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Developmental toxicity of ambroxol in zebrafish embryos/larvae: relevance of SULT-mediated sulfation of ambroxol

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Developmental Toxicity of Ambroxol in Zebrafish Embryos/Larvae: Relevance of SULT-mediated Sulfation of Ambroxol

By

Amani Al Shaban

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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An Abstract of

Developmental Toxicity of Ambroxol in Zebrafish Embryos/Larvae: Relevance of SULT-mediated Sulfation of Ambroxol

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Ambroxol is an active metabolite of bromexine that has been proven to possess a great bronchosecretolytic effect and has been used to treat infants from 0 to 6 month and children till over 12 years of age, as well as adults. My thesis research was aimed to detect potential adverse effects of ambroxol on development using zebrafish embryos/larvae as a model and to investigate the possible involvement of the zebrafish cytosolic sulfotransferases (SULTs) in the protection against the possible adverse effects. Developing eggs at 24 hpf, 48 hpf, and 72 hpf were exposed to different concentrations (1mM, 0.5 mM, 0.25 mM, 0.125 mM, and 0.05 mM) of ambroxol in triplicate and observations were made daily for eleven consecutive days. Ambroxol induced cardiac edema and bradycardia at different stages of development in a dose-dependent manner. Enzymatic assay of purified zebrafish SULTs showed significant sulfation of ambroxol by SULT2 ST1 and SULT3 ST1, 2, 3, 4, and 5. How these SULTs may be involved in protection against the adverse effects of ambroxol remains to be clarified.

Acknowledgments

I would first like to acknowledge my research supervisor for the opportunity to work on this interesting project. I would also like to thank him for his teaching, mentoring, and assistance throughout my graduate study. I would like to thanks my lab mates for their help and cooperation to accomplish this project.

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INTRODUCTION

1. Adverse Drug Reactions of Obstetric and Pediatric drugs

Although some adverse drug reactions are not very serious, others cause death, hospitalization, or serious injury of more than 2 million people in the United States each year. This issue is more serious especially during pregnancy and in the pediatric population. Some drugs have been shown to be contraindicated during pregnancy, either because they may cause developmental defects or death in extreme cases to the embryos/fetus. Teratogenicity or growth retardation and other functional defects may also manifest late in life. Any possible adverse effects on the embryos/fetus depend not only on the ability to cross the placental barrier and directly access the embryo/fetus; it could also depend on any detoxification in the embryonic/fetal liver. After birth, the safety of medication in children is a major issue. Drug development for the pediatric population especially neonates and infants is generally lacking. Most drugs are empirically administered to newborns once the efficacy has been demonstrated in adults and usefulness is suspected or demonstrated in the older pediatric population [Amanda Clarkson *et al*, 2004]. Studies regarding drug usage suggested that 90% neonates received at least 1 drug without adequate labeling to guide its use in this special population and led to serious adverse effects in this special population [George P. Giacoia, MD *et al*, 2005]. The concern about the lack of knowledge of adverse drug reactions in pediatric

population and especially the neonatal age group is well founded. For example, some studies suggest that adverse drugs reaction in pediatric population is a significant public health problem. Multiple factors may increase the susceptibility to adverse drug reactions in this population. These factors include immature drug detoxification mechanisms, multiple drug exposures, coexistence with multiple organ dysfunctions, and the use of a concentrated drug solution. One of the main factors that increase the susceptibility to adverse drug reactions in children is the immature drug detoxification mechanism which plays the major role in eliminating toxic compound and reduces their toxicity. Fetal exposure to drug is modulated to a considerable degree by the metabolic capability of the mother and the placenta during pregnancy and neonate/child liver after birth. In order to avoid certain adverse effect, efficient biotransformation of the drugs by Phase I and Phase II drug-metabolizing enzymes is an essential process. [Michael J. Blake et al, 2005].

2. Ontogeny of drug-metabolizing enzymes and the sensitivity to adverse drug reactions during development

The combination of human ontogeny and genetic constitution exerts a profound effect on both pharmacokinetics and pharmacodynamics. It is well recognized that changes in pharmacokinetics occur during human development and these changes contribute to differences in therapeutic efficacy and toxicant susceptibility of certain drugs. Although changes in drug-metabolizing enzyme (DME) expressions during development are well recognized, the knowledge needed to understand and predict therapeutic dosing and avoidance of toxicity during maturation is incomplete.

Our knowledge of the developmental expression of human Phase I drug-metabolizing enzymes is incomplete. Expression has been observed as early as organogenesis, but this observed only in few enzymes. Many of Phase I drug-metabolizing enzymes exhibit a prenatal expression changes that are regulated by mechanisms linked to birth and maturity. The different stages of ontogeny are characterized by dynamic changes in gene expression [Ronald N. Hines & Gail McCarver, 2001]. The cytochrome P450 gene superfamily is responsible for biotransformation of a large array of endogenous compound, pharmacologic agent, and environmental xenobiotics. Cytochrome P450s are responsible for the majority of Phase I reactions in human and are represented by over 50 known enzymes grouped in to families based on sequence homology. The CYP3A subfamily is the most abundant and clinically important of the CYPs enzymes in the liver and small intestine, being responsible for the metabolism of about 50% of the most commonly used drugs. The genes encoding CYP3A have been shown to include four major functional isoforms (CYP3A4, CYP3A5, CYP3A7, and CYP3A43). Total hepatic CYP3A content appears to be stable as the infant passes from fetal to neonatal life, although the expression of specific isoform will change following birth and the activity of CYP3A enzymes increases as the infant matures. The pattern of the increase appears to be clearly associated with age.

Catalytic activity of CYP3A7 has been observed in embryonic liver as early as 50-60 days gestation and expression increases throughout pregnancy, peaking at two weeks postnatal age and then decline to the lowest level. CYP3A4 is the primary hepatic CYP that is expressed in the postnatal period. It accounts for approximately 30% of the total P450 content in the liver and intestine, and it is the primary enzyme involved in

catalyzing the biotransformation of over 75 commonly used therapeutic drugs. CYP3A4 expression increases to 50% of adult values between 6 and 12 months of age with a corresponding increase in functional activity. The developmental delay in the expression of CYP3A4 has been implicated in the decreased clearance of benzodiazepine in infant up to 3 months of age [Michael J. Blake *et al*, 2005]. CYP3A5 is the primary CYP3A isoform that is expressed extrahepatically. It has been identified in fetal and adult liver with expression and functional activity showing a high degree of inter individual variability. This large variability is seen during all stages of development and no clear development pattern has been identified. CYP2E1 is highly expressed in postnatal development but less during gestation, suggesting that postnatal events are an important for enzyme expression. CYP2D6 immunoreactive protein expression is very low in fetal liver microsomes but can be identified in neonates by seven days of age.

Changes in the expression of Phase II drug-metabolizing enzyme expression levels during development, as well as the balance seen between Phase I and Phase II enzymes, can significantly alter the pharmacokinetics for a given drug or toxicant. Although our knowledge is incomplete, many of the Phase II enzymes are expressed early in development [D. Gail McCarver and Ronald N. Hines]. The Phase II reactions process conjugating xenobiotics with small molecules such as UDP-glucuronic acid, glutathione, or acetyl coenzyme A, which generally results in pharmacological inactivation or detoxification by These reactions are catalyzed by a variety of enzymes, the activities of which appear to be associated with development. The impact of ontogeny on Phase II enzymes has not been investigated to the same extent as for Phase I enzyme. Understanding their known developmental profile is important for an overall

understanding of the acquisition of metabolic competence in the neonate and the potential therapeutic implications. Glutathione S transferase, GSTA1and GSTA2 have been identified in human fetal liver tissue as early as 10 weeks gestational age with adult level not reached until the 1-2 years postnatal age while glutathione S transferase M (GSTM) is low in fetal liver and increases dramatically to adult level shortly after birth.

UDP-glucuronotransferase (UGT) is another Phase II enzyme which is responsible for glucuronidation of hundreds of endogenous and exogenous compounds. Low level of immunoreactive UGT protein is found early in gestation in liver, spleen, and kidney. After birth it increases immediately, suggesting that post natal events are essential for the expression of the gene. One of the most important groups of Phase II DMEs is the cytosolic sulfotransferases. Sulfation is catalyzed by members of cytosolic sulfotransferase (SULT) enzymes families that contain at least 11 distinct isoforms that transfer a sulfuryl moiety to a wide range of endogenous and exogenous chemicals. Sulfation generally results in a reduction in biological activity relative to the parent compound, and it is the major mechanism for protection against chemical damage during development. Additionally, the sulfation reaction plays a key role in the metabolism of endogenous compound such as steroid hormone biosynthesis, catecholamine metabolism, and thyroid hormone homeostasis. In the liver both enzyme activities and proteins levels of SULT2A1 are low at first 25 weeks of gestation and then increase to near adult levels in the neonate. The developmental expressions and activities of the major SULT enzymes responsible for the metabolism of catecholamine (SULT1A3) and thyroid hormone (SULT1A1) has been investigated. Hepatic SULT1A3 activity expressed at high level

early in the fetal and neonatal period, and then is essentially absent in adult liver [Michael J. Blake et al, 2005].

Knowledge regarding the ontogeny of DME has permitted the development of models based on their physiological and pharmacokinetic properties and improved capability to predict drug disposition in pediatric patients. Changes in pharmacokinetics parameters during development affect the therapeutic efficacy and adverse drug reaction observed in children. However, the knowledge needed for better understanding and predicting therapeutic dosing and avoidance of adverse reaction during maturation is still incomplete [Ronald N. Hines, 2008].

3. Asthma Medications

Asthma is a lung disease that makes breathing difficult for nearly 23 million Americans, including 7 million children. It causes 4000 death a year in United States. Medication such as an inhaled short acting beta-2- agonist may be used to treat acute attacks, but asthma can become persistent. Significant chronic air flow obstruction is often present despite anti-asthma therapy and can lead to more serious conditions like asthmatic bronchitis and emphysema.

In children, bronchial hyperactivity, immunological abnormalities and life threatening childhood infection can cause and/or aggravate asthmatic bronchitis. The most commonly used asthmatic bronchitis medications are Salmeterol, Fluticason, and Salbutamol. In severe cases oral inhaled steroid may be necessary to reduce the inflammation. In addition to treatment of asthmatic condition itself, a number of

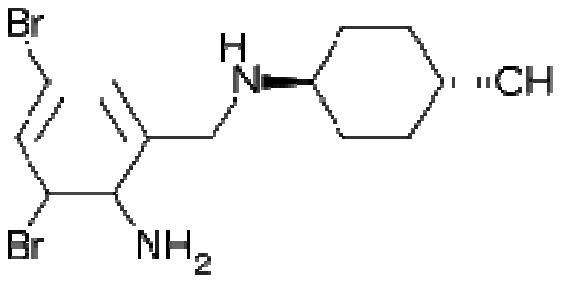
complication may arise that would require hospitalization. These include serious infection complications such as acute bronchitis, pneumonia or sinusitis. The use of other medications beside the bronchodilators and corticosteroids is necessary to prevent the exacerbation of asthma, decrease the severity, and improve the life style especially of children where the immune system is not well developed. Mucolytic drugs were proven to decrease the aggravation of asthmatic bronchitis by loosening and thinning the mucus and facilitate breathing. Many mucolytic drugs have been used such as Guanafenesin, N-acetylcystine, Dembrexine and Ambroxol. Reports of side effects from asthma drugs are poor growth, decrease bone density, glaucoma, headache, and liver test abnormalities. This lead us to wonder if we are doing more harm than good especially in early stages of life such as neonate and children. Further testing of possible side effects of these groups is required to asses any possible side effects in a dose dependent manner.

Ambroxol was proven to possess a greater bronchosecretolytic effect and has been used in different ages, from 0 to 6 month till adults and children over 12 years.

3.1 Ambroxol

Ambroxol is the active metabolite of bromexine and it has proven that this metabolite possesses a great bronchosecretolytic effect than bromhexine. It improves sputum rheology by a hydrating mechanism leading to liquefaction of the mucus and reducing dyspnea. It stimulates the production of phospholipids of surfactants by the alveolar cell, thus contributing to the lowering of superficial tension in the alveoli. The surfactant is also important to reduce the adhesion of mucus to the bronchial wall, and providing protection against bacterial aggression and irritating agents. It also reduces bronchial hyperactivity. Ambroxol produces anti-inflammatory effect by inhibiting the production of cellular cytokines and arachidonic acid metabolites. In patients with COPD, ambroxol traditionally improves airway patency. Ambroxol has also been shown to have local anesthetic properties which contribute to the soothing effect and relieving pain in acute sore throat. In addition to its anti-inflammatory effect, ambroxol is a very potent inhibitor of the neuronal Na^+ channels [Wolfram Gaida *et al*, 2005]. It also seems to have additional antioxidant properties as it protects cellular lipid from oxidative stress related to endotoxemia or inflammatory responses.

A) Chemical structure of Ambroxol



Ambroxol is used in acute and chronic diseases of upper respiratory tract concomitant with formulation of viscous and hardly separated expectoration, for therapy of asthmatic bronchitis, bronchial asthma with hard expectoration elimination, bronchiectatic disease, chronic pneumonia, and inflammatory disease of the rhinopharyngeal tract (laryngitis, pharyngitis, sinusitis, and rhinitis). It is also used in the treatment of emphysema. During acute exacerbation of bronchitis it should be given with an appropriate antibiotic.

Ambroxol is available in different formulation since the first marketing authorization in 1978. A major product is syrup with two concentrations of ambroxol available, 30 mg/ml and 15 mg/5ml, which can be given to adults and infant/children respectively. Other formulations are tablets containing 30mg or 60mg, and a pastille to be sucked with 15mg ambroxol. There is also a sustained release form with 75mg to be given just once a day to adults and children over 12 years old.

Ambroxol is also available as dry powder, inhalation solution, drops where each drop contains 6mg ambroxol and ampoules as well as effervescent tablets.

The dosage of ambroxol depends on the age and the formulation. The average daily doses of ambroxol as pediatric drops are shown in the following Tables.

Table1: Ambroxol doses in the form of pediatric drops.

Age	Dose
0 – 6 month old	0.5ml, 2 times daily
6 – 12 month old	1 ml, 2 times daily
1 – 2 year old	1.25 ml, 2 times daily

Table 2: The average daily dose of ambroxol as syrup.

Age	dose
2 -5 years old	2.5ml (½ teaspoonful) 2-3 times a day
5 – 10 years old	5 ml (1 teaspoonful) 2 -3 times a day
10 years old and adults	10 ml (2 teaspoonful), 3 times a day

Ambroxol is a metabolite of bromhexine, and its chemical described as trans-4-((2-amino-3,5dibromobenzyle) amino) cyclohexanol. It is a white to yellowish crystalline powder; slightly soluble in water and ethanol and soluble in dimethyl formamide, dimethyl sulfoxide (DMSO), and methanol, and is insoluble in chloroform and benzene.

In humans, ambroxol is metabolized to dibromoanthranilic acid (DBAA) and 6,8dibromo 3(trans-4-hydroxycyclohexyl)-1, 2, 3,4-tetrahydroquinazoline (DHTQ). The formulation of DHTQ proceeds non-enzymatically, where that of DBAA requires Nicotinamide adenine dinucleotide phosphate NADP. A study has been performed to identify the CYP isozymes involved in the formation of DBAA using human liver microsomes expressing recombinant human CYP isozyme (1A1,1A2, 2A6,2B6, 2C8, 2C9, 2C19, 2D6, 2E1, 3A4, and 4A11) showed that CYP3A4 is the only isozyme that metabolized ambroxol to DBAA [N. Ishiguro *et al*, 1999]. In the same study CYP inhibitor were examined such as furafaylline sulphaphenazole, quinidine, diethyldithiocarbamic acid and ketokinazole. Only ketoconazol inhibited the production of DBAA (>80%), these results suggest that CYP3A4 is predominantly involved in the metabolism of ambroxol to DBAA in human. Phase II drug-metabolizing enzymes involvement in the metabolism of ambroxol has not been studied in humans but the hydroxyl group in its structure suggests possible metabolism by the SULTs to prevent adverse effect and facilitate elimination. Ambroxol is well absorbed and excreted in the urine ,about 50% as gucuronide of unchanged drug, 10% as oxidized metabolized 3,5- dibromo-2-aminobenzoic acid (DBABA) and a minute amount as 6,8-dibro-3(trans-4-hydroxycyclohexyle)-1,2,3,3-tetrahydroquinazoline DHTQ.

Gastrointestinal side effects like epigastric pain may occur occasionally. Rarely allergic reaction such as skin rash, hives, and angioneurotic edema, sometimes allergic contact dermatitis and anaphylactic shock may occur. Weakness, headache, diarrhea, dry mouth and respiratory tract, exanthema, rhino-rhea, bolts and dysuria are also rarely

occurring. During prolonged application at higher doses, gastralgia, nausea and vomiting may occur.

Teratogenic and fetal toxicity studies have shown no harmful effect of ambroxol. However, it is contraindicated during pregnancy, especially during the first trimester. Safety during lactation has not been established yet. It also should not be given to a patient with hypersensitivity to ambroxol or a patient with hepatic insufficiency.

4. The cytosolic sulfotransferases (SULTs) and their possible functional roles

Biological sulfation was discovered when phenyl sulfate was isolated from the urine of a patients who had been treated with phenol as an antiseptics. Sulfation mechanism remained unknown despite the extensive studies that have been done *in vivo* until the co-substrate, adenosine 3'-phosphate 5' phosphosulfate (PAPS) was discovered. Sulfation, by definition, is the transfer of a sulfonate group (SO_3^{-2}) from PAPS to a substrate, which is catalyzed by SULT enzymes. Many endogenous and xenobiotic molecules are substrates for the SULT enzymes [Nirangali Gamage *et al,* 2005]. Different physiological processes, including, inactivation and bioactivation of xenobiotics, inactivation of hormones and catecholamines, modification of the structure and function of macromolecules, and elimination of end products of catabolism have been affected by sulfation mechanism. As mentioned above, PAPS is the co-substrate that is synthesized in the cells to create the activated form of sulfate needed for sulfation reactions.

Sulfation is an important reaction in the metabolism of numerous xenobiotics, drugs, as well as some key endogenous compounds. The addition of sulfonate moiety to a compound increases its water-solubility and decreases its biological activity in most cases. However, some of the SULT enzymes are also capable of bioactivating procarcinogenes to reactive electrophiles. Sulfation is a key reaction in the body defense against harmful chemicals and may have a major function during early development; SULTs are highly expressed in the human fetus.

The SULT gene superfamily has been categorized to several gene families based on the amino acid sequences of known vertebrate SULTs. [Werner, M.U. *et al*, 2002]. The major gene families of the SULT family are the phenol sulfotransferase (PST) family (designated SULT1) and hydroxysteroid sulfotransferase (HSST) family (designated SULT2). The PST family consists of at least four sub-families, PSTs (SULT1A), Dopa/tyrosine sulfotransferases (SULT1B), hydroxyarylamine sulfotransferases (SULT1C), and estrogen sulfotransferases (SULT1E). The HSST family comprises two subfamilies, DHEA sulfotransferases (SULT2A) and cholesterol/pregnolone sulfotransferase (SULT2B).

The SULTs are highly conserved across vertebrate species, and homologous SULT enzymes are found in different vertebrate animals due to the important roles they play in detoxification and in the homeostasis of compounds involved in the nervous and endocrine systems. Eleven SULTs have been detected and characterized in human. Eight human SULTs (SULT1A1, SULT1A2, SULT1A3, SULT 1B1, SULT1E1, SULT2A1, SULT2B1a, SULT2B1b, and SULT4A1) have been proposed to be involved either in detoxification of xenobiotics or in regulating the levels and the activities of key

endogenous substrates .However, the two SULT1C enzymes and the brain ST functions remain unclear. The functional roles proposed for the various human SULTs based primarily on their substrate specificities. Moreover, little information is available regarding the ontogeny of the SULT enzymes and the cell type, tissue, and organ-specific expression of the different SULTs. The possible coordination between the developmental expression of the SULTs that are involved in the sulfation and modulation of monoamine and catecholamine hormones, thyroid/steroid hormones and possible detoxification of xenobiotics and drugs such as ambroxol has not been investigated. To resolve these outstanding issues, we plan to perform systematic studies using zebrafish as a model.

5. Zebrafish as a model for research on developmental pharmacology

In the past decade the zebrafish (*Danio rerio*) has recently emerged as a powerful model system in developmental biology and in studies utilizing transgenic techniques. The initial interest in zebrafish as a model goes back to the early 1970s when it was selected to be the first vertebrate to study genetic screening. During the subsequent 20 years, the zebrafish model was almost exclusively used to study development. This resulted in the characterization of large number of genes involved in vertebrate pathways and the establishment of the zebrafish as a relevant model for human disease and pharmaceutical research.

There are numerous advantages for the use of the zebrafish as a model species. The main benefit of using zebrafish as development and toxicity model is the external

development with optically transparent embryos. Their optical clarity allows for easy observation of developmental stages, identification of phenotypic traits during mutagenesis screening and assessment of endpoint of toxicity during toxicity testing. The zebrafish is small in size. Adults are only approximately 1- 1.5 inch long, which allows for the maintenance of large number of animals. Therefore, only micrograms of compound are needed for screening per assay as the developing fish can live in as little as 50 µl of fluid. Moreover, one pair of adult fish is capable of laying 200-300 eggs, and if appropriately maintained, they can provide this yield every 5-7 days [Westerfield, M., 2007].

In addition to its developmental advantage, it has a great potential to serve as a model for human diseases and tremendous potential as a genetic model. The rapid maturation of zebrafish also allows for easy experimentation for transgenerational endpoint required for mutagenesis screening, establishing transgenic lines, and assessing chemicals for teratogenicity [Paul Goldsmith, 2004].

The sequencing of the zebrafish genome provides a good starting point to assess whether a target of interest is conserved in zebrafish, although it should be underscored that only about half of genes can be found readily by database crunching. This is an important limitation, because results from zebrafish must always be interpreted in terms of the similarities of any particular pathway. Protein homology and signaling pathway conservation must be considered, as well as pharmacokinetic factors that could lead to false positive or negative results.

There are many areas where zebrafish approach is particularly advantageous, e.g., analysis of environmental contaminant effects, disease model, ophthalmology, behavioral disorder, cancer, heart development and drug toxicity. The zebrafish model offers a system that can detect probable failure in the heart much earlier than it could be done with any other model [Westerfield, M. 2007]. Therefore, zebrafish embryos are particularly well suited to investigate the heart disorder and the effect of many drugs and chemicals on the heart. Human and zebrafish hearts have much in common. Both have muscles designed to pump oxygen carrying blood through valves that ensure blood flow in the single direction. And, in both cases, the heart pumps in a regular rhythmic way. The rhythmic beating of the heart in part depends on specialized heart muscle cells called myocytes that are normally highly organized part of the heart structure [Adrian J. Hill *et al*, 2005].

5.1 Stages of Embryonic development of the zebrafish

It is important to know the staging series to provide accuracy in developmental studies. This is because different embryos, even within a single clutch, develop at slightly different rates.

The first period in the development is called the zygote until the first cleavage that occurs about 40 minutes after fertilization. Following the first cleavage, after ¾ hour, about 2-7 cell cycles occur rapidly and synchronously. After 2 ¼ hours it reaches the blastula period, where the embryos enter midblastula transition.

The embryos reach glastrula period after 5 ½ hours, where morphogenic movements of involution, convergence, and extension from the epiblast, hypoblast, and embryonic axis occur. The primary organogenesis occurs after 10 hours in the segmentation periods. The extension of the tail rudiment barely begins to elongate the embryos and after 20 hours morphogenesis associated with the constriction of the yolk begins to straighten out the posterior trunk, and this, along with continued development of the tail. At the pharyngula period, 24 hours, the axis will start to straighten from early curvature with circulation, pigmentation, and finnage beginning to develop. Hatching occur after 48 hours, where the embryos will complete the rapid morphogenesis of primary organ systems like cartilage development in the head pectoral fin. By day 3, or 72 hours, the hatched larva has completed most of its organ morphogenesis, and it continues to grow rapidly. The inflation of the swim bladder and the anterior dorsal protrusion of the mouth change during the next day. Whereas during the hatching period the embryos are usually at rest, the early larva gradually begins to swim about actively, and moves its jaws and eyes. The developments produce swift escape response, the seeking of prey, and feeding. Zebrafish is DMSO tolerant and unlike invertebrates with their cuticles, it readily absorbs chemicals. Until 12-14 days post fertilization, oxygen is primarily supplied by diffusion across the skin and it seems that this is the major route for absorption of small molecules (Figure 1).

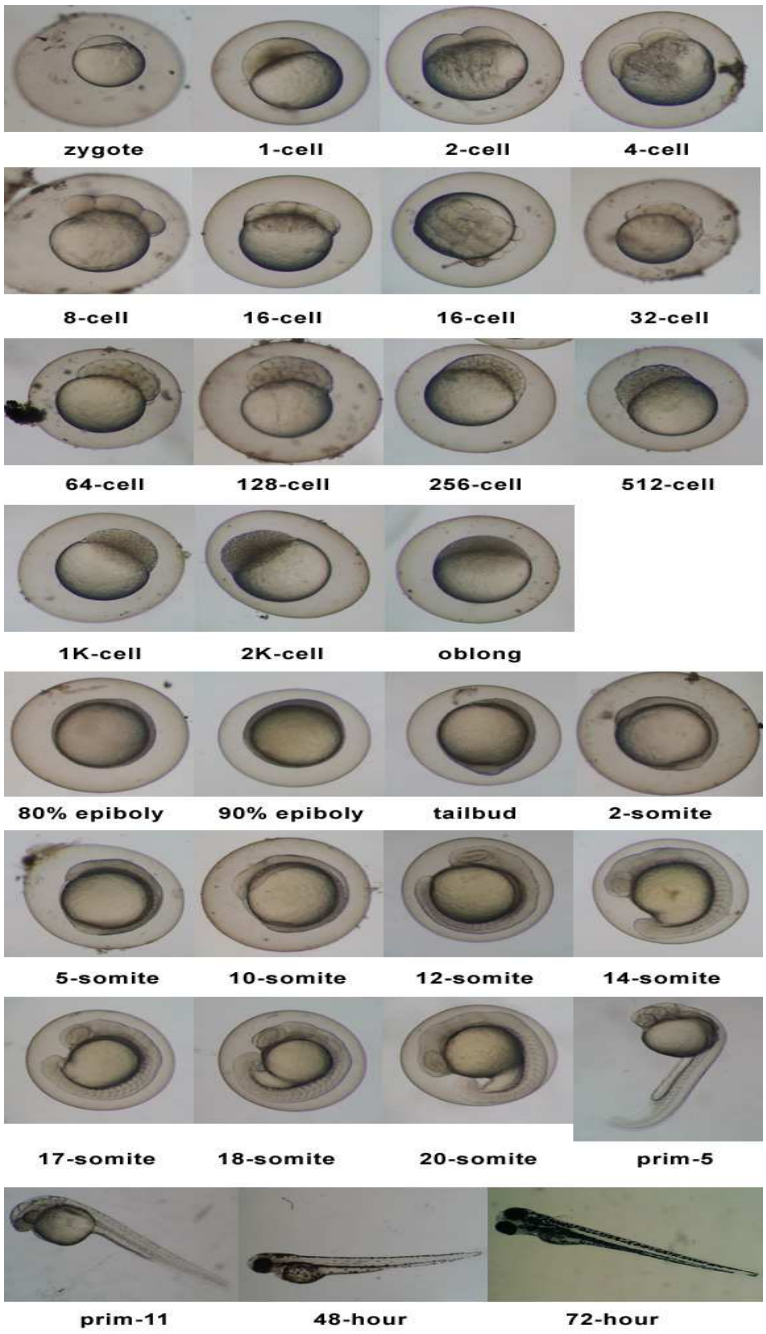


Figure1: Stages of embryonic development of the zebrafish.

5.2 Drug metabolism in zebrafish

The zebafish shows many similarities regarding the physiological function with human and it is important to identify the possible metabolic processes involved in zebrafish especially in testing chemical or drug effects on developmental toxicity studies.

CYP3A isozymes comprise the largest portion of the liver and small intestinal CYPs proteins in mammals. They are involved in the metabolism of an extensive range of endogenous substrates and xenobiotics and termination of the action of steroid hormones.

Regarding Phase I drug- metabolizing enzymes expression in zebrafish, a full-length cDNA of the CYP3A gene named CYP3A65, was cloned from zebrafish. Upon hatching of the zebrafish embryos the CYP3A65 mRNA was initially transcribed only in the liver and intestine [Hua Pin Teseng *et al*, 2004]. Moreover, a new cytochrom P450 subfamily CYP3C was also cloned from zebrafish, CYP3C1. The CYP3C1 has 44-54% identities with mammalian CYP3A and CYP 3B forms [Graham E. Corley-Smith *et al*, 2005].

Phase II drug-metabolizing enzymes are also important for drug transformation and their enzymes expressions have been studied in zebrafish. The cytosolic sulfotransferases are a family of Phase II drug-metabolizing enzymes that catalyze the transfer of a sulfonate group from 3-phosphoadenosine-5-phosphosulfate to endogenous and xenobiotic compounds. Several SULT isoforms have been identified in zebrafish. Two partial cDNA clones encoding the 5 and 3 region of putative cytosolic sulfotransferase have been identified. By analyzing the sequence data, they found that these novel zebrafish SULTs display 49, 46, and 45% amino acid sequence identity to

human SULT1A1 and they also found that SULT1 isoform 4 from the zebrafish displayed significant sulfating activity toward thyroid hormone, estrone, and dehydroepiandosterone [Ming Yih Liu *et al*, 2005]. Two of the zebrafish cDNA encoding putative cyctosolic sulfotransferase (SULTs) were also identified. The sequence analysis indicated that these two zebrafish SULTs belong to the cytosolic SULT2 gene family, designated as SULT2 ST2 and SULT2 ST3, and it showed activity toward verity of endogenous compounds like dehydroepiandosterone, corticosterone and xenobiotics [Shin Yasuda *et al*, 2006]. The unique characteristics of the zebrafish makes it an excellent model for a systematic study on the ontogeny of the enzyme expression, and physiological involvement of the SULTs, as well for studies on the adverse effects of drugs during the developmental process and the relevance of SULTs and sulfation in this process. There is relatively little information available concerning the ontogeny of SULTs expressions during development, and their physiological involvement due to the limitations in using mammalian animal models.

It is important to know the correlation between zebrafish developmental stages and stages of fetal and child development for accurate and better incorporation of the data. In human the blastocyte period which is the period between conception and embryonic stage may resembles the zebrafish blastula period, 2 ½ to 5 ½ hpf, where the blastodisc begins to look ball-like until the time of onset of gastulation. Before 24 hpf in zebrafish the tail will start to develop whereas in human, the tail has disappeared in this period which is less than 4 weeks post fertilization. The pregnancy period in the human, from fertilization to 38 weeks could be presented by the period from fertilization up to 48 hpf in zebrafish where it hatches, so at this time it's best to test the teratogenic and developmental effects

of the drugs to the embryos during pregnancy. The neonatal period in the zebrafish, 0-30 days in human, may start from 24 hpf up to 72 hpf , about up to 3 ½ days. After 72 hpf the fish is able to eat and swim, and up to 3 weeks it may represents the juvenile period in zebrafish and the pediatric period in human, 12 weeks to maturity. Drug exposure during this period could test the possible effect on pediatric population. After 3 weeks post fertilization in zebrafish may represent the adulthood in zebrafish.

OBJECTIVES AND GOALS

The cytosolic sulfotransferases (SULTs) are important in regulating the levels and activities of endogenous compound such as thyroid hormones, steroid hormones, catecholamine hormones, and cholesterol and its metabolites, and also in the detoxification of exogenous compound such as environmental toxins, and other xenobiotics including therapeutic agents [Mulder, G.J. and Jakoby, 1990; Duffel, M.W.,1997]. Based on the important role of these enzymes in the detoxification of drugs, we hypothesize that the susceptibility of developing zebrafish embryo/larva, and likewise infant/child, to any potential adverse effects of drugs may be dependent on the ontogeny and cell type/tissue/organ-specific expression of relevant drug-sulfating SULTs**.** As part of an effort to verify this hypothesis, an asthma drug, ambroxol, that is known to have a multiple mechanisms of action and been used in pediatric population, was tested on developing to zebrafish embryos/larvae. The objectives of this study were to examine possible adverse effect of ambroxol in a dose-dependent manner on developing zebrafish embryos/larvae and to analyze the ambroxol-sulfating activity of a set of zebrafish SULTs previously prepared in our laboratory. The ultimate goal is to elucidate the involvement of ambroxol-sulfating SULTs in protection against the adverse effects of this drug at different stages during zebrafish development.

MATERIALS AND METHODS

1. Study of developmental toxicity of ambroxol in zebrafish embryos/larvae

1.1 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). The fish were kept in fish tanks containing buffered water (pH 7.2) at 28ºC, and fed daily live brine shrimp naupli and Tetramin dried flake food (Tetra, Blacksburg, VA). The day: night cycle was maintained at 14 hours: 10 hours and spawning and fertilization of unexposed parent fish was stimulated by the onset of the 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 a 100 mm Petri dish and washed thoroughly with buffered 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 6-well plates and used in the experiment.

1.2 Treatment of zebrafish embryos/larvae with ambroxol

For treatment with ambroxol, three sets of freshly prepared fertilized eggs were used. Each set included six groups of 10 eggs placed in individual wells of a 6-well plate. The ambroxol treatment for the three sets of fertilized eggs began at 24, 48, and 72 hours post fertilization (hpf), respectively.

The 24 hpf set

For the 24 hpf exposure set, eggs in the six wells were exposed to, respectively, 0 mM, 0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM of ambroxol at 24 hpf when the eggs had developed normally through blastula, gastrula and segmentation stages. The experiment was performed in triplicate. Mortality and deformities of embryos/larvae, if any, were recorded at 24 hr intervals for 11 consecutive days. The solutions were changed after observation and the micro-worms were added at 72 hpf as the fish starts to eat at this stage. Morphological deformities and heart rates in embryos/larvae were closely observed using an inverted microscope (Zeiss, Axiovert25) and images were captured using Sony DSC-S75 digital/video camera. Teratogenic effects were recorded.

The 48 hpf set

For the 48 hpf exposure set, eggs were placed in the six wells were exposed to, respectively, 0 mM, 0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM of ambroxol at 48 hpf when the eggs had developed normally through blastula, gastrula, segmentation,

and pharyngula stages. The experiment was performed in triplicate. Mortality and deformities of embryos/larvae, if any, were recorded at 24 hr interval for 11 consecutive days. The solutions were changed daily after observation and the micro-worms were added at 72 hpf as the fish starts to eat at this stage. Morphological deformities and heart rates in embryos/larvae were closely observed using an inverted microscope (Zeiss, Axiovert25) and images were captured using Sony DSC-S75 digital/video camera. Teratogenic effects were recorded.

The 72 hpf set

For the 72 hpf exposure set, eggs were placed in the six wells were exposed to, respectively, 0 mM, 0.05 mM, 0.125 mM, 0.25 mM, 0.5 mM, and 1 mM of ambroxol at 48 hpf when the eggs had developed normally through blastula, gastrula, segmentation, pharyngula, and hatching stages. Testing was performed in triplicate. Mortality and deformities of larvae, if any, were recorded at 24 hr interval for 11 consecutive days. The solutions were changed daily after observation and micro-worms were added from the first day of exposure as the fish have the ability to eat at this stage. Morphological deformities and heart rate in embryos/larvae were closely observed under an inverted microscope (Zeiss, Axiovert25) and images were captured using Sony DSC-S75 digital/video camera. Teratogenic effects were recorded.

2. Sulfotransferase Assay

The sulfating activity of purified recombinant zebrafish cytosolic SULT1s (ST1, 2, 3, 4, 5, 6, 7, 8, and 9), SULT2s (ST 1, 2, and 3), SULT3s (ST 1, 2, 3, 4, and 5), and SULT 5A1 were assayed using radioactive PAP [^{35}S] as the sulfate donor. The standard assay mixture, with a final volume of 12.5 µl, containing 2.5 µl MOPS buffer at pH 7.0, 0.5 µl PAP [^{35}S] (15 Ci/mmol), 0.125 µl DTT, and 0.625µl ambroxol (1mM). DMSO or water controls were also prepared. The reaction was started by the addition of 3µl enzyme, allowed to proceed for 30 minutes at 28ºC, and terminated by heating at 100ºC for 3 minutes. The precipitates formed were cleared by centrifugation, and the supernatant was subjected to analysis of [^{35}S]-sulfated product using a previously developed TLC procedure with n-butanol/isopropanol/88%formic acid/water (3:1:1:1; by volume) as the solvent system.

RESULTS

Three sets of zebrafish embryos/larvae were exposed at different time points (24 hpf, 48 hpf, 72 hpf) during the embryonic/larval development. Observation for morphological and functional changes, heart rate, and mortality rate were made every 24 hours for 11 days. Morphological deformity and changes in heartbeat in embryos/larvae were carefully observed under an inverted microscope and images were captured. The changes described below for each of the three sets (24 hpf, 48 hpf, and 72 hpf) refer to a particular stage during the embryonic/larval development.

Gross morphological effect and cardiac edema

Cardiac edema was observed for zebrafish embryos in the 24 hpf set, while the 48 hpf and 72 hpf sets showed normal morphology at all ambroxol concentrations (1 mM,0.5 mM, 0.25 mM, 0.125 mM, and 0.05 mM) exposed groups. In the 1mM exposed group of the 24 hpf set, cardiac edema were started on the second day at 48 hpf, and lasted until the 120 hpf, and the larvae all died thereafter (Figure 2&7). For the 0.5 mM exposed group, normal morphology was observed in the first two days and cardiac edema started at 72 hpf, and lasted until 120 hpf, and the larvae all died thereafter (Figure 4&8). Both highest concentration (1 mM and 0.5 mM) showed cardiac edema in early developmental stage and the larvae failed to survive further than 120 hpf while 0.25 mM exposed group also showed cardiac edema at 72 hpf but the larvae survived till

144 hpf, (Figure 5, 9, &10). Normal morphology was observed for group exposed to the lowest concentration (0.05 Mm) and the control groups (DMSO and water) in all three (24 hpf, 48 hpf, 72 hpf) sets (Figure 3&6). No craniofacial malformations were observed among larvae in all sets throughout the experiment.

Figure 2: Zebrafish exposed to 1mM Ambroxol at 48 hour post fertilization (hpf) showed cardiac edema

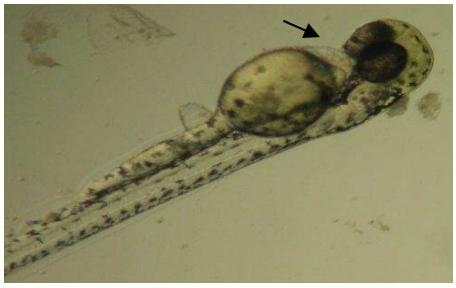


Figure 3: Control group of zebrafish larvae at 48 hpf

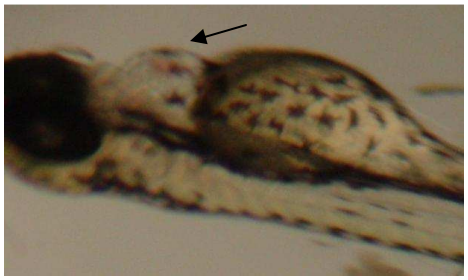


Figure 4: Zebrafish exposed to 0.5 mM Ambroxol at 72 hpf showed cardiac edema

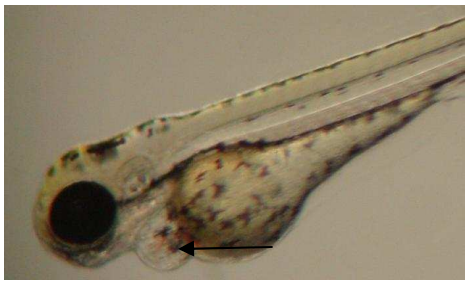


Figure 5: Zebrafish exposed to 0.25 mM Ambroxol at72 hpf showed cardiac edema

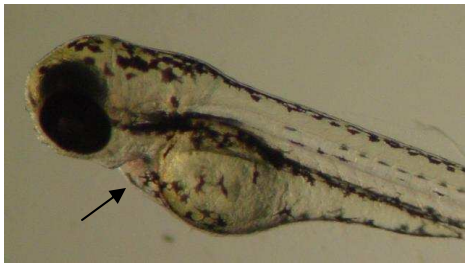


Figure 6: Control group of zebrafish larvae at 72 hpf

Figure7: Zebrafish larva exposed to 1.0 mM Ambroxol at 96 hpf showed cardiac edema

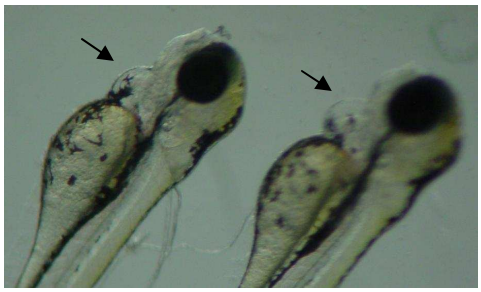


Figure 8: Zebrafish larva exposed to 0.5 mM Ambroxol at96 hpf showed cardiac edema

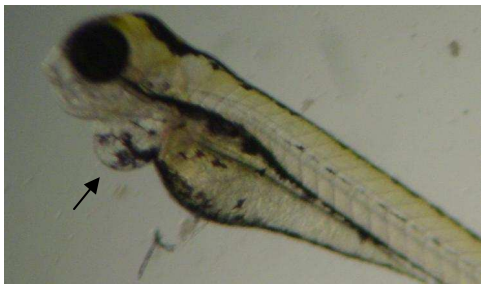


Figure 9: Zebrafish larva exposed to 0.25 mM Ambroxol at 96 hpf showed cardiac edema

Figure 10: Zebrafish larva exposed to 0.25 mM Ambroxol at 120 hpf showed cardiac edema.

Heart Rate

The heart rate was counted every 24 hours. Embryos/larvae exposed to the higher concentrations (1mM, 0.5mM, 0.25mM) of ambroxol showed significant bradycardia, at ~80 b/m, from the first day of exposure for all three (24 hpf, 48 hpf, and 72 hpf) sets. Lesser bradycardia effects, at ~115 b/m, were observed for embryos/larvae exposed to the lower concentration (0.125 mM) of ambroxol. The heart rates were found to be inversely proportional to the concentration of ambroxol to which the embryos/larvae were exposed (Figure 11, 12, &13), and the severity of bradycardia increased with time during the course of the study. The bradycardia started from the first day of exposure

until 120 hpf for all three (24 hpf, 48 hpf, and 72 hpf) sets and all larvae died thereafter. For the 0.125mM exposed group in all three sets, the bradycardia started at 120 hpf and continued to increases until 168 hpf when all larvae died. The embryos/larvae exposed to the lowest concentration (0.05mM) and those of control group (DMSO and water) showed normal heart beat throughout the experiment. The data of embryonic heart rates are presented as mean ± SD and were derived from three independent experiments (Table 3, 4, &5).

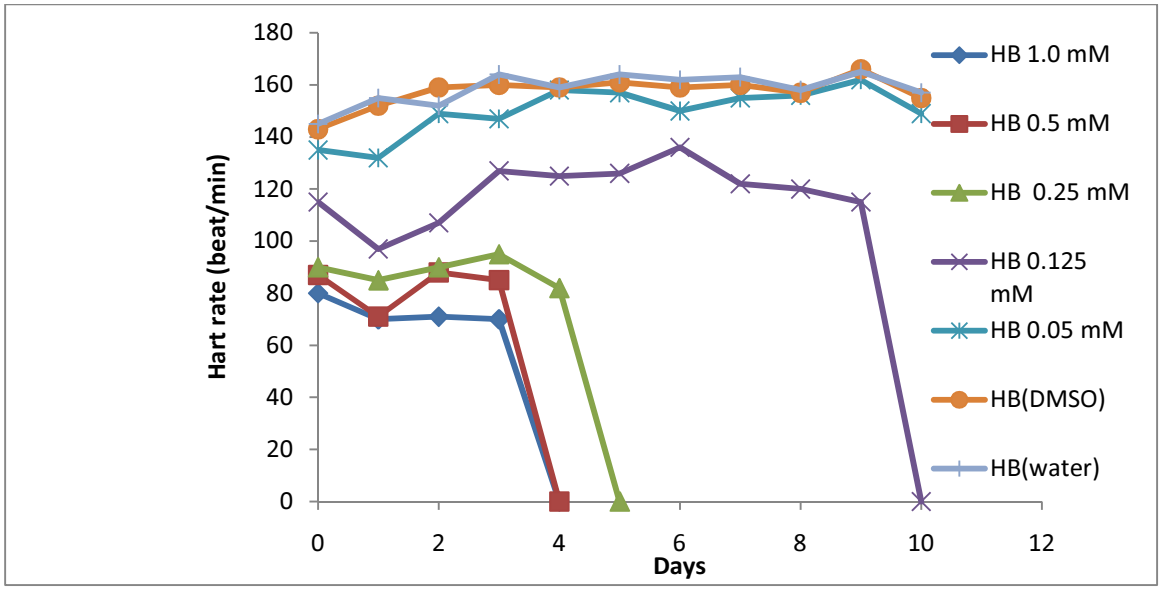


Figure 11: Heart beat chart for 24 hpf Ambroxol-exposed-zebrafis larvae showed significant bradycardia among 1 mM, 0.5 mM, and 0.25 mM Ambroxol-exposed groups

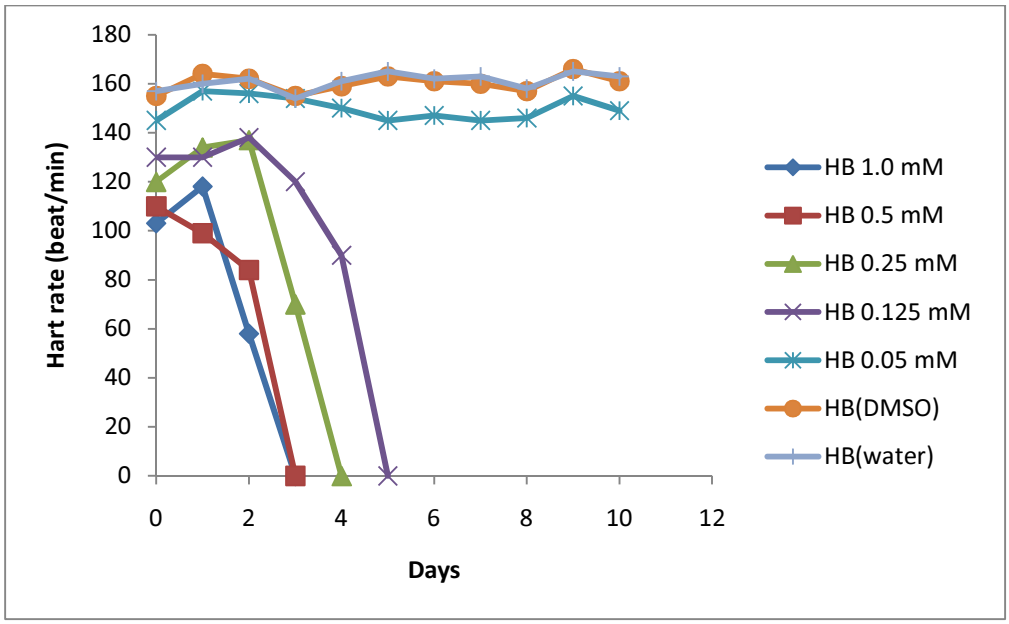


Figure 12: Heart beat chart for 48 hpf Ambroxol-exposed-zebrafish larva showed significant bradycardia among 1 mM, 0.5 mM, and 0.25 mM Ambroxol-exposed groups.

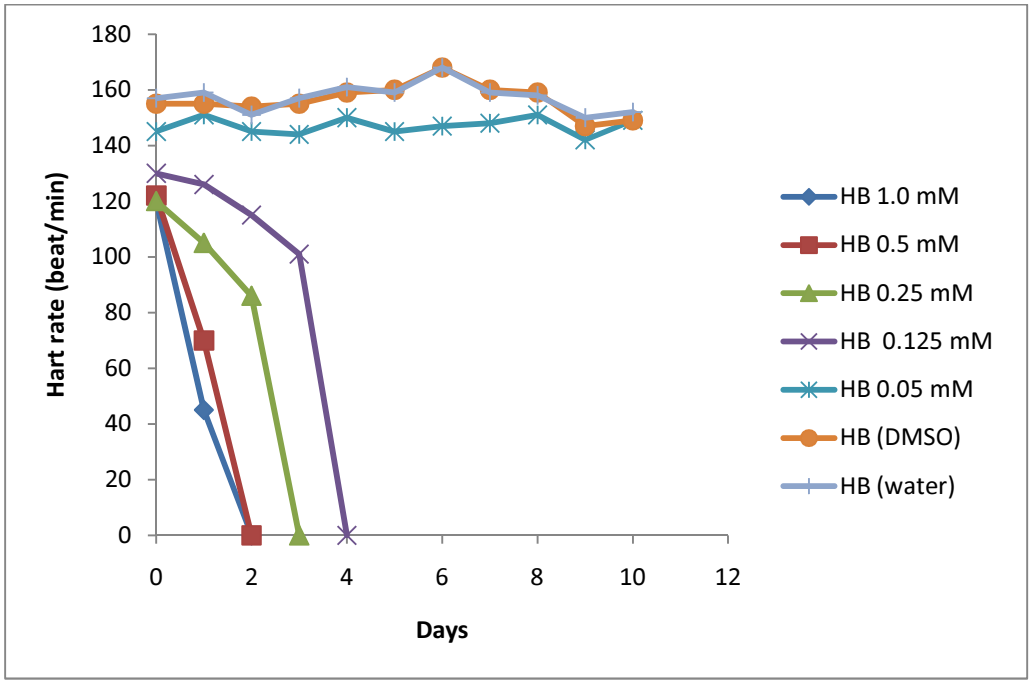


Figure 13: Heart beat chart for 72 hpf Ambroxol-exposed-zebrafish larvae showed significant bradycardia among 1 mM, 0.5 mM, 0.25 mM, and 0.125 mM Ambroxol exposed groups.

Table 3: Heart rates (beat/min) of zebrafish larvae at different developmental stages in response to different concentrations of Ambroxol. Numbers shown are the mean ± SD for the 24 hpf exposure set.

day	HB1.0 mM ±SD	HB 0.5 mM ±SD	HB 0.25 mM ±SD	HB0.125mM ±SD	HB0.05 mM ±SD	HB DMSO ±SD	HB water ±SD
1	80±0.8	87±0.7	90±0.83	115±0.7	135±1.58	143±1.9	145±0.7
2	70±1.1	71±1.1	85±0.7	97±1.4	132±1.5	152±2.2	155±1.8
3	71±0.8	88±0.7	90±1.1	107±1.1	149±1.14	159±0.7	152±2.1
4	70±1.1	85±0.7	95±0.7	127±1.1	147±0.7	160±0.7	164±0.7
5	0±0	0±0	82±1.9	125±2.1	158±2.1	159±1.14	159±1.14
6	0±0	0±0	0±0	126±2.28	157±1.1	161±1.1	164±0.7
7	0±0	0±0	0±0	136±1.94	150±1.3	159±1.5	162±1.5
8	0±0	0±0	0±0	122±1.6	155±1.2	160±1.5	163±1.6
9	0±0	0±0	0±0	120±1.6	156±3.2	157±3.3	158±3.1
10	0±0	0±0	0±0	115±0.7	162±3.2	166±1.6	165±2.1
11	0±0	0±0	0±0	0±0	149±1.58	155±0.7	157±1.14

Table 4 : Heart rates (beat/min) of zebrafish larvae at different developmental stages in response to different concentration of Ambroxol. Numbers shown are the mean ± SD for the 48 hpf exposure set

Day	HB1.0 mM ±SD	HB 0.5 mM ±SD	HB 0.25 mM ±SD	HB0.125 mM ±SD	HB 0.05 mM ±SD	HB DMSO ±SD	HB water ±SD
1	103±1.0	110±2.4	120±1.4	130±1.4	145±1.7	155±1.4	157±1.41
2	118±1.4	99±2.3	134±1.7	130±1.4	157±1.4	164±2.4	160±1.41
3	58±1.37	84±3.1	137±2.3	138±1.4	156±2.9	162±1.4	162±1.3
4	0±0	0±0	70±2.1	120±3.3	154±1.1	155±2.3	154±1.0
5	0±0	0±0	0±0	90±1.7	150±1.4	159±1.1	161±1.0
6	0±0	0±0	0±0	0±0	145±1.8	163±1.8	165±1.2
7	0±0	0±0	0±0	0±0	147±2.9	161±1.4	162±1.3
8	0±0	0±0	0±0	0±0	145±1.4	160±1.4	163±1.0
9	0±0	0±0	0±0	0±0	146±1.0	157±2.1	158±1.0
10	0±0	0±0	0±0	0±0	155±2.4	166±1.4	165±1.0
11	0±0	0±0	0±0	0±0	149±1.4	161±1.4	163±1.9

Table 5: Heart rates (beat/min) of zebrafish larvae at different developmental stages in response to different concentration of Ambroxol. Numbers shown are the mean ± SD for the 72 hpf exposure set

Day	HB 1.0 mM ±SD	HB 0.5 mM ±SD	HB 0.25 mM ±SD	HB0.125mM ±SD	HB 0.05 mM ±SD	HB DMSO ±SD	HB water ±SD
1	120±1.3	122±1.5	120±1.0	130±0.9	145±1.0	155±2.0	157±1.1
2	45±1.4	70±1.7	105±1.6	126±2.2	151±1.4	155±1.03	159±2.0
3	0±0	0±0	86±1.4	115±2.1	145±1.0	154±1.4	151±1.1
4	0±0	0±0	0±0	101±1.4	144±1.4	155±1.3	157±2.1
5	0±0	0±0	0±0	0±0	150±1.2	159±1.0	161±1.6
6	0±0	0±0	0±0	0±0	145±1.4	160±1.4	159±2.0
7	0±0	0±0	0±0	0±0	147±1.0	168±1.2	168±1.0
8	0±0	0±0	0±0	0±0	148±1.7	160±0.8	159±1.1
9	0±0	0±0	0±0	0±0	151±1.3	159±1.4	158±1.7
10	0±0	0±0	**0±0**	0±0	142±1.4	147±0.8	150±1.4
11	0±0	0±0	**0**±0	**0±0**	149±1.41	149±0.8	152±0.8

Morality Rate

For all three (24hpf, 48 hpf, and 72 hpf) sets, the larvae exposed to the highest concentrations, 1mM and 0.5mM, of ambroxol did not survive beyond 120 hpf, and the mortality rate continued to increase from the first day of exposure till it reached 100%. In contrast to the heart rates, mortality rates were proportional to the ambroxol concentration and it reached 80% at 96 hpf of the 72 hpf exposed groups, and in all three sets, the 1mM, 0.5mM, and 0.25mM ambroxol-exposed groups reached 100% at 120 hpf,

while 0.125mM ambroxol-exposed group reached 100% mortality at 144 hpf. (Figure 14, 15, & 16). The embryos/larvae exposed to the lowest concentration (0.05 mM) and those in the control group (DMSO and water) survived till the end of the experiments with no significant increase in mortality%, which was ~10%.

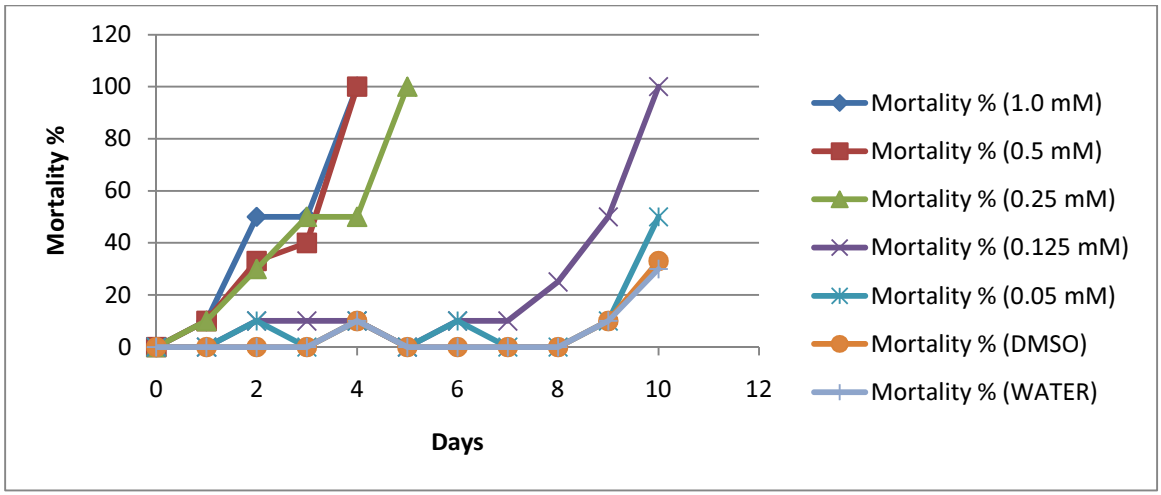


Figure 14: Mortality% chart for 24 hpf Ambroxol-exposed-zebrafish larvae showed significant increase in the mortality in 1 mM, 0.5 mM, and 0.25 mM Ambroxol-exposed groups.

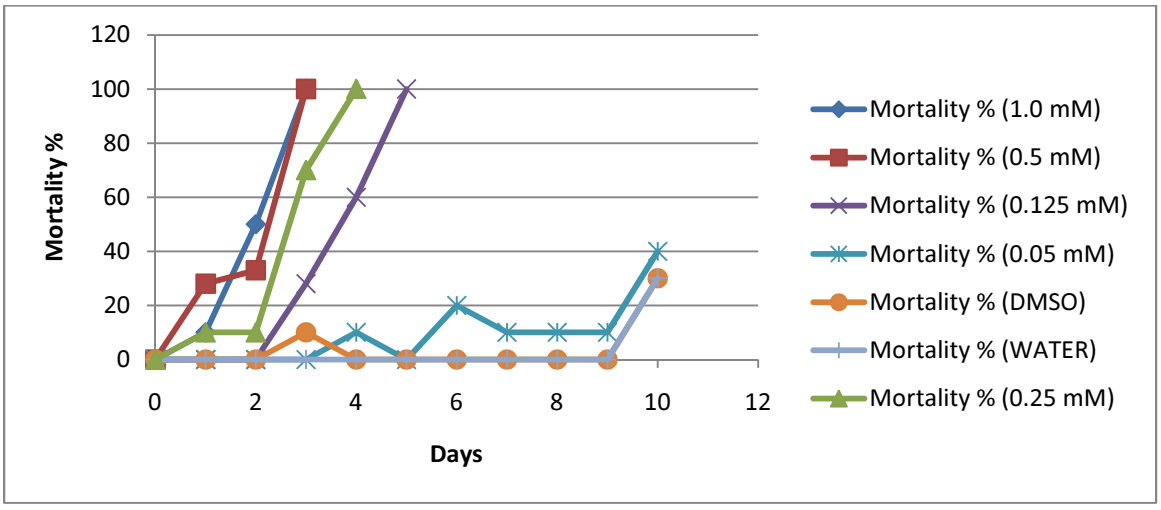


Figure 15: Mortality% chart for 48 hpf Ambroxol-exposed-zebrafish larvae showed significant increase in the mortality in 1 mM, 0.5 mM, and 0.25 mM Ambroxol-exposed groups

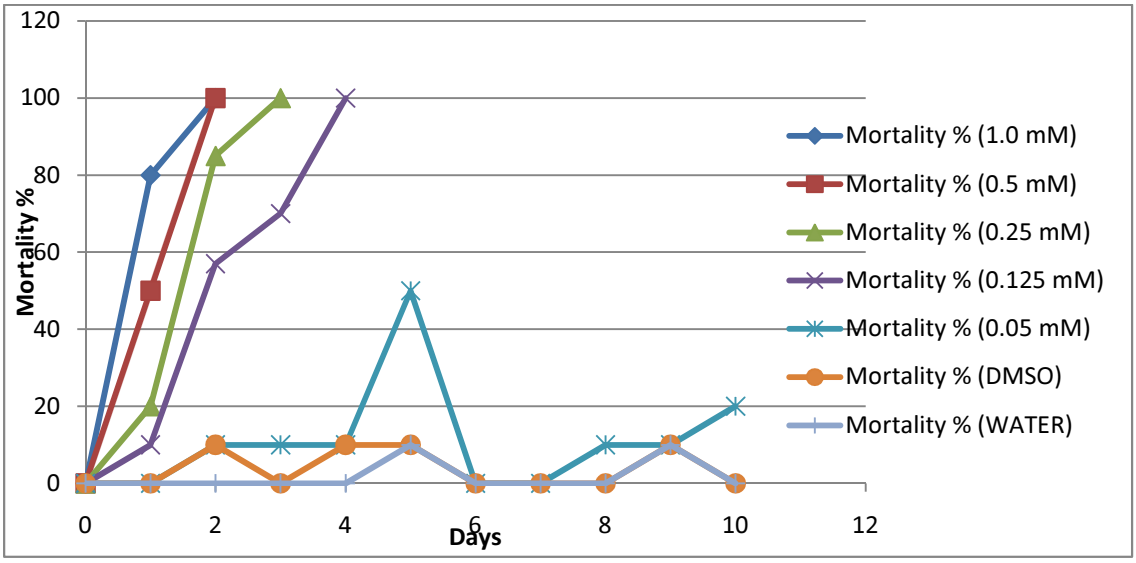


Figure 16: 72 hour post fertilization, Mortality% chart for 72 hpf Ambroxol-exposed-zebrafish larvae showed significant increase in the mortality in 1 mM, 0.5 mM, 0.25 Mm, and 0.125 mM Ambroxol-exposed groups.

Ambroxol-sulfating activity of the zebrafish SULTs

The sulfating activity of purified recombinant zebrafish cytosolic SULT, including SULT1s (ST 1, 2, 3, 4, 5, 6, 7, 8, 9), SULT2s (ST 1, 2, 3), SULT3s (ST 1, 2, 3, 4, 5), and SULT 5A1 toward ambroxol was determined using radioactive PAP[^{35}S] as the sulfate donor. The activity data revealed significant sulfating activity for SULT2 ST1, all five SULT3s (ST1, 2, 3, 4, and 5). Among the 18 zebrafish SULTs tested, SULT 3 ST 5 and 5A1 were new members of the zebrafish SULTs tested, and therefore sulfation activities toward different endogenous and xenobiotic substrates were further tested. . As shown in Table 6,SULT3 ST5 displayed significant sulfating activity toward 17α- ethynylestradiol, diethylstilbesterol, caffeic acid, butylated hydroxyanisole, acetaminophen, 17-β-estradiol, dehydroepiandrosterone, corticosterone, 4-androsterone-3,7dione, 17α-hydroxy

progetrone, β-naphthylamine, β-napthanol, mestranol, ambroxol, progesterone, prognenolone, and estrone, ;while SULT 5A1 exhibited activity toward only two compounds, dehydroepiandrosterone and progenolone.

Table 6: Specific activity of the zebrafish SULT3 ST5 and SULT 5A1 toward endogenous and xenobiotic compound as substrates. Data shown represent mean ± SD. Derived from three independent experiments. ND refers to the activity not detected.

Specific activity (nmol/min/mg)		
Drug compound	SULT3 ST5	SULT5A1
Gallic acid	ND	ND
17α-ethylenl estradiol	1.19± 0.10	ND
Diethylstilbesterol	0.55± 0.03	ND
Daidazein	ND	ND
Chlorogenic acid	ND	ND
Caffeic acid	0.09± 0.02	ND
Butylated hydroxyanisole	0.41± 0.11	ND
Acetaminophen	0.35±0.28	ND
17-β-estradiol	2.83±0.40	ND
DHEA	2.25±0.26	0.71± 0.15
Corticosterone	0.75±0.09	ND
4-androsterone-3,7dione	0.46±0.44	ND
17α-hydroxy progetrone	0.09±0.03	ND
β-naphthylamine	0.19±0.21	ND
β-naphthanol	0.09±0.03	ND
Mestranol	0.73±0.04	ND
Ambroxol	0.27±0.01	ND
Progesterone	0.52±0.24	ND
Prognenolone	0.08±0.03	0.28±0.15
Estrone	0.07±0.02	ND

DISCUSSION

Considering the important role of the SULT enzymes in the detoxification of drugs, we hypothesize that the susceptibility of a developing zebrafish embryo/larva, and likewise infant/child, to any potential adverse effects of drugs may be dependent on the ontogeny and cell type/tissue/organ-specific expression of relevant drug-sulfating SULTs**.** As part of an effort to verify this hypothesis, we tested an asthma drug, ambroxol, which is known to have a multiple mechanisms of action and has been used in pediatric population. The zebrafish was used as a model to assess the toxicity or developmental effect at different stages during embryonic and larval development.

Ambroxol is a mucolytic agent used in the treatment of respiratory disorders associated with viscid or excessive mucus such as asthmatic bronchitis and chronic pneumonia. Ambroxol has been used in infants of different age groups, ranging from 0-6 month, and children over 12 years, as well as adults. Ambroxol has many mechanisms of actions which makes it a useful anti-inflammatory and mucolytic agent, but at the same time increase the possibility of exerting sever adverse effects especially for infants and neonates. In addition to its anti-inflammatory effect, ambroxol is a potent inhibitor of the neuronal Na+ channels, which may cause adverse effects to neonates and infants. The recommended dosage of ambroxol depends on the age and the formulation.

In a normal human body, ambroxol is metabolized to dibromoanthranilic acid (DBAA) and 6,8- dibromo3(trans-4-hydroxycyclohexyl)-1,2,3,4-tetrahydroquinazoline (DHTQ). CYP3A4 is the only enzyme capable of metabolizing ambroxol to DBAA in the Phase I metabolism [N. Ishiguro *et al*, 2000]. With regard to the Phase II metabolism, our lab has recently demonstrated significant sulfating activities of SULT2 ST1, and SULT3 ST1, 2, 3, 4, and 5 toward ambroxol [Liu et al., unpublished data] which may aid in the inactivation and the excretion of ambroxol, thereby alleviating possible adverse effects. The possibility of potential adverse effects of ambroxol, however, still remains an open question, which may depend on the dose used, the developmental stage, and the ontogeny of enzymes involved in the metabolism of ambroxol. In this study we focus on exposing ambroxol to different age groups of zebrafish embryos/larvae by taking into consideration the expression of the SULT enzymes during zebrafish developmental stages and the doses for each age group.

Exposure of the first set of zebrafish embryos to ambroxol started at 24 hpf, when at this developmental stage the heart begins to beat, and the circulation and pigmentation starts to develop. For the second and third set of zebrafish embryos/larvae, the exposure to ambroxol started at 48 hpf and 72 hpf, respectively. Previous studies have demonstrated that CYP3A4 is the enzyme responsible for metabolizing ambroxol in Phase I metabolism reaction [N. Ishiguro *et al*, 1999]. A full-length cDNA encoding zebrafish CYP3A had been detected as early as 24 hpf and the expression level was found to increase upon hatching and thereafter [Ronald N. Hines & Gail McCarver, 2001]. CYP3A was detected in gill and heart and, at lower levels, in brain and eye [Hua-Pin Tseng, 2004]. Phase II drug-metabolizing enzymes involvement in the metabolism of

ambroxol have not been studied in humans but the hydroxyl group in its structure suggests possible metabolism by the SULTs. Our laboratory had previously cloned and characterized fifteen distinct zebrafish SULTs .Of these zebrafish SULTs, eight belong to the SULT1 family, three are categorized into the SULT2 gene family, three fall in the SULT3 gene family, and one appears to belong to a novel SULT gene family (SULTX) [Shin Yasuda *et al*,2009]. In my study, I carried out enzymatic assays to examine the sulfating activity of these zebrafish SULTs with ambroxol as substrate. Activity data showed significant sulfating activity of SULT2 ST1, SULT3 ST1, ST 2, ST3, ST4, and ST5 toward ambroxol. The expressions of these enzymes during zebrafish development had been studied previously [Shin Yasuda *et al*, 2006]. The mRNA encoding SULT 3 ST3 was detected in unfertilized eggs. Upon fertilization, however, no SULT3 ST3 mRNA was detected until cleavage period (1hpf) which then continues at hatching period (48 hpf) and remained high in the larval stage until maturity [Shin Yausda *et al*, 2009].

Based on my data and those reported previously [Shin Yausda *et al*, 2009; N. Ishiguro *et al*, 1999], it appears that Phase I CYP3A4, and the Phase II SULT2 ST1, and SULT 3 ST 1, 2, and 3 may be involved in the metabolism and therefore the protection against possible adverse effects of ambroxol. Such protection effect may correlate with their expression during embryonic and larval development. The developmental expression of other SULT3 enzymes, SULT3 ST4 and 5, that are capable of sulfating ambroxol, however, has not yet been studied.

Observations of the ambroxol-exposed zebrafish embryos/larvae indicated that the major toxicity is on the heart, which is in line with previous finding that ambroxol is a potent inhibitor of the neuronal Na^+ channels [Wolfram Gaida *et al*, 2005]. The results

presented in the Results section showed significant bradycardia in 1mM ambroxol-exposed groups from the first day of exposure in all three (24 hpf, 48 hpf, and 72 hpf) sets. The 24 hpf set showed cardiac edema along with the bradycardia, and according to the heart development in zebrafish, the heart begins to beat at that stage.

For all three (24hpf, 48 hpf, and 72 hpf) sets, the larvae exposed to the highest concentrations, 1mM and 0.5mM, of ambroxol did not survive beyond 120 hpf, and the mortality rate continued to increase from the first day of exposure till it reached 100%. Mortality rates were proportional to the ambroxol concentration and it reached 80% at 96 hpf of the 72 hpf exposed groups, and in all three sets, the 1 mM, 0.5 mM, and 0.25 mM ambroxol-exposed groups reached 100% at 120 hpf, while 0.125 mM ambroxol-exposed group reached 100% mortality at 144 hpf.

The expressions of the SULT 2 ST1 and SULT3 ST1, 2, and 3 that are likely involved in the metabolism of ambroxol had been studied previously during zebrafish development [Shin Yasuda *et al*, 2006], and were shown to be present during the exposure periods adopted in this study. This suggested that the protective effect of those enzymes against adverse effect like bradycardia and cardiac edema may not be sufficient. However, the developmental expression of other SULT3 enzymes, SULT3 ST4 and 5, that are capable of sulfating ambroxol, has not yet been studied. Additionally, a couple of new SULTs have been cloned and have not yet been characterized with regard to their involvement in the metabolism of ambroxol and their expressions during development of the zebrafish.

More studies will be needed in order to extrapolate these results from zebrafish to humans. Nevertheless, the dose commonly used for infants aged 0 to 6 month, 0.5 ml/2 times per day equals approximately 0.72 mM (by taking in consideration the average total body weight and the volume of distribution at this stage) is within the concentration range of ambroxol tested in this study. Any protection effect of SULTs against ambroxol in infants/young children will depend on the expression of ambroxol-metabolizing enzymes during the developmental stage. In the liver both enzyme activity and protein level of the human SULT2A1, which may be equivalent to the zebrafish SULT2 ST1, has been shown to be low at 25 weeks gestation and then increase, particularly in the last half of gestation, to near adult level in the neonate [Michael J. Blake *et al*, 2005]. More studies are warranted in order to understand the protection effects of particular SULTs that may be involved in protection against the adverse effects of ambroxol in infants and children.

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