific Activities of the Zebrafish SULT5A1 towards different Substrates.

Substrates		Specific activity (nmol/min/mg)[a]
Bile Alcohols:	5α-Cyprinol	0.18±0.04
	5β-Cyprinol	0.14±0.02
	5β- Scymnol	0.19±0.02
	5α-Mixynol	0.01±0.00
	5β-Cholestantriol	0.29±0.03

	5α-Petromyzonol (PZ)	0.71±0.09
Bile Acids:	3α, 7α, 12α- Trihydroxy-5α- Cholestan-26-oic Acid	ND
	5α-Isolithocholic Acid	0.13±0.01
	Norlithocholic Acid	ND
	Isochenodeoxycholic Acid	0.02±0.00
	Isodeoxycholic Acid	0.01±0.00
Primary Bile Acids:	Cholic Acid (CA)	0.01±0.00
	Allocholic Acid (ACA)	0.01±0.00
	Chenodeoxycholic Acid (CDCA)	ND
	Urosodeoxycholic Acid (UDCA)	ND
Conjugated Bile Acids:	Glycocholic Acid (GCA)	ND
	Glcochenodeoxycholic Acid (GCDCA)	ND
	Taurocholic Acid (TCA)	ND
	Taurochenodeoxycholic Acid (TCDCA)	ND
Secondary Bile Acids:	Deoxycholic Acid (DCA)	ND
	Lithocholic Acid (LCA)	ND
	Hyodeoxycholic Acid (HDCA)	ND
Endogenous compounds:	Dehydroepiandrosterone (DHEA)	0.28±0.03
	Pregnenolone	0.18±0.03
	Transdehydroandesterone	0.01±0.00
	25-Hydroxy- Cholesterol	ND

SULT5A1. Data represent mean±S.D. derived from three measurements. The concentration of the substrate used in the assay mixture was 50 µM. ND stands for activity not detectable. ND refers to specific activity determined being lower than the detection limit (estimated to be ~0.01 nmol/min/mg).

3.1.3 pH-dependence Studies of SULT5A1.

SULT5A1 was analyzed with regard to the pH-dependence of the sulfation of 5α-cyprinol, 5α-petromyzonol (PZ), DHEA, and pregnenolone. The pH-dependence studies were carried out under the same standard assay conditions described in the **Materials and Methods**. Results revealed that SULT5A1 with 5α-cyprinol as a substrate exhibited a narrow pH optimum spanning pH 9 to 11; with a maximum sulfating activity being at pH 10.5 (figure 3-3 A). SULT5A1 with 5α-petromyzonol (PZ) as a substrate also exhibited a narrow pH optimum spanning 9 to 11 (figure 3-3 B). In contrast, SULT5A1 with DHEA as a substrate showed no activity at pH 4.5 to 5.5. The sulfating activity was detected at pH 6 and continued to increase until reaching the maximum at pH 10. Subsequently, the sulfating activity decreased at pH 10.5 and disappeared at pH 11.5 (Figure 3-3 C). Additionally, with pregnenolone as a substrate, SULT5A1 showed no activity between pH 4.5 and 6; while a strong sulfating activity was found between pH 6.5 and 10.5; with the maximum activity at pH 10 (Figure 3-3 D).
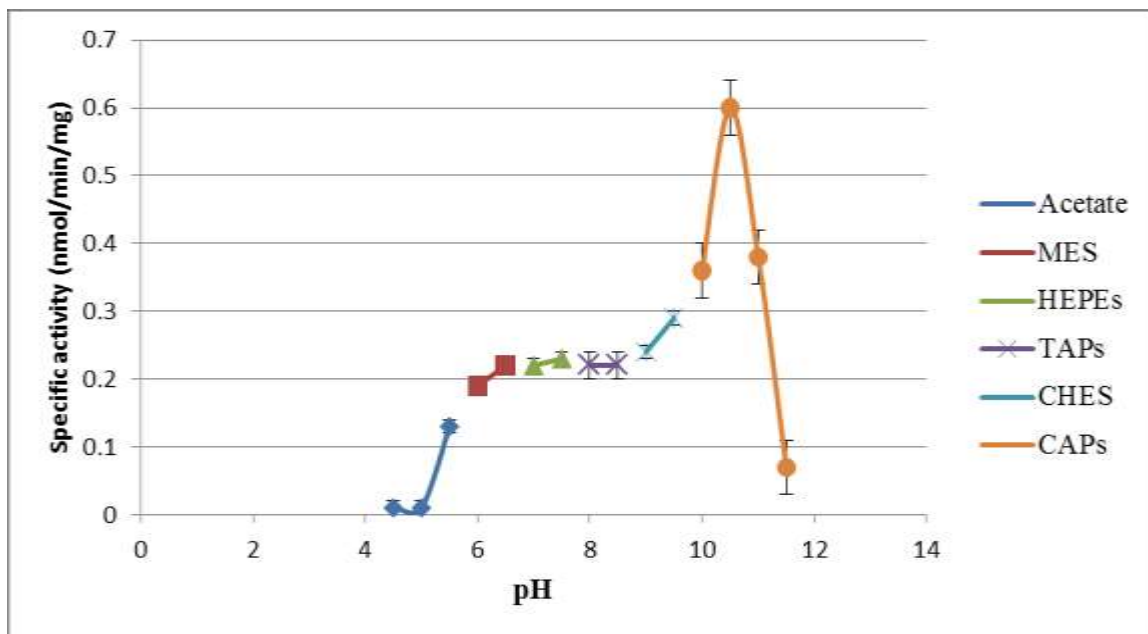
Figure 3-3 (A): pH-dependence of the sulfating activity of the zebrafish SULT5A1 with

5α-cyprinol as a substrate. Enzymatic assays were carried out under the standard

conditions as described in Materials and Methods using different buffer system as

indicated. Data shown represents calculated mean±S.D derived from three independent

experiments.

Figure 3-3 (B): pH-dependence of the sulfating activity of the zebrafish SULT5A1 with

5α-petromyzonol (PZ) as a substrate. Enzymatic assays were carried out under the

standard conditions as described in Materials and Methods using different buffer system

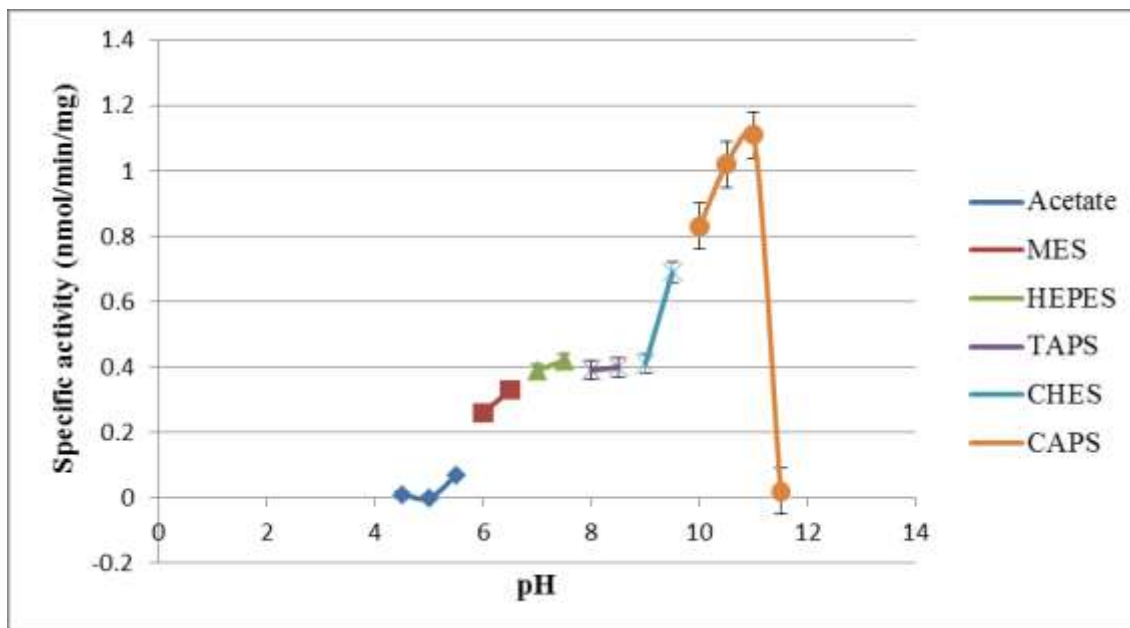as indicated. Data shown represents calculated mean±S.D derived from three independent
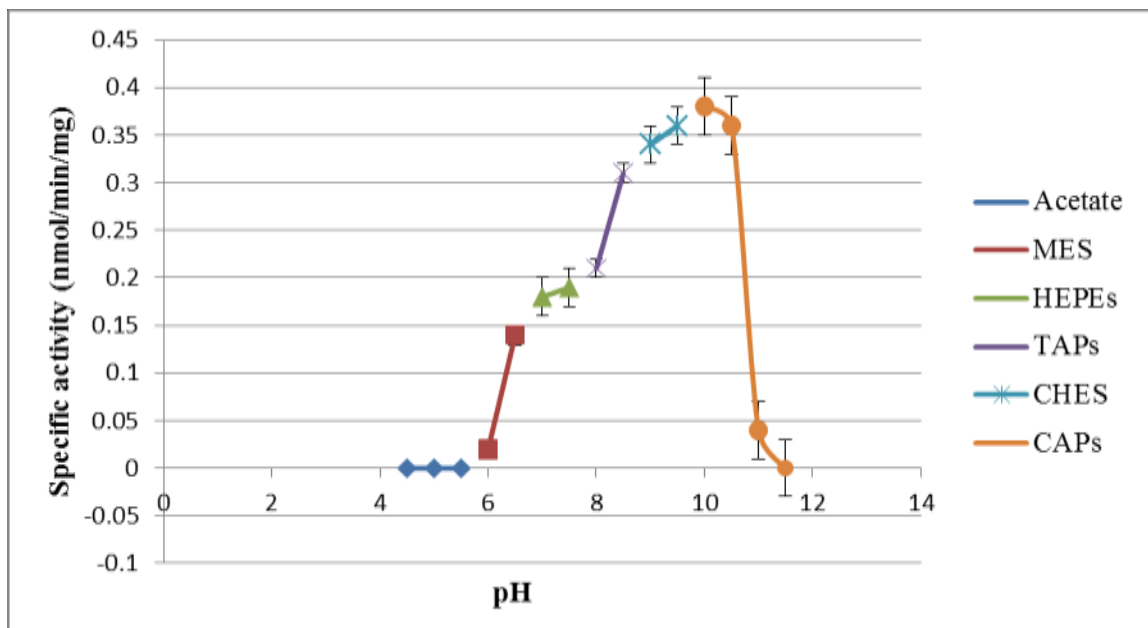
experiments.

Figure 3-3 (C): pH-dependence of the sulfating activity of the zebrafish SULT5A1 with DHEA as a substrate. Enzymatic assays were carried out under the standard conditions as described in Materials and Methods using different buffer system as indicated. Data shown represents calculated mean±S.D derived from three independent experiments.
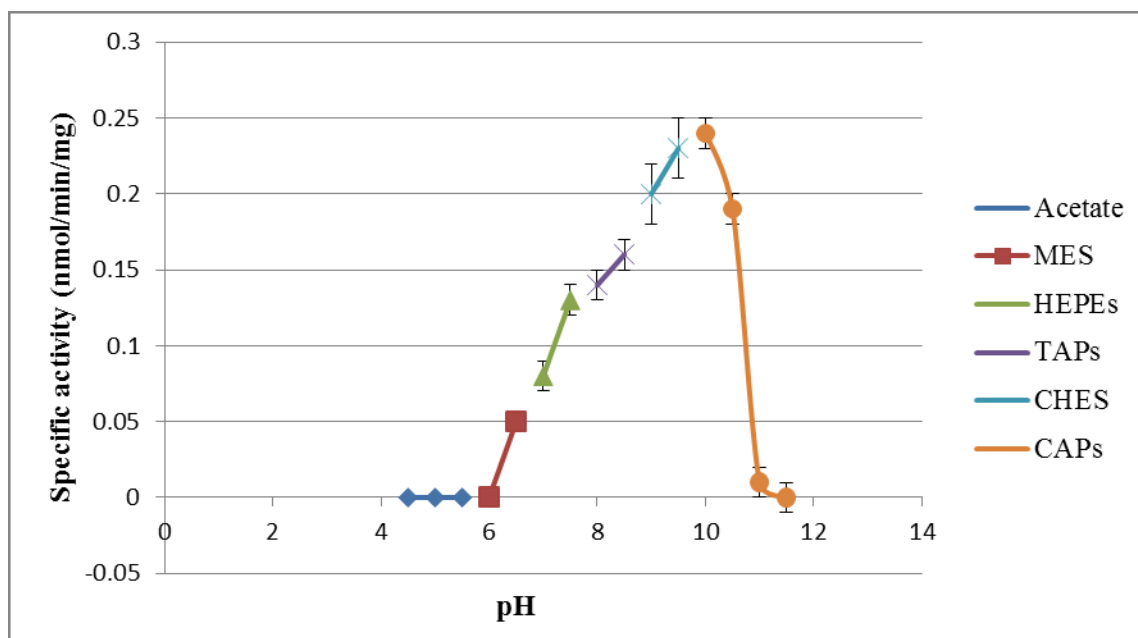
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Figure 3-3 (D): pH-dependence of the sulfating activity of the zebrafish SULT5A1 with pregnenolone as a substrate. Enzymatic assays were carried out under the standard conditions as described in Materials and Methods using different buffer system as indicated. Data shown represents calculated mean±S.D derived from three independent experiments.

3.1.4 Kinetic Studies of SULT5A1.

To investigate in more detail the sulfation of different endogenous substrates, the kinetics of sulfation of these substrates by the zebrafish SULT5A1 were analyzed. The kinetic experiments were performed using 5α-cyprinol, 5α-petromyzonol (PZ), DHEA, and pregnenolone as substrates. In these experiments, different substrate concentrations and 50 mM HEPES at pH 7.5 were used according to the enzymatic assay procedure described in the **Materials and Methods** section. The final concentrations tested were 1 to 10 µM for 5α-cyprinol, 1 to 25 µM for 5α-petromyzonol (PZ) and pregnenolone, and 1

to 100 μM for DHEA (Table 3.2). Data obtained from these experiments were processed

using the GraphPad Prism5 software program to generate the best fitting curves for the

Michaelis-Menten equation with non-linear regression in order to calculate the values of

K_m, V_{max}, and K_{cat}/K_m for the zebrafish SULT5A1 enzyme in catalyzing the sulfation of

indicated substrates. Figure 3-4 (A, B, C, and D) show the saturation kinetics of zebrafish

SULT5A1 with 5α-cyprinol, 5α-petromyzonol (PZ), DHEA, and pregnenolone as

substrates. The calculated values of K_m, V_{max}, and K_{cat}/K_m for the SULT5A1 are compiled

in Table 3.3.

Table 3.2 List of the different substrate concentrations used in the kinetic studies of

Zebrafish SULT5A1

Substrates	Final concentrations (μM)
5α-Cyprinol	1, 2.5, 5, 10
5α-Petromyzonol (PZ)	1, 2.5, 5, 10, 25
DHEA	1, 2.5, 5, 10, 25,50,100
Pregnenolone	1, 2.5, 5, 10, 25

Figure 3-4 (A): The figure shows the saturation curve analysis of the sulfation of 5α-cyprinol by the zebrafish SULT5A1. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reaction is indicated as nmol/min/mg of the enzyme. The data represent calculated mean±S.D derived from three experiments.
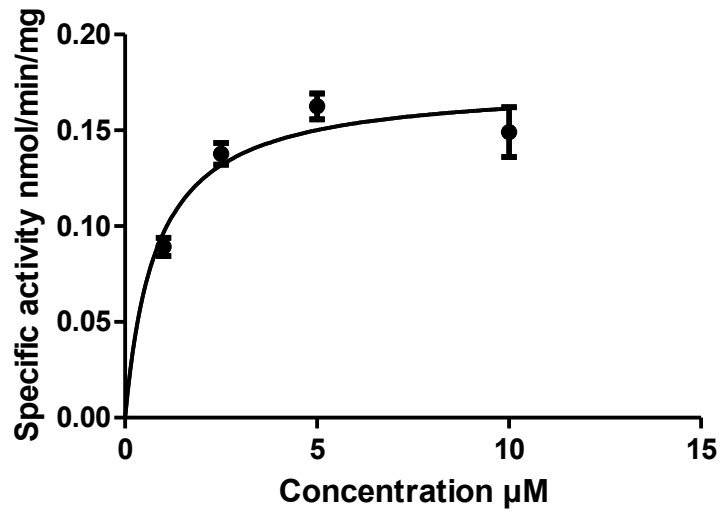
Figure 3-4 (B): The figure shows the saturation curve analysis of the sulfation of 5α-Petromyzonol by the zebrafish SULT5A1. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reactions is indicated as nmol/min/mg of the enzyme. The data represent calculated mean±S.D derived from three experiments.

Figure 3-4 (C): The figure shows the saturation curve analysis of the sulfation of DHEA by the zebrafish SULT5A1. The fitting curve was generated based on Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software. The velocity of the reactions is indicated as nmol/min/mg of the enzyme. The data represent calculated mean±S.D derived from three experiments.
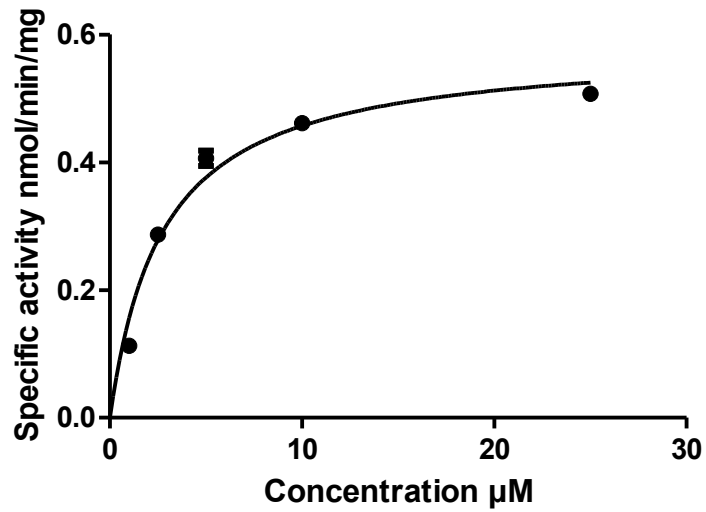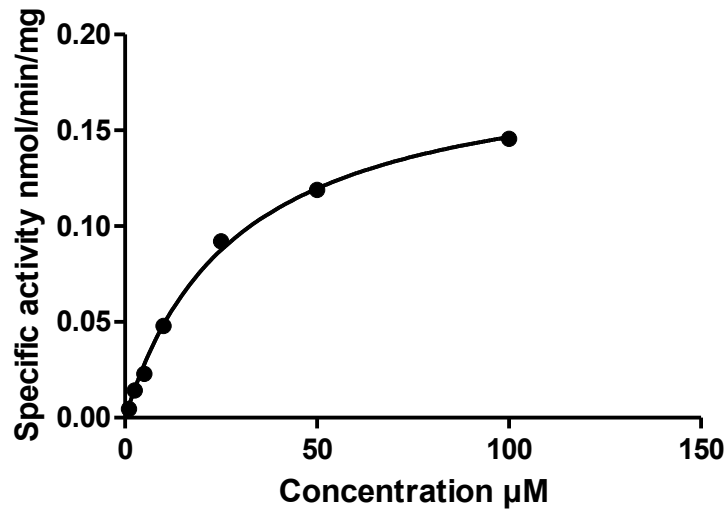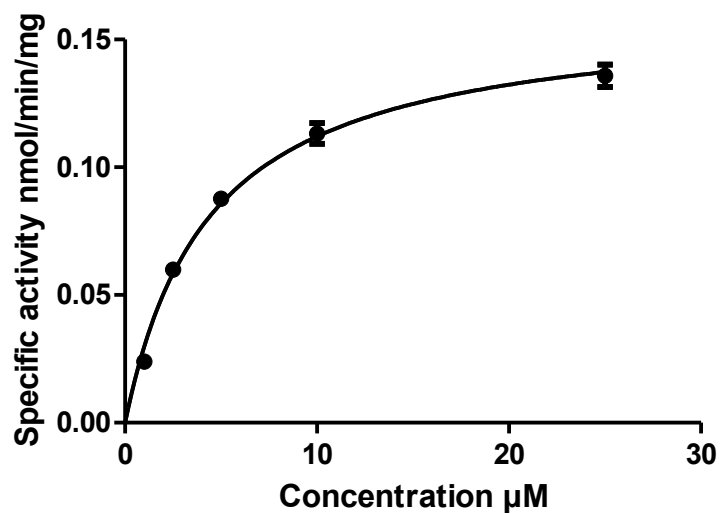
Figure 3-4 (D): The figure shows the saturation curve analysis of the sulfation of

Pregnenolone by the zebrafish SULT5A1. The fitting curve was generated based on

Michaelis-Menten equation with non-linear regression using GraphPad Prism5 software.

The velocity of the reactions is indicated as nmol/min/mg of the enzyme. The data

represent calculated mean±S.D derived from three experiments.

Table 3.3 Kinetic parameters of Zebrafish SULT5A1 with 5α-cyprinol, PZ, DHEA, and

pregnenolone as substrates

Substrates	K_m (μM)	V_{max} (nmol/min/mg)	K_{cat}/K_m
5α-Cyprinol	0.81±0.21	0.17±0	7.53
PZ	2.75±0.22	0.58±0.01	7.57
DHEA	29.03±0.5	0.19±0	0.23
Pregnenolone	4.42±0.37	0.16±0	1.3

3.2 Discussion.

The zebrafish model has been developed for studying the Phase II drug-metabolizing enzymes, particularly the SULTs. Our laboratory had previously identified nineteen distinct zebrafish SULTs enzymes. In this research, a novel zebrafish SULT5A1 was identified, cloned, purified, and characterized. The results obtained from substrate specificity studies showed that the zebrafish SULT5A1 exhibited strong sulfating activities toward 5α-cyprinol, 5β-cyprinol, 5β- scymnol, 5β-cholestantriol, PZ, and 5α-lithocholic acid, among a panel of bile alcohol and bile acid compounds that were tested as substrates. It also displayed significant sulfating activities toward endogenous compounds, including DHEA and pregnenolone. In contrast, zebrafish SULT5A1 showed no activity toward xenobiotics. As mentioned previously, the zebrafish SULT3 ST2 and SULT3 ST3 were the major enzymes previously shown to be capable of sulfating bile acids and bile alcohol, and SULT3 ST3 was suggested to be the main SULT in zebrafish for the sulfation of 5α-bile alcohol PZ. Human studies revealed that, of the thirteen known human SULTs, SULT2A1 was the only enzyme capable of sulfating the bile alcohols and bile acids. Human SULT2A1 displayed the strongest catalytic activity toward LCA, which is considered the most toxic and hydrophobic bile acid in the human bodies. However, SULT2A1 exhibited low activity toward the more hydrophilic bile acids, including UDCA, GCDCA, TCDCA, DCA, and HDCA (Kurogi, Krasowski et al. 2011). Some recent studies have demonstrated that the zebrafish SULT1 STs (ST1 through ST9) exhibited sulfating activities toward different xenobiotics (Liu, Bhuiyan et al. 2010, Mohammed, Kurogi et al. 2012).

Further characterizations of the enzymatic properties of the zebrafish SULT5A1 revealed that the pH-dependence of SULT5A1 with 5α-cyprinol and PZ showed a narrow pH optimum spanning 9 to 11. These similar pH-dependence results appeared to be due to the similarity between 5α-cyprinol and PZ with regard to 5α ('allo')-cholanoids having a flat (planer) juncture between the A and B rings. Interestingly, SULT5A1 exhibited a pH optimum spanning pH 6 to 10 with DHEA as a substrate, whereas with pregnenolone, a pH optimum spanning 6.5 to 10.5 was observed. These different pH optima of the zebrafish SULT5A1 may imply its differential catalytic activity toward different substrates in different zebrafish cell types and tissues. In a recent study, the sulfation of PZ by human SULT2A1 showed a broad pH optimum; whereas both zebrafish SULT3 ST2 and SULT3 ST3 exhibited a narrow pH optimum at pH 6 (Kurogi, Krasowski et al. 2011).

Kinetic parameters of the sulfation of 5α-cyprinol, PZ, DHEA, and pregnenolone by SULT5A1 were determined based on Michaelis-Menten equation with non-linear regression using the GraphPad Prism5 software program. The K_m for 5α-cyprinol (0.81±0.21 µM) was lower than the K_m for PZ (2.75±0.22 µM), indicating the higher affinity of SULT5A1 for 5α-cyprinol than that for PZ. The kinetic parameters of the sulfation of endogenous compounds, including DHEA, and pregnenolone by SULT5A1 revealed that the K_m for DHEA (29.03±0.5 µM) was much higher than that for pregnenolone (4.42±0.37 µM), indicating that SULT5A1 has much higher affinity toward pregnenolone. The ratio of K_{cat}/K_m is a measurement of catalytic efficiency of an enzyme in mediating a reaction at sub-maximal substrate concentrations. The calculated K_{cat}/K_m for 5α-cyprinol was almost the same as that for PZ by SULT5A1, implying that

SULT5A1 has a comparable catalytic efficiency toward these two substrates. As mentioned above, SULT5A1 displayed a higher K_m value for DHEA, the K_{cat}/K_m with DHEA (0.23) is considerably lower than that (1.3) for pregnenolone. It therefore can be concluded that SULT5A1 is more catalytically efficiency with pregnenolone than with DHEA as substrate.

In conclusion, by searching the GenBank database at NCBI, a new zebrafish SULT cDNA was identified. This new zebrafish, designated SULT5A1, was cloned, expressed, purified, and characterized. SULT5A1 was found to display sulfating activities toward cholanoids as well as endogenous compounds including DHEA and pregnenolone. These results contributed to the establishment of the zebrafish as a model for investigating the phase II metabolism of cholanoids and endogenous compounds through sulfation reaction. Further studies are needed in order to clarify further the physiological involvement of SULT5A1 in zebrafish.

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