Developmental toxicity of dextromethorphan and acetaminophen in zebrafish embryos/larvae: relevance of SULT-mediated dextromethorphan/acetaminophen sulfation

Zheng Xu

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Developmental Toxicity of Dextromethorphan and Acetaminophen in Zebrafish Embryos/Larvae: Relevance of SULT-mediated Dextromethorphan/Acetaminophen Sulfation

by

Zheng Xu

Submitted to the Graduate Faculty as a partial fulfillment of the requirement for the Master of Science in Pharmacology and Toxicology

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May 2010
Sulfation is one of the important Phase II reactions. We identified and characterized two novel zebrafish sulfotransferase SULT1 ST9 and SULT3 ST4. SULT1 ST9 showed strong sulfating activity toward substrates such as acetaminophen and 3,3',5-triiodo-L-thyronine. SULT3 ST4 exhibited strong sulfating activity towards substrate DHEA, and weaker activity toward mestranol. pH dependency assays and kinetics assays were used to characterized two enzymes’ interactions with these substrates.

Acetaminophen and dextromethorphan are widely used in over-the-counter cough and cold medications. Their efficacy and safety for infants and young children remains to be clarified. The present study was designed to use the zebrafish as a model to investigate the potential toxicity of acetaminophen and dextromethorphan during embryonic and larval development. Three sets of zebrafish embryos/larvae were exposed to
dextromethorphan at 24 hours post fertilization (hpf), 48 hpf, and 72 hpf, respectively, during embryonic/larval development. Compared with the 48 and 72 hpf exposure sets, the embryos/larvae in the 24 hpf exposure set showed much higher mortality rates which increased in a dose-dependent manner. Morphological effects of dextromethorphan exposure, including yolk sac and cardiac edema, craniofacial malformation, lordosis (curving body trunk), non-inflated swim bladder, and missing gill, were also more frequent and severe among zebrafish embryos/larvae exposed to dextromethorphan at 24 hpf. Moreover, bradycardia (30-70 heart beats/min vs. ~130 heart beats/min for normal control) was observed for the embryos/larvae treated with elevated concentrations of dextromethorphan. Another three sets of zebrafish embryos/larvae were exposed to acetaminophen at 1 hpf, 24 hpf, and 48 hpf, respectively, during embryonic/larval development. Compared with the 24 and 48 hpf sets, the embryos/larvae in the 24 hpf exposure set showed much higher mortality rates which increased in a dose-dependent manner. With increasing concentrations of acetaminophen, there was a significant dose-dependent morphological change in the developing embryos with regard to the development of the eyes and swim bladder, and distribution of pigment. There was no noticeable difference among larvae of the 1, 24 and 48 hpf sets in terms of these morphological changes. Whether the more frequent and severe developmental toxicity of dextromethorphan observed among the embryos/larvae in the 24 hpf exposure set, as compared with the 48 and 72 hpf exposure sets, was due to the developmental expression of the Phase II enzymes sulfotransferases involved in the metabolism of dextromethorphan/ acetaminophen warrants further investigation.
Acknowledgments

This research project would not have been possible without the support of many people. I wish to express my deepest gratitude to my supervisor, Dr. Ming-Cheh Liu, whose encouragement, guidance and support from the initial to the final level enabled me to finish this thesis. I am also heartily thankful to the other two members of the committee, Dr. Frederick Williams and Dr. Zahoor Shah for their knowledge and assistance.

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I want to express love and gratitude to my beloved husband Yuhan Li, my parents and in-laws; for their understanding and endless love, throughout my two years of study.

Lastly, I offer my regards and blessings to all of those who supported me in any respect during the completion of the project.
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Introduction

1.1 Overview of Developmental Pharmacology

The father of American pediatrics--Dr. Abraham Jacobi once wrote, "Pediatrics does not deal with miniature men and women, with reduced doses and the same class of disease in smaller bodies, but . . . has its own independent range and horizon." (Frank Spooner Churchill, 1911) Physicians have been taking approaches such as using discrete age points and/or allometric principles based on relative body size to assume that there are predictable, linear relations between body weight and body-surface area among infants, children, adolescents, and adults, to determine doses for pediatric drugs. However, human growth is not a linear process. Childhood, especially the neonatal and infancy periods, is a dynamic period due to the ongoing processes of rapid and significant physiologic changes. These ongoing changes in body and organ functions influence both drug effects and disposition (Novak and Allen, 2007). (see Table 1-1 for the summary of the physiological differences between children and adults on drug absorption, distribution, metabolism and excretion). The physiological differences between children and adults in drug metabolism process determine that people should not simply adjust the dosage by weight or age for use in children. While it would be the best if we can do drug testing in pediatric populations, it is not feasible due to ethical
dilemmas such as potential health risks to children participating in the research, potential exploitation of children by the drug researchers, and the need to obtain parental consent and child assent to participate in the study (Novak and Allen, 2007).

Table 1-1 Physiological differences between children and adults with regard to drug ADME (Novak and Allen, 2007).

| Absorption                  | Gastrointestinal Function: Infants have increased surface area of the GI tract, prolonged emptying time irregular peristalsis, and decreased gastric pH leading to unpredictable and irregular absorption patterns. |
|                            | Blood Flow at Administration Site: Infants and children have erratic blood flow to tissue that can cause decreased absorption rates. |
|                            | Skin Permeability: Newborns and infants have greater skin absorption due to increased hydration and thinner skin stratum than adults. |
| Distribution               | Body Composition: Newborns and infants have a higher percentage of total body water and lower percentage of body fat that affect dosing depending on hydrophilic or lipophilic nature of the medication. |
|                            | Plasma Protein Concentrations: Infants have decreased level of plasma proteins resulting in decreased ability for drug binding and a |
Physiological Barriers: Newborns have immaturity of the blood-brain barrier which results in increased CNS effects of medications and appropriate dosing is necessary.

<table>
<thead>
<tr>
<th>Metabolism</th>
<th>Primary Site: The liver is the primary organ for metabolism, but other sites include lungs, kidneys, blood, GI tract and skin also exist. Enzymatic reactions: Delay in the maturation of drug-metabolizing enzymes can lead to drug toxicity in the very young. Additionally maturation of enzymes occurs at variable rates. Metabolic Reactions: Phase I and Phase II reactions reach adult level by age three. More is known about Phase I enzymes such as the CYP450 enzymes than the Phase II enzymes.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excretion</td>
<td>Renal Excretion: Occurs primarily through filtration of active tubular secretion. It is reduced in neonates and infants due to lower glomerular filtration rate, decreased renal blood flow and decreased tubular function. Normal renal excretion is attained by one year of age.</td>
</tr>
</tbody>
</table>
1.2 Ontogeny of Drug-metabolizing Enzymes and the Possible Roles in Developmental Pharmacology

1.2.1 Ontogeny of Phase I Enzymes

Numerous human studies indicate that among the drug-metabolizing enzymes, the different cytochrome P450 isozymes of the Phase I oxidative stage may be expressed at different levels at various times during development, with some being expressed early before the start of embryogenesis and either decreasing or remaining constant later in gestation, while others are expressed only later in gestation or postnatally (Hakkola, et al., 1998; Hines, 2008; Hines, 2007; Oesterheld, 1998). One study showed that fetal liver microsomes have very limited CYP2D6 activity (approximately 1% of adult values) by using dextromethorphan O-demethylation to dextrorphan method, which is a relatively safe and useful method to determine CYP2D6 phenotype level in vivo and in vitro (Leeder and Kearns, 1997). In the latter study, immunochemically detectable microsomal CYP2D6 protein was very low or undetectable in approximately 70% of fetal liver samples but was present in all samples from newborns 7 days of age. The enzyme’s catalytic activity increases to 20% of adult by 1 month and adult competence at least by 10 years of age, and probably much earlier (Treluyer, et al., 1991). CYP3A7 is the predominant CYP isoform expressed in fetal liver. The expression peaks shortly after birth and then virtually disappears by 1-4 weeks of postnatal when CYP3A4 activity predominates (Lacroix, et al., 1997). The activity of CYP3A4, the most abundant CYP isoform in the human body, is extremely low at birth. It reaches approximately 30%-40% of adult levels by 1 month and 100% by 6 months (Lacroix, et al., 1997). The activity
may exceed adult level between 1-4 years of age and then declines after puberty. Similar patterns have also been found for CYP2C9 and CYP1A2 (Leeder and Kearns, 1997).

1.2.2 Ontogeny of Phase II Drug-metabolizing Enzymes

In addition to the cytochrome P450s, apparent age-dependence exists for several Phase II enzymes that are of quantitative importance for drug biotransformation. Compared with the Phase I cytochrome P450s, there is less information about the ontogeny of the Phase II enzymes (Darras, et al., 1999; Hines, 2008; McCarver and Hines, 2002; Richard, et al., 2001). Studies of N-acetyltransferase NAT2, using caffeine as a pharmacologic probe, demonstrated the attainment of adult activity by approximately 4 to 6 months of postnatal age (Pariente-Khayat, et al., 1991). Studies that examined the impact of age on the disposition of several UDP-glucuronosyltransferases (UGT) substrates suggest that isoform-specific, age-related differences in UGT activity occur. Acetaminophen is a substrate for UGT1A6 and UGT1A9. The glucuronidation of acetaminophen occurs to a lesser extent in newborns and young children, compared with adolescents and adults (Allegaert, et al., 2005). In some human studies, sulfation by the sulfotransferases (SULTs) appears to be more important in fetal development, such as the homeostasis of thyroid hormones (Darras, et al., 1999; Richard, et al., 2001), dehydroepiandrosterone (Barker, et al., 1994), dopamine(Richard, et al., 2001), and various estrogens and steroid hormones (Duanmu, et al., 2006), as well as for detoxification (Richard, et al., 2001) of xenobiotics and therapeutics, especially since other conjugating enzyme systems, such as the UDP-glucuronosyltransferases, are not
expressed at significant levels until the neonatal period (Richard, et al., 2001). SULTs are expressed at high levels in the human fetus. The wide tissue distribution and tight regulations of SULTs expression during human development suggest a significant role for these enzymes in the early stages of life (see Table 1-2). Some SULT forms appear to be expressed only or primarily in the prenatal period, e.g., SULT1C enzymes are only present in fetal kidney, lung, and gastrointestinal tract (Her, et al., 1997; Sakakibara, et al., 1998). The function of certain SULTs in fetal development is well established, such as the production of dehydroepiandrosterone (DHEA) sulfate by the adrenal SULT2A1 enzyme. However, the role of others is still unclear. SULT1C enzymes are believed to be involved in thyroid hormone metabolism, and this is a particularly important function in the fetus where appropriate thyroid hormone homeostasis is essential for normal brain development. Also the human fetus produces very large amounts of iodothyronine sulfates compared to an adult, the high levels of SULT1A1 in various fetal tissues may provide a protection, especially when other drug metabolizing enzymes such as the CYPs, UGTs are in general not expressed at significant levels until after birth (Coughtrie, 2002). Thus SULTs may represent a front line of chemical defense in the developing human. This has obvious implications for adverse action of drugs and would be especially important to investigate in the case of drugs with potential teratogenic effects. For this purpose, the sulfation and the responsible sulfotransferase enzymes are described in greater detail below.

1.3 Sulfation and Sulfotransferase Enzymes
1.3.1 The Overview of Sulfation.

The process of biological sulfation was discovered by Eugen Baumann in 1876. Baumann isolated and characterized phenol sulfate from the urine of a patient who had been treated with phenol as an antiseptic (Baumann, 1876). However, the mechanism of sulfate conjugation remained obscure until 1957 when Lipmann’s group discovered ‘active sulfate’ (Robbins and Lipmann, 1957) as we call it today as PAPS (3’-phosphoadenosine-5’-phosphosulfate). Since then, numerous studies have revealed that sulfation regulate the biological activities of a vast array of endogenous and foreign compounds, such as: hormones, neurotransmitter, steroids, environmental toxic agents and drugs (Adjei and Weinshilboum, 2002; Cascio, et al., 2000; Glatt and Meinl, 2004; Kitada, et al., 2003; Taskinen, et al., 2003; Thomae, et al., 2003). Among the various functional roles, sulfation i) inactivates and/or bioactivates xenobiotics, ii) inactivates of hormones and catecholamines, and iii) modifies the structure and function of macromolecules, and eliminates end products of catabolism (Klaassen and Boles, 1997).

Sulfation of drugs and xenobiotics usually leads to a loss of their biological activities. However, some compounds, such as N-hydroxy-2-acetylaminofluorene, 1’-hydroxysaphrole, N-hydroxy-4-aminobiphenyl, and 7-hydroxymethyl-12-methylbenzanthracene, have been reported to be activated to reactive electrophilic metabolites via sulfation. The electrophilic metabolites of these compounds may react with cellular nucleophiles, such as DNA, RNA, and proteins to elicit mutagenic and/or cytotoxic responses. Also, the active sulfated form of the drug, minoxidil, i.e., N- or O- sulfate ester of minoxidil, is responsible for both its therapeutic anti-hypertensive and hair
growth-stimulating effects (McCall et al, 1983; Buhl et al., 1990). Increased efficacy of minoxidil sulfate may be related to the increased hydrophilicity of minoxidil sulfate, as compared to minoxidil, due to the formation of an inner salt (McCall et al., 1983).

Sulfation, as catalyzed by the sulfotransferase enzymes, involves the transfer of a sulfonyl moiety (SO$_3^-$) from the co-substrate PAPS to hydroxyl-, amino-, sulfhydryl-, or N-oxide group of substrates (Coughtrie, 2002). The product of the sulfation reaction is a sulfoconjugate (R-OSO$_3^-$) and the adenosine-3’, 5’-diphosphate (PAP) (Figure 1-1).

\[
\text{R-OH} + \text{PAPS} \xrightarrow{\text{Sulfotransferases}} \text{R-OSO$_3^-$} + \text{PAP}
\]

Figure 1-1: General sulfation reaction

PAPS, the universal sulfate donor, is an obligatory co-substrate in the sulfation reaction. The chemical structure of PAPS is given in Figure 1-2. PAPS is an activated form of sulfate and it is synthesized in the cytosol using a two-step reaction. The first step of the reaction requires adenosine-5’-triphosphate (ATP) and inorganic sulfate (SO$_4^{2-}$) to produce adenosine-5’-phosphosulfate (APS) and pyrophosphate (PPi). The reaction is catalyzed by ATP sulfurylase in the presence of Mg$^{2+}$. The source of sulfate required for the synthesis of PAPS comes from the catabolism of the amino acids cysteine and methionine or from the diet (Klaassen and Boles, 1997). In the second step, APS reacts with another ATP molecule to form PAPS and adenosine-5’-diphosphate (ADP). This reaction is catalyzed by the enzyme APS kinase in the presence of Mg$^{2+}$. The first step of the PAPS synthesis reaction is thermodynamically unfavorable, but subsequent hydrolysis of PPi formed in the first step of the reaction and rapid utilization of the APS in the second step of the reaction relieves energy constraints and favors the forward
reaction. The second step of PAPS synthesis is thermodynamically favorable (Shailubhai, et al., 1996). In mammals, ATP sulfurylase and APS kinase exist as a single bifunctional enzyme. In bacteria and fungi, the two enzymes are present independently as distinct proteins (Mulder and Jakoby, 1990). The synthesis of PAPS may be very rapid, but the sulfation of drugs in humans may be limited by PAPS availability in certain instances (Weinshilboum and Otterness, 1994). Inorganic sulfate is essential for PAPS synthesis. Sulfate pools are replenished by the sulfoxidation of sulfur-containing amino acids (cysteine and methionine), transport-mediated intestinal absorption of inorganic sulfate, transport-mediated renal reabsorption of inorganic sulfate, degradation of sulfate-containing macromolecules, and the activity of sulfatases (Klaassen and Boles, 1997). Inorganic sulfate may be depleted by synthesis of amino acids, sulfation, and conjugation reactions. (Pang, 1990; Falany, 1997). The synthesis of PAPS also requires two molecules of ATP - it is therefore an "expensive" commodity for cells to produce. Low PAPS levels (typically no more than 20 or 30 μM) and rapid depletion is believed to limit the capacity of sulfation (Coughtrie, 2002). Sulfation, glucuronidation, acetylation, and methylation are important Phase II metabolic pathways. Many substrates that can be metabolized by sulfotransferases are also substrates for UDP-glururonosyltransferases (Burchell and Coughtrie, 1997). Though they have overlapping substrate specificity, sulfation is considered as a high affinity and low capacity Phase II reaction, which is the opposite of glucuronidation. Glucuronidation predominates at high substrate concentrations when sulfation has been saturated, e.g., acetaminophen (Lindsay, et al., 2008).
1.3.2 Cytosolic SULTs vs. Membrane-bound Sulfotransferases

Sulfotransferases can be divided into two categories; one is the cytosolic sulfotransferases (SULTs), and the other is membrane-bound sulfotransferases (Falany, 1997; Weinshilboum, et al., 1997). Cytosolic SULTs are responsible for the metabolism
of xenobiotics and small endogenous substrate such as steroids, bile acids, and
catecholamine neurotransmitters. Membrane-bound sulfotransferase is located in the
trans-Golgi apparatus and it’s responsible for the sulfation of peptides, glycoproteins,
lipids and glycosaminoglycans (Falany, 1997). Both cytosolic SULTs and membrane-
bound sulfotransferases are presented in a wide variety of plants, insects, and vertebrates.
However, the Golgi-associated sulfotransferases are generally thought not to be involved
in the drug metabolism aspects of sulfation (Falany, 1997). Cytosolic SULT enzymes are
traditionally considered to mediate Phase II pathway in drug and xenobiotic metabolism
(Weinshilboum, et al., 1997). The cytosolic SULTs are the focus of this thesis.

Like many drug metabolizing enzymes, the cytosolic SULTs comprise a large
superfamily of genes. Rat SULT2A2 was the first SULT cloned and was originally
identified as a senescence marker protein (Chatterjee, et al., 1987). Full length cDNAs
from more than 50 mammalian and avian species have since been cloned and sequenced
(Coughtrie, 2002; Nagata and Yamazoe, 2000) (Figure 1-4).
Figure 1-4: The cytosolic SULTs superfamily. (Coughtrie, 2002)

The cytosolic SULTs described to date have been classified into six families with members of each family share at least 45% amino acid sequence identity, and subfamily with members of each subfamily being at least 60% identical (Blanchard, et al., 2004; Coughtrie, 2002). SULT1 and SULT2 families are the largest and are responsible for sulfating the greatest number of endogenous and xenobiotic compounds. There are
currently 13 known isoforms of human SULT enzymes representing the SULT1, SULT2, SULT4 and SULT6 families (Coughtrie, 2002; Gamage, et al., 2006; Lindsay, et al., 2008). Coughtrie and his colleagues had summarized some of the properties, regarding to major sites of expression and specificities for endogenous and xenobiotic substrates, of known human SULTs isoforms (Coughtrie, 2002; Coughtrie, et al., 1998). Each SULT enzyme shows a distinct pattern of tissue distribution (See Table 1-2). Despite considerable research, endogenous and xenobiotic substrates for a number of SULT isoforms remain to be identified. An interesting example here is the failure to identify substrates for a highly conserved protein (SULT4A1) identified from the expressed sequence database that appears to be expressed only in mammalian brain (Coughtrie, 2002; Liyou, et al., 2003; Sakakibara, et al., 2002). This protein is prominent in a number of brain structures including the cerebral cortex, cerebellum, pituitary, and brainstem of rats and humans; however to date, no endogenous nor xenobiotic substrates have been identified for SULT4A1.

Table 1-2: Gene Location, Tissue/Organ Distribution, and Substrate Specificity of Human Cytosolic SULTs. (Freimuth, et al., 2004)

<table>
<thead>
<tr>
<th>SULT Isoforms</th>
<th>Chromosomal Location</th>
<th>Substrates</th>
<th>Major Sites of Expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULT1A1</td>
<td>16p12.1–11.2</td>
<td>4-Nitrophenol, 1-naphthol, paracetamol, minoxidil, 17β-estradiol (E2), estrone (E1),</td>
<td>Adult liver, adult gastrointestinal tract,</td>
</tr>
<tr>
<td>SULT1A2</td>
<td>16p12.1–11.2</td>
<td>2-Naphthol &gt; minoxidil, 4-nitrophenol; OH-PhIP</td>
<td>No site found yet</td>
</tr>
<tr>
<td>---------</td>
<td>--------------</td>
<td>---------------------------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>SULT1A3</td>
<td>16p11.2</td>
<td>Dopamine, norepinephrine, isoprenaline, 2-hydroxy-E2 and other catechols, numerous monocyclic phenols; vanillin, 1-naphthol; albuterol and various other β2 agonists; dopamine &gt; tyramine &gt; p-nitrocatechol, m-nitrocatechol, 4-nitrophenol, p cresol, 4-aminophenol, 4-ethylphenol; E1; minoxidil</td>
<td>Adult gastrointestinal tract, adult platelets, adult brain, placenta, fetal liver</td>
</tr>
<tr>
<td>SULT1B1</td>
<td>4q11–13</td>
<td>1-Naphthol &gt; 4-nitrophenol &gt; DES, iodothyronines</td>
<td>Adult liver, adult and fetal gastrointestinal tract</td>
</tr>
<tr>
<td>SULT1C2</td>
<td>2q11.2</td>
<td>4-Nitrophenol &gt; OH-AAF</td>
<td>Fetal kidney, lung, and gastrointestinal tract</td>
</tr>
<tr>
<td>----------</td>
<td>--------</td>
<td>------------------------</td>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>SULT1C4</td>
<td>2q11.2</td>
<td>No known substrate</td>
<td>Fetal kidney and lung</td>
</tr>
<tr>
<td>SULT1E1</td>
<td>4q13</td>
<td>E2, E1, pregnenolone, 17-ethinyl-E2, equilenin; DHEA, 1-naphthol, DES, 4-hydroxytamoxifen, naringenin, genistein; iodothyronines; minoxidil</td>
<td>Fetal liver, lung, and kidney; adult liver and endometrium</td>
</tr>
<tr>
<td>SULT2A1</td>
<td>19q13.3</td>
<td>DHEA &gt; epiandrosterone &gt; androsterone &gt; testosterone &gt; E2; cholesterol; lithocholic acid and various other bile acids; pregnenolone &gt; 17-ethinyl-E2 &gt; cortisol; minoxidil</td>
<td>Fetal and adult adrenal gland and liver</td>
</tr>
<tr>
<td>SULT2B1</td>
<td>19q13.3</td>
<td>DHEA</td>
<td>Adult skin, prostate, and placenta</td>
</tr>
<tr>
<td>SULT4A1</td>
<td>22q13.1–13.2</td>
<td>No known substrate</td>
<td>Brain</td>
</tr>
</tbody>
</table>
1.4 Toxicology of Pediatric Cough and Cold Medications

Several ingredients are present in the common cough and cold medications, such as antihistamine (acrivastine, brompheniramine, carbinoxamine, chlorpheniramine), decongestant (ephedrine, phenylephrine, pseudoephedrine), cough suppressant (dextromethorphan, codein, benzonatate), and antipyretics (acetaminophen and ibuprofen). In 2008, the total sales of over-the-counter cough and cold medications were approximately $4.1 billion in the U.S market (CHPA, 2009).

1.4.1 Adverse Effects of Cough and Cold Medications

The use of cough and cold medications is widespread, but their safety and effectiveness have remained controversial. To date, no FDA-approved dosing recommendations exist for taking over-the-counter (OTC) cough and cold medications to children aged less than 2 years. An Food and Drug Administration (FDA) advisory panel voted on Oct 19, 2007 to ban popular OTC cold products intended for children under the age of 6. Historically, a large number of pediatric medications have been prescribed off-label for children because studies were never performed in this population. Lack of data to support the use of OTC medications in young children had repeatedly been pointed out in the literature (De Sutter, et al., 2009; Paul, et al., 2004). However, 87% of the time, parents thought these products were appropriate for use in children<2 years of age (Lokker, et al., 2009). An estimated 1,519 children aged less than 2 years were treated in emergency departments for adverse events associated with cold medications during 2004-2005 (CDC,
Moreover, the Center for Disease Control and Prevention (CDC) identified three cases of infant deaths in two states during 2005 that were determined by a medical examiner or coroner to have been caused by cough and cold medications. All three infants have high levels of pseudoephedrine in postmortem blood samples. Two of the infants had detectable blood levels of dextromethorphan and acetaminophen (CDC, 2007).

In 2007, an independent panel of 8 experts from different fields such as pediatrics, pediatric critical care, pediatric toxicology, clinical toxicology, forensic toxicology, forensic pathology, studied a total of 189 U.S deaths of children younger than 12 years old associated with cough and cold medications from 1950-2007 (Dart, et al., 2009). The panel assessed the causal relationship to the medications involved, estimated the dose involved, and identified factors contributing to the poisoning. They concluded that there is a relationship between the cough and cold ingredients in 118 case, of which 33 (28%) were judged definitely related, 33 (28%) were likely related, and 52 (44%) were possibly related. Several contributing factors were identified: age younger than 2 years, use of the medication for sedation, use in a daycare setting, use of 2 medications with the same ingredient, failure to use a measuring device, product misidentification, and use of a non-prescription product intended for adult use (Dart, et al., 2009).

1.4.1.1 Dextromethorphan and Its Toxicity

Dextromethorphan is the D-isomer of levorphanol, an opioid related to codeine. Its chemical name is 3-methoxy-17-methyl-9α, 13α, 14α-morphinan. Dextromethorphan was approved by the US FDA in 1958 as a nonprescription cough suppressant and it is
now marketed throughout the world. Dextromethorphan is used clinically in the form of salt, dextromethorphan hydrobromide. Its antitussive activity is related to its action on σ-opioid receptors without significant affinity for the μ and δ receptors, which are responsible for analgesic and CNS depressant effects. Dextromethorphan is metabolized in part by CYP2D6; 85% of Americans are rapid metabolizers due to genetic polymorphism. Dextrophan, an active metabolite of dextromethorphan, antagonizes the actions of excitatory amino acids on N-methyl-d-aspartate (NMDA) receptors. Antagonism of NDMA receptors by dextromethorphan and its metabolites might also be responsible for the adrenergic effects (such as hypertension, tachycardia, diaphoresis) (Boyer, 2004). Dextromethorphan also binds to serotonergic receptors, which could contribute to its abuse potential and risk for serotonin syndrome. (Chyka, et al., 2007) At currently recommended adult doses of 10 to 30 mg orally three to six times daily, dextromethorphan is a highly effective and safe antitussive agent (Bem and Peck, 1992). If dextromethorphan is administered at higher doses (35 to 45 mg) than typically prescribed for the treatment of cough, it may be useful in the management of pain in cancer patients (Siu and Drachtman, 2007).

The adverse effects of over-the-counter (OTC) agents, such as dextromethorphan, are often overlooked. Under typical circumstances, dextromethorphan is safe when used at appropriate doses, but significant morbidity can occur. Acute intoxication with dextromethorphan usually resolves within 24 hours (Manaboriboon and Chomchai, 2005). The majority of dextromethorphan’s adverse effects occur at the level of the CNS. Neurological toxicity associated with dextromethorphan includes dystonia, fatigue,
drowsiness, and dizziness. Nystagmus, slurred speech, light-headedness, and fatigue were more commonly reported at higher doses of dextromethorphan (10 mg/kg/day) and occurred within 1 to 2 hours of administration (Chyka, et al., 2007; Pediatrics, 1997; Woo, 2008). When abused in large quantities (>2 mg/kg), the drug has been associated with a dissociative effect similar to those described by ketamine and phencyclidine abusers. Massive ingestions of the drug may be associated with untoward effects, including tachycardia, hypertension, and respiratory depression (Romanelli and Smith, 2009). Dextromethorphan can also alter cerebral blood flow (Steinberg, et al., 1991). Other non-CNS-related effects, such as dermatologic and metabolic, have also been reported. Dermatologic adverse effects, such as a specific drug eruption, can occur after therapeutic doses of dextromethorphan (Kawakami, et al., 2003; Stubb and Reitamo, 1990). Uncommon side effects associated with dextromethorphan also include hyperpyrexia, hyperglycemia, and anaphylaxis (Knowles and Weber, 1998; Konrad, et al., 2000).

1.4.1.2 Acetaminophen and Its Toxicity

N-acetyl-p-aminophenol (APAP), commonly known as acetaminophen or paracetamol, is one of the most widely used analgesic and antipyretic agents. It is available as a component of many OTC cough and cold remedies, and generally is considered to be safe when used at recommended doses. The usual dosage for adults and children age 12 and over is 650-1000 mg every four to six hours as needed. No more than 4 g (4,000 mg) should be taken in 24 hours. For children ages six to 11 years, the usual dose is 150–300
mg, three to four times a day. Parents are advised to check with a physician for dosages for children less than six years old. When consumed at higher doses, acetaminophen is a major cause of liver necrosis in humans and experimental animals (Hinson, et al., 2004), and acetaminophen overdose represents one of the most common pharmaceutical product poisonings in the United States (Litovitz, et al., 2002). Acetaminophen has also been identified as a moderate risk teratogen, with exposure associated with a modest increase in risk of gastroschisis (Werler, et al., 2002), a rare but serious congenital defect of the abdominal wall.

Table 1-3. Recommended Pediatric Dosing of Acetaminophen by Weight and Age (Temple, 1983)

<table>
<thead>
<tr>
<th>Age</th>
<th>Weight (lb)</th>
<th>Dose (mg)*</th>
<th>Recommended Daily Dose (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3 months</td>
<td>6-11</td>
<td>40</td>
<td>200</td>
</tr>
<tr>
<td>4-11 months</td>
<td>12-17</td>
<td>80</td>
<td>400</td>
</tr>
<tr>
<td>12-23 months</td>
<td>18-23</td>
<td>120</td>
<td>600</td>
</tr>
<tr>
<td>2-3 years</td>
<td>24-35</td>
<td>160</td>
<td>800</td>
</tr>
<tr>
<td>4-5 years</td>
<td>36-47</td>
<td>240</td>
<td>1200</td>
</tr>
<tr>
<td>6-8 years</td>
<td>48-59</td>
<td>320</td>
<td>1600</td>
</tr>
<tr>
<td>9-10 years</td>
<td>60-71</td>
<td>400</td>
<td>2000</td>
</tr>
</tbody>
</table>
Acetaminophen can be metabolized through several ways, such as glucuronidation, sulfation, and monooxygenase hydroxylation. The toxicity of acetaminophen associate with its hydroxylation metabolite going through rearrangement which can bind to proteins and nucleic acid and eventually results in hepatotoxicity. The major pathway for acetaminophen elimination is primarily through glucuronidation and sulfation. About 45–55% of administered acetaminophen is excreted in urine as the glucuronide conjugate (Bessems and Vermeulen, 2001), a reaction catalyzed primarily by UGTs 1A6 and 1A9 (Bock, et al., 1993). Approximately 30–35% of administered acetaminophen is excreted in urine as the sulfate conjugate (Bessems and Vermeulen, 2001), and this activity has been attributed to SULT1A1 and SULT1A3/4 (Reiter and Weinshilboum, 1982). In the fetus, UGT1A6 is essentially absent, but its expression increases postnatally from the neonatal stage to adulthood (McCarver and Hines, 2002), implying that fetal detoxification of acetaminophen would be limited to sulfation. SULT1A1 and SULT1A3/4 are expressed at variable levels throughout gestation (Richard, et al., 2001; Vietri, et al., 2001), and both SULT2A1 and SULT1E1 have also been detected in fetal liver tissue samples (Miki, et al., 2002; Richard, et al., 2001; Vietri, et al., 2001). Thus, the risk of adverse consequences associated with acetaminophen exposure may be expected to be greater in fetuses with compromised SULT activity (i.e., genetic variants associated with reduced function or loss of activity). Fetal liver is capable of

<table>
<thead>
<tr>
<th>11 years</th>
<th>72-95</th>
<th>480</th>
<th>2400</th>
</tr>
</thead>
</table>

*Doses maybe repeated every 4 hours but not more than five times daily.*
bioactivating acetaminophen to the electrophilic metabolite, Nacetyl-p-benzoquinoneimine (Rollins, et al., 1979) and CYP2E1, the predominant cytochrome P450 responsible for acetaminophen bioactivation (Gonzalez, 2005), is expressed to varying degrees in fetal liver (Johnsrud, et al., 2003). Theoretically at least, reduced sulfate conjugation could allow more acetaminophen to be available for bioactivation to the reactive metabolite and thus, thereby contribute to an increased risk of toxicity.

1.5 Zebrafish as A model for Studying Drug Metabolism

1.5.1 Zebrafish’s Advantage Compared to Other Animal Models

Due to the physical and ethical issues associated with performing experiments on humans, biomedical research utilizes primarily animal models to study biologic processes conserved between humans and lower vertebrates. The most common model organisms are rats and mice. Although these models have significant advantages, they are also expensive to maintain, difficult to manipulate embryonically, and limited for large-scale genetic studies. The zebrafish, Danio rerio, has emerged as a popular vertebrate model in different areas of research such as developmental biology, genetics, pharmacology, and toxicology in recent years (Alestrom et al., 2006; Beis and Stainier, 2006; Ingham, 2009). The zebrafish has several features that make it attractive as an animal model. As an oviparous species, zebrafish have external fertilization and development, which allow for easy detection of morphological alterations and manipulation of the transparent embryos. Zebrafish has rapid growth (with all major organs formed within 2-4 days), high egg
yield, and a short generation time. Their genetics and developmental biology have been well documented. The sequenced zebrafish genome is being assembled and annotated, and many molecular techniques have been developed to study gene function in zebrafish, including the production of transgenic and mutant fish and the use of transient antisense gene-knockdown methods (Nasevicius and Ekker, 2000; Udvadia and Linney, 2003). All these features render the zebrafish embryos/larvae as a convenient platform for pharmacological assessment and screening for potential adverse effects of drugs, particularly with regard to their potential adverse effects during the developmental process (Busquet, et al., 2008; David and Pancharatna, 2009; Gurvich, et al., 2005; Hillegass, et al., 2008; Menegola, et al., 2006; Zhang, et al., 1996)

Table 1-4: Features of the Zebrafish as a Model Laboratory Animal

<table>
<thead>
<tr>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low maintenance cost</td>
<td>Lack of extensive veterinary knowledge</td>
</tr>
<tr>
<td>Low space requirement on a per animal basis</td>
<td>New gene knockouts are available but at excess cost. Morpholino antisense oligos and targeting-induced local lesion in genomes do exist though.</td>
</tr>
<tr>
<td>Short generation time (egg to mature adult in 2-3 months)</td>
<td>Evolutionary position to extrapolate date to human</td>
</tr>
<tr>
<td>Large quantities of offspring</td>
<td></td>
</tr>
</tbody>
</table>
Well characterized developmental staging series (zebrafish book)

Transparent embryos

External fertilization and development

Easier handling for experimental manipulation and microinjection

1.5.2 Zebrafish Developmental Stages

Table 1-5: Period of Early Embryonic Development of Zebrafish (Kimmel, et al., 1995)

<table>
<thead>
<tr>
<th>Period</th>
<th>Hour post-fertilization</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zygote</td>
<td>0</td>
<td>The newly fertilized eggs through the completion of the first zygotic cell cycle</td>
</tr>
<tr>
<td>Cleavage</td>
<td>¾</td>
<td>Cell cycles 2 through 7 occur rapidly and synchronously</td>
</tr>
<tr>
<td>Blastula</td>
<td>2¼</td>
<td>Rapid, metasynchronous cell cycles (8,9) give way to lengthened, asynchronous ones at the mid-blastula</td>
</tr>
</tbody>
</table>
transition; epiboly then begins

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gastrula</td>
<td>5½</td>
<td>Morphogenetic movements of involution, convergence, and extension form the epiblast, hypoblast, and embryonic axis; through the end of epiboly.</td>
</tr>
<tr>
<td>Segmentation</td>
<td>10</td>
<td>Somites, pharyngeal arch primordia and neuromeres develop; primary organogenesis; early movements; the tail appears.</td>
</tr>
<tr>
<td>Pharyngula</td>
<td>24</td>
<td>Phylotypic-stage embryo; body axis straightens from its early curvature about the yolk sac; circulation, pigmentation, and fins begin development.</td>
</tr>
<tr>
<td>Hatching</td>
<td>48</td>
<td>Completion of rapid morphogenesis of primary organ systems; cartilage development in head and pectoral fin; hatching occurs asynchronously.</td>
</tr>
<tr>
<td>Early larva</td>
<td>72</td>
<td>Swim bladder inflates; food-seeking and active avoidance behaviors</td>
</tr>
</tbody>
</table>
1.5.3 Extrapolating Data from Zebrafish Studies to Humans

Despite of the metabolic and physiologic differences between the zebrafish and humans, the responses to chemicals that can cause endocrine disruption, reproductive toxicity, behavioral defects, teratogenesis, carcinogenesis, cardiotoxicity, ototoxicity, liver toxicity and so on, appear to be largely conserved (Brion, et al., 2004; Levin, et al., 2003;
Murakami, et al., 2003; Parng, et al., 2002; Spitsbergen, et al., 2000; Veldman and Lin, 2008). For example, zebrafish embryos are sensitive toward a range of QT-prolonging
drugs inducing severe arrhythmia (Langheinrich, et al., 2003). In one study, of 23 known
drugs that cause QT prolongation in humans, 22 were found that they consistently caused
bradycardia and AV block in the zebrafish (Milan, et al., 2003). Some experiments have
also shown that many agents such as cardiovascular, anti-angiogenic and anti-cancer
drugs produce comparable responses in zebrafish embryos to those in mammalian
systems (Hsieh and Liao, 2002; Langheinrich, 2003; Navara, et al., 2001; Sheehan, et al.,
2001). Based on these data and zebrafish’s unique advantages over other animal models,
it can be concluded that the zebrafish embryos/larvae can be utilized as a convenient
platform for pharmacological assessment and screening for potential adverse effects of
drugs, particularly with regard to their potential adverse effects during the developmental
process (Busquet, et al., 2008; David and Pancharatna, 2009; Gurvich, et al., 2005;
Hillegass, et al., 2008; Menegola, et al., 2006; Zhang, et al., 1996).
Material and methods

2.1 Materials

3,3,5-Triodo-L-thyronine (L-T₃), L-thyroxine (L-T₄), 17β-estradiol, estrone, cholesterol, dehydroepiandrosterone (DHEA), L-3,4-dihydroxyphenylalanine (L-Dopa), D-Dopa, dopamine, allopregnanolone, chlorogenic acid, kaempferol, genistein, β-naphthol, catechin, caffeic acid, daidzein, gallic acid, butylated hydroxyanisole, butylated hydroxytoluene, quercetin, myricetin, n-propyl gallate, p-nitrophenol, β-naphthylamine, acetaminophen, epicatechin, epigallocatechin gallate, mestranol, minoxidil, bisphenol A, n-octylphenol, n-nonylphenol, diethylstilbestrol, 17α-ethynylestradiol, aprotinin, adenosine 5’-triphosphate (ATP), sodium dodecyl sulfate (SDS), sodium acetate, 2-morpholinoethanesulfonic acid (MES), 3-(N-morpholino)propanesulfonic acid (MOPS), N-2-hydroxypiperazine-N2-ethanesulfonic acid (HEPES), 3-[N-tris-(hydroxymethyl)methylamino]-propanesulfonic acid (TAPS), 2-(cyclohexylamino)ethanesulfonic acid (CHES), 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS), reduced glutathione, and isopropyl-d-thiogalactopyranoside (IPTG) were products of Sigma Chemical Company (St. Louis, MO). 3-Chloro-4-biphenylol and 3,3,5,5-tetrachloro-4,4-biphenyldiol, both with a minimum purity of 95%, were obtained from Ultra
Scientific (N. Kingstown, RI). TRI Reagent was from Molecular Research Center, Inc (Cincinnati, OH). Unfertilized zebrafish eggs, embryos and larvae at different developmental stages were prepared by Scientific Hatcheries (Huntington Beach, CA). Total RNAs from zebrafish embryos and larvae at different developmental stages, as well as 3-month-old adult male or female fish, were isolated using the TRI Reagent, based on manufacturer’s instructions. Taq DNA polymerase was a product of Promega Corporation (Madison, WI), and Takara Ex Taq DNA polymerase was from Fisher Scientific (Pittsburgh, PA). T4 DNA ligase and Bam HI restriction endonuclease were from New England Biolab (Ipswich, MA). Oligonucleotide primers were synthesized by MWG Biotech (Huntsville, AL). pSTBlues-1 AcceTor Vector Kit and BL21 (DE3) competent cells were purchased from Novagen (Gibbstown, NJ). Prestained protein molecular mass standard was from Life Technologies (Gaithersburg, MD). First-strand cDNA Synthesis Kit, pGEX-2TK glutathione S-Transferase (GST) gene fusion vector, GEX-5 and GEX-3 sequencing primers, and glutathione-Sepharose 4B were products of Amersham Biosciences (Piscataway, NJ). Recombinant human bifunctional ATP sulfurylase/adenosine-5phosphosulfate kinase was prepared as described previously (Yanagisawa et al., 1998). Cellulose thin-layer chromatography (TLC) plates were products of EMD Chemicals (Gibbstown, NJ). Carrier-free sodium $[^{35}S]$sulfate, Ecolume scintillation cocktail, 17-hydroxypregnenolone, 17-hydroxyprogesterone, pregnenolone, progesterone, hydrocortisone, 4-androstene-3,17-dione, and corticosterone were from MP Biomedicals (Solon, OH). Dextromethorphan and acetaminophen were purchased from Sigma Chemical Company (St. Louis, MO). All other chemicals
were obtained from Fisher Scientific (Chicago, IL), and were of the analytical grade or the highest grade commercially available.

2.2 Methods

2.2.1 Preparation of Fertilized Zebrafish Eggs.

Adult zebrafish (*Danio rerio*) were purchased from the Zebrafish International Resource Center (ZIRC) at the University of Oregon (Eugene, OR; P40 RR012546 from NIH-NCRR). The fish were kept in fish tanks containing buffered water (pH 7.2) at 28°C, and were fed daily live brine shrimp nauplii and Tetramin dried flake food (Tetra, Blacksburg, VA). The day:night cycle was maintained at 14 hours:10 hours, and spawning and fertilization was stimulated by the onset of first light. Marbles were used to cover the bottom of the spawning tank to protect newly laid eggs and facilitate their retrieval for study. Fertilized zebrafish embryos were collected from the bottom of the tank by siphoning with a disposable pipette. The eggs were placed in Petri dishes and washed thoroughly with buffered egg water (RO water containing 60 mg sea salt (Instant Ocean, Mentor, OH) per liter of water). Groups of 10 fertilized eggs were then placed in individual wells of 24-well plates and used in the following experiments.

2.2.2 Treatment of Zebrafish Embryos/larvae with Dextromethorphan.

For treatment with dextromethorphan, three sets of freshly prepared fertilized eggs were used. Each set included six groups of 10 eggs placed in individual wells of a
24-well plate. The dextromethorphan treatment for the three sets of fertilized eggs began at 24, 48, and 72 hours post fertilization (hpf), respectively. For the 24 hpf exposure set, eggs in the six wells were exposed to, respectively, 0.1 µM, 1 µM, 25 µM, 100 µM, 1 mM, and 2 mM of dextromethorphan at 24 hpf when the eggs had developed normally through blastula, gastrula and segmentation stages. For the 48 hpf and 72 hpf exposure sets, the eggs in individual wells were exposed to above-mentioned concentrations of dextromethorphan at 48 and 72 hpf, when the eggs had developed normally through the pharyngula stage and hatching period, respectively. An untreated control group of 10 fertilized eggs was examined in parallel. Observations for any adverse developmental effects due to treatment with dextromethorphan were made at 24, 30, 48 hpf and daily thereafter until 168 hpf. Mortality, hatching rates and morphological deformities of embryos/larvae were recorded and photographed. The heart rates in beats per min of larvae were recorded. Test drug solutions were renewed daily. Dead embryos/larvae were removed immediately once observed. The above-mentioned experiments were performed in triplicate.

2.2.3 Treatment of Zebrafish Embryos/larvae with Acetaminophen

For treatment with acetaminophen, three sets of freshly prepared fertilized eggs were used. Each set included six groups of 10 eggs placed in individual wells of a 24-well plate. The acetaminophen treatment for the three sets of fertilized eggs began at 1, 24, and 48 hours post fertilization (hpf), respectively. For the 1 hpf exposure set, eggs in the six wells were exposed to, respectively 0.1, 1, 1.5, 2, 2.5 and 5mM of
acetaminophen at the 1 hpf. For the 24 hpf and 48 hpf exposure sets, the eggs in individual wells were exposed to above-mentioned concentrations of acetaminophen at 24 and 48 hpf, when the eggs had developed normally through the pharyngula stage. An untreated control group of 10 fertilized eggs was examined in parallel. Observations for any adverse developmental effects due to treatment with acetaminophen were made at 4, 12, 24, 30, 48 hpf and daily thereafter until 168 hpf. Mortality, hatching rates and morphological deformities of embryos/larvae were recorded and photographed. The heart rates in beats per min of larvae were recorded. Test drug solutions were renewed daily. Dead embryos/larvae were removed immediately once observed. The above-mentioned experiments were performed in triplicate.

2.2.4 Observation of Phenotypic Changes of Developing Zebrafish Embryos/larvae.

An inverted microscope (ZEISS Axiovert 25 CFL) fitted with a Sony DSC-S75 digital/video camera was used to examine the developmental landmarks of control and zebrafish embryos/larvae treated with dextromethorphan or acetaminophen. For embryos/larvae in the 1 hpf exposure set, observations started at 1 hpf (cleavage period) and continued at 6, 12, 18, 24, 30, 48 hpf and then daily thereafter until 168 hpf. For embryos/larvae in the 24 hpf exposure set, observations started at 24 hpf (pharyngula period) and continued at 30, 48 hpf and then daily thereafter until 168 hpf. For the 48 and 72 hpf exposure sets, observations started at 48 hpf and 72 hpf, respectively, and then daily thereafter until 168 hpf. Control and treated
embryos/larvae were observed for spontaneous movements, presence of a heartbeat, head and tail differentiation, heart rate, occurrence of edema, gross malformations of the circulatory, muscular, and nervous systems, hatching rate, expression of pigmentation, blood flow, body orientation, swimming performance, and feeding success. Photographs were taken at each time point when observations were made.

2.2.5 Preparation of Recombinant Zebrafish SULT1 ST9 and SULT3 ST4

Recombinant zebrafish SULT1 ST9 (GenBank Accession # XM_001919250) and SULT3 ST4 (GenBank Accession # XM_695052) were cloned and expressed using the pGEX-2T GST gene fusion system based on the procedure previously described (Sakakibara, et al., 2002).

2.2.6 SDS-polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed using 12% (w/v) polyacrylamide gels. The protein samples were prepared by adding an equal volume of 2x SDS sample buffer (0.5M Tris-HCl (pH 6.8), 4.4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue in distilled/deionized water) and were heat-treated for 3 minutes before loading onto the SDS gel and electrophoresed at 250 V (40 mA) for 40 minutes. After electrophoresis, the protein bands were visualized by staining the gel with Coomassie blue and destaining overnight using a destaining solution (5% methanol, 7.5% acetic acid and de-ionized water) (Laemmli, 1970).

2.2.7 Screening Substrate for SULT1 ST9 and SULT3 ST4
The sulfating activity of recombinant zebrafish SULT1 ST9 and SULT3 ST4 was assayed using radioactive PAP[^35S] as the sulfate donor. The standard assay mixture, with a final volume of 20 µl, contained 50 mM MOPS buffer at pH 7.0, 14 µM PAP[^35S] (15 Ci/mmol), 1 mM DTT, and 50 µM substrate substrate. Controls with milli Q water, in place of substrate, were also prepared. The reaction was started by the addition of the enzyme, allowed to proceed for 5 min at 28°C, and terminated by heating at 100°C for 3 min. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to the analysis of[^35S] sulfated product using the previously developed TLC procedure (Liu and Lipmann, 1984), with n-butanol/isopropanol/88% formic acid/water (3:1:1:1; by volume) as the solvent system.

2.2.8 pH–dependence Study

To examine the pH-dependence of the sulfation of acetaminophen and triiodo-L-thyronine by SULT1 ST9, DHEA and mestranol by SULT3 ST4 (in GST fusion protein form), different buffers (50mM sodium acetate at 4.5, 5.0, or 5.5; MES at 5.5, 6.0, or 6.5; MOPS at 6.5, 7.0, or 7.5; HEPES at 7.0, 7.5, 8.0; TAPS at 8.0, 8.5, 9.0; CHES at 9.0, 9.5, or 10.0; and CAPS at 10.0, 10.5, 11.0, or 11.5), instead of 50 mM MOPS (pH 7.0), were used in the reactions. The assay conditions used were the same as those described above.

2.2.9 Kinetic Studies
For the kinetic studies of the sulfation of acetaminophen and triiodo-L-thyronine (substrates of SULT1ST9), mestranol and DHEA (substrates of SULT3ST4), varying concentrations of these substrate compounds and 50mM MOPS buffer at pH 7.0 were used. Acetaminophen was dissolved in DMSO to prepare solutions of 0.2, 0.25, 0.33, 0.5, 1 and 2 mM. Triiodo-L-thyronine: 0.25, 0.5, 1, 1.33, 2, 4 mM. 0.25, Mestranol: 0.33, 0.5, 1 ,2 and 4 mM. DHEA: 0.05, 0.1, 0.2, 0.25, 0.33, 0.5 mM. The reactions were incubated for 5 min at 28 °C and terminated by heating at 100°C for 3 min. The protein concentrations of SULT1ST9 and SULT3ST4 (in GST fusion protein form) used in the final reaction mixtures in the kinetic studies were 1.04 and 0.27 mg/ml, respectively.

2.2.10 Miscellaneous Methods

PAP [35S] was synthesized from ATP and carrier-free [35S] sulfate using the bifunctional human ATP sulfurylase/APS kinase and its purity determined as previously described (Yanagisawa et al., 1998; Lin and Yang, 2000). The PAP [35S] synthesized was adjusted to the required concentration and specific activity by the addition of cold PAPS. SDS-PAGE was performed on 12% polyacrylamide gels using the method of Laemmli (1970). Protein determination was based on the method of Bradford (1976) with bovine serum albumin as the standard. The radioactive TLC plates are counted by AR-2000 Imaging scanner (Bioscan, Washington, DC)
Results

3.1 Developmental Toxicity of Dextromethorphan in Zebrafish Embryos/Larvae

Three sets of zebrafish embryos/larvae were exposed to dextromethorphan at distinct time points (24 hpf, 48 hpf, and 72 hpf) during the embryonic/larval development. Thereafter, observations for morphological and functional changes attributed to the dextromethorphan exposure were made at designated time points until 168 hpf. It is to be noted that the changes described below for embryos/larvae in each of the three (24, 48, and 72 hpf) sets refer to those observed at the particular stages during the embryonic/larval development, irrespective of the time when dextromethorphan exposure started.

3.1.1 Mortality Rate and Hatching Success.

Embryonic mortality rate was 0% among embryos/larvae in the control group. In the 24 hpf exposure set, the embryos/larvae exposed to 1 mM and 2 mM of dextromethorphan showed mortality rates of 46.7% and 96.7% at 96 hpf. The embryos/larvae exposed to the same concentrations of dextromethorphan in the 48 hpf sets showed 0% and 6.7% mortality rates at the same developmental stage (96 hpf), and similar rates were found for the embryos/larvae in the 72 hpf sets. All embryos treated with 1 or 2 mM dextromethorphan in the 24 hpf exposure set died at 120 hpf. Surprisingly, the mortality
rates for 2 mM dextromethorphan-treated larvae in the 48 and 72 hpf exposure sets also increased to 100% at 120 hpf, although most of them were still alive at 96 hpf. For the larvae in 1 mM dextromethorphan-exposed groups in these two latter set, the mortality rate remained 0% at 120 hpf, but increased to 100% at 144 hpf. In the 0.1 mM dextromethorphan-treated group of the 24 hpf exposure set, there were no dead embryos/larvae until 96 hpf, and at 144 hpf the mortality rate reached 33%, while in the 48 and 72 hpf exposure sets, the mortality rate remained 0% at the 144 hpf time point. At 168 hpf, the mortality rates increased to 63.3%, 60% and 53.3%, respectively, for the 0.1 mM dextromethorphan-treated group of the 24, 48 and 72 hpf exposure set. Groups exposed to 0.1 µM, 1 µM, and 25 µM of dextromethorphan in all three sets survived until 168 hpf. The hatching rates among surviving embryos exposed to different concentrations of dextromethorphan in each of the three sets showed no significant differences.

3.1.2 Gross Morphological and Behavioral Effects.

Developmental toxicity was not observed until 48 hpf for the 24 hpf exposure set, when the hatched embryos had completed most of their morphogenesis. For the 48 and 72 hpf exposure sets, the toxicity manifested 24 hours after the dextromethorphan treatment started. The hallmark signs of dextromethorphan developmental toxicity in zebrafish larvae were missing/reduced upper jaw, bradycardia, edema in cardiac sac, lordosis, non-inflated swim bladder, missing gill, and malformed forehead. The incidence of developmental malformations was much higher for the 24 hpf exposure set than for the
48 and 72 hpf exposure sets. For the zebrafish larvae exposed to the lowest concentration (0.1 µM) of dextromethorphan, malformations were detected in the 24 hpf exposure set, but not in the 48 or 72 hpf exposure set. The onset times for the aforementioned malformations/abnormalities are listed in Table 3-1.

Table 3-1: Endpoints of Dextromethorphan Developmental Toxicity in Zebrafish Embryos

<table>
<thead>
<tr>
<th>Progress/organ</th>
<th>Structure/function</th>
<th>Response</th>
<th>Time of onset (hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>24 hpf set</td>
</tr>
<tr>
<td>Osmoregulation</td>
<td>Pericardial sac</td>
<td>Edema</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>Edema</td>
<td>72</td>
</tr>
<tr>
<td>Heart</td>
<td>Cardiac rhythm</td>
<td>Disrupted</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Heart rate</td>
<td>Reduced</td>
<td>48</td>
</tr>
<tr>
<td>Cartilage</td>
<td>Lower jaw</td>
<td>Reduced size</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>Upper jaw</td>
<td>Reduced size</td>
<td></td>
</tr>
<tr>
<td>Swim bladder</td>
<td>Inflation</td>
<td>Blocked</td>
<td>120</td>
</tr>
<tr>
<td>Tail</td>
<td>Straight</td>
<td>Bented</td>
<td>96</td>
</tr>
<tr>
<td>Whole larva</td>
<td>Growth</td>
<td>Reduced</td>
<td>48</td>
</tr>
</tbody>
</table>
3.1.3 Yolk Sac and Cardiac Edema.

The incidence of yolk sac or cardiac edema was 0% among embryos/larvae in the control group at any of the time points examined (Figure 3-1 A). In the 24 hpf exposure set, all embryos/larvae treated with 1 and 2 mM of dextromethorphan started developing edema in the cardiac sac and yolk sac at 72 hpf, which became more extensive at 96 hpf (Figure 3-1 B). Embryos/larvae treated with the same concentrations of dextromethorphan in the other two (48 and 72 hpf) sets started developing edema at 96 hpf (24 hours later than embryos/larvae in the 24 hpf exposure set). For embryos/larvae exposed to lower concentrations of dextromethorphan in all three sets, the incidences of yolk sac and cardiac edema were low.

3.1.4 Craniofacial Malformation.

Reduced mouth and jaw malformation was initially observed in dextromethorphan-exposed zebrafish larvae at 120 hpf in all three sets. These larvae had reduced upper jaw which might have prevented them from swallowing food. Craniofacial abnormalities were not detected for zebrafish larvae in the control group. The incidence of craniofacial abnormalities was prominent among zebrafish larvae exposed to 1 µM, 25 µM, and 0.1
mM dextromethorphan at 168 hpf for all three sets. Approximately 33.3%, 93.3% and 100% of the larvae exposed to, respectively, 1 µM, 25 µM, and 0.1 mM dextromethorphan were found to exhibit shorter, malformed, or undeveloped jaws and lordosis (Figure 3-1 C). For the 0.1 µM dextromethorphan-treated group of the 24 hpf exposure set, 23.3% of the developed larvae showed craniofacial malformation at 168 hpf (Figure 3-1 D), which, however, was not found for larvae in the 0.1 µM dextromethorphan-treated group in the 48 and 72 hpf exposure sets (figures not shown).

Figure 3-1  Effects of exposure to dextromethorphan in developing zebrafish. A: non-treated control group. B: zebrafish embryo treated with 2mM dextromethorphan showed serious edema in the cardiac sac in 24, 48 and 72 hpf sets at 96 hpf time point. C. A zebrafish embryo treated with 25 µM dextromethorphan showed curving body trunk, malformed upper jaw and non-inflated swim bladder sac in 24, 48 and 72 hpf sets at 96 hpf time point. D, In the 24 hpf set, zebrafish embryos treated with 0.1 µM dextromethorphan showed similar symptoms described above. However, embryos treated with same 0.1 µM dextromethorphan in 48 and 72 hpf sets have normal jaw,
mouth and swim bladder development as E shows. The symptoms started to show in 1 µM dextromethorphan groups in 48 and 72 hpf sets.

3.1.5 Blood Flow and Heart Rate.

Dextromethorphan exerted developmental toxicity on the cardiovascular system of the zebrafish larvae by causing reduced blood flow and bradycardia. Heart rates of the dextromethorphan-treated zebrafish larvae decreased with increasing concentrations of dextromethorphan. In the 24 hpf exposure set, the heart rates of the zebrafish larvae treated with 1 mM or 2 mM dextromethorphan were reduced to approximately ~70 beats/minute, compared with ~140 beats/minute for zebrafish larvae in the control group at 48 hpf time point. The bradycardia became more severe at 96 hpf (down to ~30 beats/minute) and the larvae died shortly thereafter (Figure 3-2). Similar phenomena were observed for larvae in the 48 and 72 hpf exposure sets (data not shown). Blood flow of the larvae in these two sets appeared almost stopped by 144 hpf, although the heart was still beating.
3.1.6 Feeding and Behavior.

Feeding was observed for larvae in the control group at 96 hpf, whereas all
dextromethorphan-treated larvae with malformed mouths/jaws were unable to feed
properly at this time due to malformed mouths/jaws. Lack of feeding might have
contributed to their death. All larvae in the control group developed an inflated swim
bladder at 120 hpf, whereas 50%, 33.3%, and 33.3%, respectively, of the larvae in 0.1
µM dextromethorphan-treated group in the three sets showed no or poorly inflated swim
bladder. Only 6.7%, 16.7%, and 16.7% of the larvae treated with 1 µM
dextromethorphan in the three sets developed inflated swim bladders. None of the
zebrafish larvae in the three sets treated with dextromethorphan higher than 1 µM
concentration developed an inflated swim bladder. Possibly due to the lack of inflated
swim bladder and the bent tail, the larvae tended to lie still on their sides instead of swim
actively. Larvae in the control group were alert to tapping on the bottom of the culture
plate and showed avoidance behavior instantly, while the dextromethorphan-treated ones showed slow or no responses.

3.2 Developmental Toxicity of Acetaminophen in Zebrafish Embryos/Larvae

Three sets of zebrafish embryos/larvae were exposed to acetaminophen at distinct time points (1 hpf, 24 hpf, and 48 hpf) during the embryonic/larval development. Thereafter, observations for morphological and functional changes attributed to the acetaminophen exposure were made at designated time points until 168 hpf. It is to be noted that the changes described below for embryos/larvae in each of the three (1, 24, and 48 hpf) sets refer to those observed at the particular stages during the embryonic/larval development, irrespective of the time when acetaminophen exposure started.

3.2.1 Mortality Rate and Hatching Success.

Zebrafish embryos/larvae were observed at 1, 6, 12, 18, 24, 30, 48, 72, 96, 120, 144 and 168 hpf for developmental toxic effects as produced in response to the treatment with acetaminophen. Acetaminophen showed no significant effects on the mortality and hatching rate up to the 24 hpf time point. At 48 hpf, all embryos/larvae in 5 mM acetaminophen-treated group in three sets failed to hatch, while the hatching rates in other groups varied from 16.7% to 36.7%. All surviving embryos hatched at 72 hpf. In
the 1 hpf set, only 33.3% of the developing embryos/larvae in the 5 mM acetaminophen-treated group survived and hatched at 72 hpf, but all embryos died at 96 hpf. In contrast, there were no dead embryos in the same concentration treated group in the 24 and 48 hpf sets until 96 hpf. The mortality rate in the other 5 concentration groups in the 1 hpf set remained relatively constant from 72 hpf to 144 hpf. At 144 hpf, the numbers of dead embryos rose dramatically in 24 and 48 hpf sets, and came close to those found for the 1 hpf set at 168 hpf. Cumulative mortality rates in percentage at 168 hpf are shown in Figure 3-3.

Table 3-2: Cumulative numbers of dead zebrafish embryos treated with different concentrations of acetaminophen at different time points during embryonic/larval development. Data shown are derived from three independent experiments. Total number of embryos in each test group was 30 at the beginning of the treatment.

<table>
<thead>
<tr>
<th>hpf</th>
<th>control</th>
<th>0.1 mM</th>
<th>1 mM</th>
<th>1.5 mM</th>
<th>2 mM</th>
<th>2.5 mM</th>
<th>5 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1 hpf set</td>
<td>3</td>
<td>5</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>24 hpf set</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>48 hpf set</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>48</td>
<td>1 hpf set</td>
<td>3</td>
<td>10</td>
<td>14</td>
<td>11</td>
<td>14</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>24 hpf set</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>48 hpf set</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Time (h)</td>
<td>1 hpf set</td>
<td>11</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td>12</td>
<td>20</td>
</tr>
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</tr>
<tr>
<td>72</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>96</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>0</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>3</td>
<td>18</td>
</tr>
<tr>
<td>144</td>
<td>0</td>
<td>5</td>
<td>5</td>
<td>11</td>
<td>9</td>
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<td>22</td>
<td>29</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>11</td>
<td>15</td>
<td>15</td>
<td>17</td>
<td>20</td>
<td>30</td>
</tr>
</tbody>
</table>
3.2.2 Gross Morphological and Behavioral Effects.

Acetaminophen affected the normal embryonic development, growth, behavior and survival of zebrafish larvae. With increasing concentrations of acetaminophen, there was a significant dose-dependent morphological change in the developing embryos with regard to the development of the eyes and swim bladder, and distribution of pigment. The onset time of these changes are listed in Table 3-3. There were no noticeable differences among larvae of the 24, 48 and 72 hpf sets in terms of these morphological changes. Abundant pigmentation in the epithelial cells of the retina and in the stellate melanophores present near the cephalic and yolk sac regions were observed in the control group, as were the 0.1 mM acetaminophen-treated group. In contrast, developing embryos exposed to higher concentrations of acetaminophen (1.5 - 5 mM) displayed decreased distribution of pigmentation all over the body and retina. Also, growth
retardation in was observed in 2, 2.5, and 5 mM acetaminophen-treated zebrafish embryos/larvae. The body axis of a zebrafish larva should be straightened at 72 hpf. However, those larvae treated with >2 mM acetaminophen still showed curvature about the yolk. The eyes were not fully developed either comparing to control. In the highest concentration (5 mM) treated group, larvae showed poorly developed eyes, edema in the enlarged cardiac sac, non-inflated swim bladder, and lack of pigmentation in the retina and the cephalic and yolk sac regions (Figure 3-4). The heart rates of the embryos, nevertheless, remained within the normal range.

With regard to the behavior pattern, larvae in the control group swam actively and displayed avoidant behavior to tapping at the bottom of the plate, whereas all the acetaminophen-treated larvae tended to lay still and showed a lack of response to the stimuli.
Figure 3-4: Effects of exposure to acetaminophen in developing zebrafish larvae at 96 hpf. A: Non-treated control. B: larva from 1 mM acetaminophen-treated group showing non-inflated swim bladder. C: larva treated with 2 mM acetaminophen showing edema in the cardiac sac, non-inflated swim bladder, reduced pigmentation in the cephalic and yolk sac regions. D: larva treated with 2.5 mM acetaminophen showing edema in the cardiac sac, non-inflated swim bladder, reduced pigmentation in the retina and little pigmentation in the cephalic and yolk sac regions. E: larva treated with 5 mM acetaminophen showing edema in the cardiac sac, non-inflated swim bladder, complete lack of pigmentation in the retina and the cephalic and yolk sac regions.

Table 3-3. Endpoints of Acetaminophen Developmental Toxicity in Zebrafish Embryos/Larvae

<table>
<thead>
<tr>
<th>Progress/organ</th>
<th>Structure/function</th>
<th>Response</th>
<th>Time of Onset (hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osmoregulation</td>
<td>Pericardial sac</td>
<td>Edema</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>Edema</td>
<td>48</td>
</tr>
<tr>
<td>Pigmentation</td>
<td>Retina</td>
<td>Reduced</td>
<td>48</td>
</tr>
<tr>
<td></td>
<td>Yolk sac</td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td></td>
<td>body</td>
<td>Reduced</td>
<td></td>
</tr>
<tr>
<td>Body axis</td>
<td></td>
<td>Bent</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Inflation</td>
<td>Blockd</td>
<td>120</td>
</tr>
<tr>
<td>---------------------</td>
<td>-----------</td>
<td>--------</td>
<td>-----</td>
</tr>
<tr>
<td>Whole larva</td>
<td>Growth</td>
<td>Reduced</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>Survival</td>
<td>Reduced</td>
<td>72-240</td>
</tr>
</tbody>
</table>

3.3 Substrates Specificity of SULT1 ST9 and SULT3 ST4

Since SULTs are known to be involved in the sulfation of both endogenous and xenobiotic compounds, a number of representative endogenous and xenobiotic compounds listed in Tables 3-4 were tested as possible substrates for purified SULT1 ST9 and SULT3 ST4. These compounds were tested using the standard assay and analysis procedure as described in the Methods section. SULT1 ST9 exhibited strong sulfating activity toward acetaminophen caffeic acid, chlorogenic acid and β-napthol, weaker activity toward daidzein and gallic acid, and no detectable activity toward the other compounds, including butylated hydroxy anisole, diethylstilbesterol, mestranol, β-napthylamine and 17α-OH-progesterone. Among the endogenous compounds, SULT1ST9 exhibited sulfating activity only toward 3,3',5-triiodo-L-thyronine, whereas SULT3 ST4 exhibited strong sulfating activity toward DHEA, weaker activity toward butylated hydroxy anisole, diethylstilbesterol, daidzein, corticosterone and mestranol, and no detectable sulfating activity toward the other compounds, including caffeic acid, chlorogenic acid, 17α-ethylnylestradiol, β-naphthol, β-napthylamine,17α-OH-progesterone, 4-androstene-3,17-dione, 17β-Estradiol, estrone, pregnenolone, progesterone and 3,3',5-triiodo-L-thyronine.
Table 3-4: Specific activity of zebrafish SULT1 ST9 and SULT3 ST4 toward xenobiotic and endogenous compounds. The units of specific activity are in nmol substrate sulfated/min/mg enzyme. Data represent mean ± S.D. derived from six measurements. (ND: activity not detected.)

<table>
<thead>
<tr>
<th>Xenobiotic Compounds</th>
<th>SULT1 ST9 Specific activity (nmol/min/mg)</th>
<th>SULT3 ST4 Specific activity (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>17.5±2.7</td>
<td>ND</td>
</tr>
<tr>
<td>Butylated hydroxy anisole (BHA)</td>
<td>ND</td>
<td>1.0±0.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>15.7±2.3</td>
<td>ND</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>30.7±3.2</td>
<td>ND</td>
</tr>
<tr>
<td>Daidzein</td>
<td>7.5±2.0</td>
<td>1.2±0.1</td>
</tr>
<tr>
<td>Diethylstilbesterol (DES)</td>
<td>ND</td>
<td>1.5±0.1</td>
</tr>
<tr>
<td>17α-Ethynylestradiol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>3.4±0.9</td>
<td>ND</td>
</tr>
<tr>
<td>Mestranol</td>
<td>ND</td>
<td>6.3±1.8</td>
</tr>
<tr>
<td>Compound</td>
<td>pH-dependence</td>
<td>ND</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>----------------</td>
<td>------</td>
</tr>
<tr>
<td>β-naphthol</td>
<td>27.2±5.7</td>
<td>ND</td>
</tr>
<tr>
<td>β-naphtylamine</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>17α-OH-progesterone</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Endogenous Compounds</td>
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<td>ND</td>
</tr>
<tr>
<td>4-Androstene-3,17-dione</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>ND</td>
<td>5.1±0.8</td>
</tr>
<tr>
<td>DHEA</td>
<td>ND</td>
<td>21.1±2.8</td>
</tr>
<tr>
<td>17β-Estradiol</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Estrone</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Pregnenolone</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Progesterone</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>3,3',5-triiodo-L-thyronine</td>
<td>20.8±1.9</td>
<td>ND</td>
</tr>
</tbody>
</table>

3.4 pH-dependence
pH-dependence of the sulfating activity of SULT1 ST9 was tested using acetaminophen and 3,3',5-triiodo-L-thyronine as substrates. The results from the pH-dependence experiment revealed that SULT1 ST9 with acetaminophen as substrate exhibited a broad pH optimum spanning pH 6.5 to 9.5 with maximum sulfating activity at 8.0 (Figure 3-5 A). SULT1 ST9 with 3,3',5-triiodo-L-thyronine (Figure 3-5 B) as substrate also exhibited a broad pH optimum from 6.0 to 9.0. Interestingly, there are two pH optima, one at 6.5 the other at 8.5. SULT3 ST4 with mestranol and DHEA as substrates showed a narrow pH optimum in both cases with maximum sulfating activity at pH 7.0 and 6.0, respectively (Figure 3-5 C and D).

(A)

(B)
Figure 3-5: pH-dependency of the sulfating activity of the zebrafish SULT1 ST9 with acetaminophen (A) and 3,3',5-triiodo-L-thyronine (B) as substrates. pH-dependency of the sulfating activity of zebrafish SULT3 ST4 with mestranol (C) and DHEA (D) as substrates. The enzymatic assays were carried out under standard assay conditions as described in the Methods and Material section, using different buffer systems as indicated. The data represent calculated mean ± S.D. derived from four experiments.

3.5 Kinetic Studies

The kinetic parameters of SULT1 ST9 were determined using acetaminophen and 3,3',5-triiodo-L-thyronine as substrates. The enzymatic assays were performed using varying concentrations (2.5 to 200 µM) of each substrate (Table 3-5) as described in the Methods section. The data obtained from the kinetic experiments were used to generate Lineweaver-Burk double-reciprocal plots, where 1/[S] was plotted against 1/v. The $K_m$ and $V_{max}$ values calculated are compiled in Table 3-6. Figures 3-6 and 3-7 show the Lineweaver-Burk double-reciprocal plots of the zebrafish SULT1ST9 with acetaminophen and 3,3',5-triiodo-L-thyronine as substrates respectively. Figures 3-8 and 3-9 show Lineweaver-Burk double-reciprocal plots of the zebrafish SULT3ST4 with mestranol and DHEA as substrates respectively.

Table 3-5: List of the substrates and the concentrations used to study the kinetic properties of ZF SULT1 ST9 and SULT3 ST4.
<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrates</th>
<th>Concentrations used (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SULT1 ST9</td>
<td>Acetaminophen</td>
<td>10, 12.5, 16.7, 25,100</td>
</tr>
<tr>
<td></td>
<td>3 3,3',5-triiodo-L-thyronine</td>
<td>25, 50, 66.7, 100, 200</td>
</tr>
<tr>
<td>SULT3 ST4</td>
<td>Mestranol</td>
<td>12.5, 16.7, 25, 50, 100</td>
</tr>
<tr>
<td></td>
<td>DHEA</td>
<td>2.5, 5, 10, 12.5, 16.7</td>
</tr>
</tbody>
</table>

Table 3-6: Kinetic parameters of the zebrafish ST SULT1ST9 and SULT3ST4 determined using acetaminophen, 3 3,3',5-triiodo-L-thyronine, mestranol, DHEA as substrates.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (nmol/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetaminophen</td>
<td>85.3</td>
<td>25.7</td>
</tr>
<tr>
<td>3 3,3',5-triiodo-L-thyronine</td>
<td>61.9</td>
<td>18.7</td>
</tr>
<tr>
<td>Mestranol</td>
<td>36.0</td>
<td>19.6</td>
</tr>
<tr>
<td>DHEA</td>
<td>22.4</td>
<td>64.5</td>
</tr>
</tbody>
</table>
Figure 3-6: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 with acetaminophen as the substrate. The concentrations of acetaminophen tested were 10, 12.5, 16.7, 25, 100 µM. The velocities of the reaction are expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D. of the mean derived from four measurements.
Figure 3-7: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 using 3,3',5-triiodo-L-thyronine as the substrate. The different concentrations of 3,3',5-triiodo-L-thyronine tested were 12.5, 16.7, 25, 50, 100 µM. The velocities of the reaction are expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D. of the mean derived from four measurements.

\[
y = 1.8347x + 0.051 \\
R^2 = 0.9958
\]

Figure 3-8: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 using mestranol as the substrate. The different concentrations of mestranol tested were 25, 50, 66.7, 100, 200 µM. The velocities of the reaction are expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D. of the mean derived from four measurements.
Figure 3-9: Lineweaver-Burk double-reciprocal plot of the zebrafish SULT1 ST9 using DHEA as the substrate. The different concentrations of 3-DHEA tested were 2.5, 5, 10, 12.5, 16.7 µM. The velocities of the reaction are expressed as nmol/min/mg of enzyme. Each data point shown represents mean ± S.D. of the mean derived from four measurements.
Discussion

The zebrafish is gaining popularity as a valuable model organism for biomedical research (Alestrom, et al., 2006). A particularly attractive feature of the zebrafish for pharmacological/toxicological investigations is its potential use in high throughput screening procedures. Because of their small size, high yield, and the ease of phenotypic analysis, zebrafish embryos/larvae can be utilized as a convenient platform for pharmacological assessment and large-scale drug screening. For example, in a study conducted in 2003, 100 drug compounds were tested on zebrafish embryos/larvae. A high degree of correlation between bradycardia in zebrafish and QT prolongation in humans was observed (Milan, et al., 2003).

The present investigation represents the first study of the developmental toxicity of dextromethorphan, a commonly used cough suppressant, on developing zebrafish embryos/larvae. Results indicated that the toxicity manifested at different developmental stages in a dose-dependent manner, affecting/altering growth and survival, blood flow, heart rate, formation of mouth/jaw and swim bladder, and behavior. The bradycardia and the resulting reduced rate of blood flow were likely a main cause of the eventual death of some of the dextromethorphan-treated zebrafish larvae. The malformation of mouth/jaw, which prevented the larvae from normal feeding, might have also contributed to the death of those larvae. With regard to the latter finding, previous studies indicated that the
cardiovascular problems such as the reduced blood flow to the head area may contribute to the craniofacial malformations in zebrafish larvae that had developed beyond 96 hpf (Teraoka, et al., 2002). It is noted that several recent reports have linked the use of dextromethorphan to infant deaths (Boland, et al., 2003; Chang, et al., 2009; Chyka, et al., 2007; Dart, et al., 2009). It will be important to clarify whether and how the cardiotoxicity and other adverse effects of dextromethorphan observed in developing zebrafish larvae may have relevance to dextromethorphan-administered infants or young children.

An intriguing finding is that embryos/larvae exposed to dextromethorphan at 24 hpf resulted in a more frequent and severe morphological and functional abnormalities than embryos/larvae exposed to dextromethorphan at 48 or 72 hpf. The basis for the higher sensitivity of embryos/larvae to dextromethorphan at earlier developmental stages will be an important issue for further investigation. One possibility may lie in the differential constituents of the drug-metabolizing or detoxifying enzymes that are expressed at different stages during embryonic/larval development. Several drug-metabolizing enzymes have been reported to be involved in the metabolism and detoxification of dextromethorphan. Under normal circumstances, dextromethorphan undergoes a first-pass metabolism and becomes \(O\)-demethylated to dextrophan by CYP2D6 and \(N\)-demethylated to 3-methoxymorphinan by CYP3A4. Dextrophan and 3-methoxymorphinan can be secondarily \(N\)- or \(O\)-didemethylated to produce 3-hydroxymorphinan (Jacqz-Aigrain, et al., 1993; Kerry, et al., 1994; Van, et al., 2009). These metabolites can then be subjected to Phase II sulfation or glucuronidation (Küpfer A, 1986). Whether the developing fetus and neonate are equipped with the Phase I and
Phase II enzymes involved in the metabolism and detoxification of dextromethorphan, however, remains unclear.

Acetaminophen, a common antipyretic, was also tested for developmental toxicity on zebrafish embryos. In a recent study using zebrafish embryos/larvae (David and Pancharatna, 2009), reduced pigmentation and other morphological malformations were found in zebrafish larvae exposed to 50 µg/L (0.33 µM) acetaminophen. In our study, however, no reduced pigmentation was observed until the concentration of acetaminophen reached 1 mM. It is not clear whether the reason for this considerable difference was due to the different strains of zebrafish tested or other yet-to-be-clarified factors. Despite the concentration differences, the morphological changes observed, including the reduced pigments, growth retardation and hatch delay, tail and swim bladder malformation, were in good agreement between the two studies.

Previous studies indicated that among the drug-metabolizing enzymes, the different cytochrome P450 isozymes of the Phase I oxidative stage may be expressed at different levels at various times during development, with some being expressed early on during embryogenesis and either decreasing or remaining constant later in gestation, while others are expressed only later in gestation or postnatally (Hakkola, et al., 1998; Hines, 2008; Hines, 2007; Oesterheld, 1998). There is less information about the ontogeny of the Phase II enzymes (Barker, et al., 1994; Darras, et al., 1999; Duanmu, et al., 2006; Hines, 2008; McCarver and Hines, 2002; Richard, et al., 2001). In some human studies, sulfation by the SULTs appears to be more important in fetal development, both for the homeostasis of key endogenous compounds (Barker, et al., 1994; Darras, et al., 1999;
Duanmu, et al., 2006; Richard, et al., 2001), as well as for detoxification of xenobiotics including drugs, especially since other conjugating enzyme systems, such as the uridine diphosphate (UDP)-glucuronosyltransferases, are not expressed at significant levels until the neonatal period (Richard, et al., 2001). It therefore appears to be an important issue whether the more frequent and severe morphological and functional abnormalities observed with the zebrafish embryos/larvae exposed to dextromethorphan/acetaminophen starting at the different time points might have been due to the developmental stage-dependent expression of SULT(s) that are involved in the metabolism of dextromethorphan/acetaminophen. In a separate study, we have demonstrated that two zebrafish SULTs (designated SULT3 ST1 and SULT3 ST3) indeed were capable of catalyzing the sulfation of dextrorphan, a key metabolite of dextromethorphan (Liu et al., unpublished data). Acetaminophen had been shown to be a substrate for human SULT1A1 and SULT1E1 (Adjei, et al., 2008). In this regard, it will be important to clarify whether the developmental expression of the dextrorphan/acetaminophen-sulfating SULTs may underscore the protection against the adverse effects of dextromethorphan at later stages during the development of zebrafish embryos/larvae. In my study, two novel zebrafish sulfotransferase SULT1 ST9 and SULT3 ST4 were cloned, expressed, purified and characterized. Purified SULT1 ST9 and SULT3 ST4 were characterized with respect to their enzymatic activities. Results from the substrate specificity experiments showed that SULT1 ST9 exhibited strong sulfating activity toward acetaminophen, caffeic acid, chlorogenic acid and β–naphthol. Among the endogenous compounds tested, SULT1 ST9 was active only toward 3,3,3',5-triiodo-L-thyronine. In contrast, SULT3 ST4 exhibited stronger sulfating activity toward DHEA,
weaker sulfating activity toward xenobiotic compounds such as butylated hydroxy anisole, diethylstilbesterol, daidzein, corticosterone and mestranol.

pH-dependence study revealed that with mestranol and DHEA as substrates, the zebrafish SULT3 ST4 exhibited a narrow pH optimum at pH 6.0-7.0. With acetaminophen as the substrate, the zebrafish SULT1 ST9 exhibited a broad pH optimum range spanning pH 6.5 to 9.5, and with 3,3',5-triiodo-L-thyronine as substrate, a broad pH optimum range spanning pH 6.5 and 8.5. In the previous studies conducted in our lab on three zebrafish steroid-sulfating SULTs, SULT1 ST4 showed a pH optimum of 7.0, whereas both SULT3 ST3 displayed a pH optimum at 6.0 (Liu, et al., 2005; Yasuda, et al., 2009). It is possible that the different pH optima of the three previously identified SULT2 STs and the two SULTs identified in the current study may reflect their differential substrate specificity for different steroids and their sites of action in different cell types/tissues/organs that vary in local pH environment, and possibly at different developmental stages.

Kinetic constants for the sulfation of acetaminophen and 3,3',5-triiodo-L-thyronine by SULT1 ST9 and mestranol and DHEA by SULT3 ST4 enzyme were determined. For SULT1 ST9, the Km value of acetaminophen as substrate is higher than Km value of 3,3',5-triiodo-L-thyronine, which indicated that 3,3',5-triiodo-L-thyronine has a higher affinity to SULT1 ST9 than acetaminophen. For the same reason, DHEA has a higher affinity to SULT3 ST4 than mestranol. In terms of Vmax, acetaminophen has higher Vmax value than 3,3',5-triiodo-L-thyronine and DHEA has a higher Vmax value than
mestranol. These indicated that SULT1 ST9 and SULT3 ST4 are more efficient in sulfating acetaminophen and DHEA respectively.

In summary, this thesis research addresses two important issues: the developmental toxicity of dextromethorphan/acetaminophen using the zebrafish as a model and the enzymatic characteristics of two newly identified zebrafish SULTs. In addition to elevated mortality rates, the dextromethorphan-exposed zebrafish embryos/larvae were observed to exhibit a number of morphological/functional abnormalities. The acetaminophen-exposed zebrafish embryos/larvae exhibited a number of morphological/functional abnormalities. Whether dextromethorphan and acetaminophen also exert adverse effects on developing human fetus/neonate/child and how the adverse effects may be manifested remain to be clarified.
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