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Ontogeny of *in vitro* Cytochrome P450-mediated drug metabolism in zebrafish

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Ontogenie van *in vitro* Cytochroom P450-gemedieerd geneesmiddelenmetabolisme in de zebravis

Proefschrift voorgelegd tot het behalen van de graad van doctor in de farmaceutische wetenschappen aan de Universiteit Antwerpen, te verdedigen door

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Abbreviations

1-OHMDZ	1-hydroxymidazolam
3MM	3-methoxymorphinan
4-OHDIC	4′-hydroxydiclofenac
4-OHMDZ	4-hydroxymidazolam
5-OHDIC	5-hydroxydiclofenac
6ß-OHTST	6ß-hydroxytestosterone
ABC	ATP-binding cassette
AFB1	Aflatoxin B1
AHR	Aryl hydrocarbon receptor
ANF	A-naphthoflavone
ARNT	Aryl hydrocarbon receptor nuclear translocator
AUC	Area under curve
BaP	Benzo[a]pyrene
BCA	Pierce bicinchoninic acid
BCRP	Breast cancer resistance protein
BFC	7-benzyloxy-4-(trifluoromethyl) coumarin
BPA	Bisphenol A
BR	7-benzyloxyresorufin
BSEP	Bile salt export pump
CAR	Constitutive androstane receptor
CLZ	Clonazepam
CV	Coefficient of variation
CYP	Cytochrome P450
DBF	Dibenzylfluorescein

ddMS2	Data dependent fragmentation
DIC	Diclofenac
DMSO	Dimethyl sulfoxide
dpf	Days post fertilization
DRR	Dextrorphan
DXM	Dextromethorphan
E2	17β-estradiol
EDTA	Ethylenediaminetetraacetic acid
EM	Embryonic microsomes
ER	7-ethoxyresurofin
EROD	7-ethoxyresorufin-O-deethylase
ESC	Embryonic stem cell test
ESI	Electrospray ionization probe
FAD	Flavin adenine dinucleotide
FET	Fish embryo acute toxicity test
FLM	Zebrafish females liver microsomes
FLM	L-fluoranthene
FMN	Flavin mononucleotide
FMO	Flavin-containing monooxygenases
FWHM	Full width at half maximum
GRCz	Genome reference consortium
GSTs	Glutathione S-transferases
HCD	Higher-energy collisional dissociation
HESI	Heated electrospray ionization
HLM	Human liver microsomes

hpf	Hour post fertilization
HTS	High-throughput screening
IS	Internal standard
Km	Substrate concentration at Vmax
LOD	limit of detection
LLOD	lower limit of detection
LOB	Limits of blank
m/z	Mass/charge
MAO	Monoamine oxidases
MATE	Multidrug and toxin extrusion
MDZ	Midazolam
mEST	Mouse embryonic stem cell test
MLM	Zebrafish males liver microsomes
MR	7-methoxyresorufin
MRM	Multiple reaction monitoring
NADPH	Nicotinamide adenine dinucleotide phosphate
NP	Nonylphenol
OATP	Organic anion transporting polypeptide
OCT	Organic cation transporter
OECDTG	Organization for Economic Co-operation and Development test guideline
PAH	Polycyclic aromatic hydrocarbons
PB	Phenobarbital
PCB	Polychlorinated biphenyls
PCB126	4′, 5-pentachlorobiphenyl
PCN	Pregnenolone 16α-carbonitrile

P-gp	P-glycoprotein
pHAHs	Planar halogenated aromatic hydrocarbons
PHE	Phenacetin
PHEN	1,10-phenanthroline
PMSF	Phenylmethanesulfonyl fluoride
PN	Pregnenolone
POB	Piperonyl butoxide
PR	7-pentoxyresorufin
PXR	Pregnane X-receptor
RS	Resorufin
rWEC	Rat whole embryo culture
S	Supersomes
SD	Standard deviations
SLC	Solute carrier
SULTs	Sulfotransferases
SULTs	Sulfotransferases
TCDD	2,3,7,8-tetrachlorodibenzop-dioxin
TCPOBOP	1,4-bis [2-(3,5-dichloropyridyloxy)] benzene
TLB	Tolbutamide
TSD	Teleost-specific genome duplication
TST	Testosterone
UGTs	UDP-glucuronosyltransferases
UPLC-amMS	Ultra-performance liquid chromatography – accurate mass mass spectrometry
UV	Ultraviolet
Vmax	Vmax

WEC	Whole embryo culture
ZLM	Adult zebrafish
zMAO	Zebrafish monoamine oxidases

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General Introduction

Adapted from:

Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Van Cruchten, S., 2016. J Toxicol Sci 41, 1-11.

1.1 Zebrafish (Danio rerio) within toxicology

The zebrafish (Danio rerio) is a member of the Cyprinidae family (freshwater fishes) which belongs to the Ostariophysian superorder, Cypriniformes order, Teleostei infraclass and Actinopterygii class (ray-finned fishes). Zebrafish are omnivorous fish characterized by their small size and five blue horizontal stripes. Females have a more rounded body with a tiny genital papilla next to the anal fin (Spence et al., 2008). Interestingly, teleost fish have a unique genetic feature, which is called teleost-specific genome duplication (TSD) that results from the duplication of the whole genome in the ancestor of this group (Meyer and Schartl, 1999). As a result, zebrafish possess the highest number of protein-coding genes among the studied vertebrates (Collins et al., 2012). Consequently, the zebrafish genome contains more species-specific genes than humans and orthology between zebrafish and humans exceeds 70 %, with 47% of one-to-one relationship which makes the zebrafish an attractive translational model for humans, also within toxicology (Howe et al., 2013).

Many other characteristics make the zebrafish a promising animal model for toxicology. It is easier and cheaper to maintain compared to mammalian species and requires less amount of test compound because of its small size, which is particularly important during early drug development. Moreover, environmental and social conditions of zebrafish are easily reproduced in the laboratory, which minimizes the effect of stress on the experimental outcome (Balcombe et al., 2004). Additionally, zebrafish have a continuous breeding cycle and reach their maturity between 10 and 12 months (Detrich et al., 1999). External fertilization and development and transparency of zebrafish embryos allows for longitudinal morphological evaluation of the different organ systems during embryogenesis. Taken all these aspects together, including tolerance of up to 1% dimethyl sulfoxide (DMSO) (the solvent of choice for the majority of chemical compounds) make zebrafish an optimal model for high-throughput

screening (HTS) of chemicals and drugs in development (Maes et al., 2012). This is illustrated by the recently accepted fish embryo acute toxicity test (FET) (OECD TG no. 236 on July 26, 2013) as an alternative for the fish acute test (OECD TG 203 and OECD 1992) (OECD, 2013). Also within developmental toxicity, alternative models are being used, such as the mouse embryonic stem cell test (mEST) (Wobus and Loser, 2011) and the rat whole embryo culture (rWEC) test (Webster et al., 1997). These methods have been developed during the last years for ethical reasons in view of 3R principles, but also for economic reasons as *in vivo* assays are expensive and time consuming. Indeed, despite the fact that WEC still requires the sacrifice of pregnant dams, it reduces the number of animals used in *in vivo* rodent embryo-fetal development studies up to 60 – 70 %. Moreover, WEC offers the complexity of the whole organism in parallel with the advantages of *in vitro* assay such as flexibility in controlling the experimental environment (substrate concentration, exposure time, etc.) with an achievable assessment of effect of both parent compound and its metabolites on the cellular level (Augustine-Rauch et al., 2010).

Also the mEST has several advantages. The immortal differentiation of embryonic stem cells into ectoderm, endoderm and mesoderm has enabled their use in teratogenicity assays. A set of endpoints were chosen for EST including visual methods such as the differentiation into beating cardiomyocytes or molecular methods such as RTPCR, microarray and disruption of developmental molecular pathways (Hammoud et al., 2016; Seiler et al., 2004; van Dartel et al., 2009; zur Nieden et al., 2004). However, both EST and WEC can only detect the teratogenic potency of chemical compounds during a very limited developmental window, i.e. early organogenesis (Augustine-Rauch et al., 2010). In contrast, the zebrafish embryo assay covers the entire organogenesis from one cell stage until full organism stage at 5 dpf (days post-fertilization). Additionally, it has the physiological complexity of a vertebrate whole animal combined with the advantages of an *in vitro* system (Ali et al., 2011), and represents a similar

development to humans and other higher vertebrates (Yang et al., 2009). However, the zebrafish embryo assay has also some issues, such as the solubility and uptake of some compounds in the test solution together with the lack of a maternal component in comparison to the *in vivo* developmental toxicity studies. As such, the embryos need to rely on their intrinsic biotransformation capacity for the elimination of xenobiotics and whether these systems are already functional at that stage, still remains a point of debate and is the subject of this PhD research.

1.2 Xenobiotic biotransformation

Xenobiotic biotransformation is achieved by several phases. The main goal of the different metabolic reactions, i.e. mainly monoxidation in phase I and conjugation reactions in phase II, is increase of hydrophilicity of xenobiotics, which in turn facilitates the excretion procedures. However, in some cases these hydrophilic metabolites are more active or toxic than the parent compound (Baranczewski et al., 2006).

Phase I

Drug metabolism phase I is mainly performed by Cytochrome P450 enzymes (CYPs) but also other enzymes can be involved, such as flavin-containing monooxygenases (FMO), monoamine oxidases (MAO), alcohol dehydrogenases or aldehyde dehydrogenases.

CYPs are located on the surface of endoplasmic reticulum and the latter can be separated from the other cell organelles by several centrifugation and ultracentrifugation steps (Nilsen et al., 1998), creating vesicles of endoplasmic reticulum, the so-called microsomes. Originally, CYPs were found in rat liver microsomes as the most abundant hemoproteins. In mammals, CYPs were also found in other organs such as kidneys, intestine and brain. CYPs are in fact present in all eukaryotic organisms (such as animals, plants, and fungi) and even in some prokaryotes. They are divided into families (CYP1, 2, 3, etc.) when they exhibit an amino acid identity of

more than 40% and into subfamilies (CYP1A, 1B, 1C, etc.) when more than 55% (Brown et al., 2008).

CYPs are constructed of 400-500 amino acids. The heme molecule is attached to the protein by a cysteine residue close to the carboxyl terminus (Schenkman and Jansson, 2006). They require nicotinamide adenine dinucleotide phosphate (NADPH) CYP reductase which contains two components, namely flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), for the heme reduction and as such activity of the CYP enzymes (Isin and Guengerich, 2008).

The catalytic cycle of CYPs starts by a binding reaction between a substrate and the heme group of the enzyme leading to a displacement of the water molecule in the 6^{th} ligand resulting in a substrate-CYP. This is followed by the transfer of electrons from NADPH reductase to the heme group converting it from a ferric to ferrous state. The next step is the binding of molecular oxygen to the complex substrate-ferrous CYP followed by a transfer of an electron to the molecular oxygen resulting in a superoxide intermediate. The second electron may come from NADPH reductase to produce H_2O_2 and substrate-ferric CYP. Several steps are followed including the formation of a substrate radical, bounding of hydroxyl radical, hydroxylated substrate and regeneration of ferric CYP (figure 1). The complexity of this reaction makes it difficult to estimate the limiting step(s), which is likely to be the electron transfer to the CYP450 from NAPDH reductase (Cederbaum, 2015).

CYPs can be induced by their own substrates through the activation of the related gene and hence the increase of protein synthesis. This can be mediated by cell receptors such as the case of aryl hydrocarbon receptor (AHR) receptor in induction of CYP1A1 (Ma, 2001).

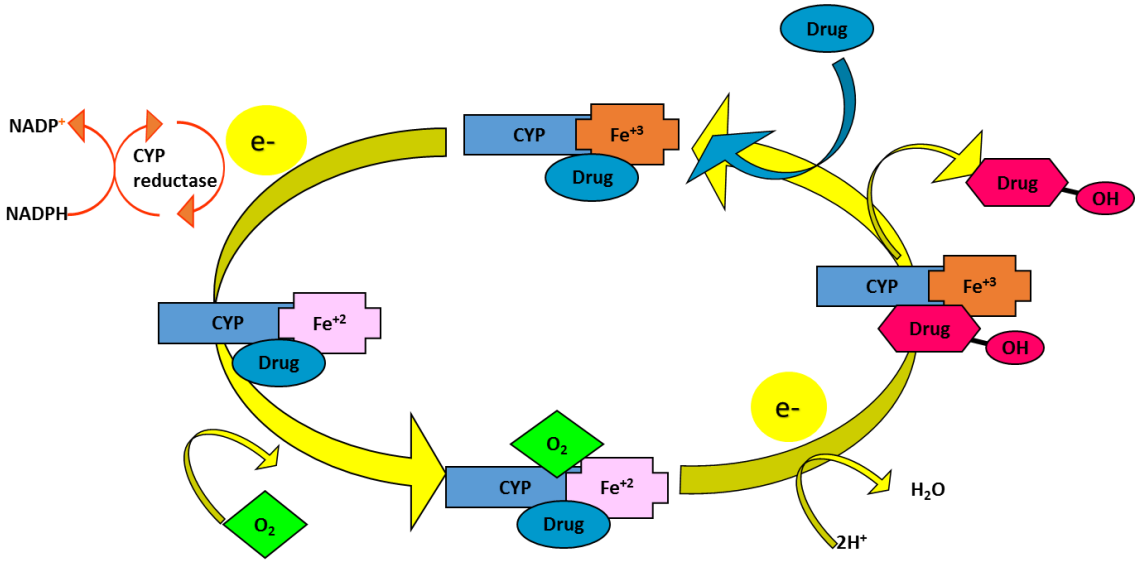

Figure 1: The catalytic cycle of CYPs (Cederbaum, 2015)

FMOs are also microsomal enzymes that share their NADPH-dependent oxidation and substrate-specificity with CYPs (Krueger and Williams, 2005). Five members of this family are recognized in humans (FMO1-5), which have around 55% of identity with the putative zebrafish FMO protein (Rodriguez-Fuentes et al., 2008). Oxidation of biogenic amines is catalyzed by MAO (mitochondrial flavoproteins). Two members of these proteins are found in humans, i.e. MAO A and MAO B (Edmondson et al., 2004). In zebrafish, only one member (zMAO) was found, which shares 70% of amino acid sequence identity with both human MAO. However, zMAO tends to be closer to MAO A in terms of functions (Aldeco et al., 2011; Arslan and Edmondson, 2010).

Phase II

The main purpose of phase II metabolism is generation of more soluble products from endogenous compounds, but also xenobiotics. This can be achieved by the catalysis of several reactions such as glucuronidation, sulfation, methylation, acetylation, glutathione and amino acid conjugations (Figure 2).

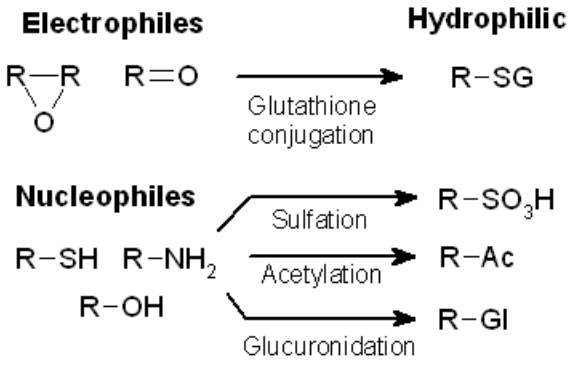

Figure 2: Main phase II conjugation reactions (Sau et al., 2010; Jancova et al., 2010; Tukey and Strassburg, 2001).

First, conjugation of a reduced glutathione to the electrophilic group on the target compound is mediated by Glutathione S-transferases (GSTs). As a result, the complex glutathione-substrate has an increased hydrophilicity which facilitates excretion (Sau et al., 2010). GSTs are divided into three families, namely mitochondrial, membrane associated and cytosolic. These families are divided according to their sequences into 10 classes.

Second, UDP-glucuronosyltransferases (UGTs) are the most abundant group of phase II drug metabolizing enzymes (Jancova et al., 2010). The metabolic reaction is achieved by adding the electrophilic group UDP-glucuronic acid on the substrate. UGTs are divided in mammals into two families, i.e. UGT1 and UGT2 (Tukey and Strassburg, 2001).

Third, sulfotransferases (SULTs) catalyze the binding of a sulfonate, hydroxyl or amino group on the target compound. Four families (SULT 1, 2, 4, 6) are recognized in humans with approximately 13 members.

As phase II reactions can impact the pharmacokinetic characteristics of a drug, inhibition studies are recommended by EMA for the main phase II-related enzymes such as UGT1A1 and UGT2B7 (EMA, 2012).

Phase III

The active excretion mechanism (phase III) is mediated by the membrane transporters. However, these proteins are also involved in the uptake procedure of drugs (phase 0). In humans, around 400 membrane transporters are characterized and divided into two major superfamilies i.e. ATP-binding cassette (ABC) and solute carrier (SLC) (Mooij et al., 2016). Inhibition or induction of these transporters by a drug can affect the bioavailability of other drugs because of the wide spectrum of substrate specificity of these transporters (International Transporter Consortium2010). Therefore, inhibition studies during drug development are recommended for compounds which inhibit any of the membrane transporters but also those which have an enzymatic induction effect mediated by a nuclear receptor. Eleven members are the main targets according to EMA guidelines, namely P-glycoprotein (P-gp), organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, organic cation transporter 2 (OCT2), OCT1, OAT1, OAT3, multidrug and toxin extrusion 1(MATE1), MATE2, breast cancer resistance protein (BCRP) and bile salt export pump (BSEP) (EMA, 2012).

1.3 Biotransformation in zebrafish

Since the toxic effects of a compound depend on the achieved exposure within an organism, a thorough knowledge of the biotransformation processes is necessary for proper safety assessment. However, the biotransformation capacity of adult zebrafish and zebrafish embryos still needs more elucidation (Beker van et al., 2013; Brannen et al., 2010; Weigt et al., 2012)

Phase I

Using the Ensembl genome browser (release 82), which incorporates the most recent annotations on the zebrafish genome from the Genome Reference Consortium (GRCz10), a total of 86 *CYP* genes that fall into 17 categories of *CYP* gene families were found (Table 1) (Genome Reference Consortium, 2015). Of these CYP families, CYP 1-3 are the main xenobiotic metabolizing enzymes responsible for drug and fatty acid metabolism, yet they display a wide variation in functions *in vivo*. It is important to note that of the 57 CYP enzymes encoded in the human genome, only around 15 are involved in xenobiotic metabolism and only five CYPs account for 95% of phase I metabolism of all marketed drugs (Guengerich, 2008).

Table 1 Zebrafish CYP families 1, 2 and 3 (Saad et al., 2016).

CYP1s	CYP2s							CYP3s
CYP1A	CYP2AA1	CYP2AE1	CYP2K6	CYP2AD2	CYP2R1	CYP2X6	CYP2Y3	CYP3A65
CYP1B1	CYP2AA2		CYP2K8	CYP2AD3	CYP2U1	CYP2X7		CYP3C1
CYP1C1	CYP2AA3		CYP2K16	CYP2AD6		CYP2X8		CYP3C2
CYP1C2	CYP2AA4		CYP2K17	CYP2J20		CYP2X9		CYP3C3
CYP1D1	CYP2AA6		CYP2K18	CYP2N13		CYP2X10.2*		CYP3C4
	CYP2AA7		CYP2K19	CYP2P6		CYP2X10.2**		
	CYP2AA8		CYP2K20	CYP2P7				
	CYP2AA9		CYP2K21	CYP2P8				
	CYP2AA11		CYP2K22	CYP2P9				
	CYP2AA12		CYP2K31	CYP2P10				
				CYP2V1				

* ENSDARG00000068283, ** ENSDARG00000006501

In the zebrafish, the full complement of *CYP* genes and their developmental expression has been well documented previously (Goldstone et al., 2010), but data on tissue distribution, modulation and activity of CYPs, which are important factors in the metabolism and clearance of xenobiotics, are scattered. Indeed, many drugs increase CYP activity by inducing the biosynthesis of CYP isoenzymes through particular signaling pathways. As such, the

metabolism and clearance of xenobiotics is also affected. In humans, constitutive androstane receptor (CAR), pregnane X-receptor (PXR) and AHR are well known transcription factors controlling *CYP* induction (Waxman, 1999). Zebrafish only exhibit two of these three regulatory mechanisms, with *CAR* being absent in zebrafish and teleost fish in general (Reschly and Krasowski, 2006). The nuclear hormone receptor PXR and *CYP3A* are induced in zebrafish with a similar mechanism as in man (Bresolin et al., 2005). However, the number of substrates that stimulate *PXR* is more limited in zebrafish (Ekins et al., 2008). Regarding AHR, this cytosolic protein complex translocates to the nucleus after ligand binding and dimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT). This leads to a high DNA binding affinity of this complex to stimulate gene transcription of the *CYP1A1* and other genes (Denison and Nagy, 2003). This AHR mechanism pathway is highly conserved across taxa, suggesting a physiological xenobiotic-independent function of the AHR (Hahn, 2002). Mammals including humans have only one functional AHR, whereas zebrafish AHR have multiple signaling members including AHR1a, AHR1b, AHR2, ARNT1, ARNT2 and two AHR repressors (Karchner et al., 2005). AHRs have affinity to a broad range of aromatic and halogenated chemicals including planar halogenated aromatic hydrocarbons (pHAH) and polycyclic aromatic hydrocarbons (PAH), which are both known as environmental contaminants (Otte et al., 2010).

In the following, we will consecutively discuss the available data on spatiotemporal distribution, modulation and activity of the CYP1, 2 and 3 families in zebrafish. These data will also be related to human CYP data when available (Table 2). The term CYP is fully capitalized in all cases except when referring to a specific gene, which is italicized, consistent with the nomenclature committee recommendations (Nelson, 2009).

Table 2 Synteny between zebrafish and human CYPs (Saad et al., 2016).

Family	Subfamily	Zebrafish CYPs	Human CYPs
CYP1	A	1A	1A1,2
	B	1B1	1B1
	C	1C1,2	-
	D	1D1	Inactive CYP1D1 gene
CYP2	AA	2AA(1-4,6-9,11,12)	-
	AE	2AE1	-
	C D	-	2C(8,9,18,19) 2D6
	E	-	2E1
	K	2K6,2K8,2K16-22, 2K31	2W1
	P,AD,V,N,J	2P10. 2J20	2J2
		2AD(2,3,6)	
		2P7, 2V1, N13	
		2P6	
	U	2U1	2U1
	R	2R1	2R1
	X	2X(6-10)	-
	Y	2Y3	2(A6,A13,B6,F1,S1)
CYP3	A	3A65	Inactive CYP3A genes
	C	3C(1-4)	3(A3,A4,A7)

CYP1 family

In zebrafish, the five known CYP1 isoforms are CYP1A, CYP1B1, CYP1C1, CYP1C2, and CYP1D1 (Goldstone et al., 2010). Except for *CYP1D1*, all of these CYP subfamilies are upregulated by AHR activation and induced by several compounds such as polychlorinated biphenyls (PCB), beta-naphthoflavone, benzo[a]pyrene (BaP), pesticides and 2,3,7,8-tetrachlorodibenzop-dioxin (TCDD) (Goldstone et al., 2009; Jonsson et al., 2007a; Jonsson et al., 2007b). Additionally, most *CYP1* isoforms are induced by exposure to oxidative stress and ultraviolet (UV) radiation (Behrendt et al., 2010). Interestingly, pregnenolone (PN), which is a specific PXR agonist, also induces zebrafish CYP1A (Kubota et al., 2015).

In humans, the activity and/or the expression of these enzymes can be downregulated by several mechanisms. For example, α-naphthoflavone (ANF) is a competitive inhibitor of CYP1A and at the same time a partial antagonist of AHR (Merchant et al., 1992; Miranda et al., 1998). L-fluoranthene (FL) lowers the CYP1A protein levels in vivo and inhibits non-competitively CYP1A in vitro, whereas piperonyl butoxide (PBO) forms a complex with the hem group leading to a nonselective inhibition of all CYPs including CYP1s (Franklin, 1977).

Humans and zebrafish show a broad spectrum of substrates metabolized by CYP1s. Many human CYP1, 2 and 3 probes are metabolized by zebrafish CYP1s. For instance, ER, 7-methoxyresorufin (MR), 17β-estradiol (E2), and benzo[a]pyrene (BaP) (human CYP1 probes) (Shimada et al., 1996), and 7-benzyloxyresorufin (BR) (CYP2 and 3 probe) (Niwa et al., 2003); are metabolized by zebrafish CYP1s (Scornaienchi et al., 2010a, b). Surprisingly, 7-benzyloxy-4-(trifluoromethyl) coumarin (BFC), which is a selective substrate for CYP3A in humans (Crespi and Stresser, 2000), is metabolized by zebrafish CYP1A more efficiently than zebrafish CYP3A65 (Scornaienchi et al., 2010b). Additionally, E2 which is metabolized by the human CYP1B1 is metabolized more effectively in zebrafish by CYP1C1 and CYP1A rather than CYP1B1 (Scornaienchi et al., 2010a).

CYP1A

In adult zebrafish, the highest basal expression of CYP1A mRNA can be found in liver and gut tissue. Compared to other CYP1s, CYP1A mRNA levels are the highest in organs of the abdominal cavity and the heart (Jonsson et al., 2007b), with expression mainly in the cardiovascular system during the embryonic development (Otte et al., 2010).

In this respect, CYP1A mRNA expression is low during early development and increases drastically after hatching around 72 hpf (Goldstone et al., 2009). Because of this gap in expression, Mattingly and Toscano (2001) suggested that a posttranscriptional silencing mechanism exists in embryos until 72 hpf. This silencing is believed to be a protective mechanism in early developmental stages against cardiovascular abnormalities caused by the CYP1A biotransformation of some environmental pollutants (Mattingly and Toscano, 2001). In contrast, Otte *et al.* (2010) used 7-ethoxyresorufin-*O*-deethylase (EROD) assays for CYP1A activity and found peak levels at 8 hpf. This activity reached the lowest level at 36 hpf and then increased until another peak at 104 hpf (Otte et al., 2010).

Planar halogenated aromatic hydrocarbons (pHAHs), like TCDD, have been proven to induce *CYP1A* and cause vascular abnormalities in early zebrafish developmental stages (Andreasen et al., 2002; Henry et al., 1997). The localization of AHR2 and ARNT2 in parallel to CYP1A in the vasculature of early embryos, suggests an important role for those two proteins in the induction of *CYP1A* by TCDD (Andreasen et al., 2002). However, the relation between *CYP1A* on one hand and the induction pathways AHR and ARNT2 and xenobiotics on the other hand remains complicated. Teraoka *et al.* (2003) showed that TCDD abnormalities can be prevented by *CYP1A* knockdown (Teraoka et al., 2003), while other research groups completely contradict this finding (Carney et al., 2004). Other organic toxins (e.g. tricyclic PAHs) must have an *AHR*- and *CYP1A*-independent pathway of toxicity, since *AHR* and *CYP1A* knockdown zebrafish embryos showed no decreased toxicity (Incardona et al., 2005). Surprisingly, the

toxicity of tetracyclic PAH compounds is increased significantly by utilizing CYP1A morphants and reduced by AHR2 morphants. This indicates a protective influence of CYP1A towards such pollutants with an AHR2-dependent mechanism of toxicity (Billiard et al., 2006). Still, much debate is ongoing about the role of *CYP1A* and *AHR* pathway in this observed toxicity. Furthermore, PXR may also be involved in *CYP1A* induction as a trend towards a reduced CYP1A expression is noted in zebrafish embryos when *PXR* is knocked down (Kubota et al., 2015).

Regardless of the underlying regulatory processes, it is clear that zebrafish CYP1A metabolizes environmental pollutants, but also many other human CYP substrates including ER, MR, 7-pentoxyresorufin (PR), and BR (Scornaienchi et al., 2010b). Furthermore, CYP1A in zebrafish, as in humans, is the sole CYP1 that shows 16-α hydroxylation activity of E2, despite the fact that the overall metabolism rate in zebrafish is much lower than that of human CYP1A1 (Scornaienchi et al., 2010a).

CYP1B

The only isoform of this subfamily in zebrafish is CYP1B1 (Godard et al., 2005), which is expressed in adults particularly in the eyes and the heart (Jonsson et al., 2007b). *CYP1B1* expression showed a peak between 30 and 48 hpf and subsequent expression after hatching at 72 hpf (Yin et al., 2008). Such a distinct spatio-temporal CYP1B1 pattern with considerable levels around 30 hpf suggests an inherent role in eye development, as the retina begins to develop at this time (Easter and Nicola, 1996).

CYP1B1 modulation follows an AHR2 independent pathway in the eye and the brain before hatching and an AHR2 dependent pathway in the branchial arches and the heart after hatching (Yin et al., 2008). However, the AHR2 independent pathway still needs AHR2 as a modulating factor. This dependency on AHR2 explains the absence of *CYP1B1* expression in *AHR2* knockdown embryos (Yin et al., 2008). Therefore, the induction by *AHR* agonists such as

TCDD and 3, 3′, 4, 4′, 5-pentachlorobiphenyl (PCB126) is observed in both post-hatched embryos and adults (Jonsson et al., 2007b).

When compared to CYP1A, CYP1B1 showed much lower (Scornaienchi et al., 2010b) or even negligible (Stegeman et al., 2015) EROD activity *in vitro.* This is in accordance with data from Timme-Laragy *et al*. (2008), who showed that knocking down of *CYP1B1* in zebrafish larvae had no effect on EROD activity (Timme-Laragy et al., 2008). The latter confirms the contribution of other CYP1s to the observed high EROD activity *in vivo*.

The mechanisms of CYP1B1 action are similar across taxa. For example, the ratio of ER and E2 biotransformation by CYP1B1 to CYP1A in zebrafish is similar to the biotransformation by CYP1B1 to CYP1A1 in humans (Scornaienchi et al., 2010a). Furthermore, there is a high conservation of the E2 4-hydroxylation activation site in CYP1B1 proteins in both zebrafish and humans (Lewis et al., 1999). However, the ratio of E2 4-hydroxylation to E2 2-hydroxylation by zebrafish CYP1B1 is higher than this ratio by its human ortholog. This means that the main metabolite of E2 by zebrafish CYP1B1 is 4-hydroxy E2 while it is 2-hydroxy E2 by human CYP1B1 (Scornaienchi et al., 2010a). Additionally, zebrafish CYP1B1 can also metabolize dibenzylfluorescein (DBF), which is a CYP2C8, CYP2C9 and CYP3A4 substrate in human (Scornaienchi et al., 2010b). This indicates for distinctive metabolic characteristics of zebrafish CYP1B1 compared to human CYP1B1.

CYP1Cs

This subfamily is composed of two members in zebrafish, CYP1C1 and CYP1C2 (Godard et al., 2005), yet it has no counterpart in humans (Uno et al., 2012). The gene expression of *CYP1C*s is higher than all other CYP1s at 80 hpf (Jonsson et al., 2007b). The induction of these genes by AHR agonists reaches a peak at 4 days post fertilization (dpf) for *CYP1C1*, which remains at a high level during adulthood (Jonsson et al., 2007a; Jonsson et al., 2007b; Timme-Laragy et al., 2008; Wang et al., 2006). Moreover, the induction of *CYP1Cs* by AHR agonists

shows a spatiotemporal trend. Hence, *CYP1C1* is induced strongly by 3,3′,4,4′,5-pentachlorobiphenyl in adults and embryos while it is negligible for *CYP1C2* in adults (Jonsson et al., 2007b). This induction is much higher in the mesenteric artery than in the liver (Bugiak and Weber, 2009). Both *CYP1C1* and *CYP1C2* have the highest basal expression in the heart and the eyes and the lowest in the ovary. In particular, *CYP1C1* and *CYP1C2* also have high basal expressions in the gills and the kidneys, respectively (Jonsson et al., 2007b). This suggests an important biological and developmental role of this subfamily. Furthermore, vascular toxicity is reduced by silencing either *CYP1C1* or *CYP1C2*, which indicates complementary functions of those two isoforms in embryos (Kubota et al., 2011).

Similar to CYP1A, CYP1C1 metabolizes E2 at the same rate (Scornaienchi et al., 2010a). Additionally, CYP1C1 and CYP1C2, at varying rates, can also metabolize numerous substrates and environmental pollutants. For instance, both subfamilies metabolize human CYP1 substrates, such as ER, MR and PR, and human CYP2 substrates, such as BR and the environmental contaminant BaP (Scornaienchi et al., 2010b; Stegeman et al., 2015). Interestingly, biotransformation of BaP by zebrafish CYP1Cs leads to metabolites that are similar to the ones formed by human CYP1A1. Furthermore, these CYP1C enzymes metabolize testosterone mainly to 6ß-OH-testosterone, which is a specific human CYP3A metabolite (Stegeman et al., 2015). This wide spectrum of substrates indicates an important role of this subfamily, which has no ortholog in man, in the biotransformation of endogenous substrates and detoxification of environmental pollutants in zebrafish.

CYP1D

In 2008, Goldstone *et al.* cloned a new CYP1 subfamily with one gene, namely *CYP1D1*, in the freshwater fish species medaka *(Oryzias latipes)*. It was recently proven to be transcribed in many organs of adult zebrafish and in early stages of zebrafish development (Goldstone et al., 2009; Goldstone and Stegeman, 2008). A high level of CYP1D1 expression is detected in

embryos at 9 hpf, but it reaches the highest level in adult zebrafish, more specifically in liver and brain (Goldstone et al., 2009). This gene has no functional protein in man (Nelson et al., 2004), though it does have in other mammals (Uno et al., 2011). Except for humans, the main function of this subfamily seems to be similar in mammals and other vertebrates (Goldstone et al., 2009).

Unlike other *CYP1*s, *CYP1D1* is not induced by *AHR* agonists such as 6-formylindolo[3,2-b]carbazole (Jonsson et al., 2009) or by UV radiation exposure (Behrendt et al., 2010). It was not induced by PCB126 in killifish (Zanette et al., 2009), however, it was induced in Cynomolgus monkey hepatocytes by omeprazole and rifampicin (Uno et al., 2011). Furthermore, CYP1D1 has low EROD and MROD activities, which are not affected by adding oxidative activators such as cytochrome b5 (Goldstone et al., 2009) and no activity towards other coumarin substrates (Stegeman et al., 2015). Subsequently, it does not metabolize E2 (Scornaienchi et al., 2010a), while there are low activities towards BaP (Scornaienchi et al., 2010b). The same is also observed with caffeine in some mammals rather than humans such as monkeys (Uno et al., 2011). Surprisingly, CYP1D1 metabolizes testosterone to 6ß-OH-testosterone at much lower concentrations than CYP1Cs, but it forms two other undefined testosterone metabolites at higher concentrations than what is detected for CYP1Cs (Stegeman et al., 2015). These unique characteristics of CYP1D1 in zebrafish, and especially its low response towards different inducers and substrates, raise doubts about its role in the metabolism of xenobiotics.

CYP2 family

The *CYP2* family comprises the largest CYP gene family in zebrafish, with 41 genes (Genome Reference Consortium, 2015). It is also the largest CYP gene family in humans containing 16 genes.

In both man and zebrafish, each *CYP2* gene consists of nine exons, except for *CYP2R* and *CYP2U*, which are composed of five exons. These exons show identical size in mammalian and ray-finned fishes, including zebrafish (Kirischian et al., 2011). In fish, *CYP2s* seem to be regulated via PXR and AHR (Kubota et al., 2013; Kubota et al., 2015; Mosadeghi et al., 2007; Yuan et al., 2013). In contrast, several substrates tend to induce *CYP2* genes in mammals, including humans, by the nuclear receptor CAR (Handschin and Meyer, 2003; Waxman, 1999).

CYP2AA

In zebrafish, the CYP2AA subfamily consists of 10 members (CYP2AA1-4, 6-9, 11, 12) (Genome Reference Consortium, 2015). Genes within this subfamily show 65-85% similarity to each other according to The Gene Wise algorithm, but no homology to mammalian *CYP2*s (Goldstone et al., 2010). The amino acids sequences of the predicted proteins of CYP2AA1 and 2AA2 are similar to the mammalian CYP2Bs, with a similarity of approximately 38-41% (Kubota et al., 2013).

In adult zebrafish, CYP2AA1 is expressed in most tissues, with the intestine showing the highest expression levels. This gene has a variable response to PXR agonists. For instance, its mRNA levels increased after treatment with pregnenolone 16α-carbonitrile (PCN), but not with phenobarbital (PB), 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), or PCB126 (Kubota et al., 2013; Kubota et al., 2015). In contrast, CYP2AA2 expression, which is highly abundant in the kidneys, was significantly upregulated by TCPOBOP and slightly by PB, but not by PCN or by PCB126 (Kubota et al., 2013). However, CYP2AA12 was induced by both PN and PCB126, and in both cases the induction could be partially suppressed by using morpholinos of PXR or AHR, respectively (Kubota et al., 2015). This suggests a role of the PXR pathway in the induction of both CYP2AA1 and CYP2AA12 but not CYP2AA2, and the AHR pathway in regulation of CYP2AA12 but not CYP2AA1 and AA2.

In zebrafish embryos, CYP2AA7-9 and 2AA12 mRNA levels show peak expression at 24 hpf, followed by a decrease until 36 hpf, when it starts increasing slowly again. Moreover, CYP2AA4 is detected in unfertilized eggs (Goldstone et al., 2010). However, the functional meaning of these spatiotemporal patterns still needs to be further elucidated.

CYP2AE

This subfamily is unique to zebrafish and contains only one gene, i.e. *CYP2AE1* (Genome Reference Consortium, 2015). It shares no synteny with human CYPs or CYPs from other fish species (Kirischian et al., 2011) and its function is unknown yet.

CYP2K

This subfamily consists of 10 members (*CYP2K6, 8, 16-22, 31*) (Genome Reference Consortium, 2015), which in general share synteny with the human *CYP2W1* (Goldstone et al., 2010). The individual isoforms show different spatio-temporal patterns and a variable homology to other species. For example, CYP2K6 mRNA is only detected in liver and ovary of adult zebrafish with a late onset of expression in the embryos. It is poorly expressed at 3 dpf and only reaches high levels at 5 dpf in embryos/larvae. In contrast, CYP2K22 has earlier peaks of expression, i.e. at 3 hpf and 48 hpf (Goldstone et al., 2010). Furthermore, the amino acid sequence of zebrafish CYP2K6 is 63% identical to rainbow trout CYP2K1 (Wang-Buhler et al., 2005).

Despite the orthological relationship between CYP2K1 in rainbow trout and CYP2K6 in zebrafish, their metabolic features are not completely the same. Both of them metabolize aflatoxin B1 (AFB1) to the carcinogenic exo-8, 9-AFB1 epoxide, whereas only rainbow trout CYP2K1 can metabolize lauric acid (Wang-Buhler et al., 2005). Though CYP2K6 mRNA is not detected in embryos until 5 dpf (Wang-Buhler et al., 2005), Weigt *et al.* (2011) showed that different AFB1-induced malformations were already observed at 1 dpf (Weigt et al., 2011). It

is unclear whether these malformations result from direct or indirect mutagenic effects of AFB1 itself or due to biotransformation of AFB1 by other CYPs than CYP2K6.

CYP2AD, CYP2N, CYP2P, CYP2J, CYP2V

The localization of the 11 genes (*CYP2AD2,3,6, CYP2N13, CYP2P 6-10, CYP2J20, CYP2V1*) in these subfamilies on chromosome 20 suggests a synteny with human *CYP2J2* (Genome Reference Consortium, 2015; Goldstone et al., 2010), which is responsible for the biotransformation of arachidonic acid to cis-epoxyeicosatrienoic acids (Wang et al., 2005). In particular, the sequence of CYP2P6 putative protein is 50% similar to that of human CYP2J2, which makes it a good candidate for the biosynthesis of cis-epoxyeicosatrienoic acids (Wang et al., 2007b). CYP2P6 has a peak at 12 hpf, then decreasing to very low levels and increasing gradually again at 36 hpf. This isoform is expressed mainly in brain and gonads of adult zebrafish and slightly in liver, heart and kidneys. Its expression in adults fluctuates during the ovarian follicular maturation with high levels in parallel to the FSH and LH production stages. Moreover, it has high levels at the early oocyte developmental stages, namely stage I and II, and reaches undetectable levels at stage III when the yolk proteins start to appear. Such expression patterns may be related to a female reproductive function of CYP2P6. Interestingly, silencing of the *CYP2P6* gene has no effect on embryonic development, whereas the overexpression of this gene causes lethality, malformations in dorsal cords or the cardiovascular system and curved tails (Wang et al., 2007b).

For the rest of this group of genes, little is known about their function in zebrafish. CYP2P10, CYP2J20 and CYP2AD6 have peaks at 3 hpf, CYP2P7 and CYP2V1 have increased levels between 6-12 hpf, whereas CYP2N13 has two peaks, i.e. at 3 and 48 hpf. The early detected expression suggests a role in the transition from maternally- to oocyte-derived transcript (Goldstone et al., 2010).

In other fish species, such as killifish (*Fundulus heteroclitus*), CYP2N1 and 2N2 were observed in the liver and the intestine, and the heart and the brain, respectively. Both of them show efficient epoxidation of arachidonic acid, as it is suggested for zebrafish CYP2P6, but also N-demethylation of benzphetamine activities, and low O-dealkylase activities towards BR and PR (Oleksiak et al., 2000). On the other hand, CYP2P3 has a higher activity towards the previously mentioned resorufin substrates and benzphetamine than CYP2Ns (Oleksiak et al., 2003). Both CYP2Ns and CYP2P3 are suppressed by 12-O-tetradecanoylphorbol-13-acetate (Oleksiak et al., 2000; Oleksiak et al., 2003). Hence, it is possible for the zebrafish homologous isoforms to have identical metabolic characters as those in killifish.

CYP2R and CYP2U

These two subfamilies exist in most of the studied vertebrate (Kirischian et al., 2011; Nelson et al., 2004) and invertebrate species (Zheng et al., 2013). In zebrafish, sequence analysis of *CYP2R1* and *CYP2U1* shows that both are classified as orthologs of human CYP genes, as they have very similar sequences and show synteny with their human counterparts (Goldstone et al., 2010).

Both isoforms play an important role in biotransformation, which is well documented in humans. Human CYP2R1 is involved in the metabolism of vitamin D into active metabolites (Cheng et al., 2003; Yasuda et al., 2013) and prostate carcinogenesis (Ellfolk et al., 2009), while CYP2U1 shares the activation of many fatty acids such as arachidonic acid (Strushkevich et al., 2008).

Interestingly, zebrafish embryos show higher CYP2R1 levels in low Ca^{+2} water, which also indicates a possible role of this isoform in vitamin D synthesis in zebrafish embryos (Lin et al., 2012).

CYP2X

Most ray-finned species express only one gene of this subfamily. In contrast, zebrafish has six genes, namely *CYP2X6-9* and *CYP2X10* that shows two similar copies (Genome Reference Consortium, 2015; Goldstone et al., 2010). These genes do not share synteny with any mammalian CYP (Goldstone et al., 2010; Nelson, 2009).

The function of this subfamily in zebrafish is unknown yet. However, some isoforms are well documented in other fish species, which suggests possible functions in zebrafish as well.

In channel catfish (*Ictalurus punctatus*), CYP2X1 has a broad spectrum distribution throughout the organism but low biotransformation activities and induction responses. This isoform is detected in all tissues but especially in the liver and the gills. However, it has a very low N-demethylation activity towards aminopyrine and benzphetamine when compared to other CYP2 subfamilies in fish. Furthermore, it has no activity towards alkoxyresorufin substrates and p-nitrophenol, which are usually general CYP2 substrates. Except the significant suppression of mRNA expression in the kidneys, no changes were observed with other CYP inducers and suppressors such as ethanol, clofibric acid, rifampin and pyridine (Mosadeghi et al., 2007).

On the other hand, the expression of CYP2X10 in goldfish (*Carassius auratus*) increased significantly after exposure to environmental pollutants, which indicates a possible role of this subfamily in biotransformation of xenobiotics (Wang et al., 2007a).

CYP2Y

CYP2Y3 is the only member of this subfamily in zebrafish (Genome Reference Consortium, 2015). It shares synteny with human *CYP2A6*, *CYP2A13*, *CYP2B6*, *CYP2F1*, and *CYP2S1* (Goldstone et al., 2010). Zebrafish CYP2Y3 is identical to the CYP2Y3 found in Atlantic cod (Olsvik et al., 2009). Though little is known about the activity of this subfamily in zebrafish, several studies detected the effects of different toxins on CYP2Y3 expression in other fish

species. Many differences were observed in the response to different substrates, inducers and suppressors between this subfamily in fish and its mammalian homologs. For instance, nonylphenol (NP), which is a PXR-mediated CYP-inducer in mice (Mota et al., 2011), and bisphenol A (BPA), which is a suppressor for some rat CYPs (Pfeiffer and Metzler, 2004), cause a reduction in CYP2Y3 mRNAs levels in Atlantic cod (Olsvik et al., 2009). Another example for differential responses between species is 2,2',4,4'tetrabromodiphenyl ether PBDE-47, an environmental toxin. It induces human *CYP2B6* by PXR and CAR pathways (Sueyoshi et al., 2014) and is also significantly metabolized by human CYP2B6 (Feo et al., 2013). However, this compound does not affect the expression of CYP2Y3 in Atlantic cod (Olsvik et al., 2009), despite the synteny between *CYP2Y3* and human *CYP2B6* (Goldstone et al., 2010). This suggests that different regulatory mechanisms may be involved for this subfamily in fish species rather than PXR and CAR induction pathways. Yuan *et al*. (2013) showed some evidence of Chinese rare minnow *CYP2Y3* induction, which shares 75% pair-wise identity with zebrafish CYP2Y3 putative protein, by BaP, which is an AHR agonist. This suggests a role for AHR-linked induction of *CYP2Y3*, but this needs further investigation.

CYP3 family

In zebrafish, this family consists out of only five members, i.e. CYP3A65 and CYP3C1-4 (Goldstone et al., 2010). Numerous human CYP3 substrates are metabolized by zebrafish, though differences in metabolization profiles are reported, e.g. for testosterone (Chng et al., 2012). The metabolization profile of testosterone is also different from zebrafish adult compared to larvae (Chng et al., 2012). This may be due to the variable expression of CYPs throughout the development. In general, in zebrafish, only 25% of CYPs has an elevated trend during the first 2 dpf, while the rest shows various temporal patterns (Goldstone et al., 2010). Similar to human CYP3As, zebrafish CYP3As are involved in the biotransformation of drugs such as emodin, an herbal compound for diverse human therapeutic indications. However, toxic

metabolites of emodin are reported in zebrafish, whereas this compound is safe in man (He et al., 2012). Additionally, Alderton *et al.* (2010) showed that for midazolam, which is a well-known human CYP3A tool compound, no metabolites could be detected in zebrafish larvae (Alderton et al., 2010). This makes CYP3 activity in zebrafish still a controversial issue.

CYP3A

CYP3A65 is the only known isoform of this subfamily in zebrafish. It is detected at low levels at 24 hpf, with an increasing trend after 72 hpf typically in the foregut, and later in the liver and the intestine of adults. Other organs such as the brain, the gills, and the eyes express low levels of CYP3A65 (Tseng et al., 2005).

The nuclear receptor PXR is an important transcriptional pathway of *CYP3A*s in human being and mammals in general, which can be induced by several xenobiotics such as rifampicin, clotrimazole and nifedipine in man and dexamethasone and pregnenolone 16a-carboninitrile (PCN) in mice (Coumoul et al., 2002; Guengerich, 1999; Liddle and Goodwin, 2002; Moore et al., 2002). In zebrafish larvae, *CYP3A65* is also induced by pregnenolone (PN), rifampicin and dexamethasone (Kubota et al., 2015; Tseng et al., 2005). Clotrimazole and nifedipine show no effect on CYP3A65 mRNA levels in adult liver, whereas PCN increases significantly these levels (Bresolin et al., 2005). However, both basal and TCDD-induced levels of CYP3A65 mRNA in zebrafish were negligible in AHR2 morpholino embryos (Tseng et al., 2005). In contrast, Kubota *et al*. (2015) showed elevated levels of CYP3A65 mRNA after exposure to PCB126 and these induced levels were decreased in AHR knockdown embryos. These findings indicate that CYP3A65 expression is regulated by AHR2 and PXR pathways (Chang et al., 2013; Kubota et al., 2015).

Similar to human CYP3As, zebrafish CYP3A65 plays an important role in the detoxification of environmental hepatotoxins and in drug metabolism such as microcystin and testosterone (Chng et al., 2012; Li et al., 2013). Also 2-hydroxy E2 is the main metabolite of E2 via CYP3A

in both zebrafish and humans (Scornaienchi et al., 2010a). Still differences are reported. For instance, BFC, which is a specific human CYP3A substrate, is biotransformed more efficiently in zebrafish by CYP1A rather than CYP3A65 (Scornaienchi et al., 2010b).

CYP3C

This subfamily consists of four genes (*CYP3C1-4*) (Goldstone et al., 2010) and shares synteny with human *CYP3A3*, *3A4* and *3A7* (Qiu et al., 2008). Corley-Smith *et al*. (2006) showed CYP3C1 mRNA levels in embryos at 12 hpf, which are distributed in the whole embryo until 48 hpf. At 120 hpf, CYP3C1 concentrates in the brain and appears in the pharynx and the gastrointestinal system. However, in adults, CYP3C1 mRNA levels are absent in the brain, low in the heart and the eyes and high in the liver, the intestines, and the ovaries (Corley-Smith et al., 2006).

In zebrafish embryos, Corley-Smith *et al.* (2006) suggested that *CYP3C1* modulation is independent from the PXR and the AHR pathways as its mRNA levels were not affected by TCDD, dexamethasone or rifampicin (Corley-Smith et al., 2006). In contrast, Kubota *et al*. (2015) showed a possible role of PXR and AHR pathways in CYP3C1 regulation as it was induced by both PN and PCB126, and this induction was partially reversed when using PXR and AHR morpholinos, respectively (Kubota et al., 2015). This discrepancy in results on CYP3C1 regulation may be due to the difference in substrates that were used by both groups. Additionally, Corley-Smith *et al*. (2006) applied a continuous exposure of fertilized eggs until 120 hpf at relatively high substrate concentrations, whereas Kubota *et al*. (2015) exposed different developmental stages for only 24h at relatively low substrate concentrations. The latter exposure window was specifically chosen as preliminary experiments had shown a lower induction of CYPs and receptors when 48 hpf embryos were exposed to PN for 48h instead of 24h. Interestingly, knockdown of Krüppel-like factor 6 caused a decrease in CYP3C1 expression in 96 hpf zebrafish embryos (Zhao et al., 2010). However, as these morphants also

have small livers, the low expression levels of CYP3C1 may be rather due to the low number of hepatocytes in these embryos than caused by a direct effect of Krüppel-like factor 6 on CYP3C1.

Phase II

Gene expression and intrinsic activity of several phase II enzymes has been detected in zebrafish starting from very early embryonic stages (Alazizi et al., 2011; Alderton et al., 2010; Brox et al., 2016; Christen and Fent, 2014; Jones et al., 2010; Kurogi et al., 2013).

In zebrafish, 9 classes of GST family were detected. Although synteny between mammalian and zebrafish GSTs is present, different functions are suggested due to spatio-temporal differences (Glisic et al., 2015). At early developmental stages, GSTs were detected, which indicates an important physiological role of these enzymes during embryogenesis (Hansen and Harris, 2015; Timme-Laragy et al., 2013; Wiegand et al., 2001). Also glutathione conjugates of atrazine and clofibric acid were detected in zebrafish embryos (Wiegand et al., 2001).

Both UGT1 and UGT2 families were detected in zebrafish together with the UGT5 family which exists only in teleosts and amphibians (Christen and Fent, 2014; Huang and Wu, 2010), while zebrafish *SULTs* are divided into six families containing 20 members (Kurogi et al., 2013). Glucuronidation and sulfation conjugates were detected for several compounds at several developmental stages e.g. calycosin and testosterone in the zebrafish larval stage (Alderton et al., 2010), benzophenone-2 and the bisphenol A substitute bisphenol S in zebrafish liver cells (Le Fol et al., 2015) and clofibric acid starting from 48 hpf (Brox et al., 2016). Other phase II activities were detected at early embryonic stages such as the conjugations of taurine, aminomethanesulfonic acid, and carnitine (Brox et al., 2016), indicating early phase II activity in zebrafish.

Phase III

Little is known about the function of drug transporters in zebrafish. Despite the fact that several drug transporters were detected in zebrafish embryos, namely P-gp, mrp1, mrp2, mrp5 at 2, 1, 72, and 12 hpf, respectively. Some evidences were found for the role of these transporters in detoxification of toxic chemicals such as cadmium chloride and PAH like β-naphthoflavone (Fischer et al., 2013; Long et al., 2011; Yin et al., 2016).

1.4 Conclusion

The zebrafish (embryo) is a promising animal/alternative model in drug discovery and toxicology. Therefore, knowledge on its biotransformation capacity and its ontogeny is key for a correct interpretation of toxicity data, in particular for human safety assessment. Generally, zebrafish CYPs, as the most important enzymes in biotransformation, show a strong evolutionary and orthological relationship to humans. Regarding CYP activity, several studies revealed shared metabolic characteristics with humans. Yet, important differences in the metabolite profile of many substrates, gene expression inhibition and induction have also been reported.

Taken altogether, these data indicate a distinct paucity in information regarding xenobiotic CYP activity within family 1-3. Uncovering these elusive mechanisms will aid in a better understanding of (developmental) toxicity data. This also applies for Phase II enzymes and drug transporters in zebrafish but falls out of the scope of this PhD research.

With increasing knowledge on the biotransformation processes in zebrafish at different developmental stages, the zebrafish can be further validated and strengthened as a vertebrate model in drug discovery and toxicology.

1.5 References

Alazizi, A., Liu, M.Y., Williams, F.E., Kurogi, K., Sakakibara, Y., Suiko, M., Liu, M.C., 2011. Identification, characterization, and ontogenic study of a catechol O-methyltransferase from zebrafish. Aquat Toxicol 102, 18-23.

Aldeco, M., Arslan, B.K., Edmondson, D.E., 2011. Catalytic and inhibitor binding properties of zebrafish monoamine oxidase (zMAO): comparisons with human MAO A and MAO B. Comp Biochem Physiol B Biochem Mol Biol 159, 78-83.

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Ali, S., Champagne, D.L., Spaink, H.P., Richardson, M.K., 2011. Zebrafish embryos and larvae: a new generation of disease models and drug screens. Birth Defects Res C Embryo Today 93, 115-133.

Andreasen, E.A., Spitsbergen, J.M., Tanguay, R.L., Stegeman, J.J., Heideman, W., Peterson, R.E., 2002. Tissue-specific expression of AHR2, ARNT2, and CYP1A in zebrafish embryos and larvae: effects of developmental stage and 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure. Toxicol. Sci 68, 403-419.

Arslan, B.K., Edmondson, D.E., 2010. Expression of zebrafish (Danio rerio) monoamine oxidase (MAO) in Pichia pastoris: purification and comparison with human MAO A and MAO B. Protein Expr Purif 70, 290-297.

Augustine-Rauch, K., Zhang, C.X., Panzica-Kelly, J.M., 2010. In vitro developmental toxicology assays: A review of the state of the science of rodent and zebrafish whole embryo culture and embryonic stem cell assays. Birth Defects Res C Embryo Today 90, 87-98.

Balcombe, J.P., Barnard, N.D., Sandusky, C., 2004. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 43, 42-51.

Baranczewski, P., Stanczak, A., Sundberg, K., Svensson, R., Wallin, A., Jansson, J., Garberg, P., Postlind, H., 2006. Introduction to in vitro estimation of metabolic stability and drug interactions of new chemical entities in drug discovery and development. Pharmacol Rep 58, 453-472.

Behrendt, L., Jonsson, M.E., Goldstone, J.V., Stegeman, J.J., 2010. Induction of cytochrome P450 1 genes and stress response genes in developing zebrafish exposed to ultraviolet radiation. Aquat. Toxicol 98, 74-82.

Beker van, W.A., Wolterbeek, A., Te, B.L., Snel, C., Menke, A., Rubingh, C., de, G.D., Kroese, D., 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reprod. Toxicol 41, 35-44.

Billiard, S.M., Timme-Laragy, A.R., Wassenberg, D.M., Cockman, C., Di Giulio, R.T., 2006. The role of the aryl hydrocarbon receptor pathway in mediating synergistic developmental toxicity of polycyclic aromatic hydrocarbons to zebrafish. Toxicol. Sci 92, 526-536.

Brannen, K.C., Panzica-Kelly, J.M., Danberry, T.L., Augustine-Rauch, K.A., 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res. B Dev. Reprod. Toxicol 89, 66-77.

Bresolin, T., de Freitas, R.M., Celso Dias, B.A., 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. Comp Biochem. Physiol C. Toxicol. Pharmacol 140, 403-407.

Brown, C.M., Reisfeld, B., Mayeno, A.N., 2008. Cytochromes P450: a structure-based summary of biotransformations using representative substrates. Drug Metab Rev 40, 1-100.

Brox, S., Seiwert, B., Haase, N., Kuster, E., Reemtsma, T., 2016. Metabolism of clofibric acid in zebrafish embryos (Danio rerio) as determined by liquid chromatography-high resolution-mass spectrometry. Comp Biochem Physiol C Toxicol Pharmacol 185-186, 20-28.

Bugiak, B., Weber, L.P., 2009. Hepatic and vascular mRNA expression in adult zebrafish (Danio rerio) following exposure to benzo-a-pyrene and 2,3,7,8-tetrachlorodibenzo-p-dioxin. Aquat. Toxicol 95, 299-306.

Carney, S.A., Peterson, R.E., Heideman, W., 2004. 2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish. Mol. Pharmacol 66, 512-521.

Cederbaum, A.I., 2015. Molecular mechanisms of the microsomal mixed function oxidases and biological and pathological implications. Redox Biol 4, 60-73.

Chang, C.T., Chung, H.Y., Su, H.T., Tseng, H.P., Tzou, W.S., Hu, C.H., 2013. Regulation of zebrafish CYP3A65 transcription by AHR2. Toxicol. Appl. Pharmacol 270, 174-184.

Cheng, J.B., Motola, D.L., Mangelsdorf, D.J., Russell, D.W., 2003. De-orphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxilase. J. Biol. Chem 278, 38084-38093.

Chng, H.T., Ho, H.K., Yap, C.W., Lam, S.H., Chan, E.C., 2012. An investigation of the bioactivation potential and metabolism profile of Zebrafish versus human. J. Biomol. Screen 17, 974-986.

Christen, V., Fent, K., 2014. Tissue-, sex- and development-specific transcription profiles of eight UDP-glucuronosyltransferase genes in zebrafish (Danio rerio) and their regulation by activator of aryl hydrocarbon receptor. Aquat Toxicol 150, 93-102.

Collins, J.E., White, S., Searle, S.M., Stemple, D.L., 2012. Incorporating RNA-seq data into the zebrafish Ensembl genebuild. Genome Res 22, 2067-2078.

Corley-Smith, G.E., Su, H.T., Wang-Buhler, J.L., Tseng, H.P., Hu, C.H., Hoang, T., Chung, W.G., Buhler, D.R., 2006. CYP3C1, the first member of a new cytochrome P450 subfamily found in zebrafish (Danio rerio). Biochem. Biophys. Res. Commun 340, 1039-1046.

Coumoul, X., Diry, M., Barouki, R., 2002. PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. Biochem. Pharmacol 64, 1513-1519.

Crespi, C.L., Stresser, D.M., 2000. Fluorometric screening for metabolism-based drug—drug interactions. J. Pharmacol. Toxicol. Methods 44, 325-331.

Denison, M.S., Nagy, S.R., 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu. Rev. Pharmacol. Toxicol 43, 309-334.

Detrich, H.W., 3rd, Westerfield, M., Zon, L.I., 1999. Overview of the Zebrafish system. Methods Cell Biol 59, 3-10.

Easter, S.S., Jr., Nicola, G.N., 1996. The development of vision in the zebrafish (Danio rerio). Dev Biol 180, 646-663.

Edmondson, D.E., Mattevi, A., Binda, C., Li, M., Hubalek, F., 2004. Structure and mechanism of monoamine oxidase. Curr Med Chem 11, 1983-1993.

Ekins, S., Reschly, E.J., Hagey, L.R., Krasowski, M.D., 2008. Evolution of pharmacologic specificity in the pregnane X receptor. BMC. Evol. Biol 8, 103.

Ellfolk, M., Norlin, M., Gyllensten, K., Wikvall, K., 2009. Regulation of human vitamin D(3) 25-hydroxylases in dermal fibroblasts and prostate cancer LNCaP cells. Mol. Pharmacol 75, 1392-1399.

EMA, 2012. Guideline on the investigation of drug interactions. www.ema.europa.eu/ema/pages/includes/document/open_document.jsp?webContentId=WC500129606.

Feo, M.L., Gross, M.S., McGarrigle, B.P., Eljarrat, E., Barcelo, D., Aga, D.S., Olson, J.R., 2013. Biotransformation of BDE-47 to potentially toxic metabolites is predominantly mediated by human CYP2B6. Environ. Health Perspect 121, 440-447.

Fischer, S., Kluver, N., Burkhardt-Medicke, K., Pietsch, M., Schmidt, A.M., Wellner, P., Schirmer, K., Luckenbach, T., 2013. Abcb4 acts as multixenobiotic transporter and active barrier against chemical uptake in zebrafish (Danio rerio) embryos. BMC Biol 11, 69.

Franklin, M.R., 1977. Toxic effects. Science 198, 1029-1030.

Genome Reference Consortium, 2015. GRCz10

Glisic, B., Mihaljevic, I., Popovic, M., Zaja, R., Loncar, J., Fent, K., Kovacevic, R., Smital, T., 2015. Characterization of glutathione-S-transferases in zebrafish (Danio rerio). Aquat Toxicol 158, 50-62.

Godard, C.A., Goldstone, J.V., Said, M.R., Dickerson, R.L., Woodin, B.R., Stegeman, J.J., 2005. The new vertebrate CYP1C family: cloning of new subfamily members and phylogenetic analysis. Biochem. Biophys. Res. Commun 331, 1016-1024.

Goldstone, J.V., Jonsson, M.E., Behrendt, L., Woodin, B.R., Jenny, M.J., Nelson, D.R., Stegeman, J.J., 2009. Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. Arch. Biochem. Biophys 482, 7-16.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC. Genomics 11, 643.

Goldstone, J.V., Stegeman, J.J., 2008. Gene structure of the novel cytochrome P4501D1 genes in stickleback (Gasterosteus aculeatus) and medaka (Oryzias latipes). Mar. Environ. Res 66, 19-20.

Guengerich, F.P., 1999. Cytochrome P-450 3A4: regulation and role in drug metabolism. Annu. Rev. Pharmacol. Toxicol 39, 1-17.

Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chem Res Toxicol 21, 70-83.

Hahn, M.E., 2002. Aryl hydrocarbon receptors: diversity and evolution. Chem Biol Interact 141, 131-160.

Hammoud, L., Adams, J.R., Loch, A.J., Marcellus, R.C., Uehling, D.E., Aman, A., Fladd, C., McKee, T.D., Jo, C.E., Al-Awar, R., Egan, S.E., Rossant, J., 2016. Identification of RSK and TTK as Modulators of Blood Vessel Morphogenesis Using an Embryonic Stem Cell-Based Vascular Differentiation Assay. Stem Cell Reports 7, 787-801.

Handschin, C., Meyer, U.A., 2003. Induction of drug metabolism: the role of nuclear receptors. Pharmacol. Rev 55, 649-673.

Hansen, J.M., Harris, C., 2015. Glutathione during embryonic development. Biochim Biophys Acta 1850, 1527-1542.

He, Q., Liu, K., Wang, S., Hou, H., Yuan, Y., Wang, X., 2012. Toxicity induced by emodin on zebrafish embryos. Drug Chem. Toxicol 35, 149-154.

Henry, T.R., Spitsbergen, J.M., Hornung, M.W., Abnet, C.C., Peterson, R.E., 1997. Early life stage toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish (Danio rerio). Toxicol. Appl. Pharmacol 142, 56-68.

Howe, K., Clark, M.D., Torroja, C.F., Torrance, J., Berthelot, C., Muffato, M., Collins, J.E., 2013. The zebrafish reference genome sequence and its relationship to the human genome. Nature 496, 498-503.

Huang, H., Wu, Q., 2010. Cloning and comparative analyses of the zebrafish Ugt repertoire reveal its evolutionary diversity. PloS One 5, e9144.

Incardona, J.P., Carls, M.G., Teraoka, H., Sloan, C.A., Collier, T.K., Scholz, N.L., 2005. Aryl hydrocarbon receptor-independent toxicity of weathered crude oil during fish development. Environ. Health Perspect 113, 1755-1762.

International Transporter, C., Giacomini, K.M., Huang, S.M., Tweedie, D.J., Benet, L.Z., Brouwer, K.L., Chu, X., Dahlin, A., Evers, R., Fischer, V., Hillgren, K.M., Hoffmaster, K.A., Ishikawa, T., Keppler, D., Kim, R.B., Lee, C.A., Niemi, M., Polli, J.W., Sugiyama, Y., Swaan, P.W., Ware, J.A., Wright, S.H., Yee, S.W., Zamek-Gliszczynski, M.J., Zhang, L., 2010. Membrane transporters in drug development. Nat Rev Drug Discov 9, 215-236.

Isin, E.M., Guengerich, F.P., 2008. Substrate binding to cytochromes P450. Anal Bioanal Chem 392, 1019-1030.

Jancova, P., Anzenbacher, P., Anzenbacherova, E., 2010. Phase II drug metabolizing enzymes. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 154, 103-116.

Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae in vivo. Zebrafish 7, 23-30.

Jonsson, M.E., Franks, D.G., Woodin, B.R., Jenny, M.J., Garrick, R.A., Behrendt, L., Hahn, M.E., Stegeman, J.J., 2009. The tryptophan photoproduct 6-formylindolo[3,2-b]carbazole (FICZ) binds multiple AHRs and induces multiple CYP1 genes via AHR2 in zebrafish. Chem. Biol. Interact 181, 447-454.

Jonsson, M.E., Jenny, M.J., Woodin, B.R., Hahn, M.E., Stegeman, J.J., 2007a. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Sci 100, 180-193.

Jonsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007b. Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicol. Appl. Pharmacol 221, 29-41.

Karchner, S.I., Franks, D.G., Hahn, M.E., 2005. AHR1B, a new functional aryl hydrocarbon receptor in zebrafish: tandem arrangement of ahr1b and ahr2 genes. Biochem. J 392, 153-161.

Kirischian, N., McArthur, A.G., Jesuthasan, C., Krattenmacher, B., Wilson, J.Y., 2011. Phylogenetic and functional analysis of the vertebrate cytochrome p450 2 family. J. Mol. Evol 72, 56-71.

Krueger, S.K., Williams, D.E., 2005. Mammalian flavin-containing monooxygenases: structure/function, genetic polymorphisms and role in drug metabolism. Pharmacol Ther 106, 357-387.

Kubota, A., Bainy, A.C., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2013. The cytochrome P450 2AA gene cluster in zebrafish (Danio rerio): expression of CYP2AA1 and CYP2AA2 and response to phenobarbital-type inducers. Toxicol Appl Pharmacol 272, 172-179.

Kubota, A., Goldstone, J.V., Lemaire, B., Takata, M., Woodin, B.R., Stegeman, J.J., 2015. Role of pregnane X receptor and aryl hydrocarbon receptor in transcriptional regulation of pxr, CYP2, and CYP3 genes in developing zebrafish. Toxicol Sci 143, 398-407.

Kubota, A., Stegeman, J.J., Woodin, B.R., Iwanaga, T., Harano, R., Peterson, R.E., Hiraga, T., Teraoka, H., 2011. Role of zebrafish cytochrome P450 CYP1C genes in the reduced mesencephalic vein blood flow caused by activation of AHR2. Toxicol. Appl. Pharmacol 253, 244-252.

Kurogi, K., Liu, T.A., Sakakibara, Y., Suiko, M., Liu, M.C., 2013. The use of zebrafish as a model system for investigating the role of the SULTs in the metabolism of endogenous compounds and xenobiotics. Drug Metab Rev 45, 431-440.

Le Fol, V., Ait-Aissa, S., Cabaton, N., Dolo, L., Grimaldi, M., Balaguer, P., Perdu, E., Debrauwer, L., Brion, F., Zalko, D., 2015. Cell-specific biotransformation of benzophenone-2 and bisphenol-s in zebrafish and human in vitro models used for toxicity and estrogenicity screening. Environ Sci Technol 49, 3860-3868.

Lewis, D.F., Lake, B.G., George, S.G., Dickins, M., Eddershaw, P.J., Tarbit, M.H., Beresford, A.P., Goldfarb, P.S., Guengerich, F.P., 1999. Molecular modelling of CYP1 family enzymes

CYP1A1, CYP1A2, CYP1A6 and CYP1B1 based on sequence homology with CYP102. Toxicology 139, 53-79.

Li, X., Ma, J., Fang, Q., Li, Y., 2013. Transcription alterations of microRNAs, cytochrome P4501A1 and 3A65, and AhR and PXR in the liver of zebrafish exposed to crude microcystins. Toxicon 73, 17-22.

Liddle, C., Goodwin, B., 2002. Regulation of hepatic drug metabolism: role of the nuclear receptors PXR and CAR. Semin. Liver Dis 22, 115-122.

Lin, C.H., Su, C.H., Tseng, D.Y., Ding, F.C., Hwang, P.P., 2012. Action of vitamin D and the receptor, VDRa, in calcium handling in zebrafish (Danio rerio). PloS One 7, e45650.

Long, Y., Li, Q., Cui, Z., 2011. Molecular analysis and heavy metal detoxification of ABCC1/MRP1 in zebrafish. Mol Biol Rep 38, 1703-1711.

Ma, Q., 2001. Induction of CYP1A1. The AhR/DRE paradigm: transcription, receptor regulation, and expanding biological roles. Curr Drug Metab 2, 149-164.

Maes, J., Verlooy, L., Buenafe, O.E., de Witte, P.A., Esguerra, C.V., Crawford, A.D., 2012. Evaluation of 14 organic solvents and carriers for screening applications in zebrafish embryos and larvae. PloS One 7, e43850.

Mattingly, C.J., Toscano, W.A., 2001. Posttranscriptional silencing of cytochrome P4501A1 (CYP1A1) during zebrafish (Danio rerio) development. Dev. Dyn 222, 645-654.

Merchant, M., Wang, X., Kamps, C., Rosengren, R., Morrison, V., Safe, S., 1992. Mechanism of benzo[a]pyrene-induced Cyp1a-1 gene expression in mouse Hepa 1c1c7 cells: role of the nuclear 6 s and 4 s proteins. Arch. Biochem. Biophys 292, 250-257.

Meyer, A., Schartl, M., 1999. Gene and genome duplications in vertebrates: the one-to-four (-to-eight in fish) rule and the evolution of novel gene functions. Curr Opin Cell Biol 11, 699-704.

Miranda, C.L., Henderson, M.C., Buhler, D.R., 1998. Evaluation of chemicals as inhibitors of trout cytochrome P450s. Toxicol. Appl. Pharmacol 148, 237-244.

Mooij, M.G., Nies, A.T., Knibbe, C.A., Schaeffeler, E., Tibboel, D., Schwab, M., de Wildt, S.N., 2016. Development of Human Membrane Transporters: Drug Disposition and Pharmacogenetics. Clin Pharmacokinet 55, 507-524.

Moore, L.B., Maglich, J.M., McKee, D.D., Wisely, B., Willson, T.M., Kliewer, S.A., Lambert, M.H., Moore, J.T., 2002. Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. Mol. Endocrinol 16, 977-986.

Mosadeghi, S., Furnes, B., Matsuo, A.Y., Schlenk, D., 2007. Expression and characterization of cytochrome P450 2X1 in channel catfish (Ictalurus punctatus). Biochim. Biophys. Acta 1770, 1045-1052.

Mota, L.C., Barfield, C., Hernandez, J.P., Baldwin, W.S., 2011. Nonylphenol-mediated CYP induction is PXR-dependent: The use of humanized mice and human hepatocytes suggests that hPXR is less sensitive than mouse PXR to nonylphenol treatment. Toxicol. Appl. Pharmacol 252, 259-267.

Nelson, D.R., 2009. The cytochrome p450 homepage. Hum Genomics 4, 59-65.

Nelson, D.R., Zeldin, D.C., Hoffman, S.M., Maltais, L.J., Wain, H.M., Nebert, D.W., 2004. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. Pharmacogenetics 14, 1-18.

Nilsen, B.M., Berg, K., Goksoyr, A., 1998. Induction of cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. Methods Mol Biol 107, 423-438.

Niwa, T., Shiraga, T., Yamasaki, S., Ishibashi, K., Ohno, Y., Kagayama, A., 2003. In vitro activation of 7-benzyloxyresorufin O-debenzylation and nifedipine oxidation in human liver microsomes. Xenobiotica 33, 717-729.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Publishing.

Oleksiak, M.F., Wu, S., Parker, C., Karchner, S.I., Stegeman, J.J., Zeldin, D.C., 2000. Identification, functional characterization, and regulation of a new cytochrome P450 subfamily, the CYP2Ns. J. Biol. Chem 275, 2312-2321.

Oleksiak, M.F., Wu, S., Parker, C., Qu, W., Cox, R., Zeldin, D.C., Stegeman, J.J., 2003. Identification and regulation of a new vertebrate cytochrome P450 subfamily, the CYP2Ps, and functional characterization of CYP2P3, a conserved arachidonic acid epoxygenase/19-hydroxylase. Arch. Biochem. Biophys 411, 223-234.

Olsvik, P.A., Lie, K.K., Sturve, J., Hasselberg, L., Andersen, O.K., 2009. Transcriptional effects of nonylphenol, bisphenol A and PBDE-47 in liver of juvenile Atlantic cod (Gadus morhua). Chemosphere 75, 360-367.

Otte, J.C., Schmidt, A.D., Hollert, H., Braunbeck, T., 2010. Spatio-temporal development of CYP1 activity in early life-stages of zebrafish (Danio rerio). Aquat. Toxicol 100, 38-50.

Pfeiffer, E., Metzler, M., 2004. Effect of bisphenol A on drug metabolising enzymes in rat hepatic microsomes and precision-cut rat liver slices. Arch. Toxicol 78, 369-377.

Qiu, H., Taudien, S., Herlyn, H., Schmitz, J., Zhou, Y., Chen, G., Roberto, R., Rocchi, M., Platzer, M., Wojnowski, L., 2008. CYP3 phylogenomics: evidence for positive selection of CYP3A4 and CYP3A7. Pharmacogenet. Genomics 18, 53-66.

Reschly, E.J., Krasowski, M.D., 2006. Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. Curr. Drug Metab 7, 349-365.

Rodriguez-Fuentes, G., Aparicio-Fabre, R., Li, Q., Schlenk, D., 2008. Osmotic regulation of a novel flavin-containing monooxygenase in primary cultured cells from rainbow trout (Oncorhynchus mykiss). Drug Metab Dispos 36, 1212-1217.

Sau, A., Pellizzari Tregno, F., Valentino, F., Federici, G., Caccuri, A.M., 2010. Glutathione transferases and development of new principles to overcome drug resistance. Arch Biochem Biophys 500, 116-122.

Schenkman, J.B., Jansson, I., 2006. Spectral analyses of cytochromes P450. Methods Mol Biol 320, 11-18.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010a. Cytochrome P450-mediated 17beta-estradiol metabolism in zebrafish (Danio rerio). J. Endocrinol 206, 317-325.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010b. Functional differences in the cytochrome P450 1 family enzymes from zebrafish (Danio rerio) using heterologously expressed proteins. Arch. Biochem. Biophys 502, 17-22.

Seiler, A., Visan, A., Buesen, R., Genschow, E., Spielmann, H., 2004. Improvement of an in vitro stem cell assay for developmental toxicity: the use of molecular endpoints in the embryonic stem cell test. Reprod Toxicol 18, 231-240.

Shimada, T., Yamazaki, H., Mimura, M., Wakamiya, N., Ueng, Y.F., Guengerich, F.P., Inui, Y., 1996. Characterization of microsomal cytochrome P450 enzymes involved in the oxidation of xenobiotic chemicals in human fetal liver and adult lungs. Drug Metab Dispos 24, 515-522.

Spence, R., Gerlach, G., Lawrence, C., Smith, C., 2008. The behaviour and ecology of the zebrafish, Danio rerio. Biol Rev Camb Philos Soc 83, 13-34.

Stegeman, J.J., Behrendt, L., Woodin, B.R., Kubota, A., Lemaire, B., Pompon, D., Goldstone, J.V., Urban, P., 2015. Functional characterization of zebrafish cytochrome P450 1 family proteins expressed in yeast. Biochim Biophys Acta 1850, 2340-2352.

Strushkevich, N., Usanov, S.A., Plotnikov, A.N., Jones, G., Park, H.W., 2008. Structural analysis of CYP2R1 in complex with vitamin D3. J. Mol. Biol 380, 95-106.

Sueyoshi, T., Li, L., Wang, H., Moore, R., Kodavanti, P.R., Lehmler, H.J., Negishi, M., Birnbaum, L.S., 2014. Flame retardant BDE-47 effectively activates nuclear receptor CAR in human primary hepatocytes. Toxicol Sci 137, 292-302.

Teraoka, H., Dong, W., Tsujimoto, Y., Iwasa, H., Endoh, D., Ueno, N., Stegeman, J.J., Peterson, R.E., Hiraga, T., 2003. Induction of cytochrome P450 1A is required for circulation failure and edema by 2,3,7,8-tetrachlorodibenzo-p-dioxin in zebrafish. Biochem. Biophys. Res. Commun 304, 223-228.

Timme-Laragy, A.R., Goldstone, J.V., Imhoff, B.R., Stegeman, J.J., Hahn, M.E., Hansen, J.M., 2013. Glutathione redox dynamics and expression of glutathione-related genes in the developing embryo. Free Radic Biol Med 65, 89-101.

Timme-Laragy, A.R., Noyes, P.D., Buhler, D.R., Di Giulio, R.T., 2008. CYP1B1 knockdown does not alter synergistic developmental toxicity of polycyclic aromatic hydrocarbons in zebrafish (Danio rerio). Mar. Environ. Res 66, 85-87.

Tseng, H.P., Hseu, T.H., Buhler, D.R., Wang, W.D., Hu, C.H., 2005. Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. Toxicol. Appl. Pharmacol 205, 247-258.

Tukey, R.H., Strassburg, C.P., 2001. Genetic multiplicity of the human UDP-glucuronosyltransferases and regulation in the gastrointestinal tract. Mol Pharmacol 59, 405-414.

Uno, T., Ishizuka, M., Itakura, T., 2012. Cytochrome P450 (CYP) in fish. Environ Toxicol Pharmacol 34, 1-13.

Uno, Y., Uehara, S., Murayama, N., Yamazaki, H., 2011. CYP1D1, pseudogenized in human, is expressed and encodes a functional drug-metabolizing enzyme in cynomolgus monkey. Biochem. Pharmacol 81, 442-450.

Van Dartel, D.A., Pennings, J.L., Hendriksen, P.J., van Schooten, F.J., Piersma, A.H., 2009. Early gene expression changes during embryonic stem cell differentiation into cardiomyocytes and their modulation by monobutyl phthalate. Reprod Toxicol 27, 93-102.

Wang-Buhler, J.L., Lee, S.J., Chung, W.G., Stevens, J.F., Tseng, H.P., Hseu, T.H., Hu, C.H., Westerfield, M., Yang, Y.H., Miranda, C.L., Buhler, D.R., 2005. CYP2K6 from zebrafish (Danio rerio): cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. Comp Biochem. Physiol C. Toxicol. Pharmacol 140, 207-219.

Wang, J., Wei, Y., Li, X., Xu, M., Dai, J., 2007a. Identification of differentially expressed genes from contaminant and thermal exposed goldfish Carassius auratus in Gaobeidian Lake in Beijing, China. Ecotoxicology 16, 525-532.

Wang, L., Scheffler, B.E., Willett, K.L., 2006. CYP1C1 messenger RNA expression is inducible by benzo[a]pyrene in Fundulus heteroclitus embryos and adults. Toxicol. Sci 93, 331-340.

Wang, L., Yao, J., Chen, L., Chen, J., Xue, J., Jia, W., 2007b. Expression and possible functional roles of cytochromes P450 2J1 (zfCyp 2J1) in zebrafish. Biochem Biophys Res Commun 352, 850-855.

Wang, Y., Wei, X., Xiao, X., Hui, R., Card, J.W., Carey, M.A., Wang, D.W., Zeldin, D.C., 2005. Arachidonic acid epoxygenase metabolites stimulate endothelial cell growth and angiogenesis via mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt signaling pathways. J Pharmacol Exp Ther 314, 522-532.

Waxman, D.J., 1999. P450 gene induction by structurally diverse xenochemicals: central role of nuclear receptors CAR, PXR, and PPAR. Arch. Biochem. Biophys 369, 11-23.

Webster, W.S., Brown-Woodman, P.D., Ritchie, H.E., 1997. A review of the contribution of whole embryo culture to the determination of hazard and risk in teratogenicity testing. Int J Dev Biol 41, 329-335.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology 281, 25-36.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2012. Developmental effects of coumarin and the anticoagulant coumarin derivative warfarin on zebrafish (Danio rerio) embryos. Reprod. Toxicol 33, 133-141.

Wiegand, C., Krause, E., Steinberg, C., Pflugmacher, S., 2001. Toxicokinetics of atrazine in embryos of the zebrafish (Danio rerio). Ecotoxicol Environ Saf 49, 199-205.

Wobus, A.M., Loser, P., 2011. Present state and future perspectives of using pluripotent stem cells in toxicology research. Arch Toxicol 85, 79-117.

Yang, L., Ho, N.Y., Alshut, R., Legradi, J., Weiss, C., Reischl, M., Mikut, R., Liebel, U., Muller, F., Strahle, U., 2009. Zebrafish embryos as models for embryotoxic and teratological effects of chemicals. Reprod Toxicol 28, 245-253.

Yasuda, K., Endo, M., Ikushiro, S., Kamakura, M., Ohta, M., Sakaki, T., 2013. UV-dependent production of 25-hydroxyvitamin D2 in the recombinant yeast cells expressing human CYP2R1. Biochem. Biophys. Res. Commun 434, 311-315.

Yin, H., Bai, P., Miao, P., Chen, M., Hu, J., Deng, X., Yin, J., 2016. Functional expressions of adenosine triphosphate-binding cassette transporters during the development of zebrafish embryos and their effects on the detoxification of cadmium chloride and beta-naphthoflavone. J Appl Toxicol 36, 925-935.

Yin, H.C., Tseng, H.P., Chung, H.Y., Ko, C.Y., Tzou, W.S., Buhler, D.R., Hu, C.H., 2008. Influence of TCDD on zebrafish CYP1B1 transcription during development. Toxicol. Sci 103, 158-168.

Yuan, L., Lv, B., Zha, J., Wang, Z., Wang, W., Li, W., Zhu, L., 2013. New cytochrome P450 1B1, 1C1, 2Aa, 2Y3, and 2K genes from Chinese rare minnow (Gobiocypris rarus): Molecular characterization, basal expression and response of rare minnow CYP1s and CYP2s mRNA exposed to the AHR agonist benzo[a]pyrene. Chemosphere 93, 209-216.

Zanette, J., Jenny, M.J., Goldstone, J.V., Woodin, B.R., Watka, L.A., Bainy, A.C., Stegeman, J.J., 2009. New cytochrome P450 1B1, 1C2 and 1D1 genes in the killifish Fundulus heteroclitus: Basal expression and response of five killifish CYP1s to the AHR agonist PCB126. Aquat. Toxicol 93, 234-243.

Zhao, X., Monson, C., Gao, C., Gouon-Evans, V., Matsumoto, N., Sadler, K.C., Friedman, S.L., 2010. Klf6/copeb is required for hepatic outgrowth in zebrafish and for hepatocyte specification in mouse ES cells. Dev. Biol 344, 79-93.

Zheng, S., Chen, B., Qiu, X., Lin, K., Yu, X., 2013. Three novel cytochrome P450 genes identified in the marine polychaete Perinereis nuntia and their transcriptional response to xenobiotics. Aquat. Toxicol 134-135, 11-22.

Zur Nieden, N.I., Kempka, G., Ahr, H.J., 2004. Molecular multiple endpoint embryonic stem cell test—a possible approach to test for the teratogenic potential of compounds. Toxicol Appl Pharmacol 194, 257-269.

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Aims and Objectives

The need for alternative screening assays in toxicology that reduce costs, time, and use of animals is increasing. Zebrafish represent a promising animal model in view of the 3R principle, i.e. Reduce, Replace, Refine, in addition to several advantageous characteristics such as synteny to humans. In general, biotransformation (in which cytochrome P450 enzymes play a fundamental role) changes the chemical characteristics of xenobiotics and consequently their pharmacological and toxic nature. Differences in CYP–mediated biotransformation have already been reported between humans and conventionally used animals for non-clinical studies such as rats, mice and dogs but also monkeys (Bogaards et al., 2000). As zebrafish are also becoming an important preclinical species for toxicity assessment, the assessment of xenobiotic biotransformation, and particularly CYP activity, in adult zebrafish and its potential differences with man (Figure 1) is critical for a correct interpretation of toxicity data. Additionally, as zebrafish embryos are being used for teratogenicity studies, CYP ontogeny in zebrafish plays a critical role in the (in)activation of xenobiotics. Lack of CYP activities were detected in humans during the early developmental stages. This lack of metabolic activity is compensated by the fully equipped metabolism system in the mother in case of mammals. However, no such compensation is present in zebrafish embryos due to the external fertilization.

In this regard, the following research hypotheses were tested:

- Zebrafish show a similar CYP-mediated drug metabolism as humans.
- Zebrafish embryos show no CYP-mediated drug metabolism during organogenesis.

To test these hypotheses, an *in vitro* approach, i.e. a microsomal stability assay (using liver homogenates from adult zebrafish and whole embryo body homogenates for the embryonic microsomal proteins at several developmental stages) was chosen for different reasons. First, *in vitro* approaches avoid confounding factors such as uptake, distribution and excretion which are mainly found in *in vivo* methods. Second, microsomal stability assay has the advantages

of easiness, reproducibility and CYP-selectivity compared to other *in vitro* assays such as recombinant enzymes and hepatocytes or liver slices (Jia and Liu, 2007). Additionally, the high concentration of CYPs in the microsomal protein fraction increases the depletion rates of substrates and production rates of metabolites, and subsequently improves the sensitivity of the assay (Jia and Liu, 2007). However, in this *in vitro* approach the CYP activity may be masked in zebrafish embryos due to dilution of the signal when using whole body homogenates and this should be taken into account when comparing the embryonic data with the adult data that are derived from liver homogenates, which contain highly concentrated CYPs.

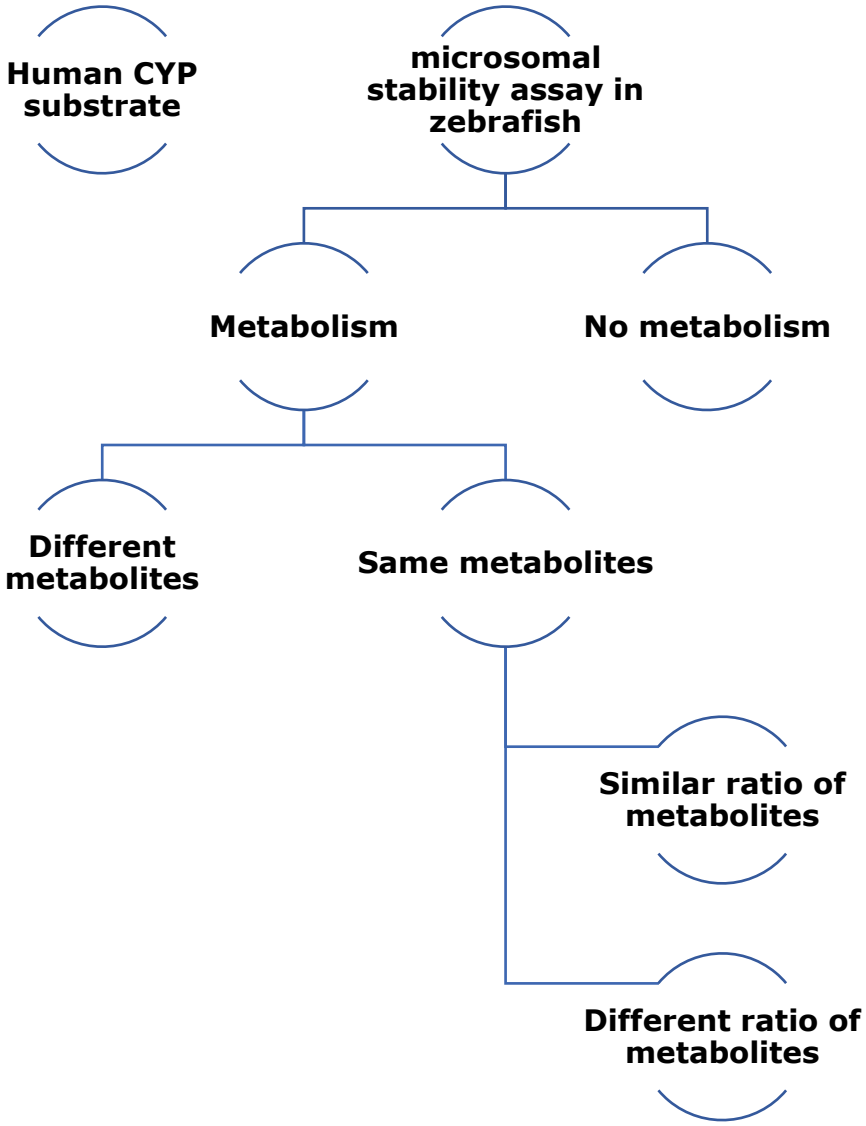

Figure 1: All possible scenarios in *in vitro* biotransformation of xenobiotics in zebrafish compared to man

As a first step the protocol of the microsomal stability assay was optimized for the zebrafish, including microsomal protein preparation, substrate and protein concentrations and reaction temperature.

In contrast to all previous studies, we aimed to quantify the biotransformation capacity in zebrafish using pharmacokinetic parameters such as V_{max} (maximum velocity) and K_m (substrate concentration at V_{max}). Five substrates were selected to be used for microsomal stability assay, i.e. 7-ethoxyresurofin (ER), diclofenac (DIC), dextromethorphan (DXM), testosterone (TST) and midazolam (MDZ), which are selective substrates for human CYP1A2, 2C9, 2D6 and 3A3/4. These five CYP isozymes are responsible for the biotransformation of approximately 75% of the commercial drugs in humans (Zanger and Schwab, 2013). Additionally, phenacetin (PHE) and tolbutamide (TLB), which are probes for human CYP1A2 and CYP2C9, respectively, were used during the initial experiments.

To test our research hypotheses, the following research objectives were formulated:

1- A state-of-the-art review on zebrafish CYP families 1, 2 and 3 focusing on spatiotemporal distribution, modulation and activity (chapter 1).
2- Assess CYP-mediated drug metabolism in adult zebrafish and zebrafish embryos/larvae at different developmental stages, and compare it to human drug metabolism using several human CYP-specific substrates for CYP1A, CYP2C9, CYP2D6 and CYP3A4 (chapters 3 and 4).
3- Evaluate any sex-related differences in zebrafish regarding protein yield and CYP-mediated drug metabolism as such determine whether studies can be limited to one gender or not (chapters 3 and 4).

4- Investigate in detail testosterone metabolism in adult zebrafish and 96 hpf embryos, and compare it to humans as testosterone was metabolized in both species but the main human metabolite was totally absent in zebrafish (chapter 5).
5- Compare CYP-mediated drug metabolism in zebrafish with other non-clinical species (chapter 5).

References

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., van Bladeren, P.J., Walther, B., 2000. Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 30, 1131-1152.

Jia, L., Liu, X., 2007. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 8, 822-829.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 138, 103-141.

3

In vitro CYP1A activity in the zebrafish

Adapted from:

In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage. Saad, M., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Maes, L., Cos, P., Van Cruchten, S., 2016. Reprod Toxicol 64, 50-56.

3.1 Abstract

The zebrafish (*Danio rerio*) is increasingly used as a screening model for acute, chronic and developmental toxicity. More specifically, the embryo is currently investigated as a replacement of *in vivo* developmental toxicity studies, although its biotransformation capacity remains a point of debate. As the cytochrome P450 1 (CYP1) family plays an important role in the biotransformation of several pollutants and drugs, a quantitative *in vitro* protocol was refined to assess gender- and age-related CYP1A activity in the zebrafish using the ethoxyresorufin-o-deethylase (EROD) assay. Microsomal protein fractions were prepared from livers of adult males and females, ovaries and whole embryo homogenates of different developmental stages. A large biological variation but no gender-related difference in CYP1A activity was observed in adult zebrafish. Embryos showed distinct temporal but low CYP1A activity during organogenesis. These *in vitro* data raise questions on the bioactivation capacity of zebrafish embryos in developmental toxicity studies.

3.2 Introduction

The zebrafish embryo is gaining a lot of interest as an in vitro model to assess the teratogenic liability of drugs and chemicals. Initially intended as a screening tool (Chapin et al., 2008), the focus on the zebrafish embryo is now more directed towards a reduction or even replacement of in vivo tests for developmental toxicity (Beker van et al., 2013; Panzica-Kelly et al., 2012). The recent acceptance of this model as a validated alternative for acute toxicity testing of chemicals (Test Guideline 236) (OECD) also triggered its exploration as an alternative for other types of toxicity testing (Di Paolo et al., 2015; Wang et al., 2015). As toxic and pharmacological effects may also be exerted by metabolites of the parent compound, knowledge on the bioactivation potential and overall biotransformation capacity is pivotal for a correct interpretation of toxicity studies in zebrafish. Differences in drug metabolism between zebrafish and man have been reported previously (Saad et al., 2016), and this has also been shown for other preclinical species, such as rat and dog (Martignoni et al., 2006). However, the biotransformation capacity of zebrafish embryos and possible gender differences in adult zebrafish are still a point of debate and controversy (Gagnaire et al., 2013; Halden et al., 2011). Gender-dependent differences in xenobiotic metabolism have been clearly observed in rats, which justify, for this species, the use of both genders in risk assessment. Differences have also been reported between women and men, but inter-individual variability is considered to be more important in humans (Mugford and Kedderis, 1998; Waxman and Holloway, 2009). Therefore, knowledge on the presence or lack of gender-related biotransformation in the zebrafish may help in deciding whether one gender would be representative for the population or whether both genders are required in toxicity studies. Also for the zebrafish embryo, knowledge on its biotransformation capacity during organogenesis is key for developmental toxicity studies, as there is no maternal metabolism of the drug or chemical in contrast to *in*

vivo studies in mammals. Zebrafish embryos are directly exposed to the compound via the medium and as such a lack of or negligible bioactivation of the parent compound may lead to false negative results in the case of proteratogens, which impacts human risk assessment. The ultimate proof of lack or presence of bioactivation in the zebrafish embryo would be the absence or presence of malformations in embryos exposed to proteratogens. This has been done before (Weigt et al., 2011). However, the data are difficult to interpret, as one would need to be sure that only the metabolite(s) is/are teratogenic for the zebrafish embryo and not the parent compound itself. Additionally, other factors than bioactivation, such as absorption, distribution and excretion of the compound may complicate the results of a developmental toxicity study in the zebrafish. Therefore, we decided to first investigate the intrinsic biotransformation potential of zebrafish *in vitro*, i.e. by using microsomes of whole embryo homogenates and adult livers. These microsomes, which are subcellular fractions of endoplasmic reticulum obtained by (ultra)centrifugation steps, contain highly concentrated cytochrome P450 enzymes (CYPs) (Jia and Liu, 2007).

CYPs, and in particular the CYP1, CYP2 and CYP3 families are the most important groups of enzymes for bioactivation of xenobiotics (Guengerich, 2008). For the zebrafish, the genetic features and synteny are well characterized for the different CYP isoforms, as is their expression in different organs. However, CYP activity data are either scarce or conflicting (Saad et al., 2016). The CYP1 family is of a particular interest as it has a broad spectrum of substrates, including drugs and environmental pollutants (Zanger and Schwab, 2013). The ethoxyresorufin-o-deethylase (EROD) assay is one of the most commonly used tests to assess CYP1 activity in humans and fish, including zebrafish (Hegelund et al., 2004; Shimada et al., 1998), especially for CYP1A1 and CYP1A, respectively (Scornaienchi et al., 2010; Shimada et al., 1997). The high affinity of zebrafish CYP1A for ethoxyresorufin (ER) has been clearly demonstrated, as EROD activity was much lower in CYP1A morpholinos than in CYP1B1

morpholinos (Carney et al., 2004; Timme-Laragy et al., 2008). However, differences in study design, including applied substrate concentration, gender, developmental stage, incubation temperature, etc. make the interpretation of the available EROD data for adult zebrafish and embryos very difficult (Table 1).

The aims of the present study were to set-up a standardized *in vitro* protocol to assess CYP1A activity in zebrafish and to apply this assay to different developmental stages during organogenesis to determine the biotransformation potential of zebrafish embryos during their critical window for teratogens. In addition, possible gender differences were assessed in adult zebrafish as this may explain discrepancies between studies and as such also help deciding whether both genders should be used in toxicity studies or not.

Table 1: Published *in vitro* studies on EROD kinetics (velocity) in adult zebrafish (liver or whole body) and/or embryos, including number (n) of samples, gender (F: female, M: male), incubation temperature, ethoxyresorufin (ER) concentration and (ultra)centrifugation protocol (ND: not determined, ?: not known, hpf: hours post-fertilization, *: radius of rotation not mentioned).

Study	Velocity (pmol/min/mg protein)		Gender (Batches x n)	Temperature	ER concentration	(Ultra)centrifugation protocol
	Adult liver	Embryos				
Troxel *et al*. (1997) (Troxel et al., 1997)	23 ± 5	ND	3 x 2F	30°C	2 µM	1000 x g for 10 min
Mattingly and Toscano (2001) (Mattingly and Toscano, 2001)	ND	≈ 0 at 24 and 48 hpf ≈ 0.5 at 72 hpf ≈ 0.5 at 96 hpf	ND	30°C	≈ 10 µM	2500 rpm* for 10 min
Arukwe *et al*. (2008) (Arukwe et al., 2008)	≈ 40 (whole body)	ND	? (3 x 1)	37°C	1.2 µM	12 000 x g for 20 min 100 000 x g for 60 min
Jonsson *et al*. (2009) (Jonsson et al., 2009)	260 ± 180	ND	? (3 x 5-7)	Room temperature	≈ 10 µM	10 000 x g for 15 min 105 000 x g for 60 min
Otte *et al*. (2010) (Otte et al., 2010)	ND	0.3 at 8 hpf 0.1 at 32 hpf 0.2 at 56 hpf 0.2 at 80 hpf 0.3 at 104 hpf 0.2 at 128 hpf	ND	Room temperature	≈ 11.4 µM	10 000 x g for 15 min
Halden *et al*. (2011) (Halden et al., 2011)	≈ 9 in F ≈ 10 in M	ND	4 x 2F 4 x 2M	Room temperature	0.5 µM	10 000 x g for 20 min
Pauka *et al*. (2011) (Pauka et al., 2011)	≈ 18 (whole body – 2 weeks old)	ND	?	37°C	?	10 000 x g for 30 min
Gagnaire *et al.* (2013) (Gagnaire et al., 2013)	5 in F 9 in M	ND	3 x 1F 3 x 1M	27±1°C	2 µM	9 000 x g for 10 min

3.3 Material and methods

Chemicals

Phenylmethanesulfonyl fluoride (PMSF), 1,10-phenanthroline (PHEN), Ethylenediaminetetraacetic acid (EDTA), dimethyl sulfoxide (DMSO), Oil Red O, ER and resorufin (RS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). KH_2PO_4/K_2HPO_4 buffer was purchased from BD Gentest (Woburn, MA, USA). NADPH regenerating system solution A and B were obtained from Corning (Woburn, MA, USA). Total protein content was determined using the Pierce bicinchoninic acid assay (BCA Assay; Pierce Chemical, Rockford, IL, USA). This company also delivered the Halt Protease Inhibitor Cocktail®. Stock solutions of RS and ER were made by dissolving 1 mg in 50 ml and 5 ml DMSO, respectively. Aliquots were made and stored in dark reaction tubes (Greiner Bio-One GmbH, Frickenhausen, Germany) at -80°C. The absorbance was measured at 572 nm for RS and 482 nm for ER after diluting the stock solution 1:3 and 1:100 in 100 mM KH_2PO_4/K_2HPO_4 buffer. The exact concentrations were calculated using the extinction coefficient (ε RS =73.2 $M^{-1}.CM^{-1}$, ε ER =73.2 $M^{-1}.CM^{-1}$) (Klotz et al., 1984; Prough et al., 1978).

Animals and breeding

Adult (one to two years old) zebrafish (*Danio rerio*, in house wild type AB zebrafish line) were kept in glass aquaria of 60 L at a density of 1 fish/L, using a filtered system and day-night rhythm of 14/10 hours. The water parameters were 28 ± 1°C, 500 ± 40 $\mu S.cm^{-1}$ and 7.5 ± 0.3 of temperature, conductivity and pH. The water was renewed at least once in a fortnight to keep the levels of ammonia, nitrite and nitrate below the detection limits. Fish were fed three times daily: twice daily with thawed food (*Artemia nauplii*, *Daphnia* or *Chironomidae* larvae) and once daily with granulated food (Biogran medium; Prodac International, Cittadella, Italy). For embryo collection, the fish were put in spawning tanks before the light was turned on. Embryos

were collected 45 minutes after the start of spawning. Feces and coagulated eggs were removed and the embryos were gently washed using freshly prepared embryo solution, i.e. Instant Ocean® Sea Salt (Blacksburg, VA, USA) and sodium bicarbonate (VWR, Leuven, Belgium) dissolved in reverse osmosis water (conductivity 500 $\mu S.cm^{-1}$; pH 7.5). The embryos were kept in embryo solution under the same environmental conditions of light and temperature as for the adults. The embryo solution was renewed daily and dead embryos were removed. When the embryos reached the desired developmental stage, they were frozen in liquid nitrogen and stored at -80 °C to be used for the preparation of the embryo microsomal protein.

Tissue sampling

For the preparation of adult liver microsomes, each batch consisted of 10 females or 10 males. In total, five batches of each gender were prepared. Animals were fasted for 48 hours before they were euthanized by decapitation followed by a rapid destruction of the brains (Recommendation 2007/526/EC, Species Specific Section, Humane Killing Fish, p89). Livers were rinsed during the dissection process with pre-cooled washing buffer (100 mM KCl, 1 mM EDTA and 10 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4) to remove the remaining blood and avoid bile contamination. Additionally, both ovaries of 20 adult zebrafish were collected for microsomal protein preparation. For the embryo microsomal protein, five batches of embryos were collected for each of the six different developmental stages, i.e. 5, 24, 48, 72, 96 and 120 hpf (hours post-fertilization). In order to have a sufficient yield of microsomal protein, each batch consisted of at least 1500 embryos. The livers, ovaries and whole embryos were stored at -80°C until further use. The Ethical Committee of Animal Experimentation from the University of Antwerp (Belgium) approved the use of the animals in this study (ECD 2015-49).

Microsomal protein preparation

The protocol was adapted from Nilsen *et al*. (1998) (Nilsen et al., 1998). All steps of the microsomal protein preparation were performed at 4°C. Homogenization buffer (100 mM KCl, 1 mM EDTA, 0.1 mM PMSF, 0.1 mM PHEN, 1 unit of protease inhibitor cocktail per 15 ml of the final volume and 100 mM KH_2PO_4/K_2HPO_4 buffer at pH 7.4) was added 1:1 v/v for all tissues, which were then homogenized using a Potter-Elvehjem Tissue Grinder (VWR, Radnor, USA) at 580 rpm. Ultrasonication was applied five times for five seconds with intervals of 10 seconds and 75% amplitude using Ultrasonic Processor VCX 130 (Sonics, Newtown, USA). Homogenates were centrifuged at 12,000 × g for 20 minutes and the supernatants were collected. During the first step of centrifugation of the liver homogenates, a high fat load was present on the surface of the supernatant, particularly in females. In the case of the embryos, a high load of melanophores contaminated the supernatant after the first centrifugation step, especially from 48 hpf onwards. Therefore, the protocol was adjusted with an additional centrifugation at 12,000 × g for 20 minutes to obtain more pure and clear supernatants. After removal of the remaining lipid layer from each sample, the supernatants were ultracentrifuged, using Optima™ MAX-TL ultracentrifuge (Beckman Coulter, Brea, USA), at 105,000 × g for 1 hour, after which the pellets were resuspended in the homogenization buffer. This ultracentrifugation step was repeated. The final microsomal pellets were suspended in a storage buffer (homogenization buffer with 20% glycerol w/v), aliquoted and stored at -80ºC. The aliquots were used for EROD activity after determination of the protein concentration using the BCA Protein Assay. The reproducibility of this procedure was determined by evaluating different aliquots of one homogenate of liver tissues for protein content and EROD activity. Insect cell control Supersomes (BD Gentest, Woburn, MA, USA) and a pool of human liver microsomes (HLM) from 50 donors (HMMCPL-PL050B, Life Technologies, Thermo Fisher Scientific, Rockford, USA) were used as negative and positive controls, respectively.

Oil Red O staining

The livers of two males and two females were snap-frozen in liquid nitrogen. Cryosections were made and stained with Oil Red O to assess qualitatively possible gender differences in lipid content (Penney et al., 2002).

EROD assay

The incubation mixtures contained microsomal protein in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 with 5% and 1 % of NADPH regenerating system reagents A and B, respectively. Immediately after the start of the reaction that was induced by addition of the substrate (ER), fluorescence was measured at 28.5°C for 2 hours with intervals of 3 minutes using a Tecan GENios microplate reader (Tecan, Männedorf, Switzerland) at λ_{ex} 550 nm and λ_{em} 590 nm. A reaction temperature of 28.5°C was chosen as 26°C to 28.5°C is the optimal temperature range for zebrafish (embryos) (Matthews et al., 2002). For HLM, the EROD assay was performed at 28.5°C and 37°C to verify whether 28.5°C influenced the CYP activity, as 37°C is the body temperature for man. EROD activity is expressed as pmol of RS per mg microsomal protein per minute (pmol $RS.mg^{-1}.min^{-1}$). Standard concentrations of RS were made by diluting the stock solution in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4. Each experiment was performed in triplicate at different days.

Based upon the papers from Mattingly and Toscano (2001) and Otte *et al*. (2010), an initial concentration of 10 µM of ER was chosen to determine the optimal protein concentration for the final experiments (Mattingly and Toscano, 2001; Otte et al., 2010). This was conducted by incubation of 10 µM ER with 10 to 500 µg microsomal protein/ml (from 50 pooled livers of adult male and female zebrafish). The enzyme kinetic parameters (K_m and V_{max}) of ER biotransformation in adult zebrafish liver microsomes were determined using different ER concentrations ranging from 0 to 40 µM.

Data analysis

The values of the negative control were subtracted at each time point for all samples. Velocity of all reactions was calculated within the linear part of the reaction curve and data are presented as mean ± SD. The limit of detection (LOD) was calculated according to Armbuster and Pry (2008) (Armbruster and Pry, 2008). The kinetic parameters of ER biotransformation were determined using non-linear least-squares regression analysis of untransformed data (GraphPad Software, La Jolla, CA, USA) and data were fit to Michaelis Menten kinetics with an uncompetitive substrate inhibition model. The estimated parameters were the maximum reaction velocity (V_{max}) and the substrate concentration corresponding to 50% of the V_{max} (K_m). To assess normal distribution of the data and homogeneity of variances, the one-sample Kolmogorov-Smirnov test and Levene's test were used, respectively. A one-way ANOVA test (embryos) or *t* test (adults) was applied for data that showed a normal distribution and no significant differences among variances. When the data did not show a normal distribution, the Kruskal-Wallis test for non-parametric data was applied and pairwise comparisons were used to detect differences. Differences were considered significant when $p \leq 0.05$. All statistical analyses were performed by means of SPSS statistics version 20 (IBM, Armonk, NY, USA).

3.4 Results

Microsomal protein yield

The reproducibility of the preparation procedure for the microsomal protein from the same batch of zebrafish livers and its associated EROD activity was more than 90%. For the adult liver, which tended have larger volumes in females than in males, large inter-batch differences but no gender differences were present, i.e. 4.96 ± 2.84 and 4.57 ± 1.86 mg microsomal protein per 10 female and 10 male fish, respectively. For the ovaries, the microsomal protein yield was

1.07 ± 0.25 mg per fish. The yield of microsomal protein in the embryos increased significantly between 5 hpf and 72 hpf, after which it tended to slightly decrease again (Fig. 1).

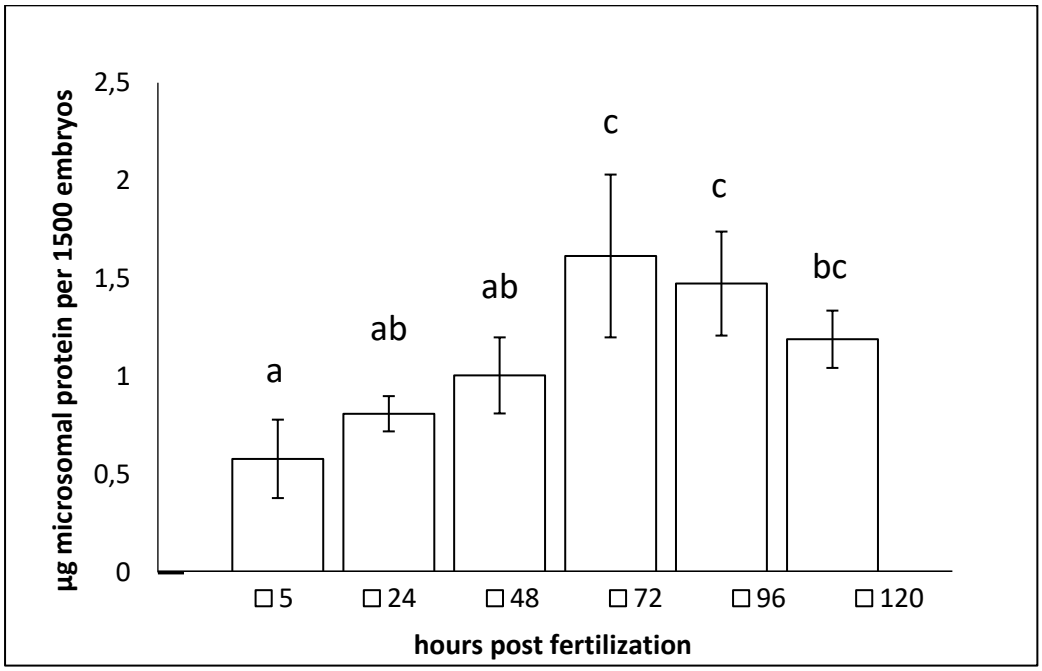

Figure 1: Microsomal protein yield of zebrafish embryos at different developmental stages (5, 24, 48, 72, 96 and 120 hpf). Bars represent the yield at each stage (mean ± SD, n = 5 batches of 1500 embryos). Significant differences ($p \leq 0.05$) between developmental stages are indicated by different letters.

Oil Red O staining

Female livers showed the presence of large lipid droplets in the hepatocytes whereas males showed a more condensed liver parenchyma (Fig. 2).

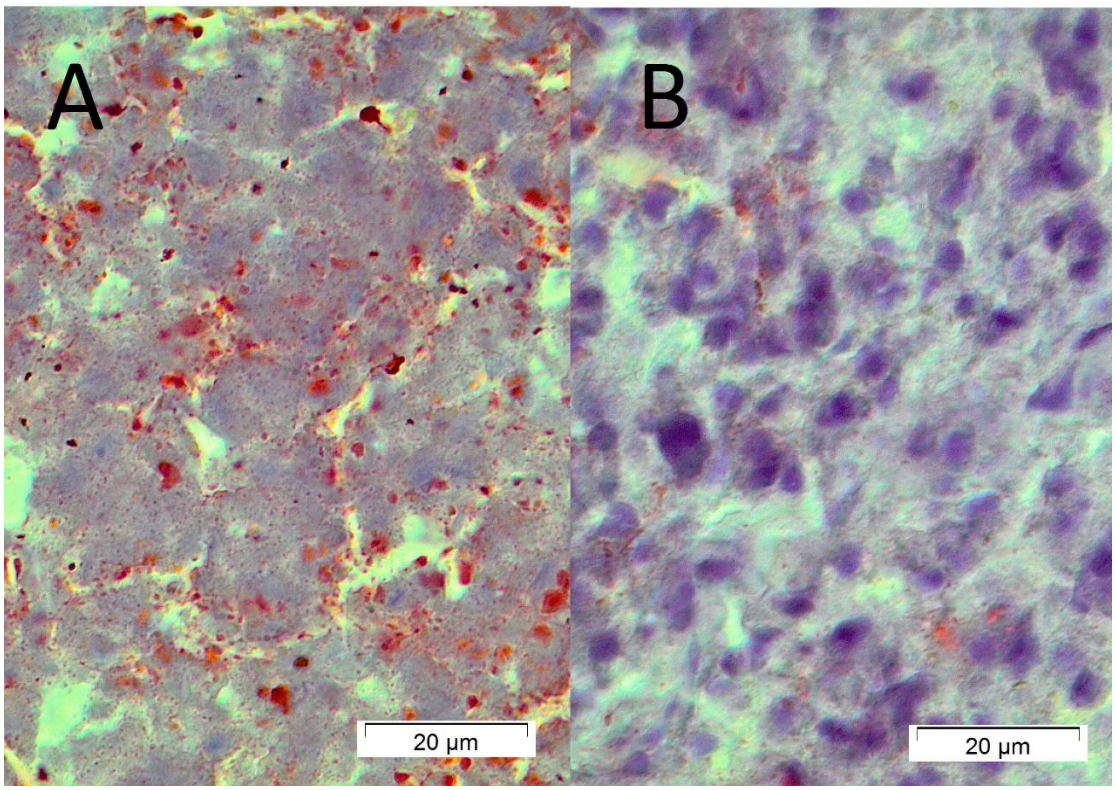

Figure 2: Oil Red O staining of adult zebrafish liver. A: Female liver tissue with high fat content (red stained dots). B: Male liver tissue with only scarce fat distribution and clear basophilic nuclei.

EROD assay

For the technical replicates, the repeatability of the EROD assay was more than 95% (Fig. 3). Additionally, HLM showed a similar EROD activity at 28.5°C and 37°C (difference less than 10%; data not shown). The LOD was 0.28 nM RS.

For the adult zebrafish liver microsomes, the optimal microsomal protein concentration was 100 µg/ml and enzyme kinetics showed a K_m of 0.63 ± 0.26 µM and a V_{max} 32.53 ± 5.04 pmol RS.mg^{-1}.min^{-1}. In view of the V_{max}, 1.2 µM ER (2-fold K_m) was used as substrate concentration for the final microsomal protein incubations of the different developmental stages, ovaries and different batches of adult female and male liver microsomes. EROD activity in the latter was 21.60 ± 4.70 and 17.60 ± 5.10 pmol RS.mg^{-1}.min^{-1} with inter-batch variations of 21.6% and 28.8%, respectively. No gender differences were noted. HLM showed a lower EROD activity

under the same conditions, i.e. 6.80 ± 0.31 pmol RS.mg^{-1}.min^{-1} (Fig. 3). The EROD activity in ovarian microsomes was 0.57 ± 0.33 pmol RS.mg^{-1}.min^{-1} with large inter-individual differences.

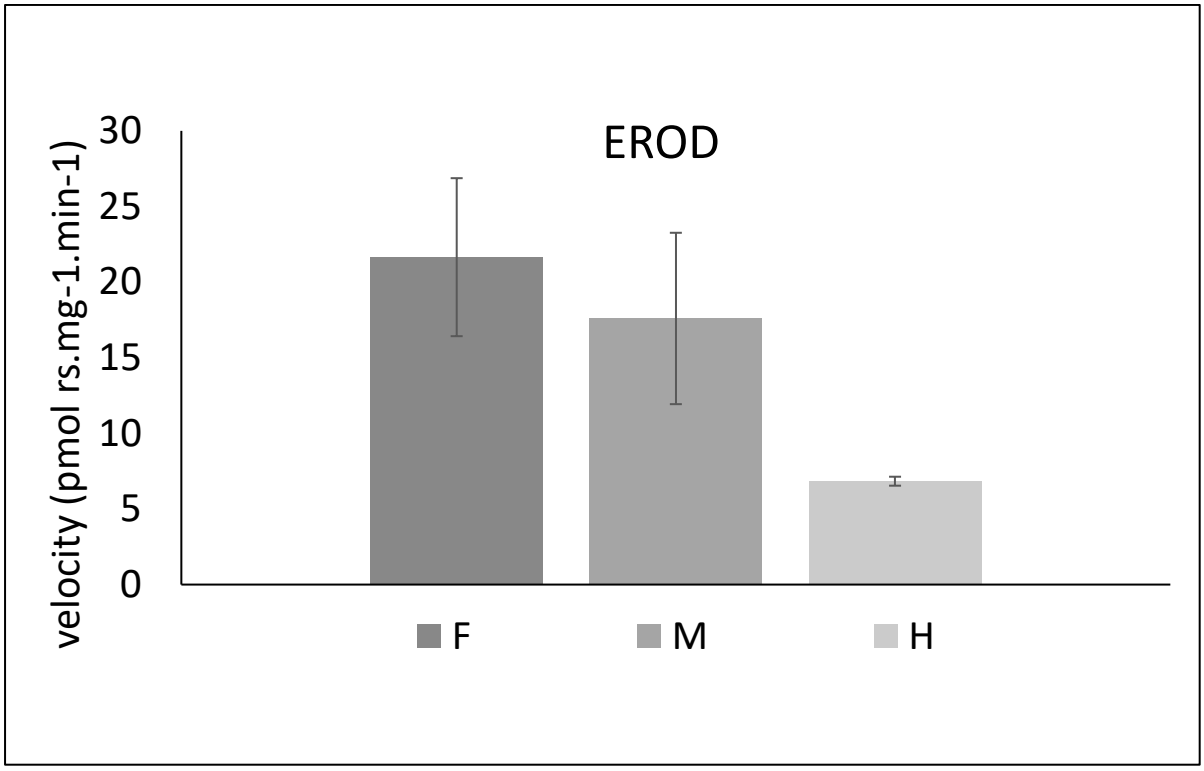

Figure 3: EROD activity in adult female (F) and male (M) zebrafish and human (H) liver microsomes as reference value. Bars of (F) and (M) represent the mean ± SD of 5 batches for each gender (10 fish in each batch). No gender difference was observed. For H, bars represent the mean ± SD of 3 technical replicates.

The reaction velocity was much lower in the different developmental stages than in the adult zebrafish, but a temporal trend was present (Fig. 4). The highest EROD activity was detected at 5 hpf with a large inter-batch variation (1.50 ± 1.40 pmol RS.mg^{-1}.min^{-1}). At 24 hpf and 48 hpf, EROD activity dropped to negligible levels (0.33 ± 0.29 and 0.14 ± 0.15 pmol RS.mg^{-1}.min^{-1}), whereas EROD activity tended to increase again at 72 hpf (0.60 ± 0.50 pmol RS.mg^{-1}.min^{-1}) and even further at the end of organogenesis, i.e. 96 hpf (0.91 ± 0.47 pmol RS.mg^{-1}.min^{-1}). At 120 hpf, negligible EROD activity, i.e. 0.31 ± 0.20 pmol RS.mg^{-1}.min^{-1} was

observed. EROD activity was significantly lower at 48 hpf than at 5 hpf ($p \leq 0.05$) whereas 96 hpf showed a trend towards higher levels compared to 48 hpf ($p = 0.068$).

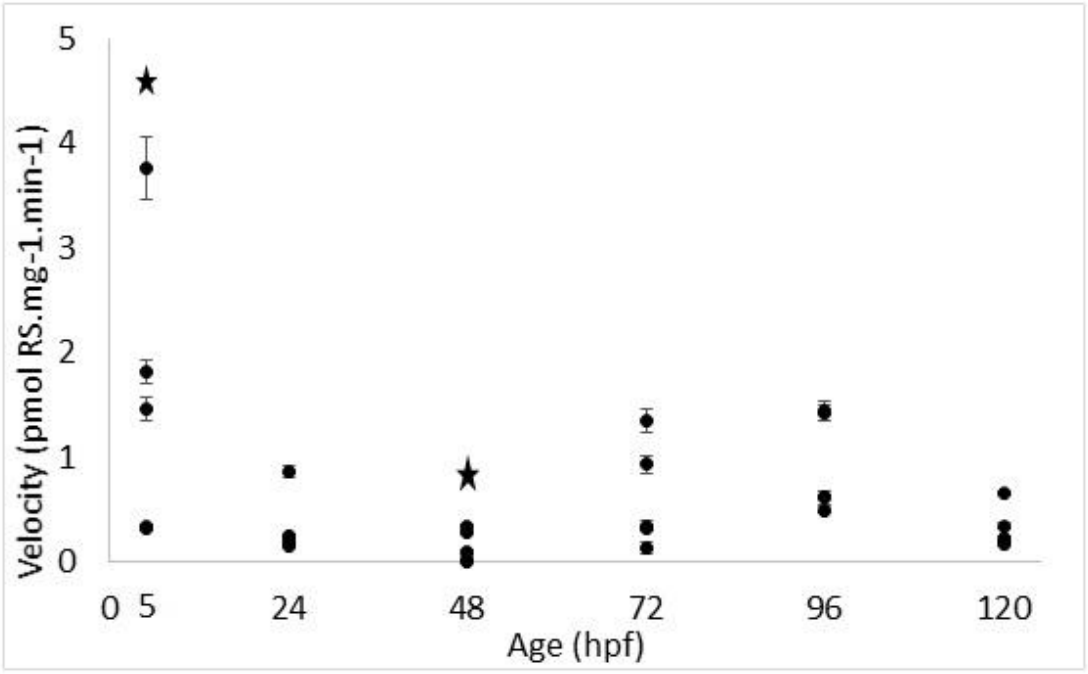

Figure 4: EROD activity of whole embryo microsomes at different developmental stages (5, 24, 48, 72, 96 and 120 hours post-fertilization (hpf)). Each developmental stage consists of 5 batches of pooled embryos (n= 1500/batch). The reaction velocity of each batch of embryos is plotted as a dot (i.e. mean ± SD of the 3 technical replicates). Stars represent significant differences ($p \leq 0.05$) between age groups.

3.5 Discussion

In vitro drug metabolism studies are part of the drug development process and can help in species selection for toxicity studies, i.e. the species with a metabolite profile the closest to man. However, other factors such as the expression of the target, pharmacokinetic data etc. are important as well. The most often used *in vitro* methods are: recombinant enzymes for reaction phenotyping, subcellular fractions such as microsomes or S9 fractions, and hepatocytes or liver slices. The use of recombinant enzymes is an effective way to further unravel metabolic

pathways but is too labor intensive and specific for a general screening of drug metabolism in several species. Cells and tissue slices of the liver are physiologically the most relevant samples to measure drug metabolism but enzymatic activities are not stable and obtaining and culturing cells from other organs can be very challenging (Jia and Liu, 2007). Therefore, the most commonly used tools for *in vitro* drug metabolism studies are the S9 fraction and microsomes. The S9 fraction, which contains microsomes and cytosol, gives a more complete picture of drug metabolism, as not only CYPs but also a wide variety of other enzymes, including Phase II enzymes, flavin-monooxygenases etc. are present. However, for CYP activity studies microsomes are the preferred method as they contain almost exclusively CYPs (Jia and Liu, 2007). This results in an approximately 4- to 5-fold higher CYP activity in the microsomal fraction than in the corresponding S9 fraction (Jia and Liu, 2007; Zhang et al., 2012). Therefore, we also opted to use the microsomal protein fraction in our *in vitro* drug metabolism protocol and this also explains why lower EROD velocities were observed for the adult male and female liver (Gagnaire et al., 2013; Halden et al., 2011) and zebrafish embryos (Otte et al., 2010) in studies using the S9 fraction. As such, the S9 fraction may underestimate the CYP activity in zebrafish embryos and adults. Furthermore, our standardized protocol showed a high reproducibility (> 90%) for the preparation procedure of zebrafish microsomes, which makes it a very reliable technique to also investigate other CYP isoforms in this species.

Interestingly, the liver of male and female zebrafish showed a similar microsomal protein yield despite the fact that female livers tended to be larger. The latter was related to the higher lipid content compared to males, which became evident in the first centrifugation step after liver homogenization and was also confirmed histologically by the Oil Red O staining. These findings are in accordance with Vliegenthart *et al.* (2014) who showed more vacuolated hepatocytes on paraffin sections in female zebrafish than in males (Vliegenthart et al., 2014), and with Peute *et al*. (1978) who studied the ultrastructure of female zebrafish livers and also

found a high lipid content, which they related to female reproductive function, i.e. vitellogenin synthesis (Peute et al., 1978). Besides the similar microsomal protein yield, we also noted similar EROD activity in both genders, but with a large biological variation. The latter could explain the large differences between earlier reported results on EROD activity in adult zebrafish liver (Table 1). As these studies only used a small number of male and/or female zebrafish, i.e. 3 to 4 groups of 2 to 3 adult fish (Gagnaire et al., 2013; Halden et al., 2011; Troxel et al., 1997), low or high EROD activity in one fish will obviously influence the mean of the group. Large inter-individual differences in CYP activity, often due to polymorphisms in CYP isoforms, have also been reported in man (Marinkovic et al., 2013) and are considered to be more important than gender-related differences (Mugford and Kedderis, 1998). This is in accordance with our CYP1A data and as such the zebrafish appears to be representative for human risk assessment, regardless of the gender. However, this should be further substantiated for other CYP isoforms. Furthermore, to avoid inter-individual variability as a confounder in EROD studies with different treatment groups, we recommend using at least 5 batches of (livers from) 10 fish (male, female or mixed gender) per group as we noted that the mean EROD activity of these batches was in the same range of the Vmax that was calculated from pooled liver microsomes of 50 male and female zebrafish.

For the embryos, the temporal increase in microsomal protein yield up to 72 hpf is not surprising as the embryonic genome gets activated around gastrulation (5.25 hpf) leading towards increased transcription and consecutive translation into proteins (Giraldez et al., 2006), which are important for embryonic growth and development. However, the decrease of the microsomal protein yield at later stages, i.e. 96 hpf and 120 hpf is intriguing. Subfractionation (by using density gradients) of microsomes (Beaufay et al., 1974) from whole embryo homogenates at different developmental stages could help to elucidate whether the lower protein yield at 96 hpf and 120 hpf is due to a lower absolute amount of microsomes or to a

different composition of the microsomal fraction as microsomes also contain phospholipids and RNA (Amar-Costesec et al., 1974).

A temporal trend was also observed for CYP1A activity in the zebrafish embryos. However, the activity was low during organogenesis, questioning the overall biotransformation capacity of embryos in view of human risk assessment. Indeed, when taking the inter-batch variation into account, the metabolite concentrations were often just above the LOD in several of the developmental stages. Despite this fact, subtle changes during development must be ongoing as Otte *et al.* (2010), who investigated CYP1 activity directly in homogenate supernatants of a limited number of zebrafish embryos (n = 25), showed a strikingly similar temporal trend for embryos at 8, 32, 56, 80, 104 and 128 hpf (Otte et al., 2010). The embryos at 8 hpf and 104 hpf had the highest CYP1 activity, which corresponds with our 5 hpf and 96 hpf data. This trend was further substantiated by strong EROD activity *in vivo* at these developmental stages. Indeed, intense RS fluorescence was noted in the envelope layer at 8 hpf and in the liver, intestine and circulatory system at 104 hpf, whereas a much milder staining was observed in the other stages, especially at 32 and 56 hpf (Otte et al., 2010). The EROD activity in the earliest embryos is not surprising as it is mainly maternally derived, which is further substantiated by the detection of CYP1A mRNA in unfertilized eggs (Goldstone et al., 2010) and zebrafish ovaries, although CYP1 mRNA levels were low in these latter (Jonsson et al., 2007b). This was also reflected by the low CYP1 ovarian activity that was observed in our study, which may be due to a dilution effect by the stromal cells in the ovary. The negligible CYP1 activity at 24 hpf and 48 hpf and the gradual increase at 72 hpf and 96 hpf also corresponds well with the ontogeny of CYP1 mRNA in the zebrafish (Jonsson et al., 2007a; Jonsson et al., 2007b). In these studies, CYP1 mRNA was detected as early as 8 hpf with a decrease at 24 hpf and a peak at 72 hpf. These results are to some extent in accordance with the findings of Mattingly & Toscano (2001) who could not detect any CYP1A activity or proteins until 72 hpf, the time

when the embryos hatched (Mattingly and Toscano, 2001). The higher CYP1 activity at 96 hpf, the end of organogenesis, and the clear drop at 120 hpf remains intriguing, as this was also observed *in vivo* by Otte *et al.* (2010). At 120 hpf the RS fluorescence was much less in the circulatory system (including the branchial arches, i.e. the future gills) and almost absent in the liver compared to the high intensity in these important metabolizing organs at 104 hpf (Otte et al., 2010). The reason for this drop in CYP1A activity remains unclear and it is also in contrast with a study of Goldstone *et al.* (2009) who showed a peak of CYP1A mRNA at 120 hpf (Goldstone et al., 2009). Furthermore, it is unknown when exactly zebrafish larvae attain adult CYP1A activity. From Pauka *et al.* (2011) we know that 2-week old zebrafish showed EROD activity that was similar to our adult data, whereas Alderton *et al.* (2010) showed only very low metabolite levels for several CYP substrates in 7-day old larvae (Alderton et al., 2010; Pauka et al., 2011). Hence, we can cautiously assume that CYP1A activity is fully mature in the zebrafish by 2 weeks of age.

In conclusion, our study showed that there is no gender difference in CYP1A activity in adult zebrafish but that large biological variability occurs both in adults and in embryos during organogenesis. Although a temporal trend in CYP1A activity was present, biotransformation remained low during organogenesis, which questions the bioactivation capacity of zebrafish embryos in developmental toxicity studies.

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3.7 References

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., Berthet, J., 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. J Cell Biol 61, 201-212.

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29 Suppl 1, S49-52.

Arukwe, A., Nordtug, T., Kortner, T.M., Mortensen, A.S., Brakstad, O.G., 2008. Modulation of steroidogenesis and xenobiotic biotransformation responses in zebrafish (Danio rerio) exposed to water-soluble fraction of crude oil. Environ Res 107, 362-370.

Beaufay, H., Amar-Costesec, A., Thines-Sempoux, D., Wibo, M., Robbi, M., Berthet, J., 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. 3. Subfractionation of the microsomal fraction by isopycnic and differential centrifugation in density gradients. J Cell Biol 61, 213-231.

Beker van, W.A., Wolterbeek, A., Te, B.L., Snel, C., Menke, A., Rubingh, C., de, G.D., Kroese, D., 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reprod. Toxicol 41, 35-44.

Carney, S.A., Peterson, R.E., Heideman, W., 2004. 2,3,7,8-Tetrachlorodibenzo-p-dioxin activation of the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator pathway causes developmental toxicity through a CYP1A-independent mechanism in zebrafish. Mol. Pharmacol 66, 512-521.

Chapin, R., Augustine-Rauch, K., Beyer, B., Daston, G., Finnell, R., Flynn, T., Hunter, S., Mirkes, P., O'Shea, K.S., Piersma, A., Sandler, D., Vanparys, P., Van Maele-Fabry, G., 2008. State of the art in developmental toxicity screening methods and a way forward: a meeting report addressing embryonic stem cells, whole embryo culture, and zebrafish. Birth Defects Res B Dev Reprod Toxicol 83, 446-456.

Di Paolo, C., Groh, K.J., Zennegg, M., Vermeirssen, E.L., Murk, A.J., Eggen, R.I., Hollert, H., Werner, I., Schirmer, K., 2015. Early life exposure to PCB126 results in delayed mortality and growth impairment in the zebrafish larvae. Aquat Toxicol 169, 168-178.

Gagnaire, B., Cavalie, I., Camilleri, V., Adam-Guillermin, C., 2013. Effects of depleted uranium on oxidative stress, detoxification, and defence parameters of zebrafish Danio rerio. Arch Environ Contam Toxicol 64, 140-150.

Giraldez, A.J., Mishima, Y., Rihel, J., Grocock, R.J., Van Dongen, S., Inoue, K., Enright, A.J., Schier, A.F., 2006. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312, 75-79.

Goldstone, J.V., Jonsson, M.E., Behrendt, L., Woodin, B.R., Jenny, M.J., Nelson, D.R., Stegeman, J.J., 2009. Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. Arch. Biochem. Biophys 482, 7-16.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC. Genomics 11, 643.

Guengerich, F.P., 2008. Cytochrome P450 and chemical toxicology. Chem Res Toxicol 21, 70-83.

Halden, A.N., Arnoldsson, K., Haglund, P., Mattsson, A., Ulleras, E., Sturve, J., Norrgren, L., 2011. Retention and maternal transfer of brominated dioxins in zebrafish (Danio rerio) and

effects on reproduction, aryl hydrocarbon receptor-regulated genes, and ethoxyresorufin-O-deethylase (EROD) activity. Aquat Toxicol 102, 150-161.

Hegelund, T., Ottosson, K., Radinger, M., Tomberg, P., Celander, M.C., 2004. Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. Environ Toxicol Chem 23, 1326-1334.

Jia, L., Liu, X., 2007. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 8, 822-829.

Jonsson, M.E., Brunstrom, B., Brandt, I., 2009. The zebrafish gill model: induction of CYP1A, EROD and PAH adduct formation. Aquat Toxicol 91, 62-70.

Jonsson, M.E., Jenny, M.J., Woodin, B.R., Hahn, M.E., Stegeman, J.J., 2007a. Role of AHR2 in the expression of novel cytochrome P450 1 family genes, cell cycle genes, and morphological defects in developing zebra fish exposed to 3,3',4,4',5-pentachlorobiphenyl or 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol. Sci 100, 180-193.

Jonsson, M.E., Orrego, R., Woodin, B.R., Goldstone, J.V., Stegeman, J.J., 2007b. Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. Toxicol. Appl. Pharmacol 221, 29-41.

Klotz, A.V., Stegeman, J.J., Walsh, C., 1984. An alternative 7-ethoxyresorufin O-deethylase activity assay: a continuous visible spectrophotometric method for measurement of cytochrome P-450 monooxygenase activity. Anal Biochem 140, 138-145.

Marinkovic, N., Pasalic, D., Potocki, S., 2013. Polymorphisms of genes involved in polycyclic aromatic hydrocarbons' biotransformation and atherosclerosis. Biochem Med (Zagreb) 23, 255-265.

Martignoni, M., Groothuis, G.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol 2, 875-894.

Matthews, M., Trevarrow, B., Matthews, J., 2002. A virtual tour of the Guide for zebrafish users. Lab Anim (NY) 31, 34-40.

Mattingly, C.J., Toscano, W.A., 2001. Posttranscriptional silencing of cytochrome P4501A1 (CYP1A1) during zebrafish (Danio rerio) development. Dev. Dyn 222, 645-654.

Mugford, C.A., Kedderis, G.L., 1998. Sex-dependent metabolism of xenobiotics. Drug Metab Rev 30, 441-498.

Nilsen, B.M., Berg, K., Goksoyr, A., 1998. Induction of cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. Methods Mol Biol 107, 423-438.

OECD, Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Publishing.

Otte, J.C., Schmidt, A.D., Hollert, H., Braunbeck, T., 2010. Spatio-temporal development of CYP1 activity in early life-stages of zebrafish (Danio rerio). Aquat. Toxicol 100, 38-50.

Panzica-Kelly, J.M., Zhang, C.X., Augustine-Rauch, K., 2012. Zebrafish embryo developmental toxicology assay. Methods Mol Biol 889, 25-50.

Pauka, L.M., Maceno, M., Rossi, S.C., Silva de Assis, H.C., 2011. Embryotoxicity and biotransformation responses in zebrafish exposed to water-soluble fraction of crude oil. Bull Environ Contam Toxicol 86, 389-393.

Penney, D.P., Powers, J.M., Frank, M., Willis, C., Churukian, C., 2002. Analysis and testing of biological stains--the Biological Stain Commission Procedures. Biotech Histochem 77, 237-275.

Peute, J., van der Gaag, M.A., Lambert, J.G., 1978. Ultrastructure and lipid content of the liver of the zebrafish, Brachydanio rerio, related to vitellogenin synthesis. Cell Tissue Res 186, 297-308.

Prough, R.A., Burke, M.D., Mayer, R.T., 1978. Direct fluorometric methods for measuring mixed function oxidase activity. Methods Enzymol 52, 372-377.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Ginneken, C.V., Cruchten, S.V., 2016. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. J Toxicol Sci 41, 1-11.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010. Functional differences in the cytochrome P450 1 family enzymes from zebrafish (Danio rerio) using heterologously expressed proteins. Arch. Biochem. Biophys 502, 17-22.

Shimada, T., Gillam, E.M., Sutter, T.R., Strickland, P.T., Guengerich, F.P., Yamazaki, H., 1997. Oxidation of xenobiotics by recombinant human cytochrome P450 1B1. Drug Metab Dispos 25, 617-622.

Shimada, T., Yamazaki, H., Foroozesh, M., Hopkins, N.E., Alworth, W.L., Guengerich, F.P., 1998. Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1. Chem Res Toxicol 11, 1048-1056.

Timme-Laragy, A.R., Noyes, P.D., Buhler, D.R., Di Giulio, R.T., 2008. CYP1B1 knockdown does not alter synergistic developmental toxicity of polycyclic aromatic hydrocarbons in zebrafish (Danio rerio). Mar. Environ. Res 66, 85-87.

Troxel, C.M., Buhler, D.R., Hendricks, J.D., Bailey, G.S., 1997. CYP1A induction by beta-naphthoflavone, Aroclor 1254, and 2,3,7,8-tetrachlorodibenzo-p-dioxin and its influence on aflatoxin B1 metabolism and DNA adduction in zebrafish. Toxicol Appl Pharmacol 146, 69-78.

Vliegenthart, A.D., Tucker, C.S., Del Pozo, J., Dear, J.W., 2014. Zebrafish as model organisms for studying drug-induced liver injury. Br J Clin Pharmacol 78, 1217-1227.

Wang, W.D., Chen, G.T., Hsu, H.J., Wu, C.Y., 2015. Aryl hydrocarbon receptor 2 mediates the toxicity of Paclobutrazol on the digestive system of zebrafish embryos. Aquat Toxicol 159, 13-22.

Waxman, D.J., Holloway, M.G., 2009. Sex differences in the expression of hepatic drug metabolizing enzymes. Mol Pharmacol 76, 215-228.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology 281, 25-36.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 138, 103-141.

Zhang, D., Luo, G., Ding, X., Lu, C., 2012. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. Acta Pharmaceutica Sinica B 2, 549-561.

4

In vitro CYP2 and 3 activity in the zebrafish

Adapted from:

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4.1 Abstract

The increasing use of zebrafish embryos as an alternative model for toxicological and pharmacological studies necessitates a better understanding of xenobiotic biotransformation in this species. As cytochrome P450 enzymes (CYPs) play an essential role in this process, *in vitro* drug metabolism of four human CYP-specific substrates, i.e. dextromethorphan (DXM), diclofenac (DIC), testosterone (TST) and midazolam (MDZ) was investigated in adult male and female zebrafish, and in zebrafish embryos and larvae up to 120 hours post-fertilization. Substrate depletion and production of their respective metabolites were measured using tandem quadrupole UPLC-MS/MS. Human liver microsomes were used as positive control. Adult zebrafish produced the two major human metabolites of DIC and DXM. For DIC the metabolite ratio was similar to that in man, whereas it was different for DXM. For TST, the major human metabolite could not be detected and MDZ was not metabolized. No sex-related differences were detected, except for the higher TST depletion rate in adult females. Zebrafish embryos and larvae showed no or only low biotransformation capacity. In conclusion, *in vitro* CYP-mediated drug metabolism in adult zebrafish shows differences compared to man and appears to be lacking in the early zebrafish life stages. As CYP-mediated drug metabolism in zebrafish may not be predictive for the one in man, we recommend including the zebrafish in metabolic stability testing of new compounds when considering non-clinical species for human risk assessment.

4.2 Introduction

Zebrafish and in particular their embryos/larvae are increasingly used as a model for pharmacological and toxicological studies. Indeed, due to their small size, low compound needs, high fecundity and external embryonic development, zebrafish represent a promising tool for preclinical risk assessment. Furthermore, within Europe zebrafish embryos are not considered as laboratory animals until the stage of independent feeding at 120 hours post-fertilization (hpf), which makes them ideal candidates for alternative testing (EC, 2010). As such, the zebrafish embryo has already been accepted as a validated alternative for the acute fish toxicity test (OECD TG236). Several efforts are ongoing to develop the zebrafish embryo assay as an alternative for developmental toxicity testing in mammals (Beker van et al., 2013; Brannen et al., 2010; Panzica-Kelly et al., 2012), but to fully reach that objective, the model needs further characterization. One of the remaining issues is their biotransformation capacity (Saad et al., 2016a), as embryos from oviparous species lack the maternal metabolism of the dosed drug or chemical that is present in the *in vivo* mammalian studies. This is particularly important for compounds that require bioactivation to exert their toxic effect, i.e. biotransformation of the parent compound into one or more active metabolites. For developmental toxicity, these compounds are called proteratogens. So, if the zebrafish embryo lacks this bioactivation capacity of proteratogens, false negative results will occur in the zebrafish embryo assay and this will impact human risk assessment.

Cytochrome P450 enzymes (CYPs), a superfamily of mono-oxygenases, and in particular the CYP1, 2 and 3 families, are key in the biotransformation and bioactivation of xenobiotics. In humans, five CYP isoforms, namely CYP1A2, CYP2C9, CYP2D6 and CYP3A4/5, are responsible for the oxidative biotransformation of about 70% of most clinically used drugs (Zanger and Schwab, 2013) and comprise around 65% of the CYP content in the liver (Shimada

et al., 1994). It is well-known that factors such as sex, age and inter-individual differences can affect CYP activity in mammals and may differ between species. Sex differences in CYP-related drug metabolism are less pronounced in humans, dogs, rabbits and monkeys compared to rats and mice (Waxman and Holloway, 2009). In contrast, inter-individual differences are more pronounced in man, which can be explained by the larger genetic variability compared to purpose-bred and more genetically uniform and defined laboratory animals (Shimada et al., 1994). In this respect, large groups are required to estimate a population effect of a compound when inter-individual differences exist (Waxman and Holloway, 2009). Age also appears to be an important factor, especially when considering the embryo-foetal development and risk assessments for the paediatric population. In several mammalian species including man, CYP-related drug metabolism tends to be significantly lower during early life stages (Hakkola et al., 1998; Hines and McCarver, 2002; Parkinson et al., 2004).

For the zebrafish, data on drug metabolism are scarce and fragmented. The majority of CYP-related studies have been focusing on gene expression profiles (reviewed in (Saad et al., 2016a)). However, as CYP activity is not necessarily correlated to its gene expression or even protein levels (Goldstein, 2001), more focus on the CYP activity is needed. In one of our previous studies, CYP1 activity in zebrafish was investigated using the *in vitro* ethoxyresorufin-o-deethylase (EROD) assay (Saad et al., 2016b), a fast, reproducible and easy screening tool. The present study aims to provide a more comprehensive view on the ontogeny of CYP activity during zebrafish organogenesis and on the possible differences in the *in vitro* CYP-mediated drug metabolism between zebrafish and man. This was conducted using the microsomal stability assay that has the advantages of simplicity, speed and sensitivity when compared to other *in vitro* methods such as liver slices, cell lines or primary hepatocytes. In addition, this *in vitro* approach avoids potential confounding factors that are present in *in vivo* models, such as absorption and distribution issues. For this purpose, we chose four model

substrates (dextromethorphan (DXM), diclofenac (DIC), testosterone (TST) and midazolam (MDZ)), that are directed towards four human CYPs considered key in human drug metabolism, including CYP2C9, CYP2D6 and CYP3A4/5 (EMA, 2012; FDA, 2016). In particular, the formation of 4′-hydroxydiclofenac (4-OHDIC) and dextrorphan (DRR) is known to be mediated by CYP2C9 and CYP2D6 (Zanger and Schwab, 2013), whereas the CYP3A4/5 pathway is mediating the formation of 5-hydroxydiclofenac (5-OHDIC), 6ß-hydroxytestosterone (6ß-OHTST), 1-hydroxymidazolam (1-OHMDZ), 4-hydroxymidazolam (4-OHMDZ) and 3-methoxymorphinan (3MM) (Bogaards et al., 2000; Jacqz-Aigrain et al., 1993; Patki et al., 2003; Shen et al., 1999). As such, this study will provide information that is relevant for human risk assessment.

4.3 Material and methods

Chemicals

Dimethyl sulfoxide (DMSO), DXM, DRR, 3MM, DIC, 4-OHDIC, TST, 6ß-OHTST, 1-OHMDZ, 4-OHMDZ and clonazepam (CLZ) were purchased from Sigma–Aldrich (St. Louis, MO, USA). 5-OHDIC was obtained from Santa Cruz Biotechnology (Texas, USA), MDZ from Hoffmann-La Roche AG (Basel, Switzerland), and HPLC gradient water (H-water) from Fisher Scientific (Loughborough, UK). KH_2PO_4/K_2HPO_4 buffer and NADPH regenerating system solution A and B were delivered by BD Gentest (Woburn, MA, USA). The Pierce bicinchoninic acid assay (BCA Assay) used for the determination of total protein content, and the Halt™ Protease Inhibitor Cocktail were obtained from Pierce Chemical (Rockford, IL, USA). All substrates were dissolved in DMSO to prepare the compound stock solutions and stored in aliquots at -20°C. The final concentration of the organic solvent was less than 0.5% (v/v) in all microsomal stability assays to prevent any effect on CYP activity (Chauret et al., 1998).

Animals and breeding

Zebrafish (*Danio rerio*, in-house wild-type AB zebrafish line) were housed as previously described (Saad et al., 2016b). Briefly, they were kept in a day-night rhythm of 14/10 hours while the temperature, conductivity and pH were 28 ± 1°C, 500 ± 40 µS.cm^{-1} and 7.5 ± 0.3. Embryos were kept under the same environmental conditions. The Ethical Committee for Animal Experimentation from University of Antwerp (Belgium) approved the use of the animals (ECD 2015-49).

Microsomal proteins preparation

Adult fish were euthanized by decapitation followed by a rapid destruction of the brains (Recommendation 2007/526/EC, p89). In order to obtain sufficient microsomal proteins, each batch consisted of 10 adults or approximately 1500 embryos. Ten batches of adult zebrafish liver microsomes (ZLM), five of each gender, in addition to microsomes of a liver homogenate of 50 adult zebrafish were prepared as described previously (Saad et al., 2016b). Five batches of whole embryo microsomes (EM), harvested at different developmental stages, i.e. 5, 24, 48, 72, 96 and 120 hpf, were prepared as mentioned above. Additionally, insect cell control Supersomes (S) (BD Gentest, Woburn, MA, USA) and a pool of human liver microsomes (HLM) from 50 donors (HMMCPL-PL050B, Life Technologies, Thermo Fisher Scientific, Rockford, USA) were used as negative and positive controls, respectively.

Microsomal stability assay

Each substrate was incubated with the microsomal proteins at 28.5°C in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 with 5% and 1% of NADPH regenerating system reagents A and B (Saad et al., 2016b). The initial experiments were conducted using 1 µM of substrate and a range from 10 to 500 µg microsomal proteins/ml of adult zebrafish to determine the optimal protein concentration. The latter concentration was incubated with a range from 0.1 to 80 µM

substrate for estimation of the kinetic parameters, namely maximum reaction velocity (V_{max}) and the substrate concentration corresponding to 50% of the V_{max} (K_m). Microsomal stability assays of female ZLM (FLM), male ZLM (MLM), and EM were conducted by incubation of K_m from each substrate with the optimal protein concentration. For identification of TST metabolites (Saad et al., 2017 accepted) and separation of DIC metabolites, 40 µM of TST or DIC and 1000 µg/ml protein of HLM, MLM, FLM and EM (at 96 hpf) were used to attain high concentrations of metabolites to avoid measurement sensitivity issues.

Sample preparation

Immediately after the start of the microsomal stability assay, aliquots from the main reaction mixture were added at several time points to cooled ACN (4°C) (which already contained the internal standard (IS)) to stop the reaction and denature the microsomal proteins. IS was chosen for each substrate according to the best multiple reaction monitoring (MRM) where no interferences were observed (table 1).

Table 1: Substrates used in the microsomal stability assay and their relevant internal standard.

Substrate	Internal Standard (IS)
Dextromethorphan (DXM)	1-hydroxymidazolam (1-OHMDZ)
Testosterone (TST)	Diclofenac (DIC)
Diclofenac (DIC)	Dextrorphan (DRR)
Midazolam (MDZ)	Clonazepam (CLZ)

Centrifugation at 10 000 ×g for 10 min was applied to precipitate the denatured proteins before the supernatants were diluted by H-water to reach the required percentage of ACN, which was 10% for all samples that were prepared for quantitative analysis. The same steps were applied for the calibration standard, which was diluted in 100 mM KH_2PO_4/K_2HPO_4 buffer pH 7.4 to minimize matrix or extraction process effects. The samples for TST metabolite identification (qualitative analysis) were diluted to 30% ACN to reduce the dilution effect on the measurement sensitivity (Saad et al., 2017 Accepted).

Quantitative UPLC-MS/MS analysis

Microsomal stability assays and sample preparation were performed in duplicate at different days, and the analysis of each sample was also performed in duplicate at the same day. A volume of 5 µl of each sample was injected into a tandem quadrupole UPLC-MS/MS system (Waters® ACQUITY UPLC® – Xevo™ TQ MS, Milford, USA) fitted with an electrospray ionization probe (ESI). Data were acquired in positive or negative ion mode depending on the substrate using MRM. Nitrogen was used as nebulizer, desolvation and collision gas. Chromatographic separation was executed using a reversed phase Acquity® UPLC BEH C18 Column, 2.1 mm × 50 mm, 1.7 µm particle size (Waters, Milford, USA) with an Acquity® UPLC BEH C18 VanGuard Pre-column, 2.1 mm × 5 mm, 1.7 µm particle size (Waters, Milford, USA). The mobile phase consisted of a mixture of two solvents (Solvent A: 95:5 water:ACN + 0.1% formic acid, solvent B: ACN + 0.1% formic acid) with a flow rate of 0.6 ml/min and a gradient as described in table 2.

Table 2: The gradient of the mobile phase (10% ACN) for the quantitative analysis. Solvent A: 95:5 water: ACN + 0.1% formic acid, solvent B: ACN + 0.1% formic acid.

Time (minute)	**%A**	**%B**
0	90	10
0.50	90	10
2.25	0	100
3.25	0	100
4.00	90	10

Applied temperatures for the source, desolvation, autosampler and column were 150, 600, 10 and 30°C, respectively. The gas flow rate was 1000 and 50 L/hour for desolvation and cone. MS parameters such as transitions, cone voltage, collision energy and retention time were optimized for each analyte by conducting full scan acquisitions with a flow rate of 200 µl/min of 500 ng/ml of analyte in the mobile phase (table 3).

Table 3: Capillary and collision settings and retention time of each analyte for ACQUITY UPLC® – Xevo™ TQ MS.

Compound	Parent (*m/z*)	Transitions (*m/z*)	Cone (V)	Collision (V)	Retention time (min)
Dextromethorphan (DXM)	272.09	215.15	38	22	1.32
3-methoxymorphinan (3MM)	258.08	147.05	34	32	1.30
Dextrorphan (DRR)	258.08	157.00	40	36	0.96
Testosterone (TST)	289.24	109.00	30	22	1.63
6ß-hydroxytestosterone (6ß-OHTST)	305.17	269.02	28	16	1.26
Midazolam (MDZ)	325.98	291.08	42	26	1.32
1-hydroxymidazolam (1-OHMDZ)	342.16	203.04	36	26	1.30
4-hydroxymidazolam (4-OHMDZ)	342.16	234.05	40	20	1.24
Diclofenac (DIC)	295.86	250.00	20	12	1.85
Hydroxydiclofenac (OHDIC)	312.04	166.02	22	50	1.58
OHDIC	312.04	230.78	22	30	1.58
OHDIC	312.04	265.89	22	18	1.58
Clonazepam (CLZ)	316.13	269.84	44	28	1.51

Since the above mentioned method did not allow separating 4-OHDIC and 5-OHDIC chromatographically, another UPLC-MS/MS method was developed using an ACQUITY UPLC TQ detector (Waters, Milford, USA). As a TQ Detector is less sensitive than a Xevo TQ MS, higher concentrations of DIC (40 µM) and 1000 µg/ml of HLM, ZLM and EM (at 96 hpf) were used for a microsomal stability assay for 60 minutes to reach higher concentrations of the metabolites. The samples were injected in full loop mode (10 µL) on an XSELECT CSH C18 2.5 µm 2.1 x 75 mm column XP (Waters Milford, USA) and thermostatically (40°C) eluted with mobile phase solvents consisting of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), and a gradient as described in table 4. Data were acquired in ESI positive or negative ion mode depending on the substrate using MRM. Capillary voltage was set at 4.5 kV. Two transitions were monitored for DIC, namely 296 → 215 and 296 → 250 with cone voltage set at 26 V and collision energy at 19 V and 13 V, respectively. Two transitions were monitored

for OHDIC, namely 312 → 231 and 312 → 266 with collision energy set at 19 V and 13 V and cone voltage at 26 V. The source and desolvation temperatures were set at 120 and 450°C. Nitrogen was used as nebulizer and desolvation gas. Argon was used as collision gas. The desolvation and cone gas flow rates were set at 850 and 50 L/h.

Table 4: The gradient of the mobile phase (10% ACN) for the separation of 4OH-DIC and 5OH-DIC. Solvent A: water +0.1% formic acid, solvent B: ACN + 0.1 % formic acid

Time (minute)	%A	%B
0.00	90	10
0.30	67	33
2.00	60	40
2.50	50	50
3.50	0	100
4.00	0	100
4.10	90	10
6.00	90	10

Qualitative ultra-performance liquid chromatography – accurate mass mass spectrometry (UPLC-amMS)

The protocol is described in *(*Saad et al.*, 2017 Accepted)*.

Data processing following quantitative analysis

All experiments were performed in duplicate, and all microsomal stability assays samples, QC samples and blanks were measured two, four and 16 times, respectively. Data acquisition and analysis were conducted using Waters MassLynx™ Software, Version 4.1. The response of each sample was calculated by dividing the area under curve (AUC) of the analyte peak by the AUC of the corresponding IS. Limits of blank (LOB) and lower limits of detection (LLOD) were calculated according to Armbruster and Pry (2008) (Armbruster and Pry, 2008) and accuracy was calculated using the QC samples as:

$$Accuracy\ \% = \frac{mean\ measured\ concentration}{nominal\ concentration} \times 100$$

The coefficient of variation (CV) was used to express the precision percentage (SD: standard deviations):

$$CV = \frac{SD\ of\ the\ measured\ concentration}{mean\ of\ the\ measured\ concentration} \times 100$$

The data of accuracy, precision, LOB and LLOD are presented in tables 5-8.

Table 5: Accuracy and coefficient of variation (CV) of quality control samples (QC) of diclofenac (DIC) and hydroxydiclofenac (OHDIC). NA: lower than lower limits of detection (LLOD).

n=4	QC	OHDIC265	OHDIC230	OHDIC166	DIC
accuracy	6.25	NA	104.8	NA	101.4
	25	106.8	88.3	90.9	102.3
	100	100	93.3	88.2	103.1
CV	6.25	NA	8.6	NA	6.7
	25	13.3	4.3	13.9	3.4
	100	4.5	3.3	7.2	4.5

Table 6: Accuracy and coefficient of variation (CV) of quality control samples (QC) of testosterone (TST) and 6-betahydroxytestosterone (6β-OHTST). NA: lower than lower limits of detection (LLOD).

n=4	QC	6β-OHTST	TST
accuracy	3.1	NA	83.8
	12.5	119.2	96.6
	50.0	109.9	97.8
CV	3.1	NA	2.0
	12.5	18.5	2.1
	50.0	13.0	3.5

Table 7: Accuracy and coefficient of variation (CV) of quality control samples (QC) of dextrorphan (DRR), 3-methoxymorphinan (3MM) and dextromethorphan (DXM). NA: lower than lower limits of detection (LLOD).

n=4	QC for DRR and 3MM	DRR	3MM	QC for DXM	DXM
accuracy	0.04	114.5	NA	0.16	NA
	0.64	89.6	100.7	2.56	93.0
	10.24	100.8	85.5	40.96	108.0

CV	0.04	8.7	NA	0.16	NA
	0.64	2.5	2.1	2.56	2.0
	10.24	16.9	20.1	40.96	4.0

Table 8: Standard curve parameters of the substrates and the metabolites as calculated from the quantitative analysis. STD: standard deviation. LLOD: lower limits of detection. LOB limits of blank.

n=16	STD	LOB ng/ml	LLOD ng/ml	Equation	R^2
Dextromethorphan (DXM)	0.02	0.14	0.18	$y = 1.0868x - 3.5885$	0.9979
Dextrorphan (DRR)	0.0021	0.009	0.0126	$y = 0.0602x^2 + 0.5866x - 2.8665$	0.9973
3-Methoxymorphinan (3MM)	0.08	0.32	0.46	$y = 0.3194x^2 - 1.2925x + 0.558$	0.9979
Diclofenac (DIC)	0.13	0.46	0.69	$y = 0.9811x + 2.7036$	0.9985
Hydroxydiclofenac (OHDIC265)	0.38	9.39	10.03	$y = -0.2056x^2 + 0.8079x + 3.0783$	0.9925
OHDIC230	0.93	2.6	4.15	$y = 0.1392x^2 + 1.4351x + 3.4724$	0.9947
OHDIC166	0.76	2.4	3.65	$y = 0.2508x^2 + 2.1596x + 4.0236$	0.9943
Testosterone (TST)	0.25	0.84	1.25	$y = 0.8296x + 1.4547$	0.9990
Hydroxytestosterone (OHTST)	1.37	6.3	8.56	$y = -0.1699x^2 + 0.8833x + 2.7384$	0.9968

The velocity of all reactions was calculated within the linear part of the reaction curve and data were presented as mean ± SD (n=3). The kinetic parameters of all substrates were determined using non-linear least-squares regression analysis of untransformed data (GraphPad Software, La Jolla, CA, USA). To assess normal distribution of the data and homogeneity of variances, the one-sample Kolmogorov-Smirnov test and Levene's test were used, respectively. A one-way ANOVA test (embryos) or *t* test (adults) was applied for data that showed a normal distribution and no significant differences among variances. When data did not show a normal distribution, the Kruskal-Wallis test for non-parametric data was applied and post-hoc test was conducted to detect differences. Differences were considered significant when $p < 0.05$. All statistical analyses were accomplished by means of SPSS statistics version 20 (IBM, Armonk, NY, USA).

4.4 Results

DXM

For ZLM, V_{max} and K_m for 3MM and DRR were 28.18 ± 1.48 pmol.mg^{-1}.min^{-1}, 9.43 ± 1.06 µM and 34.60 ± 5.70 pmol.mg^{-1}.min^{-1}, 10.11 ± 3.48 µM, respectively. Both metabolites followed Michaelis-Menten kinetics with R^2 of 0.98 and 0.89 (Figure 1). A concentration of 10 µM (≈ K_m) was chosen for the final experiments in adults and embryos.

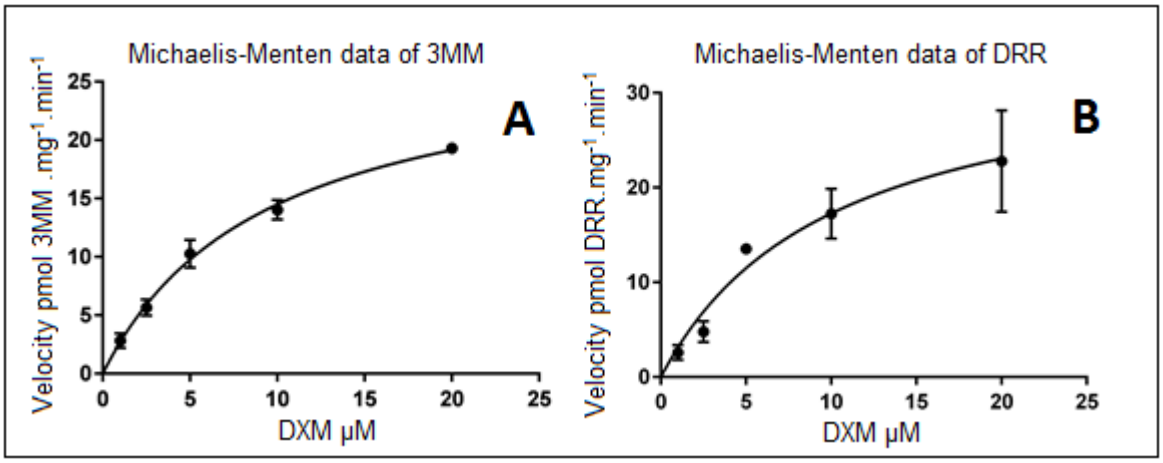

Figure 1: Velocity of 3-methoxymorphinan (3MM) (A) and dextrorphan (DRR) (B) formation at different concentrations of dextromethorphan (DXM) using 200 µg/ml ZLM. Each data point represents the mean of three independent experiments each with duplicate measurements.

No significant differences were observed for the production rates of 3MM and DRR between FLM and MLM with 20.73 ± 7.67 and 9.19 ± 4.13 pmol.mg^{-1}.min^{-1} for FLM, and 14.88 ± 4.71 and 11.47 ± 4.78 pmol.mg^{-1}.min^{-1} for MLM, respectively. Under these experimental conditions, HLM showed lower 3MM levels (4.57 ± 1.71 pmol.mg^{-1}.min^{-1}) and higher DRR levels (48.27 ± 1.50 pmol.mg^{-1}.min^{-1}) than ZLM (Figure 2).

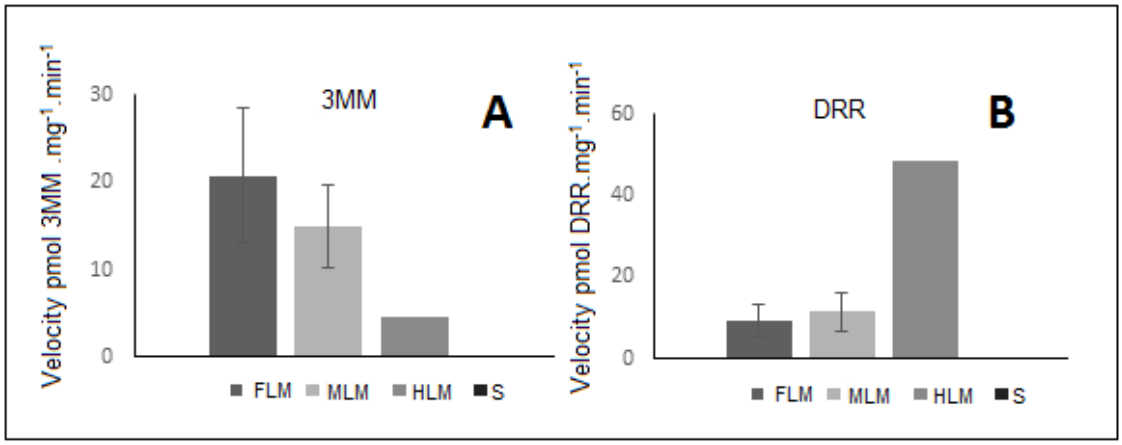

Figure 2: Velocity of 3-methoxymorphinan (3MM) (A) and dextrorphan (DRR) (B) formation in female zebrafish liver microsomes (FLM) (five batches), male zebrafish liver microsomes (MLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

Levels of DRR in EM at 5, 24 and 48 hpf were lower than the LLOD. EM at 96 hpf showed significantly higher DRR levels (0.37 ± 0.25 pmol.mg^{-1}.min^{-1}) than at 120 hpf (0.12 ± 0.10 pmol.mg^{-1}.min^{-1}) but not at 72 hpf (0.12 ± 0.06 pmol.mg^{-1}.min^{-1}) (Figure 3). For 3MM, concentrations were much lower than LLOD in all embryonic stages except for 96 hpf at which some levels of the metabolite could be observed but not quantified (higher than LOB but lower than LLOD).

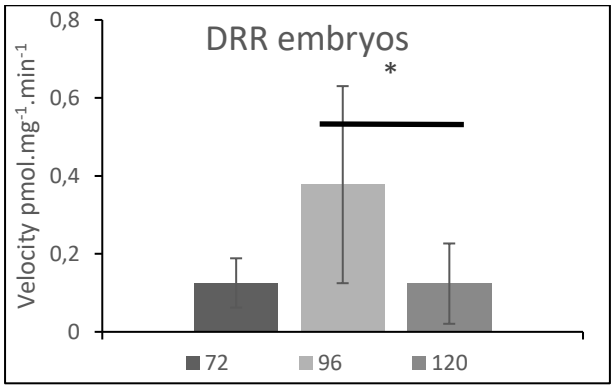

Figure 3: Velocity of dextrorphan (DRR) in microsomes of whole body homogenates of zebrafish embryos (EM) at 72, 96 and 120 hpf (five batches of each). Statistical differences ($P < 0.05$) between developmental stages are indicated by (*).

DIC

It was not possible to distinguish the two metabolites of DIC, i.e. 4-OHDIC and 5-OHDIC from each other using the UPLC BEH C18 column, 2.1 mm × 50 mm, 1.7 µm particle size as both had a very close retention time and the same ionization products (312.04 → 166.02; 312.04 → 230.79 and 312.04 → 265.90). The abundant peak at *m/z* 230.79 was selected for quantitative assessment as it showed the best repeatability. V_{max} and K_m of OHDIC production were 659.10 ± 60.23 pmol.mg^{-1}.min^{-1} and 11.73 ± 5.65 µM with the Michaelis-Menten kinetic model and an R^2 of 0.92 (Figure 4A). A concentration of 12 µM (≈ K_m) of DIC was used for the final experiments. No significant differences were observed between MLM (154.64 ± 63.15 pmol.mg^{-1}.min^{-1}) and FLM (153.97 ± 30.25 pmol.mg^{-1}.min^{-1}) and lower levels of OHDIC were detected in HLM (58.06 ± 16.40 pmol.mg^{-1}.min^{-1}) under these experimental conditions (Figure 4B).

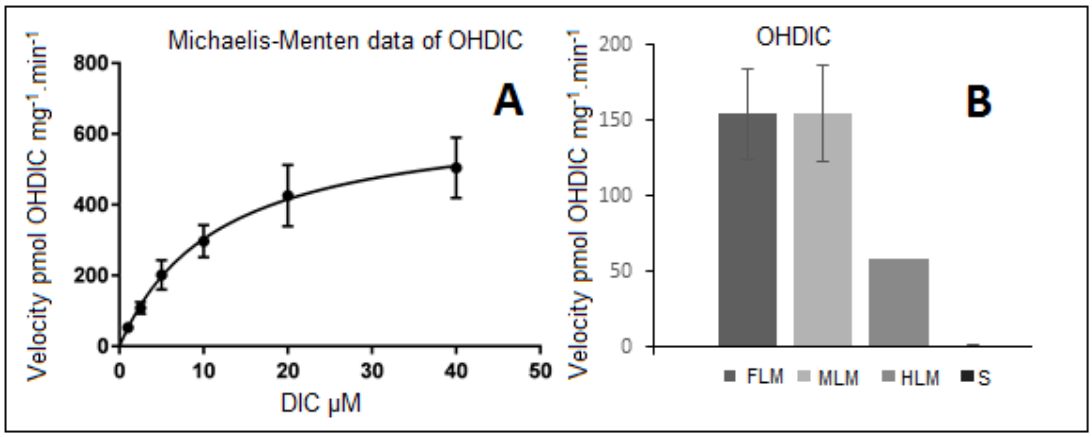

Figure 4: (A) Velocity of hydroxydiclofenac OHDIC formation at different concentrations of diclofenac (DIC) using 200 µg/ml of ZLM. Each data point represents the mean of three independent experiments, each with duplicate measurements. (B) Velocity of OHDIC formation in female zebrafish liver microsomes (FLM) (five batches), male zebrafish liver microsomes (MLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

No metabolites were detected in EM, except in two 96 hpf-batches in which levels close to the LLOD were present. Using the ACQUITY UPLC-TQ Detector system and XSELECT CSH C18 2.5 µm 2.1x75mm column XP, both 4-OHDIC and 5-OHDIC could be separated. Increasing the concentrations of both substrate and microsomal proteins resulted in an increase in the concentrations of both metabolites that exceeded the LLOD in all samples, i.e. MLM, FLM, HLM and EM at 96 hpf. The concentrations of both metabolites in EM samples at 96 hpf were 10 times lower than those in ZLM and the ratio 4-OHDIC/5-OHDIC in HLM was very close to the ratio in ZLM (Figure 5).

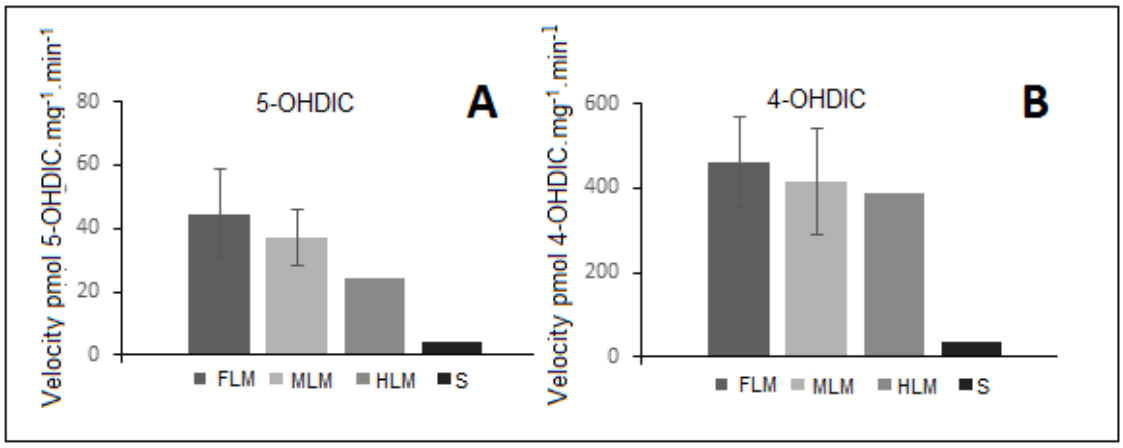

Figure 5: Velocity of 5-hydroxydiclofenac (5-OHDIC) (A) and 4′-hydroxydiclofenac (4-OHDIC) (B) formation in female zebrafish liver microsomes (FLM) (five batches), male zebrafish liver microsomes (MLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

MDZ

No depletion of MDZ was observed in FLM and MLM, although it was very pronounced in HLM (Figure 6). Therefore, higher substrate (0.1-10 µM) and microsomal protein concentrations (50-1000 µg/ml) were used to detect possible depletion of the substrate or formation of metabolites. Even at these high concentrations no depletion of MDZ was observed in zebrafish samples (Figure 6A) and only very low, non-quantifiable, concentrations of 1-

OHMDZ were observed in ZLM after 90 minutes of incubation (Figure 6B). High levels of 1-OHMDZ (Figure 6B) and much lower levels of 4-OHMDZ (Figure 6C) were detected in HLM.

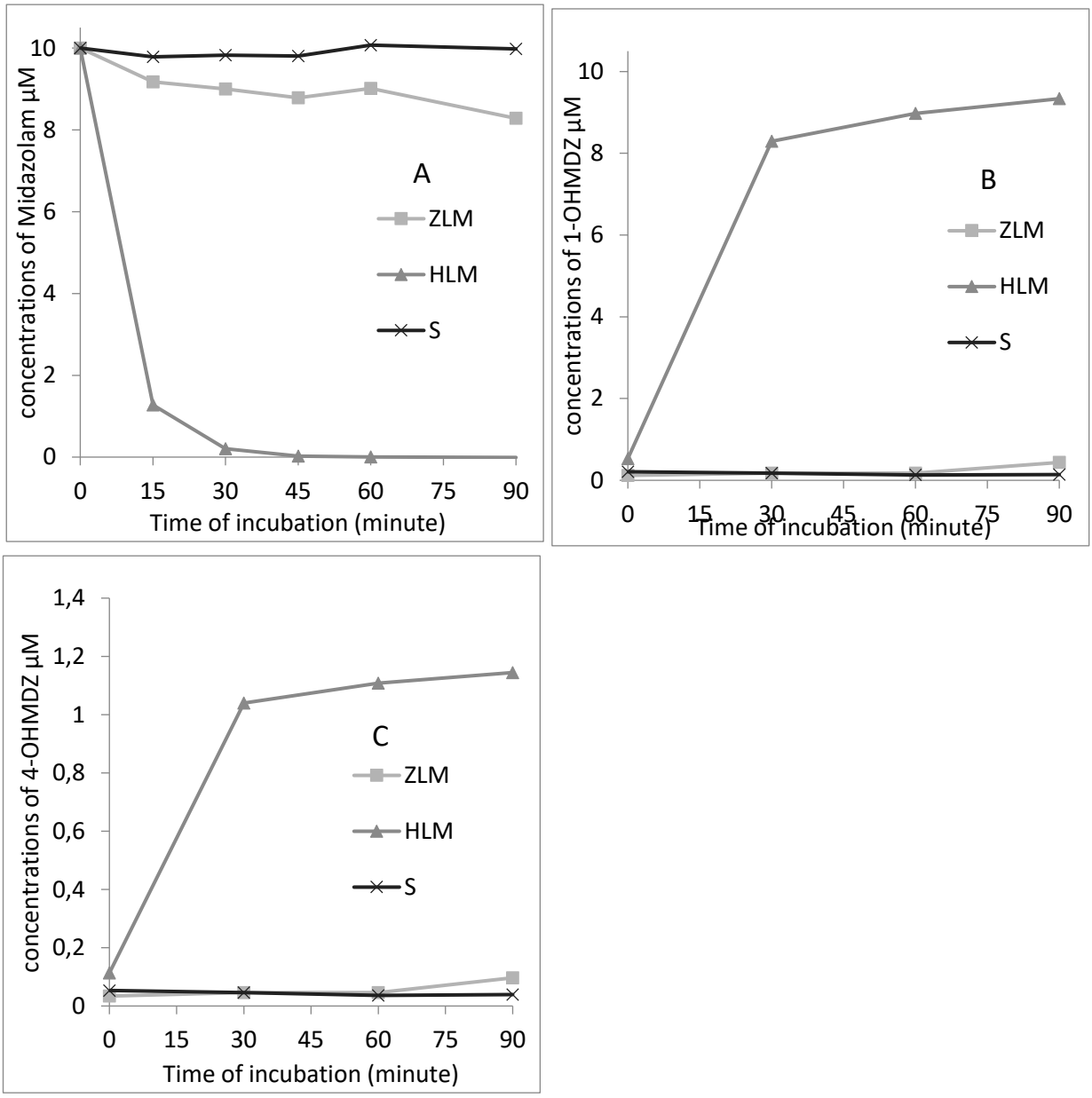

Figure 6: (A) Midazolam (MDZ) depletion, (B) 1-OHMDZ and (C) 4-OHMDZ formation in zebrafish liver microsomes (ZLM), human liver microsomes (HLM) and Supersomes (S). Protein concentration: 1000 µg/ml and MDZ concentration: 10 µM.

TST

TST was metabolized by the zebrafish. However, the first experiments did not show any detectable concentration of 6ß-OHTST, which is the main metabolite of TST in humans. Therefore, an UPLC-amMS system (Saad et al., 2017 Accepted) was used in an attempt to identify other potential metabolites. No main metabolite(s) could be detected in ZLM. However, six minor metabolites with several isomers (three in positive and three in negative ionization mode) could be detected in ZLM samples after 120 minutes of incubation. These metabolites were absent at time zero and in blank samples as well (Saad et al., 2017 Accepted), but they could not be quantified because of the absence of standards. Therefore, the depletion of TST was used for the determination of the kinetic parameters of TST biotransformation. V_{max} and K_m were 301.70 ± 21.19 pmol.mg^{-1}.min^{-1} and 9.70 ± 1.88 µM. The biotransformation of TST showed Michaelis-Menten kinetics with an R^2 of 0.94 (Figure 7A). A concentration of 10 µM of TST (≈K_m) was used for the final experiments.

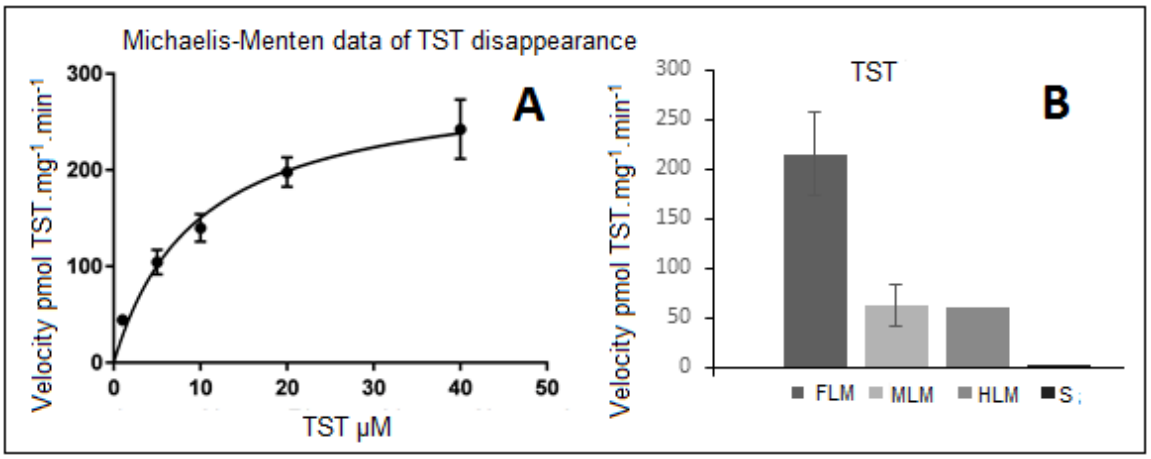

Figure 7: (A) Velocity of testosterone (TST) depletion at different concentrations of TST using 200 µg/ml of ZLM. Each data point represents the mean of three independent experiments each with duplicate measurements. (B) Velocity of TST depletion in female zebrafish liver

microsomes (FLM) (five batches), male zebrafish liver microsomes (MLM) (five batches), human liver microsomes (HLM) (one batch) and Supersomes (S) (one batch).

FLM showed a significantly higher velocity (215.30 ± 42.09 pmol.mg^{-1}.min^{-1}) than MLM (63.22 ± 21.07 pmol.mg^{-1}.min^{-1}), whereas the latter was comparable to the velocity in HLM (60.57 ± 11.26 pmol.mg^{-1}.min^{-1}) (Figure 7B). No substrate consumption was detected in any of the different embryonic stages.

4.5 Discussion

Adult zebrafish

In the present study, we detected several CYP-related reactions in zebrafish using human CYP2- and CYP3-specific substrates. Interestingly, the biotransformation rates were mostly higher in zebrafish compared to humans, except for CYP2D6-like activity. Sex-related differences were only present for TST.

DXM, which has sedative, dissociative and stimulant properties and is used mainly as a cough suppressant, was metabolized by ZLM into DRR and 3MM. In man, DXM is O-demethylated by CYP2D6 to DRR and at a lower rate N-demethylated by CYP3A4 to 3MM (Figure 8). Both metabolites can be further N- or O-demethylated to 3-hydroxymorphinan (Jacqz-Aigrain et al., 1993).

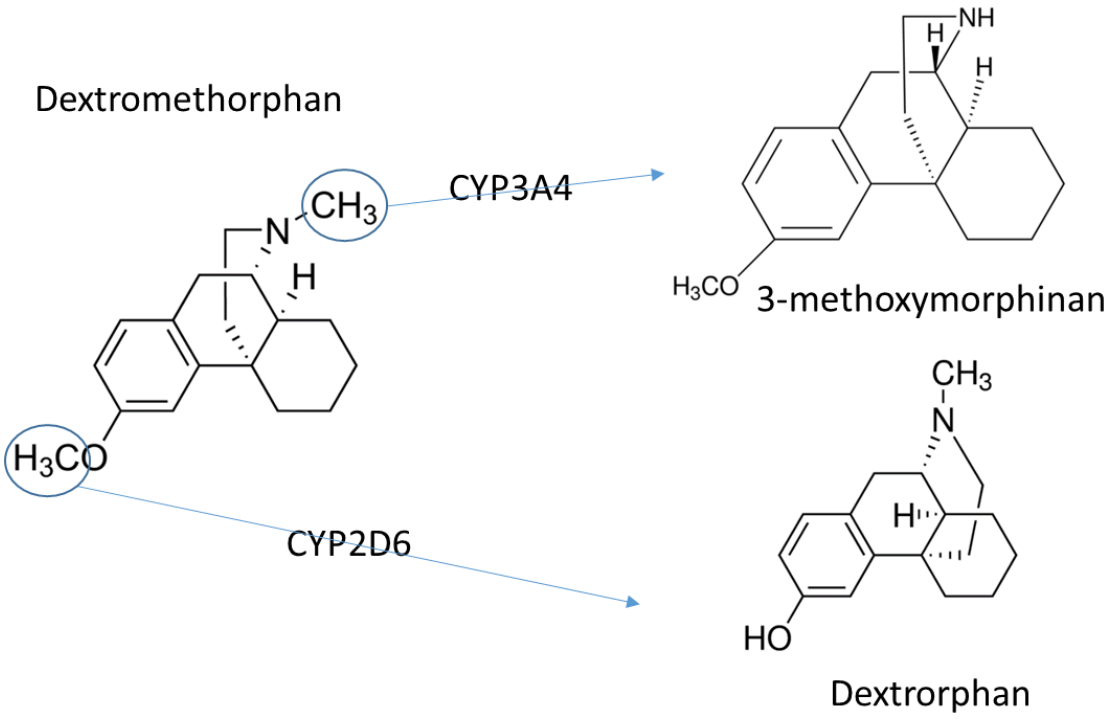

Figure 8: Dextromethorphan metabolism in humans

Although no homologue of human *CYP2D6* has been found in zebrafish (Goldstone et al., 2010), the present study showed that zebrafish do metabolize DXM to DRR, in addition to 3MM, but the ratio between both metabolite concentrations was different than in man. DRR is produced with a much higher affinity than 3MM in humans (von Moltke et al., 1998) and in other mammals such as rats for example (Hendrickson et al., 2003). In zebrafish, both metabolites are characterized by similar K_m values while the total consumption rate of DXM showed no significant difference between ZLM and HLM under our experimental conditions. Furthermore, the present study failed to detect any sex-related difference in DXM metabolism in zebrafish. In man, the data are contradictory (Hagg et al., 2001; Labbe et al., 2000; McCune et al., 2001; Parkinson et al., 2004; Tamminga et al., 1999), which is most likely related to the genetic polymorphism of *CYP2D6* portrayed by more than 80 allelic variants (Teh and Bertilsson, 2012).

Also for DIC (a nonsteroidal anti-inflammatory drug), we showed high concentrations of 4-OHDIC in ZLM and 11 times lower concentrations of 5-OHDIC, despite the absence of any

homolog of CYP2C9 in zebrafish (Figure 9) (Goldstone et al., 2010). 5-OHDIC is most likely responsible for the formation of adducted proteins, which is the initial step in (idiosyncratic) hepatotoxicity of DIC in humans and rats (Boerma et al., 2012; Kishida et al., 2012). This makes the identification of the DIC metabolism profile essential for a precise interpretation of toxic effects of this compound, also in the zebrafish.

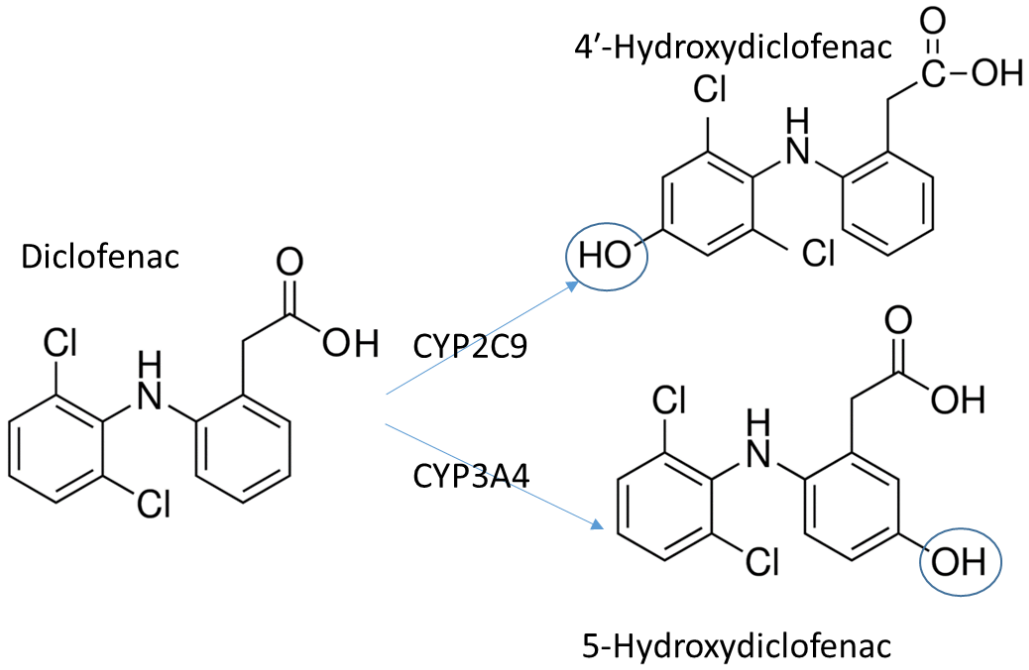

Figure 9: Diclofenac metabolism in humans (Bogaards et al., 2000)

No significant differences were detected between FLM and MLM in 4- and 5-OHDIC production, which is in agreement with published reports on 4-OHDIC formation by HLM (Bogaards et al., 2000) and with the observation that *in vivo* CYP2C9 activity showed no sex-related differences in humans when other substrates such as losartan were used (Cabaleiro et al., 2013; Hatta et al., 2015). Interestingly, the increase of DIC concentrations up to 4xK_m for metabolites separation purpose, had increased velocities by different proportions in both humans and zebrafish. This can be related to different K_m values in both species which can reach up to 52 µM in HLM, whereas it was around 12 µM in ZLM (Spaggiari et al., 2014). Hence, ZLM can reach a saturation phase faster than HLM.

In contrast to the former substrates, our study clearly showed differences in biotransformation of MDZ and TST between zebrafish and man. For MDZ (a short-acting benzodiazepine), neither consumption nor metabolites could be detected in ZLM (above the LLOD), whereas this substrate was largely metabolized into 1-OHMDZ and, to a much lesser extent, into 4-OHMDZ in HLM (Figure 10). Also Alderton *et al.* (2010) could not detect any metabolism of MDZ in zebrafish larvae at 7 dpf. As a result, MDZ, which is a typical substrate for CYP3A in man, appeared to have no affinity for any of the zebrafish CYPs.

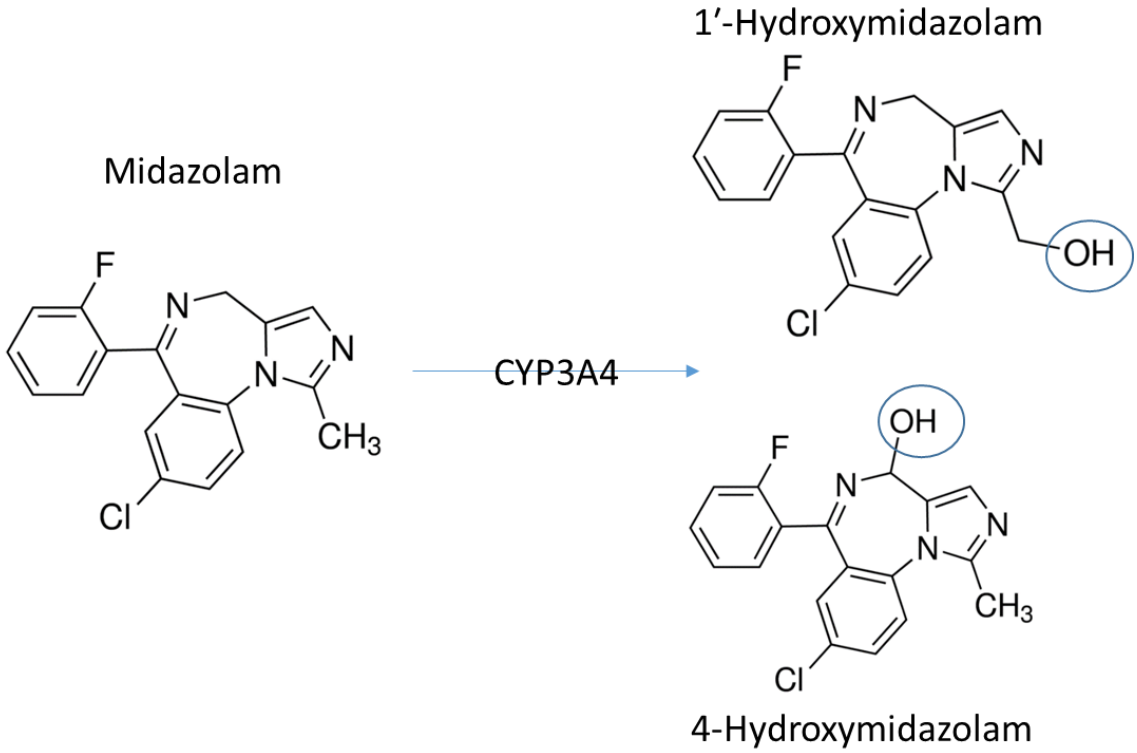

Figure 10: Midazolam metabolism in humans (Spaggiari et al., 2014).

For TST (an anabolic androgen), the major human CYP3A metabolite, i.e. 6ß-OHTST (Figure 11), could not be detected (Saad et al., 2017 accepted), although consumption of TST was evident in ZLM. This contrasts with the results obtained by Chng *et al.* (2012) who detected seven mono-hydroxylated metabolites of TST in FLM of which 6ß-OHTST appeared to be predominant. Reschly *et al*. (2007) also detected this metabolite in addition to three different

mono-hydroxylated metabolites in zebrafish hepatocytes. These authors reported that the levels of 6ß-OHTST increased after exposure to different PXR inducers (Reschly et al., 2007).

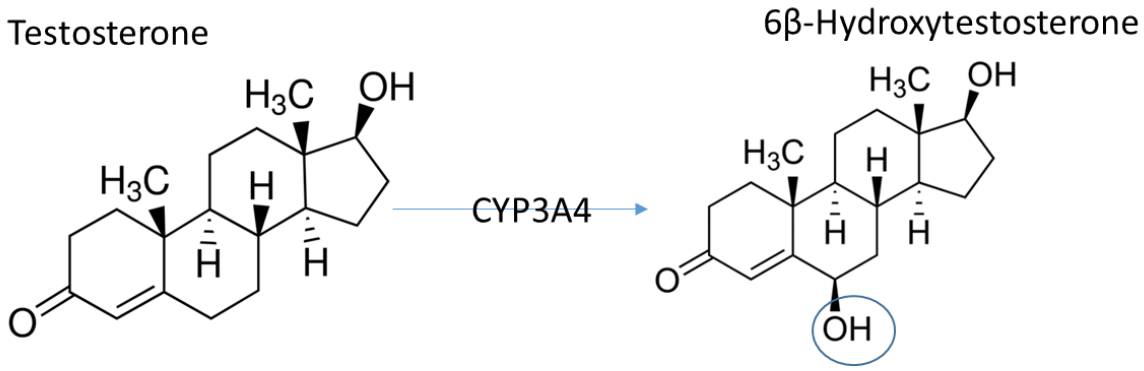

Figure 11: Testosterone metabolism in humans (Reschly et al., 2007)

To ascertain that no 6ß-OHTST was formed in our zebrafish samples, we performed an experiment at very high concentrations of both microsomal proteins and substrate with a long incubation time (1000 µg/ml and 40µM, 120 minutes, respectively). No oxidation activity could be detected but several other abundant peaks were visible after biotransformation by ZLM (Saad et al., 2017 Accepted). Even if traces of 6ß-OHTST under the LLOD were present in ZLM, we can ensure that these presumptive levels are negligible when compared to those in humans (Saad et al., 2017 Accepted). As Chng *et al*. (2012) also noted much higher 6ß-OHTST levels in HLM than in FLM, the relevance of this metabolite in zebrafish is questionable (Chng et al., 2012). Nevertheless, a remarkable depletion of TST was observed in ZLM in the microsomal stability assay and, in contrast to the other substrates, TST consumption showed large differences between FLM and MLM, which was also reflected in some of the observed metabolites peaks (Saad et al., 2017 Accepted). Our results are in accordance with another study on fathead minnows in which females biotransformed TST at higher rates compared to males (Parks and LeBlanc, 1998). These sex differences, which are also present in other species like rats and mice (Niwa et al., 1995; Wilson et al., 1999), are critical for the maintenance of androgen homeostasis, which is responsible for reproduction and sexual differentiation

(Mangochi, 2010). Significant sex-related differences in TST biotransformation have not been reported in humans possibly due to high inter-individual variations.

So, although adult zebrafish showed for some CYP-probes reactions that also occur in man, e.g. dextromethorphan O-demethylation, dextromethorphan N-demethylation and 4- and 5-diclofenac hydroxylation, also clear differences between these two species were observed for other human CYP-probes. As such, CYP-mediated drug metabolism in zebrafish may not be predictive for the one in man and should be investigated for each new compound when using this animal model for human risk assessment.

Zebrafish embryos/larvae

As zebrafish embryos are gaining interest as an alternative model for developmental toxicity studies, several research groups are evaluating their predictive potential for teratogenic compounds (Brannen et al., 2010; Panzica-Kelly et al., 2012). Weigt *et al.* (2010) showed that zebrafish embryos developed malformations when they were exposed to mammalian proteratogens in the absence of an external metabolizing system, suggesting intrinsic bioactivation of these compounds by the embryos (Weigt et al., 2011). However, as no metabolite analysis was performed, it cannot be excluded that the presumed proteratogens were teratogenic by themselves in the zebrafish and did not require bioactivation. This is in line with a study from Mattsson *et al.* (2012) who showed that albendazole, an anthelmintic drug, was very toxic for zebrafish embryos and that co-incubation with an external metabolizing system, i.e. rat liver microsomes, drastically decreased the toxicity due to consumption of the parent compound and formation of the non-toxic metabolite albendazole sulfoxide (Mattsson et al., 2012). Also for DXM, mortality and diverse morphological and physiological malformations were inversely correlated to the age of the embryos/larvae (Xu et al., 2011). The observed lower toxicity with increasing age might be, due to higher CYP activity at later stages, responsible for the detoxification of DXM. This is in accordance with our *in vitro* study, in which we

observed a lack in biotransformation of DXM during the embryonic stages. Only at the larval stage (from 72 hpf onwards) and more specifically at 96 hpf, metabolites above the LLOD were detected for most CYP-probes. This is also in agreement with *in vivo* data in 96 hpf or 120 hpf larvae, which showed *in vivo* EROD, ECOD and OOMR activities and TST metabolism (Jones et al., 2010). Alderton *et al.* (2010) also recorded several metabolic activities in 7 dpf larvae including glucuronidation of TST, N- and O-demethylation as well as hydroxylation of DXM, and sulphate conjugation of cisapride. Additionally, 12 metabolites of verapamil were detected in 7 dpf larvae and 10 of them could already be detected at 3 dpf. These authors also detected low levels of OHTST and OHDIC at 7 dpf, but exact determination of their chemical structure was unfeasible. However, even at this later stage of development the authors questioned the biological relevance of the detected metabolite concentrations as they were still very low (Alderton et al., 2010).

In summary, no *in vitro* biotransformation of the tested substrates was observed during zebrafish embryonic development. At 96 hpf, metabolic activation occurred for some CYP probes but, as this developmental stage is the end of organogenesis in this species, the embryo appears to lack the biotransformation and bioactivation potential during the sensitive period for developmental toxicants. As such, this limitation of the zebrafish embryo assay should be taken into account when setting-up and interpreting results from developmental toxicity studies.

Impact of the study

To our knowledge, this is the first study that assessed qualitative and quantitative differences in *in vitro* CYP-mediated drug metabolism between man, adult zebrafish and zebrafish embryos/larvae using human reference compounds. Four distinctive biotransformation patterns were observed in zebrafish compared to humans. The first pattern indicated similar biotransformation pathways and their potency as shown by the production of similar main metabolites at similar ratios in both species, i.e. DIC biotransformation into 4- and 5-OHDIC.

This pattern represents the ideal situation for extrapolation of *in vitro* drug metabolism data from zebrafish to humans. In the second pattern, substrates were also biotransformed into similar main metabolites as in humans, but with altered ratios as shown for DXM (DDR/3MM). The third pattern indicated different pathways as totally different metabolites were observed between zebrafish and man, e.g. for TST (6ß-OHTST). As such, different pharmacological/toxicological effects can be expected in both scenarios and especially in the third pattern. In the last pattern, in which the substrate was not metabolized by zebrafish, e.g. MDZ, there is the risk of overestimation of toxic effects by the parent compound and metabolite-mediated effects can be overlooked. The fourth scenario also applies for zebrafish embryos, as the lack of metabolic activity during a major part of organogenesis limits the extrapolation of developmental toxicity. So, from our study we can conclude that CYP-mediated drug metabolism can be substantially different in zebrafish compared with man. As such, results from toxicity studies in zebrafish should be interpreted with caution when used for human risk assessment. We recommend including zebrafish liver microsomes/hepatocytes in the *in vitro* drug metabolism testing battery during the non-clinical/discovery phase in addition to the currently used mouse, rat, dog, non-human primate and human liver microsomes/hepatocytes when considering this species for human risk assessment data to humans.

4.6 References

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Armbruster, D.A., Pry, T., 2008. Limit of blank, limit of detection and limit of quantitation. Clin Biochem Rev 29 Suppl 1, S49-52.

Beker van, W.A., Wolterbeek, A., Te, B.L., Snel, C., Menke, A., Rubingh, C., de, G.D., Kroese, D., 2013. A category approach to predicting the developmental (neuro) toxicity of organotin compounds: the value of the zebrafish (Danio rerio) embryotoxicity test (ZET). Reprod. Toxicol 41, 35-44.

Boerma, J.S., Dragovic, S., Vermeulen, N.P., Commandeur, J.N., 2012. Mass spectrometric characterization of protein adducts of multiple P450-dependent reactive intermediates of diclofenac to human glutathione-S-transferase P1-1. Chem Res Toxicol 25, 2532-2541.

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., van Bladeren, P.J., Walther, B., 2000. Determining the best animal model for human cytochrome P450 activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 30, 1131-1152.

Brannen, K.C., Panzica-Kelly, J.M., Danberry, T.L., Augustine-Rauch, K.A., 2010. Development of a zebrafish embryo teratogenicity assay and quantitative prediction model. Birth Defects Res. B Dev. Reprod. Toxicol 89, 66-77.

Cabaleiro, T., Roman, M., Ochoa, D., Talegon, M., Prieto-Perez, R., Wojnicz, A., Lopez-Rodriguez, R., Novalbos, J., Abad-Santos, F., 2013. Evaluation of the relationship between sex, polymorphisms in CYP2C8 and CYP2C9, and pharmacokinetics of angiotensin receptor blockers. Drug Metab Dispos 41, 224-229.

Chauret, N., Gauthier, A., Nicoll-Griffith, D.A., 1998. Effect of common organic solvents on in vitro cytochrome P450-mediated metabolic activities in human liver microsomes. Drug Metab Dispos 26, 1-4.

Chng, H.T., Ho, H.K., Yap, C.W., Lam, S.H., Chan, E.C., 2012. An investigation of the bioactivation potential and metabolism profile of Zebrafish versus human. J. Biomol. Screen 17, 974-986.

EMA, 2012. Guideline on the investigation of drug interactions. http://www.ema.europa.eu/ema/index.jsp?curl=pages/news_and_events/news/2012/2007/news_detail_001561.jsp&mid=WC001560b001501ac058004d001565c001561.

FDA, 2016. Drug Development and Drug Interactions: Table of Substrates, Inhibitors and Inducers. http://www.fda.gov/Drugs/DevelopmentApprovalProcess/DevelopmentResources/DrugInteractionsLabeling/ucm093664.htm.

Goldstein, J.A., 2001. Clinical relevance of genetic polymorphisms in the human CYP2C subfamily. Br J Clin Pharmacol 52, 349-355.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC. Genomics 11, 643.

Hagg, S., Spigset, O., Dahlqvist, R., 2001. Influence of gender and oral contraceptives on CYP2D6 and CYP2C19 activity in healthy volunteers. Br J Clin Pharmacol 51, 169-173.

Hakkola, J., Tanaka, E., Pelkonen, O., 1998. Developmental expression of cytochrome P450 enzymes in human liver. Pharmacol Toxicol 82, 209-217.

Hatta, F.H., Lundblad, M., Ramsjo, M., Kang, J.H., Roh, H.K., Bertilsson, L., Eliasson, E., Aklillu, E., 2015. Differences in CYP2C9 Genotype and Enzyme Activity Between Swedes and Koreans of Relevance for Personalized Medicine: Role of Ethnicity, Genotype, Smoking, Age, and Sex. OMICS 19, 346-353.

Hendrickson, H.P., Gurley, B.J., Wessinger, W.D., 2003. Determination of dextromethorphan and its metabolites in rat serum by liquid-liquid extraction and liquid chromatography with fluorescence detection. J Chromatogr B Analyt Technol Biomed Life Sci 788, 261-268.

Hines, R.N., McCarver, D.G., 2002. The ontogeny of human drug-metabolizing enzymes: phase I oxidative enzymes. J Pharmacol Exp Ther 300, 355-360.

Jacqz-Aigrain, E., Funck-Brentano, C., Cresteil, T., 1993. CYP2D6- and CYP3A-dependent metabolism of dextromethorphan in humans. Pharmacogenetics 3, 197-204.

Jones, H.S., Panter, G.H., Hutchinson, T.H., Chipman, J.K., 2010. Oxidative and conjugative xenobiotic metabolism in zebrafish larvae in vivo. Zebrafish 7, 23-30.

Kishida, T., Onozato, T., Kanazawa, T., Tanaka, S., Kuroda, J., 2012. Increase in covalent binding of 5-hydroxydiclofenac to hepatic tissues in rats co-treated with lipopolysaccharide and diclofenac: involvement in the onset of diclofenac-induced idiosyncratic hepatotoxicity. J Toxicol Sci 37, 1143-1156.

Labbe, L., Sirois, C., Pilote, S., Arseneault, M., Robitaille, N.M., Turgeon, J., Hamelin, B.A., 2000. Effect of gender, sex hormones, time variables and physiological urinary pH on apparent CYP2D6 activity as assessed by metabolic ratios of marker substrates. Pharmacogenetics 10, 425-438.

Mangochi, P., 2010. Endocrine distrupting chemicals and human health: the plausibility of research results on DDT and reproductive health. Malawi Med J 22, 42-45.

Mattsson, A., Ulleras, E., Patring, J., Oskarsson, A., 2012. Albendazole causes stage-dependent developmental toxicity and is deactivated by a mammalian metabolization system in a modified zebrafish embryotoxicity test. Reprod. Toxicol 34, 31-42.

McCune, J.S., Lindley, C., Decker, J.L., Williamson, K.M., Meadowcroft, A.M., Graff, D., Sawyer, W.T., Blough, D.K., Pieper, J.A., 2001. Lack of gender differences and large intrasubject variability in cytochrome P450 activity measured by phenotyping with dextromethorphan. J Clin Pharmacol 41, 723-731.

Niwa, T., Kaneko, H., Naritomi, Y., Togawa, A., Shiraga, T., Iwasaki, K., Tozuka, Z., Hata, T., 1995. Species and sex differences of testosterone and nifedipine oxidation in liver microsomes of rat, dog and monkey. Xenobiotica 25, 1041-1049.

Panzica-Kelly, J.M., Zhang, C.X., Augustine-Rauch, K., 2012. Zebrafish embryo developmental toxicology assay. Methods Mol Biol 889, 25-50.

Parkinson, A., Mudra, D.R., Johnson, C., Dwyer, A., Carroll, K.M., 2004. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. Toxicol Appl Pharmacol 199, 193-209.

Parks, L.G., LeBlanc, G.A., 1998. Involvement of multiple biotransformation processes in the metabolic elimination of testosterone by juvenile and adult fathead minnows (Pimephales promelas). Gen Comp Endocrinol 112, 69-79.

Patki, K.C., Von Moltke, L.L., Greenblatt, D.J., 2003. In vitro metabolism of midazolam, triazolam, nifedipine, and testosterone by human liver microsomes and recombinant cytochromes p450: role of cyp3a4 and cyp3a5. Drug Metab Dispos 31, 938-944.

Reschly, E.J., Bainy, A.C., Mattos, J.J., Hagey, L.R., Bahary, N., Mada, S.R., Ou, J., Venkataramanan, R., Krasowski, M.D., 2007. Functional evolution of the vitamin D and pregnane X receptors. BMC Evol Biol 7, 222.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Ginneken, C.V., Cruchten, S.V., 2016a. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. J Toxicol Sci 41, 1-11.

Saad, M., Matheeussen, A., Bijttebier, S., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Apers, S., Maes, L., Cos, P., Van Cruchten, S., 2017 Accepted . Identification of testosterone metabolites using microsomal proteins from zebrafish adults and embryos compared to humans. Data in Brief.

Saad, M., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Maes, L., Cos, P., Van Cruchten, S., 2016b. In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage. Reprod Toxicol.

Shen, S., Marchick, M.R., Davis, M.R., Doss, G.A., Pohl, L.R., 1999. Metabolic activation of diclofenac by human cytochrome P450 3A4: role of 5-hydroxydiclofenac. Chem Res Toxicol 12, 214-222.

Shimada, T., Yamazaki, H., Mimura, M., Inui, Y., Guengerich, F.P., 1994. Interindividual variations in human liver cytochrome P-450 enzymes involved in the oxidation of drugs, carcinogens and toxic chemicals: studies with liver microsomes of 30 Japanese and 30 Caucasians. J Pharmacol Exp Ther 270, 414-423.

Spaggiari, D., Geiser, L., Daali, Y., Rudaz, S., 2014. A cocktail approach for assessing the in vitro activity of human cytochrome P450s: an overview of current methodologies. J Pharm Biomed Anal 101, 221-237.

Tamminga, W.J., Wemer, J., Oosterhuis, B., Weiling, J., Wilffert, B., de Leij, L.F., de Zeeuw, R.A., Jonkman, J.H., 1999. CYP2D6 and CYP2C19 activity in a large population of Dutch healthy volunteers: indications for oral contraceptive-related gender differences. Eur J Clin Pharmacol 55, 177-184.

Teh, L.K., Bertilsson, L., 2012. Pharmacogenomics of CYP2D6: molecular genetics, interethnic differences and clinical importance. Drug Metab Pharmacokinet 27, 55-67.

von Moltke, L.L., Greenblatt, D.J., Grassi, J.M., Granda, B.W., Venkatakrishnan, K., Schmider, J., Harmatz, J.S., Shader, R.I., 1998. Multiple human cytochromes contribute to biotransformation of dextromethorphan in-vitro: role of CYP2C9, CYP2C19, CYP2D6, and CYP3A. J Pharm Pharmacol 50, 997-1004.

Waxman, D.J., Holloway, M.G., 2009. Sex differences in the expression of hepatic drug metabolizing enzymes. Mol Pharmacol 76, 215-228.

Weigt, S., Huebler, N., Strecker, R., Braunbeck, T., Broschard, T.H., 2011. Zebrafish (Danio rerio) embryos as a model for testing proteratogens. Toxicology 281, 25-36.

Wilson, V.S., McLachlan, J.B., Falls, J.G., LeBlanc, G.A., 1999. Alteration in sexually dimorphic testosterone biotransformation profiles as a biomarker of chemically induced androgen disruption in mice. Environ Health Perspect 107, 377-384.

Xu, Z., Williams, F.E., Liu, M.C., 2011. Developmental toxicity of dextromethorphan in zebrafish embryos/larvae. J Appl Toxicol 31, 157-163.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol Ther 138, 103-141.

5

Identification of testosterone metabolites

Adapted from

Identification of testosterone metabolites using microsomal proteins from zebrafish adults and embryos compared to humans. Saad, M., Matheeussen, A., Bijttebier, S., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Maes, L., Cos, P., Van Cruchten, S.

Data in Brief, accepted.

5.1 Abstract

In our previous study, the major metabolite of testosterone (TST) in humans, 6β-hydroxytestosterone, could not be detected in the zebrafish microsomal stability assay (Saad et al., 2017). Consequently, the observed depletion of TST in zebrafish must have resulted in the production of other metabolites. Therefore, an UPLC-amMS system was used in an attempt to identify these metabolites. A full spectrum scan was conducted for TST metabolites from the microsomal stability assay in zebrafish and humans. The microsomal proteins were extracted from adult zebrafish male (MLM) and female (FLM) livers, whole body homogenates of 96 hour post fertilization larvae (EM) and a pool of human liver microsomes from 50 donors (HLM). Several minor metabolites were detected in adult zebrafish with gender related differences, but no hydroxyl metabolites were observed. In zebrafish larvae at 96 hpf these hydroxyl metabolites were also absent and in general no or only very low metabolite concentrations were detected at this developmental stage.

5.2 Specifications Table

Subject area	Biology
More specific subject area	Toxicology
Type of data	Table, text file, figure
How data was acquired	Mass spectroscopy
Data format	Analyzed
Experimental factors	Microsomal proteins were extracted from livers of 10 male and 10 female zebrafish, 1500 whole body larvae and 50 human donors livers.
Experimental features	Microsomal stability assay was performed by incubation of 1 mg microsomal proteins with 40 µM testosterone for two hours at 28.5°C. The proteins were denatured using 70% acetonitrile, centrifuged at 10 000 g for 10 minutes and diluted in water till 30 % acetonitrile.
Data source location	Flemish Institute for Technological Research (VITO), Business Unit Separation and Conversion Technology (SCT), Boeretang 200, 2400 Mol, Belgium
Data accessibility	Data are presented in this article

5.3 Value of the data

Our data showed differences in metabolite profile of TST between man and zebrafish. These differences can result in different pharmacodynamic or toxicological effects depending on the pharmacological characteristics of the metabolites. Moreover, the low concentrations of

metabolites in larvae at 96 hours post fertilization (hpf) indicate a low metabolic capacity at this early developmental stage. This can influence the use of zebrafish embryos and larvae as alternative model in toxicity testing.

5.4 Results

Table 1: Abundance of different metabolites of TST in zebrafish female liver microsomal proteins (FLM), zebrafish male liver microsomal proteins (MLM), human liver microsomal proteins (HLM) and microsomal proteins of whole body homogenates of 96 hour post fertilization zebrafish larvae (EM). (*) positive ionization mode, (**) negative ionization mode

m/z	Most probable molecular formula	Abundance							
		HLM		FLM		MLM		EM	
		Time: 0	Time: 120	Time: 0	Time: 120	Time: 0	Time: 120	Time: 0	Time: 120
*275.23	$C_{19}H_{30}O$	0	1.29E+04	0	5.37E+05	0	7.54E+05	0	4.69E+04
*257.22	$C_{19}H_{28}$	0	2.87E+04	0	1.28E+06	0	2.25E+06	0	5.45E+04
*291.23	$C_{19}H_{30}O_2$	0	2.56E+04	0	2.92E+05	0	1.24E+05	0	6.01E+03
*331.22	$C_{19}H_{32}O_3$	0	2.11E+04	0	1.74E+05	0	1.82E+04	0	4.56E+03
*305.21	$C_{19}H_{28}O_3$	1.41E+05	1.47E+07	3.36E+04	3.01E+04	3.74E+04	3.74E+04	3.25E+04	8.98E+04
**467.26	$C_{25}H_{38}O_8$	0	0	0	2.41E+05	0	2.51E+04	0	0
**497.27	$C_{18}H_{40}O_9$	0	0	0	2.74E+04	0	5.89E+05	0	3.65E+04
**499.29	$C_{26}H_{42}O_9$	0	0	0	1.85E+05	0	4.53E+05	0	9.28E+03

Table 2: Approximate log differences between time 0 and time 120 min.

m/z	Most probable molecular formula	HLM	FLM	MLM	EM
*275.23	$C_{19}H_{30}O$	0	1	1	0
*257.22	$C_{19}H_{28}$	0	2	2	0
*291.23	$C_{19}H_{30}O_2$	0	1	1	0
*331.22	$C_{19}H_{32}O_3$	0	1	0	0
*305.21	$C_{19}H_{28}O_3$	2	0	0	0
**467.26	C_{25} H_{38} O_8	0	1	0	0
**497.27	C_{18} H_{40} O_9	0	0	1	0
**499.29	$C_{26}H_{42}$ O_9	0	1	1	0

Two isomeric metabolites were detected in FLM and MLM samples at *m/z* 275.23, most probably corresponding to the molecular formula of $C_{19}H_{31}O$ ($[M+H]^+$), and only minor amounts of these metabolites were formed in HLM and EM. No differences were found in the fragmentation spectra that were detected at *m/z* 275.23691. A very intense signal was observed at *m/z* 257.22 ($[M+H-H_2O]^+$), indicating the loss of one water molecule. This product ion was also formed by in-source fragmentation resulting in a much intense peak than the protonated molecule at *m/z* 275.23. Minor product ions were formed at *m/z* 109 and 123 for these metabolites. However, accurate mass data revealed a mass difference between the product ions formed for TST (123.08 and 109.06 corresponding to $C_8H_{11}O$ and C_7H_9O, respectively) and for the two isomers (109.10 and 123.11 corresponding to C_8H_{13} and C_9H_{15}, respectively).

Six other low abundant isomeric metabolites were detected at *m/z* 291.23, corresponding to a molecular formula of $C_{19}H_{31}O_2$ ($[M+H]^+$). The highest abundance of these isomers was found in FLM and MLM samples, while only minimal amounts were formed in EM and HLM. The

relative abundance of these isomers differed significantly between FLM and MLM samples. Both in-source and HCD fragmentation of five of the isomeric metabolites at *m*/*z* 291.23 resulted in high abundant peaks at *m*/*z* 273.22 and 255.21 due to the loss of two water molecules.

Two very low abundant peaks were detected at *m*/*z* 331.22, most probably corresponding to $C_{19}H_{32}O_3Na$ ($[M+Na]^+$), which indicates a gain of one oxygen and four hydrogen atoms with respect to TST. The highest abundance of these isomers was found in FLM samples, while only minimal amounts were formed in MLM and HLM and none in EM samples. Because of the low abundance of the metabolites at *m*/*z* 331.22, selective fragmentation did not produce any significant product ions.

Nine metabolic isomers at *m*/*z* 305.21 corresponding to $C_{19}H_{29}O_3$ ($[M+H]^+$) were detected in positive ion mode, but only in HLM. The most intense isomer (metabolite 4) was identified as 6β-hydroxytestosterone (6β-OHTST) by comparison with an analytical standard. Selective fragmentation of the OHTST metabolites using HCD resulted in clear fragmentation spectra of OHTST isomers 1, 4, 6, 7, 8 and 9. No fragmentation spectra could be obtained for isomers 2, 3 and 5 because of their low abundances. As expected, only very low abundant peaks were detected at *m*/*z* 97 and 109 for the 6β-OHTST isomer (metabolite 4) (Williams et al., 1999). Similarly, fragmentation of isomers 6 and 9 resulted in low abundant spectral peaks at *m*/*z* 97 and 109. In contrast, high abundant signals at *m*/*z* 97 and 109 were detected in the product ion spectra of isomers 1, 7 and 8 (Figure 1).

Very low abundant peaks of OHTST isomers were detected in all samples at time zero with no significant changes after 120 minutes in ZLM samples (Figure 2). Moreover, these minute peaks were detected to a much lesser extent in the blank samples.

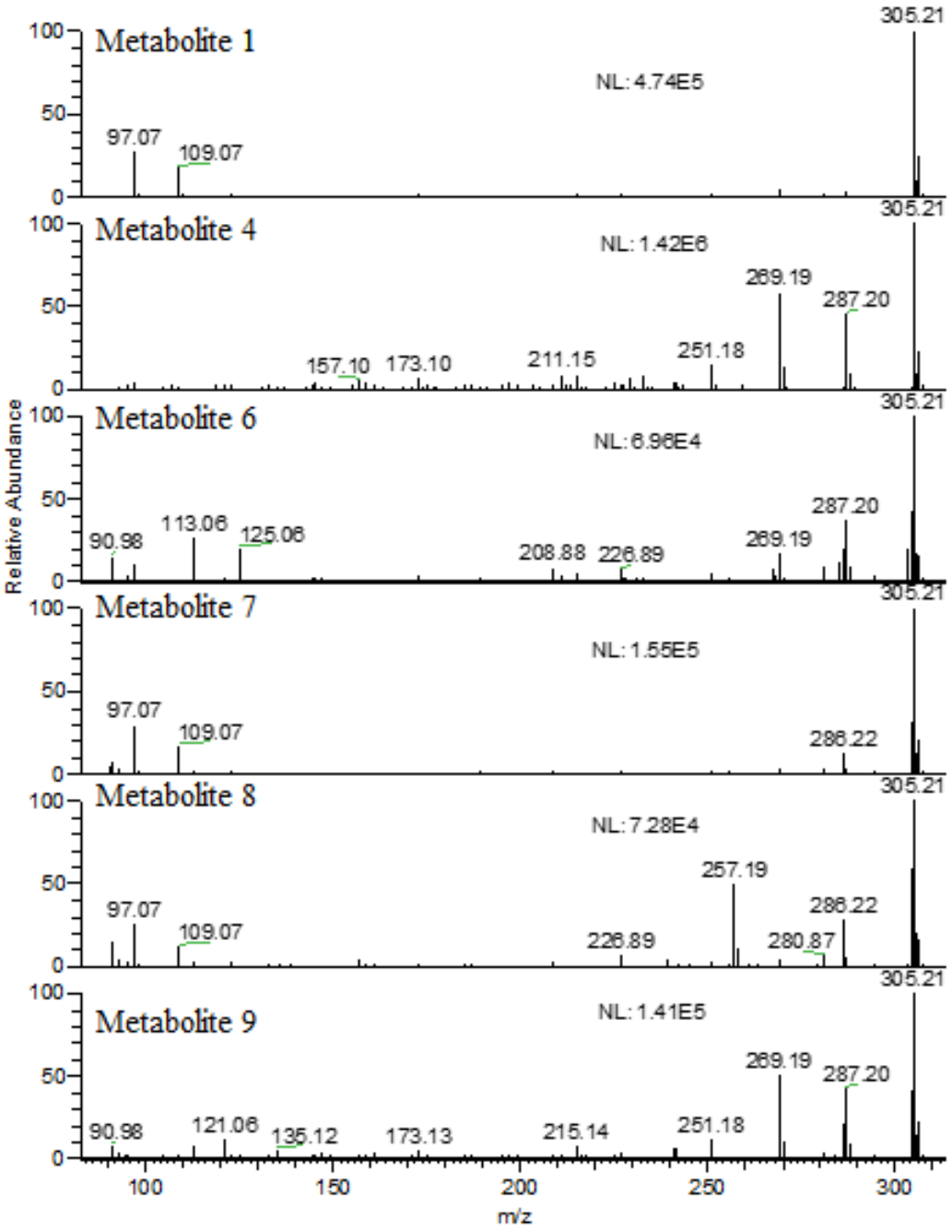

Figure 1 Selective fragmentation spectra of the isomeric metabolites of hydroxyl testosterone in positive ion mode.

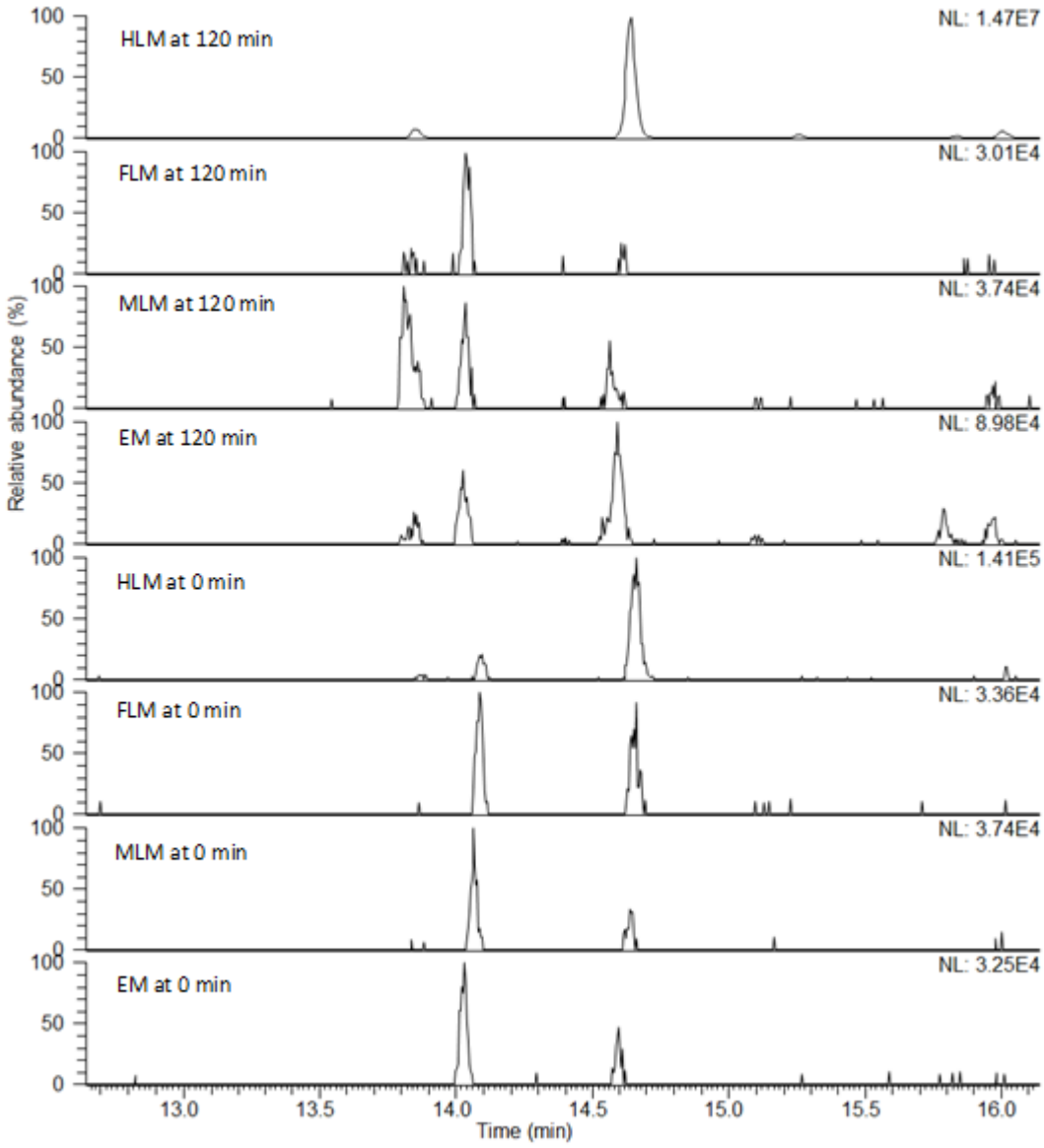

Figure 2: Extracted ion chromatogram of the metabolites at m/z 305.2 (hydroxytestosterone) in zebrafish female liver microsomal proteins (FLM), zebrafish male liver microsomal proteins (MLM), human liver microsomal proteins (HLM) and microsomal proteins of whole body homogenates of 96 hour post fertilization zebrafish larvae (EM) at time 0 and 120 minutes in the microsomal stability assay of TST.

Very low abundant peaks were detected in negative ionization mode. Three very low abundant peaks at *m/z* 467.26 and two low abundant peaks at *m/z* 499.29 corresponding to $C_{25}H_{39}O_8$ and $C_{26}H_{43}O_9$, respectively, were detected in FLM and MLM samples. One isomer of *m/z* 499.29

was also detected in EM. Additionally, one isomer was detected at *m/z* 497.27 (corresponding to $C_{18}H_{40}O_9$) in negative ionization mode in FLM and MLM, while several isomers of this metabolite were detected in EM samples (Table 1).

5.5 Experimental Design, Materials and Methods

Microsomal protein stability assay and sample extraction were conducted as stated in (Saad et al., 2017). The extracts were analyzed as described previously (Bijttebier et al., 2016). Briefly, a volume of 5 µL of extract was injected with a CTC PAL™ autosampler (CTC Analytics) on a Waters Acquity UPLC BEH SHIELD RP18 column (3.0 mm × 150 mm, 1.7 µm) and thermostatically (40°C) eluted with an Accela™ quaternary solvent manager and a 'Hot Pocket' column oven (Thermo Fisher Scientific). The mobile phase solvents consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), and a gradient as described in Table 3. For detection, an accurate mass spectrometer (amMS, Q Exactive™; Thermo Fisher Scientific) was used with heated electrospray ionization (HESI). During a first analysis, full scan data were acquired using polarity switching with an *m/z* range of 120-1800 and resolving power set at 70,000 at full width at half maximum (FWHM). Spray voltage was set at ± 2.5 kV, sheath gas and auxiliary gas at 47 and 15 (adimensional) and capillary temperature at 350°C. Fragmentation data were also recorded using higher-energy collisional dissociation (HCD) and data dependent fragmentation ($ddMS^2$) in positive and negative ionization mode (one analysis per mode) to obtain additional structural information (resolving power set at 17,500 FWHM, stepped collision energy 10, 30, 50 V, isolation window: 4 mass/charge (*m*/*z*), top 10 of most abundant ions selected for fragmentation). If fragmentation was not triggered due to low abundance of metabolites, targeted MS^2 was carried out in an effort to get diagnostic fragmentation spectra.

Table 3: The gradient of the mobile phase 30% acetonitrile for the identification of testosterone metabolites. Solvent A: water + 0.1% formic acid, solvent B: acetonitrile + 0.1% formic acid

Time (minute)	%A	%B
0	99	1
9.91	74	26
18.51	35	65
18.76	0	100
20.76	0	100
20.88	99	1
23.00	99	1

SIEVE software for differential analysis (Thermo Fisher Scientific) and Xcalibur software (Thermo Fisher Scientific) were used for processing the raw data. Comparison of TST samples before and after metabolism and blank samples (no addition of substrate) allowed to locate the metabolites that were formed. Structures were assigned to unknown peaks only when both the *m/z*-ratios and molecular formulae of the precursor and product ions were in agreement.

5.6 Discussion

In contrast to earlier studies in zebrafish (Alderton et al., 2010; Chng et al., 2012), no 6β-hydroxytestosterone was detected in our present study. The latter is the main TST metabolite in mammals, including man (Chauret et al., 1997; Krauser et al., 2004; Williams and Borghoff, 2000). Moreover, no main metabolite(s) could be detected in ZLM. However, six minor metabolites with several isomers (three in positive and three in negative ionization mode) could be detected in ZLM samples after 120 minutes of incubation, which were absent at time zero and in blank samples. The two isomers detected at m/z 275.23 could not be distinguished, as the same product ions were formed for both. The most probable molecular formula of this metabolite is C19H31O ([M+H]+), suggesting a loss of an oxygen and a gain of two hydrogen atoms with respect to the molecular formula of TST. This could potentially be caused by the loss of the carbonyl group of TST, or alternatively the hydroxyl group, and saturation of the

double bond. Interestingly, the intense signal at m/z 257.22 ([M+H-H2O]+) corresponded to the loss of water. This easy loss of water suggests the presence of a hydroxyl group in the metabolite structure. Additionally, Williams et al. (1999) reported that fragmentation of TST itself leads to the formation of major product ions, mainly at m/z 123.08 and 109.06 (corresponding to C8H11O and C7H9O, respectively). These product ions are formed by protonation of the carbonyl group of TST and subsequent fragmentation into product ions containing this carbonyl group (Williams et al., 1999). However, the formation of different product ions of C19H31O metabolites (namely C8H13 and C9H15) confirms that these isomeric metabolites are formed by the loss of the carbonyl group of TST during metabolization. This was unexpected, as oxidation reactions ought to occur predominantly during the NADPH-dependent metabolization by microsomes. Additionally, a multitude of product ions was formed during fragmentation, corresponding to molecular formulae consisting of only carbon and hydrogen, which are most probable. These product ions are most likely the result of progressive fragmentation of the alkyl structure.

Six isomeric metabolites at m/z 291.23 with a most probable molecular formula of C19H30O2 were detected. This molecular formula suggests a saturation of the double bond of TST (into dihydrotestosterone or androsterone (Kuuranne et al., 2014)) or a change of the carbonyl group into a hydroxyl moiety. The straightforward loss of two water molecules during the fragmentation implies the presence of two hydroxyl groups (reduction of the carbonyl group), which is again in opposition to our prediction of oxidized metabolites. However, the precise identity of these isomers could not be revealed on the basis of the fragmentation spectra since the abundance was too low.

The two isomers at m/z 331.22 corresponding to C19H32O3Na ([M+Na]+) most probably resulted from the saturation of the double bond, hydrogenation of the ketone functional group

into a hydroxyl functional group, and an additional hydroxylation. Nevertheless, further research is needed to elucidate the precise structure.

As expected, several isomers at m/z 305.21 were detected after HLM metabolization, which corresponds to OHTST of C19H29O3 ([M+H]+). Williams et al. (1999) investigated if OHTST isomers can be distinguished based on their product ion spectra. They reported that as hydroxylation of TST progressed from the D ring toward the A ring, the product ions at m/z 97 and 109 diminished in abundance relative to the total ion response in the spectra (Williams et al., 1999). This is because, with respect to TST fragmentation, the additional hydroxy group present in the monohydroxy TST is lost during fragmentation and the position of the resulting double bond influences the mechanism of product ion formation (Williams et al., 1999). According to the fragmentation data collected , it is suggested that the additional hydroxyl group of isomers 6 and 9 and metabolites 1, 7 and 8 is most probably located at or close to the A and D ring, respectively. It is, however, not possible to determine the position of the additional hydroxy group based solely on MS fragmentation spectra (Williams et al., 1999). Nevertheless, the precise structures of these metabolites were already investigated (Choi et al., 2005). On the other hand, no changes in OHTST levels were observed in ZLM, which contradicts previous studies (Alderton et al., 2010; Chng et al., 2012; Reschly et al., 2007). This contradiction could be explained by the differences in the experimental set up. In our study, we tried to exclude any false positive peaks that could be seen in the blank method regarding the noise in signal.

Regarding the metabolites that were detected in the negative ionization mode, no data-dependent fragmentation spectra were available because of the low abundance of the preceding metabolites. As a consequence, a more concentrated sample is needed to obtain more structural information.

From the data presented above, no oxidation activity could be detected. Nevertheless, several abundant peaks were visible after biotransformation by ZLM. Moreover, differences between FLM and MLM concerning the levels of some metabolites were also observed.

5.7 References

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Chauret, N., Gauthier, A., Martin, J., Nicoll-Griffith, D.A., 1997. In vitro comparison of cytochrome P450-mediated metabolic activities in human, dog, cat, and horse. Drug Metab Dispos 25, 1130-1136.

Chng, H.T., Ho, H.K., Yap, C.W., Lam, S.H., Chan, E.C., 2012. An investigation of the bioactivation potential and metabolism profile of Zebrafish versus human. J. Biomol. Screen 17, 974-986.

Choi, M.H., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 2005. Characterization of testosterone 11 beta-hydroxylation catalyzed by human liver microsomal cytochromes P450. Drug Metab Dispos 33, 714-718.

Krauser, J.A., Voehler, M., Tseng, L.H., Schefer, A.B., Godejohann, M., Guengerich, F.P., 2004. Testosterone 1 beta-hydroxylation by human cytochrome P450 3A4. Eur J Biochem 271, 3962-3969.

Kuuranne, T., Saugy, M., Baume, N., 2014. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. Br J Sports Med 48, 848-855.

Reschly, E.J., Bainy, A.C., Mattos, J.J., Hagey, L.R., Bahary, N., Mada, S.R., Ou, J., Venkataramanan, R., Krasowski, M.D., 2007. Functional evolution of the vitamin D and pregnane X receptors. BMC Evol Biol 7, 222.

Saad, M., Matheeussen, A., Bijttebier, S., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Apers, S., Maes, L., Cos, P., Van Cruchten, S., 2017. In vitro CYP-mediated drug metabolism in the zebrafish (embryo) using human reference compounds. Toxicol In Vitro 42, 329-336.

Williams, T.M., Borghoff, S.J., 2000. Induction of testosterone biotransformation enzymes following oral administration of methyl tert-butyl ether to male Sprague-Dawley rats. Toxicol Sci 57, 147-155.

Williams, T.M., Kind, A.J., Houghton, E., Hill, D.W., 1999. Electrospray collision-induced dissociation of testosterone and testosterone hydroxy analogs. J Mass Spectrom 34, 206-216.

6

General discussion

For the correct interpretation of pharmacological and toxicity data in the zebrafish, a thorough knowledge on CYP-related metabolism of drugs in this species is key. Therefore, we tried to answer the following research questions in this doctoral thesis:

- What are the differences in CYP-related activities between humans and zebrafish?
- Are there sex-related differences in CYP-mediated drug metabolism in zebrafish?
- Does the zebrafish embryo express CYP-related activities during the sensitive period for developmental toxicants?

Prior to discussing the obtained results for CYP activity in zebrafish during the adult and embryonic/larval stage, some words on the optimization of the technique are necessary because the above questions were addressed by an *in vitro* approach, i.e. by using microsomes.

6.1 Optimization of the in vitro drug metabolism assay

In comparison with other *in vitro* methods for studying drug metabolism, e.g S9 fractions (Otte et al., 2010), microsomal proteins have the advantage that they contain highly concentrated CYPs leading to less dilution of CYP-related activity (4-5 times higher than in S9 fractions) (Jia and Liu, 2007). However, in our study we observed a high fat content in the zebrafish liver homogenates and presence of melanophores in the embryonic tissue homogenates. As these factors would affect the reproducibility of our CYP activity assay due to turbidity of the sample, we duplicated the first centrifugation step in order to remove the high fat content and melanophores in the liver tissues and embryonic homogenates, respectively. To determine the total microsomal protein concentration, the BCA Protein Assay represents a fast and easy assay with a broad linear range (Nilsen et al., 1998). However, several dilutions of the microsomal protein fractions were made prior to performing the protein assay because the concentration of copper ions in the BCA kit was affected by EDTA molecules in the storage buffer which act as chelating agents and as such lead to an incorrect estimation of the protein concentration in the

samples (Rodger and Sanders, 2010). A dilution factor of 50x minimized the EDTA interaction significantly and kept the measurement within the linear part of the assay

A matrix effect was also noted in our study. Buffer as well as protein content in our samples had a large influence on the measurement methods. The effect of the buffer content could be easily calculated by adding the equivalent concentrations of buffer to the standard curve samples, while this was not possible for the protein effect due to the high cost of protein preparations. In LC-MS, however, the latter effect could be significantly reduced by dilution and extraction steps, taking into account that high dilution factors would affect, to some extent, the sensitivity of the measurement (Dams et al., 2003). For EROD, the use of supersomes as a blank decreased the matrix effect of the proteins. Furthermore, the use of an identical protein concentration for all samples at one run minimized inter-samples variation. Other authors used resorufin as an internal standard to avoid the matrix effect (Nilsen et al., 1998), but this increases the number of steps and duration of the EROD assay, increasing as such the risk for errors. In our study, we also noted that CYP activity by HLM was similar at 28.5°C as body temperature (37°C). As such, HLM could be included as positive controls in the same well-plate as the zebrafish samples.

6.2 Zebrafish adult data

Despite the fact that zebrafish females have a bigger mass of liver compared to males, the total protein yield per organism was similar in both genders and the number of organisms required for different assays can be approximately predicted. Consequently, no gender preference is recommended for the microsomal protein preparation. This can be explained by the high content of fats which was observed in females during the preparation process. To elucidate this difference, oil red O staining was conducted on liver samples from both genders. Large lipid droplets were detected in a higher density in female samples compared to male. This fatty

content had no effect on microsomal stability assay as we removed it during the preparation process but it should be kept in mind when precision-cut liver slices are used for the metabolic assessment. Parenchymal fat in the liver is known as storage of metabolites and thus could slow down the distribution and exposure of the potential toxic metabolites to the target organs. These effects, which can only be seen *in vivo*, are already reported in other species, particularly, for pathological liver cases such as steatosis or non-alcoholic fatty liver disease. In this regard, a possible effect could be predicted on activity of some CYPs such as CYP3A and CYP2E1 (Leclercq et al., 1998; Merrell and Cherrington, 2011). However, this is irrelevant to our study as we used normally fed non-obese zebrafish.

In our study CYP1, 2 and 3 –related activities were detected, namely EROD, diclofenac 4' and 5-hydroxylation and dextromethorphan N- and O-demethylation, whereas other activities were totally absent such as midazolam 1- and 4-hydroxylation and testosterone 6-ß hydroxylation. Furthermore, quantitative similarities but also differences were already observed in CYP activity between humans and zebrafish.

First, several human CYP1 substrates were metabolized by zebrafish CYP1. However, the latter could metabolize some human CYP2 and 3 which makes selectivity of this family less predictable. For instance, zebrafish CYP1A and to a much lesser extent CYP3A65 could metabolize 7-benzyloxy-4-(trifluoromethyl) coumarin (BFC) (a selective human CYP3A substrate) (Crespi and Stresser, 2000; Scornaienchi et al., 2010b). Additionally, E2 which is a human CYP1B1 substrate, is metabolized by CYP1C1 and CYP1A in zebrafish more efficiently than zebrafish CYP1B1 itself, however, human and zebrafish CYP3A metabolized E2 to 2-hydroxy E2 (Scornaienchi et al., 2010a). Surprisingly, Stegeman *et al.* (2015) detected 6ß-OH-testosterone as a main metabolite of testosterone by zebrafish CYP1C, which is a specific metabolic pathway of human CYP3A (Stegeman et al., 2015). In our study, EROD activity, which is CYP1A-related activity, was detected in zebrafish at a higher rate than in

humans. Regardless the differences in substrate-selectivity, zebrafish a promising candidate for (pro)carcinogenic environmental compounds which are (de)activated by CYP1A enzymes.

Second, little is known about the enzymatic activity of CYP2 and 3 families in zebrafish. Zebrafish CYP2K6 is known for catalysing aflatoxin B1 into its carcinogenic metabolite, i.e. exo-8, 9-AFB1 epoxide (Wang-Buhler et al., 2005). Additionally, other CYP3A-related activities were detected in zebrafish such as metabolism of emodin (He et al., 2012) and microcystin (Li et al., 2013). Two metabolites of tramadol were detected in zebrafish, namely O- and N-desmethyltramadol (Zhuo et al., 2016), which indicates for CYP2 and 3-related activities as tramadol is O–demethylated by CYP2D6 and is O–demethylated by CYP2B6 and CYP3A4 in humans (Subrahmanyam et al., 2001). In our study we used as CYP2 and 3-related activity substrate DXM which is metabolized in humans to DRR and, to a lesser extent, 3MM, and the former metabolite is responsible for the abuse effect. Zebrafish produced both metabolites equally which makes, for instance, the evaluation of the abuse effect of DXM more difficult as a significant pharmacological effect will be related to 3MM as well as to DRR.

This is not the case for DIC of which the main metabolites, i.e. 4-OHDIC and 5-OHDIC were present in the same ratio in both species. This similarity makes zebrafish a more conventional model for DIC than rats (for example) in which 5-OHDIC is produced at a higher ratio (Masubuchi et al., 2001).

In contrast, the absence of a major metabolization pathway in zebrafish, such as hydroxylation of TST (especially 6-ß hydroxylation), can result in important pharmacological consequences. Additionally, the absence of MDZ metabolism increases the possibility of accumulative toxicity in zebrafish (figure 1).

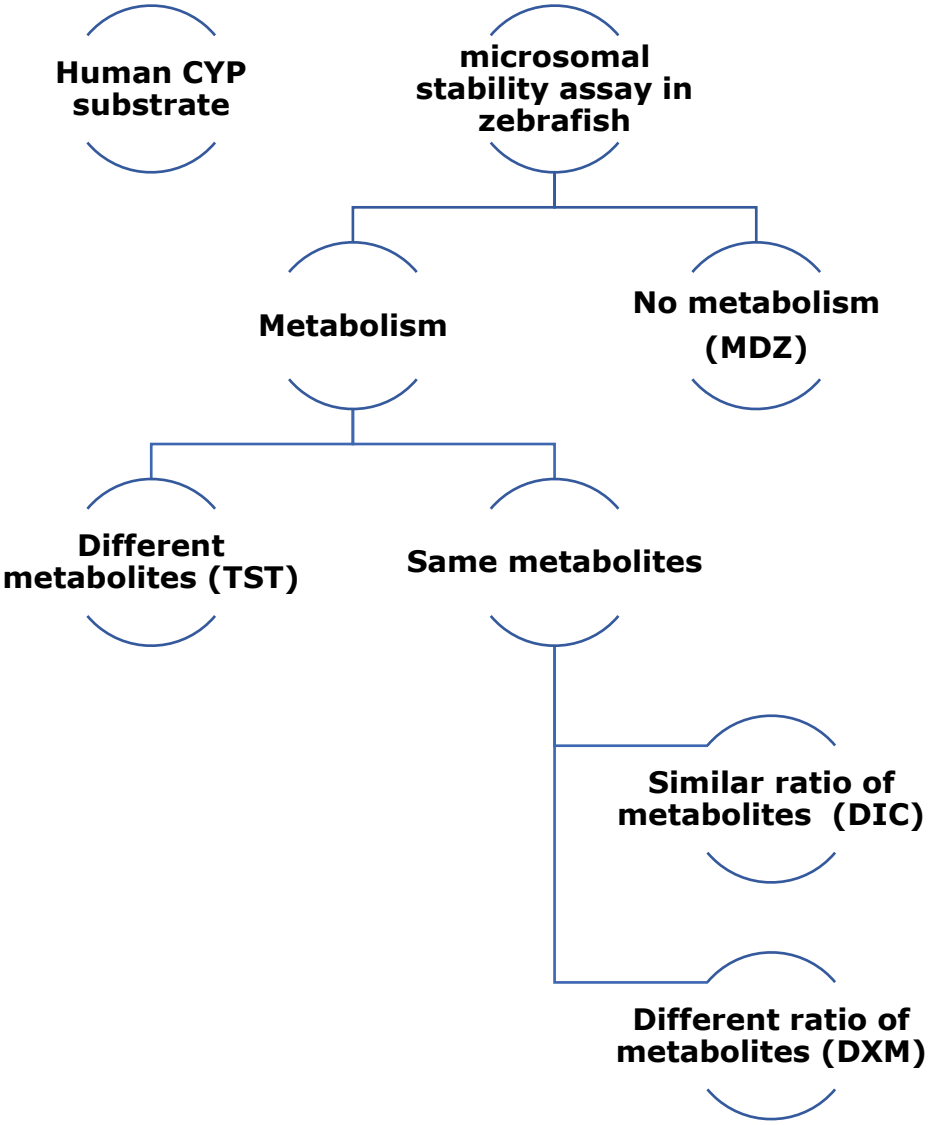

Figure 1: All possible scenarios in *in vitro* biotransformation of xenobiotics in zebrafish compared to man

Recently, we also found that zebrafish do not metabolize another CYP3 key substrate, namely Luciferin-IPA (Verbueken et al., 2017). Based on the above CYP3-related activity in zebrafish is doubtful and it remains to be assessed whether DIC 5-hydroxylation and DXM N-demethylation are CYP3-related activities. Therefore, further studies should be conducted on recombinant zebrafish CYP3 isoenzymes to determine their potential role in metabolism.

The main human metabolites of PHE and TLB i.e. acetaminophen and 4- hydroxytolbutamide were detected in FLM and MLM samples at rates of five to four times less than in HLM. The

latter substrates have a similar case as in DXM i.e. similar metabolite but different rates. No further experiments were conducted for these two substrates or any others as we already had all four possible options of metabolism similarities/differences between humans and zebrafish. Nevertheless, PHE and TLB represent additional examples of CYP1A2 and CYP2C9-related activities in zebrafish.

No significant sex-related differences were observed in the tested substrates except for TST. The latter substrate was metabolized faster in FLM compared to MLM. Consequently, a different abundance were observed in TST metabolites in both genders. These differences were reported in some mammals like rat but not in others such as the dog, monkey and humans (Bogaards et al., 2000). As a result, it is then recommended to use a mix of both genders for microsomal stability assay in zebrafish. Additionally, inter-individual differences are observed in zebrafish samples. However, batches of 10 fish each, gave acceptable levels of homogeneity. In humans, high inter-individual variations are observed as well (Parkinson et al., 2004).

Substrate-substrate interaction was assessed using a concentration of K_m of each, i.e. DXM, DIC, MDZ and TST. No significant interaction was recognized. However, further method validation should be performed prior to drawing conclusions. This investigation can be conducted in future research work.

6.3 CYPs and drug metabolism in lab animals

Several non-clinical species are used in the testing strategies of drug screening, (eco) toxicology and disease modeling, such as the non-human primates (monkeys), rodents (e.g. rat), non-rodent mammals (e.g. dog) and fish (e.g. zebrafish). Generalization from animal models to humans in these aspects, can be affected deeply by the metabolic capacity of the tested animal. CYPS play a fundamental role in xenobiotics biotransformation. Recently, 86

CYP Gene were identified in the zebrafish, 58 in humans , 114 in Rhesus monkeys, 48 in dogs, 102 in mice and 89 in rats (Pan et al., 2016; Saad et al., 2016a).

The main problem in comparing the xenobiotic metabolism systems among these species is the use of amino acid sequencing to identify the homology to human enzymes rather than the intrinsic metabolic activity. It was proven that amino acid sequence method is not sufficient as the same pathways can be conducted via non-homologous enzymes (e.g. diclofenac 5-hydroxylation is performed by CYP3A in humans and CYP2C11 in rat). Therefore, assessment of the intrinsic metabolic activity is highly required for a convenient cross-species evaluation. In this regard, several trails were conducted in order to determine the best animal model for human CYPs depending on enzymatic kinetic parameters and specific inhibition profiles. To this end, several mammal species were tested using microsomal stability assay to determine the best metabolic model compared to humans. As expected, the main metabolites of all tested substrates in humans were mostly detected in other mammals. However, quantitative differences were found depending on K_m and V_{max} but also inhibition profiles. Briefly, CYP1A, CYP2C, CYP2D, and CYP3A are better representative to humans by mouse, monkey, dog and male rat, respectively, whereas CYP2E1 did not show large differences among these species (Bogaards et al., 2000; Zuber et al., 2002). Nevertheless, some qualitative differences were also reported among mammals. For example, bromopride was metabolised in humans to 8 metabolites of which two were recognised as human-specific metabolites. However, 12 metabolites were detected in mouse, rat, rabbit, dog and monkey, but not in humans. For this particular compound, monkey represented the best model for man according to the number of shared metabolites (Dunne et al., 2013). Another example is Honokiol (poly-phenolic compound with neuroprotective properties) which was metabolised to 32 metabolites of which 17 were common among mouse, rat, dog, monkey and humans, whereas 15 were different (Jeong et al., 2016).

CYP1-related activity

CYP1As were first isolated in rat and share 80% of identity with their human counterparts. This subfamily is well studied because of its role in metabolism of a wide spectrum of environmental carcinogens such as aromatic amines and polycyclic aromatic hydrocarbons. In both rats and humans, it is induced by polycyclic aromatic hydrocarbons and polychlorinated biphenyls (Guengerich, 1997). However, the regulation mechanisms for CYP1As in monkey and dog are different from humans (Bogaards et al., 2000; Nishimura et al., 2007). Nevertheless, EROD activity is similar in rat, monkey and humans, whereas it has higher levels in dog which could be explained by the interference of other dog CYPs in this activity (Bogaards et al., 2000). In zebrafish EROD activity shows higher levels than in humans which is also can be explained by the interference of other CYPs in this activity (Saad et al., 2016b; Scornaienchi et al., 2010b).

CYP2-related activity

CYP2E1, which is the only member of this subfamily, plays an important role in detoxification of different compounds such as ethanol (Zuber et al., 2002). Rat CYPE1 shares 80% of identity with its human ortholog and it is induced by ethanol and acetone in both species (Martignoni et al., 2006). This makes rat a preferred model for CYP2E1-related substrates. Generally, other species do not show big differences in CYP2E1 related activities from humans (Bogaards et al., 2000). However, other CYP2-related activities showed more differences compared to humans. For example, some human CYP3A-related activities are performed by CYP2C in rat such as steroids hydroxylation (Nedelcheva and Gut, 1994). Quinidine, which is a specific CYP2D6 inhibitor in humans, could not affect the activity of the rat ortholog i.e. CYP2D1 (Nedelcheva and Gut, 1994). Additionally, k_m of diclofenac 4'-hydroxylation in rat and humans are so close, however, no inhibition was detected in rat via sulphaphenazole (specific human CYP2C9 inhibitor) (Bogaards et al., 2000). Additionally, the latter inhibitor did not have any

effect on tolbutamide 4-hydroxylation in rat (Eagling et al., 1998). Diclofenac was also metabolized by CYP3A to 5-hydroxydiclofenac in humans at very low levels, whereas rat CYP2C11 metabolized diclofenac to 4'- and 5-hydroxydiclofenac at similar ratios (Masubuchi et al., 2001). Monkey CYP2C represents similar substrate selectivity with a close regulation mechanism to their human counterparts (Hosaka et al., 2015; Iwasaki et al., 2010; Martignoni et al., 2006), while CYP2D6 in monkey has a wider substrate selectivity (Iwasaki et al., 2010). Propofol (CYP2-related substrate) was metabolized by the same CYP2 orthologs in humans and dog (Court et al., 2001; Hay Kraus et al., 2000). More similarities were reported in enzymatic activity between human CYP2D6 and its dog ortholog CYP2D15 (Roussel et al., 1998) which makes dog a promising model for CYP2D6- related substrates. Zebrafish showed relatively higher diclofenac 4'-hydroxylation activity compared to humans (Saad et al., 2017). In conclusion, it is more difficult to find an optimal animal model for human CYP2-related activity.

CYP3-related activity

CYP3A1 in rat is not induced by human CYP3A inducers such as rifampicin (Lu and Li, 2001) and does not metabolize nifedipine as it is the case in human CYP3A (Guengerich, 1997). Midazolam 1- and 4- hydroxylation (CYP3A-related pathways) were detected in rat as it is the case in man with different affinities and susceptibility to ketoconazole (Kotegawa et al., 2002). Midazolam 1'-hydroxylation pathway was induced by Efavirenz in monkey and humans but not in rat (Kosugi and Takahashi, 2015). In contrast, midazolam was metabolized by CYP2s in dogs (Mills et al., 2010). Zebrafish could not metabolize midazolam at all. Furthermore, TST was metabolized in mammals mainly to 6-ßhydroxytestosterone which is not the case in zebrafish (Saad et al., 2017). Interestingly, rat male has a similar TST metabolism profile to man regarding the quantitative parameters but also ketoconazole inhibition pattern (Bogaards

et al., 2000). In conclusion, several differences were detected among different species regarding CYP3-related activity which necessitate caution in extrapolation to humans.

All the above mentioned differences between man and animals provoked new solutions to find/create an optimal animal model. For instance, engineered mouse models, where mouse *CYP*s were replaced by human *CYP*s, are being increasingly used (Zhang et al., 2012). Moreover, humanized zebrafish represents a promising animal model for toxicology studies (Poon et al., 2017).

6.4 Zebrafish embryo data

Zebrafish embryo goes through several developmental stages to reach the exogenous feeding stage at 120 hpf, namely gastrula (5hpf), pharyngula (24 hpf), hatching (48 hpf) and larvae (After 72 hpf) (Kimmel et al., 1995). In general, transcription of the zygotic genome in zebrafish starts prior to mid-blastula (3.0 - 4.5 hpf) with temporal patterns (Mathavan et al., 2005). Particularly, transcription of *CYP*s was reported for several isozymes starting around 3 hpf (Goldstone et al., 2010). However, enzymatic activity is not necessarily related to the mRNA levels only as other posttranscriptional factors may interfere. Therefore, assessment of CYP intrinsic activity during early developmental stages is invaluable as most of toxicity and teratogenicity assays are conducted within the embryogenesis period.

In our study, a decrease in the microsomal protein yield was observed starting at 96 hpf, which may be due to a relative larger contribution of tissues with cells that contain either less endoplasmic reticulum or less proteins in the endoplasmic reticulum (Amar-Costesec et al., 1974). However, this finding needs further investigation. The intrinsic CYP-related activity of the majority of the tested compounds were undetectable until the larvae stage with an exception of CYP1 activity which was observed at 5 hpf. Interestingly, CYP1 activity reached the lowest

levels at 48 hpf which indicates for a maternally derived activity as this activity was already detected in the ovaries.

A temporal trend of CYP2D6 related activity, namely DXM O-demethylation, was detected in EM samples starting from 72 hpf onwards. At 96 hpf, low levels of DXM N-demethylation detected but not quantified, which is in line with a previous study (Alderton et al., 2010). The temporal trend of CYP2D6-related activity observed in the present study has already been reported in human embryos, in which DXM O-demethylation was undetectable during the first and second trimester of gestation. In the 3rd trimester O-demethylation activity was observed and a significant increase was reported at seven days postnatally (Stevens et al., 2008). However, CYP2D6 mRNA levels were lower in adulthood than early life stages despite the fact that DRR production rates were much higher in adults. Hence, no clear correlation was detected between CYP2D6 mRNA levels and DXM O-demethylation activity, suggesting a posttranslational regulation (Treluyer et al., 1991). A possible concordance between gene expression and the intrinsic activity could not be assessed in zebrafish, as the responsible isozyme for DXM O-demethylation is not defined yet in this species.

For DIC, only very low levels of metabolites could be detected in EM samples of 96 hpf. DIC hydroxylation has already been reported in 7 dpf zebrafish larvae (Alderton et al., 2010). In our study, considerable levels of 4- and 5-OHDIC (higher than the LLOD) were detected in EM at 96 hpf when high concentrations of the substrate (DIC) and the microsomal proteins were applied. The ratio 4-OHDIC/5-OHDIC was similar in EM at 96 hpf, zebrafish adults and humans, however, velocities in EM samples were much lower. Consequently, the levels of both metabolites at early developmental stages (from 5 till 72 hpf) were negligible. This is consistent with human data, in which low DIC hydroxylation was detected at early developmental stages and increased drastically during the first five months of age (Koukouritaki et al., 2004).

In conclusion, zebrafish embryo at early developmental stages did not express any CYP2 and 3-related activities and a gap in CYP1-related activity was reported between 24 and 48 hpf. Our results are consistent with data from human and rat CYP ontogeny where a lack/absence of CYP activity was detected (de Zwart et al., 2008; van den Berg et al., 2012). However, this lack/absence of CYP activity in human embryo is compensated by the maternal metabolism, which is not the case in zebrafish embryo. Therefore, the use of zebrafish embryo for (pro) teratology effects can be risky. Some solutions are suggested such as the use of exogenous metabolism system, as it is the case in metabolic *Danio rerio* Test (mDarT) (Weigt et al., 2010). The latter can provide a convenient solution, taking into account other disadvantages of this model such as oxidative stress (Pype et al., 2017).

6.5 Conclusion

Zebrafish presents a promising model for CYP1-related compounds and to less extent for CYP2 compounds. However, CYP3-related compounds seem to be of a high risk to be assessed in zebrafish. Therefore, a microsomal stability assay is a must, prior to a safe generalization from zebrafish to humans. Our *in vitro* model represents a convenient tool for such assessment. Zebrafish embryo lacks biotransformation capacity. Particularly for (pro) teratology assessment, the lack of metabolic capacity should be compensated by adding the final metabolites directly to the test medium or by the use of external metabolic system (mDarT) if possible.

6.6 Future perspectives

Our research was focused on the intrinsic CYP activity in zebrafish adults and embryos using microsomal stability assay of four human CYP substrates. However, only five CYPs were considered in this research. Other important CYP-related activities can be tested in this model

especially CYP2 family such as CYP2B6, CYP2C8, CYP2C19 and CYP2E1. Some observations from the protein yield in adults and embryos need further investigation especially the constant protein yield per embryo after 72 hpf. Additionally, the observed decrease in CYP activity after 96 hpf, as the case in DRR production (CYP2D6-like activity) or even the decreasing trend in EROD (CYP1A-like activity), could not be explained. Therefore, further research on protein content and CYP gene transcription and enzymatic activity in later developmental stages until the juvenile stage is warranted.

Additionally, phase II enzymes which have an important role in metabolism, can be assessed in this model after some changes in the reagents which were used in the microsomal stability assay.

Finally, despite the fact that we used specific human CYP substrates, from our study we can not conclude which zebrafish CYPs are involved in the observed reactions, e.g. CYP2D6 and CYP2C9, and CYP3A4 substrates in humans showed significant differences in zebrafish. Inhibition studies and recombinant CYP enzymes could provide an answer to this question and is part of other PhD research in our research group.

6.7 References

Alderton, W., Berghmans, S., Butler, P., Chassaing, H., Fleming, A., Golder, Z., Richards, F., Gardner, I., 2010. Accumulation and metabolism of drugs and CYP probe substrates in zebrafish larvae. Xenobiotica 40, 547-557.

Amar-Costesec, A., Beaufay, H., Wibo, M., Thines-Sempoux, D., Feytmans, E., Robbi, M., Berthet, J., 1974. Analytical study of microsomes and isolated subcellular membranes from rat liver. II. Preparation and composition of the microsomal fraction. J Cell Biol 61, 201-212.

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., van Bladeren, P.J., Walther, B., 2000. Determining the best animal model for human cytochrome P450 activities:

a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica 30, 1131-1152.

Chauret, N., Gauthier, A., Martin, J., Nicoll-Griffith, D.A., 1997. In vitro comparison of cytochrome P450-mediated metabolic activities in human, dog, cat, and horse. Drug Metab Dispos 25, 1130-1136.

Chng, H.T., Ho, H.K., Yap, C.W., Lam, S.H., Chan, E.C., 2012. An investigation of the bioactivation potential and metabolism profile of Zebrafish versus human. J. Biomol. Screen 17, 974-986.

Choi, M.H., Skipper, P.L., Wishnok, J.S., Tannenbaum, S.R., 2005. Characterization of testosterone 11 beta-hydroxylation catalyzed by human liver microsomal cytochromes P450. Drug Metab Dispos 33, 714-718.

Court, M.H., Duan, S.X., Hesse, L.M., Venkatakrishnan, K., Greenblatt, D.J., 2001. Cytochrome P-450 2B6 is responsible for interindividual variability of propofol hydroxylation by human liver microsomes. Anesthesiology 94, 110-119.

Crespi, C.L., Stresser, D.M., 2000. Fluorometric screening for metabolism-based drug--drug interactions. J. Pharmacol. Toxicol. Methods 44, 325-331.

Dams, R., Huestis, M.A., Lambert, W.E., Murphy, C.M., 2003. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: influence of ionization type, sample preparation, and biofluid. J Am Soc Mass Spectrom 14, 1290-1294.

de Zwart, L., Scholten, M., Monbaliu, J.G., Annaert, P.P., Van Houdt, J.M., Van den Wyngaert, I., De Schaepdrijver, L.M., Bailey, G.P., Coogan, T.P., Coussement, W.C., Mannens, G.S., 2008. The ontogeny of drug metabolizing enzymes and transporters in the rat. Reprod Toxicol 26, 220-230.

Dunne, C.E., Bushee, J.L., Argikar, U.A., 2013. Metabolism of bromopride in mouse, rat, rabbit, dog, monkey, and human hepatocytes. Drug Metab Pharmacokinet 28, 453-461.

Eagling, V.A., Tjia, J.F., Back, D.J., 1998. Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. Br J Clin Pharmacol 45, 107-114.

Goldstone, J.V., McArthur, A.G., Kubota, A., Zanette, J., Parente, T., Jonsson, M.E., Nelson, D.R., Stegeman, J.J., 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. BMC. Genomics 11, 643.

Guengerich, F.P., 1997. Comparisons of catalytic selectivity of cytochrome P450 subfamily enzymes from different species. Chem Biol Interact 106, 161-182.

Hay Kraus, B.L., Greenblatt, D.J., Venkatakrishnan, K., Court, M.H., 2000. Evidence for propofol hydroxylation by cytochrome P4502B11 in canine liver microsomes: breed and gender differences. Xenobiotica 30, 575-588.

He, Q., Liu, K., Wang, S., Hou, H., Yuan, Y., Wang, X., 2012. Toxicity induced by emodin on zebrafish embryos. Drug Chem. Toxicol 35, 149-154.

Hosaka, S., Murayama, N., Satsukawa, M., Uehara, S., Shimizu, M., Iwasaki, K., Iwano, S., Uno, Y., Yamazaki, H., 2015. Similar substrate specificity of cynomolgus monkey cytochrome P450 2C19 to reported human P450 2C counterpart enzymes by evaluation of 89 drug clearances. Biopharm Drug Dispos 36, 636-643.

Iwasaki, K., Murayama, N., Koizumi, R., Uno, Y., Yamazaki, H., 2010. Comparison of cytochrome P450 3A enzymes in cynomolgus monkeys and humans. Drug Metab Pharmacokinet 25, 388-391.

Jeong, H.U., Kim, J.H., Kong, T.Y., Choi, W.G., Lee, H.S., 2016. Comparative metabolism of honokiol in mouse, rat, dog, monkey, and human hepatocytes. Arch Pharm Res 39, 516-530.

Jia, L., Liu, X., 2007. The conduct of drug metabolism studies considered good practice (II): in vitro experiments. Curr Drug Metab 8, 822-829.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev Dyn 203, 253-310.

Kotegawa, T., Laurijssens, B.E., Von Moltke, L.L., Cotreau, M.M., Perloff, M.D., Venkatakrishnan, K., Warrington, J.S., Granda, B.W., Harmatz, J.S., Greenblatt, D.J., 2002. In vitro, pharmacokinetic, and pharmacodynamic interactions of ketoconazole and midazolam in the rat. J Pharmacol Exp Ther 302, 1228-1237.

Koukouritaki, S.B., Manro, J.R., Marsh, S.A., Stevens, J.C., Rettie, A.E., McCarver, D.G., Hines, R.N., 2004. Developmental expression of human hepatic CYP2C9 and CYP2C19. J Pharmacol Exp Ther 308, 965-974.

Krauser, J.A., Voehler, M., Tseng, L.H., Schefer, A.B., Godejohann, M., Guengerich, F.P., 2004. Testosterone 1 beta-hydroxylation by human cytochrome P450 3A4. Eur J Biochem 271, 3962-3969.

Kuuranne, T., Saugy, M., Baume, N., 2014. Confounding factors and genetic polymorphism in the evaluation of individual steroid profiling. Br J Sports Med 48, 848-855.

Leclercq, I., Horsmans, Y., Desager, J.P., Delzenne, N., Geubel, A.P., 1998. Reduction in hepatic cytochrome P-450 is correlated to the degree of liver fat content in animal models of steatosis in the absence of inflammation. J Hepatol 28, 410-416.

Li, X., Ma, J., Fang, Q., Li, Y., 2013. Transcription alterations of microRNAs, cytochrome P4501A1 and 3A65, and AhR and PXR in the liver of zebrafish exposed to crude microcystins. Toxicon 73, 17-22.

Lu, C., Li, A.P., 2001. Species comparison in P450 induction: effects of dexamethasone, omeprazole, and rifampin on P450 isoforms 1A and 3A in primary cultured hepatocytes from man, Sprague-Dawley rat, minipig, and beagle dog. Chem Biol Interact 134, 271-281.

Martignoni, M., Groothuis, G.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert Opin Drug Metab Toxicol 2, 875-894.

Masubuchi, Y., Ose, A., Horie, T., 2001. Mechanism-based inactivation of CYP2C11 by diclofenac. Drug Metab Dispos 29, 1190-1195.

Mathavan, S., Lee, S.G., Mak, A., Miller, L.D., Murthy, K.R., Govindarajan, K.R., Tong, Y., Wu, Y.L., Lam, S.H., Yang, H., Ruan, Y., Korzh, V., Gong, Z., Liu, E.T., Lufkin, T., 2005. Transcriptome analysis of zebrafish embryogenesis using microarrays. PLoS Genet 1, 260-276.

Merrell, M.D., Cherrington, N.J., 2011. Drug metabolism alterations in nonalcoholic fatty liver disease. Drug Metab Rev 43, 317-334.

Mills, B.M., Zaya, M.J., Walters, R.R., Feenstra, K.L., White, J.A., Gagne, J., Locuson, C.W., 2010. Current cytochrome P450 phenotyping methods applied to metabolic drug-drug interaction prediction in dogs. Drug Metab Dispos 38, 396-404.

Nedelcheva, V., Gut, I., 1994. P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer. Xenobiotica 24, 1151-1175.

Nilsen, B.M., Berg, K., Goksoyr, A., 1998. Induction of cytochrome P450 1A (CYP1A) in fish. A biomarker for environmental pollution. Methods Mol Biol 107, 423-438.

Nishimura, M., Koeda, A., Suganuma, Y., Suzuki, E., Shimizu, T., Nakayama, M., Satoh, T., Narimatsu, S., Naito, S., 2007. Comparison of inducibility of CYP1A and CYP3A mRNAs by prototypical inducers in primary cultures of human, cynomolgus monkey, and rat hepatocytes. Drug Metab Pharmacokinet 22, 178-186.

Otte, J.C., Schmidt, A.D., Hollert, H., Braunbeck, T., 2010. Spatio-temporal development of CYP1 activity in early life-stages of zebrafish (Danio rerio). Aquat. Toxicol 100, 38-50.

Pan, S.T., Xue, D., Li, Z.L., Zhou, Z.W., He, Z.X., Yang, Y., Yang, T., Qiu, J.X., Zhou, S.F., 2016. Computational Identification of the Paralogs and Orthologs of Human Cytochrome P450 Superfamily and the Implication in Drug Discovery. Int J Mol Sci 17.

Parkinson, A., Mudra, D.R., Johnson, C., Dwyer, A., Carroll, K.M., 2004. The effects of gender, age, ethnicity, and liver cirrhosis on cytochrome P450 enzyme activity in human liver microsomes and inducibility in cultured human hepatocytes. Toxicol Appl Pharmacol 199, 193-209.

Poon, K.L., Wang, X., Ng, A.S., Goh, W.H., McGinnis, C., Fowler, S., Carney, T.J., Wang, H., Ingham, P.W., 2017. Humanizing the zebrafish liver shifts drug metabolic profiles and improves pharmacokinetics of CYP3A4 substrates. Arch Toxicol 91, 1187-1197.

Pype, C., Verbueken, E., Saad, M.A., Bars, C., Van Ginneken, C.J., Knapen, D., Van Cruchten, S.J., 2017. Antioxidants reduce reactive oxygen species but not embryotoxicity in the metabolic Danio rerio test (mDarT). Reprod Toxicol.

Reschly, E.J., Bainy, A.C., Mattos, J.J., Hagey, L.R., Bahary, N., Mada, S.R., Ou, J., Venkataramanan, R., Krasowski, M.D., 2007. Functional evolution of the vitamin D and pregnane X receptors. BMC Evol Biol 7, 222.

Rodger, A., Sanders, K., 2010. Biomacromolecular Applications of UV-Visible Absorption Spectroscopy. Encyclopedia of Spectroscopy and Spectrometry 2, 166–173.

Roussel, F., Duignan, D.B., Lawton, M.P., Obach, R.S., Strick, C.A., Tweedie, D.J., 1998. Expression and characterization of canine cytochrome P450 2D15. Arch Biochem Biophys 357, 27-36.

Saad, M., Cavanaugh, K., Verbueken, E., Pype, C., Casteleyn, C., Ginneken, C.V., Cruchten, S.V., 2016a. Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. J Toxicol Sci 41, 1-11.

Saad, M., Matheeussen, A., Bijttebier, S., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Apers, S., Maes, L., Cos, P., Van Cruchten, S., 2017. In vitro CYP-mediated drug metabolism in the zebrafish (embryo) using human reference compounds. Toxicol In Vitro 42, 329-336.

Saad, M., Matheeussen, A., Bijttebier, S., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Apers, S., Maes, L., Cos, P., Van Cruchten, S., 2017 Accepted . Identification of testosterone metabolites using microsomal proteins from zebrafish adults and embryos compared to humans. Data in Brief.

Saad, M., Verbueken, E., Pype, C., Casteleyn, C., Van Ginneken, C., Maes, L., Cos, P., Van Cruchten, S., 2016b. In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage. Reprod Toxicol.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010a. Cytochrome P450-mediated 17beta-estradiol metabolism in zebrafish (Danio rerio). J. Endocrinol 206, 317-325.

Scornaienchi, M.L., Thornton, C., Willett, K.L., Wilson, J.Y., 2010b. Functional differences in the cytochrome P450 1 family enzymes from zebrafish (Danio rerio) using heterologously expressed proteins. Arch. Biochem. Biophys 502, 17-22.

Stegeman, J.J., Behrendt, L., Woodin, B.R., Kubota, A., Lemaire, B., Pompon, D., Goldstone, J.V., Urban, P., 2015. Functional characterization of zebrafish cytochrome P450 1 family proteins expressed in yeast. Biochim Biophys Acta 1850, 2340-2352.

Stevens, J.C., Marsh, S.A., Zaya, M.J., Regina, K.J., Divakaran, K., Le, M., Hines, R.N., 2008. Developmental changes in human liver CYP2D6 expression. Drug Metab Dispos 36, 1587-1593.

Subrahmanyam, V., Renwick, A.B., Walters, D.G., Young, P.J., Price, R.J., Tonelli, A.P., Lake, B.G., 2001. Identification of cytochrome P-450 isoforms responsible for cis-tramadol metabolism in human liver microsomes. Drug Metab Dispos 29, 1146-1155.

Treluyer, J.M., Jacqz-Aigrain, E., Alvarez, F., Cresteil, T., 1991. Expression of CYP2D6 in developing human liver. Eur J Biochem 202, 583-588.

van den Berg, H., van den Anker, J.N., Beijnen, J.H., 2012. Cytostatic drugs in infants: a review on pharmacokinetic data in infants. Cancer Treat Rev 38, 3-26.

Verbueken, E., Alsop, D., Saad, M.A., Pype, C., Van Peer, E.M., Casteleyn, C.R., Van Ginneken, C.J., Wilson, J., Van Cruchten, S.J., 2017. In Vitro Biotransformation of Two Human CYP3A Probe Substrates and Their Inhibition during Early Zebrafish Development. Int J Mol Sci 18.

Wang-Buhler, J.L., Lee, S.J., Chung, W.G., Stevens, J.F., Tseng, H.P., Hseu, T.H., Hu, C.H., Westerfield, M., Yang, Y.H., Miranda, C.L., Buhler, D.R., 2005. CYP2K6 from zebrafish (Danio rerio): cloning, mapping, developmental/tissue expression, and aflatoxin B1 activation by baculovirus expressed enzyme. Comp Biochem. Physiol C. Toxicol. Pharmacol 140, 207-219.

Weigt, S., Huebler, N., Braunbeck, T., von Landenberg, F., Broschard, T.H., 2010. Zebrafish teratogenicity test with metabolic activation (mDarT): effects of phase I activation of acetaminophen on zebrafish Danio rerio embryos. Toxicology 275, 36-49.

Williams, T.M., Borghoff, S.J., 2000. Induction of testosterone biotransformation enzymes following oral administration of methyl tert-butyl ether to male Sprague-Dawley rats. Toxicol Sci 57, 147-155.

Williams, T.M., Kind, A.J., Houghton, E., Hill, D.W., 1999. Electrospray collision-induced dissociation of testosterone and testosterone hydroxy analogs. J Mass Spectrom 34, 206-216.

Zhang, D., Luo, G., Ding, X., Lu, C., 2012. Preclinical experimental models of drug metabolism and disposition in drug discovery and development. Acta Pharmaceutica Sinica B 2, 549-561.

Zhuo, H., Jin, H., Peng, H., Huang, H., 2016. Distribution, pharmacokinetics and primary metabolism model of tramadol in zebrafish. Mol Med Rep 14, 5644-5652.

Zuber, R., Anzenbacherova, E., Anzenbacher, P., 2002. Cytochromes P450 and experimental models of drug metabolism. J Cell Mol Med 6, 189-198.

7 Summary

Summary

In this research, we investigated potential differences and similarities in CYP-related drug metabolism between zebrafish and man, as well as the ontogeny of CYP activity in zebrafish. To this end, we compared *in vitro* alternative models for teratogenicity studies, including the zebrafish embryo in **Chapter** 1. This comparison showed the importance of the zebrafish embryo as a promising alternative model. Additionally, we compiled all available zebrafish CYP data regarding spatiotemporal gene expression, protein levels and intrinsic activity. Mainly gene expression data are available and to a lesser extent intrinsic activity. Therefore, further studies concerning the intrinsic CYP activity are needed, especially during organogenesis in view teratogenicity testing.

Chapter 2 describes in detail our research hypotheses and objectives.

The first step in this research was to optimise our experimental design including tissues collection, protein fractions' preparation and microsomal protein stability assay. In **Chapter 3** we assessed CYP1 activity in adult zebrafish and zebrafish embryos using the EROD assay as it is an easy, cheap and fast assay. The aim was first to establish standard experimental conditions that minimize any confounding factors in EROD activity. Afterwards, any potential gender differences in liver CYP1 activity in adult zebrafish and the ontogeny of CYP1 activity during organogenesis were assessed. Our EROD data showed a lack in CYP1 activity in zebrafish embryos compared to adults. No sex-related differences were detected. The high reproducibility of microsomal protein fraction preparation and EROD assay was encouraging to proceed with other CYP activity-related tests.

CYP2 and 3–related activity was tested in **Chapter 4** using human CYP-specific substrates. Despite the fact that the major metabolites of diclofenac showed a very similar ratio in zebrafish compared to humans, dextromethorphan showed differences in its metabolite ratio for these species. Testosterone was metabolized in both species but into totally different metabolites.

Surprisingly, midazolam was not metabolized at all in zebrafish. The last two substrates show that toxicity studies in zebrafish need to be interpreted with caution for human risk assessment when pharmacokinetics play a key role. Moreover, zebrafish embryo showed no or very low CYP activity during the early developmental stages. This finding is of critical value when considering the zebrafish embryo for teratogenicity studies.

In **Chapter 4** we also showed that testosterone was metabolized in both species. However, the main human metabolite was not detected in zebrafish. Therefore, a sensitive LC-MS/MS method was applied on testosterone microsomal stability assay samples to compare the metabolites in both species **(in Chapter 5)**. Apparently, zebrafish and humans do not share the same main metabolites for testosterone. Interestingly, testosterone is the only substrate that showed sex-related differences in zebrafish which is in accordance with the data in man.

Finally, we discussed our research results in **Chapter 6** and compared them to the available data from other conventionally used animals in toxicity and pharmacological studies. In conclusion, significant differences in biotransformation of xenobiotics can be expected between humans and zebrafish, which must be taken in consideration during human risk assessment.

Samenvatting

In dit onderzoek bekeken we mogelijke verschillen en overeenkomsten in het CYP-gemedieerd geneesmiddelenmetabolisme tussen de zebravis en de mens, evenals de ontogenie van de CYP-activiteit in de zebravis.

Hierbij maakten we gebruik van een alternatief model voor teratogeniciteitsstudies, nl. het zebravisembryo, wat beschreven wordt in **hoofdstuk 1**. Hierin wordt het belang van het zebravisembryo als een veelbelovend alternatief model besproken. Daarnaast zijn alle beschikbare CYP-gegevens van de zebravis verzameld met betrekking tot spatiotemporele gen- en eiwitexpressie en intrinsieke activiteit. Er zijn hoofdzakelijk gegevens over genexpressie beschikbaar en in mindere mate over intrinsieke activiteit. Daarom zijn verdere studies met betrekking tot de intrinsieke CYP-activiteit nodig, vooral tijdens de organogenese met het oog op teratogeniciteitstesten.

Hoofdstuk 2 beschrijft in detail de onderzoekshypothesen en –doelstellingen van dit doctoraal proefschrift.

De eerste stap in dit onderzoek was het optimaliseren van het in vitro protocol, waaronder de verzameling van weefsels, de bereiding van eiwitfracties en de microsomale eiwitstabiliteitsassay behoorden. In **hoofdstuk 3** hebben we de CYP1-activiteit in vitro, nl. in de microsomale fracties, bij volwassen zebravissen en zebravisembryo's bepaald met behulp van de EROD-test, aangezien het een eenvoudige, goedkope en snelle test is. Het doel was eerst om de experimentele omstandigheden te standardiseren. Daarna werden eventuele potentiële geslachtsverschillen in CYP1-activiteit van de lever in de volwassen zebravis en de ontogenie van CYP1-activiteit tijdens de organogenese beoordeeld. Onze EROD-gegevens toonden een lage tot zeer lage CYP1-activiteit in zebravisembryo's vergeleken met volwassenen. Er werden geen geslachtsgerelateerde verschillen gedetecteerd. De hoge

reproduceerbaarheid van microsomale eiwitfractiebereiding en EROD-assay was bemoedigend om door te gaan met andere in vitro CYP-activiteitsgerelateerde testen.

CYP2 en 3-gerelateerde activiteit werd bepaald in **hoofdstuk 4** met behulp van humane CYP-specifieke substraten, nl. diclofenac, dextromethorfan, midazolam en testosteron. Ondanks het feit dat de belangrijkste metabolieten van diclofenac in de zebravis een gelijkaardige verhouding vertoonden in vergelijking met de mens, toonde dextromethorfan verschillen in zijn metabolietratio voor deze species. Testosteron werd in beide species gemetaboliseerd, maar in andere metabolieten. Verrassend genoeg werd midazolam in de zebravis helemaal niet gemetaboliseerd. De laatste twee substraten laten zien dat toxiciteitsstudies in de zebravis voorzichtig moeten worden geïnterpreteerd in het kader van humane geneesmiddelenontwikkeling wanneer de farmacokinetiek een sleutelrol speelt. Bovendien vertoonde het zebravisembryo geen of zeer lage CYP-activiteit tijdens de vroege ontwikkelingsstadia. Deze bevinding is van cruciaal belang bij het gebruik van het zebravisembryo bij teratogeniciteitsstudies.

In **hoofdstuk 5** wordt de metabolisatie van testosteron bij de mens en de zebravis verder besproken door het gebruik van een gevoelige LC-MS/MS-methode die toegepast werd op testosteron-microsomale stabiliteitstestmonsters om de metabolieten in beide species te vergelijken. Blijkbaar delen zebravissen en mensen niet dezelfde belangrijke metabolieten voor testosteron. Interessant is dat testosteron het enige substraat was dat geslachtsgebonden verschillen vertoont in de zebravis, wat ook het geval is bij de mens.

Tenslotte bespreken we onze onderzoeksresultaten in **hoofdstuk 6** en worden ze vergeleken met de beschikbare gegevens van andere conventioneel gebruikte dieren in toxiciteits- en farmacologische studies. Concluderend kan er worden verwacht dat er belangrijke verschillen zijn in de CYP-gemedieerde omzetting van xenobiotica in mensen en zebravissen, waarmee rekening moet worden gehouden bij de beoordeling van toxiciteitsstudies.

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Bibliography

Publications

- Antioxidants reduce reactive oxygen species but not embryotoxicity in the metabolic Danio rerio test (mDarT).Pype C, Verbueken E, Saad MA, Bars C, Van Ginneken CJ, Knapen D, Van Cruchten SJ. Reprod Toxicol. 2017 Sep;72:62-73. doi: 10.1016/j.reprotox.2017.06.132. Epub 2017 Jun 27.
- In vitro CYP-mediated drug metabolism in the zebrafish (embryo) using human reference compounds. Saad M, Matheeussen A, Bijttebier S, Verbueken E, Pype C, Casteleyn C, Van Ginneken C, Apers S, Maes L, Cos P, Van Cruchten S.Toxicol In Vitro. 2017 Aug;42:329-336. doi: 10.1016/j.tiv.2017.05.009. Epub 2017 May 12.
- In Vitro Biotransformation of Two Human CYP3A Probe Substrates and Their Inhibition during Early Zebrafish Development. Verbueken E, Alsop D, Saad MA, Pype C, Van Peer EM, Casteleyn CR, Van Ginneken CJ, Wilson J, Van Cruchten SJ. Int J Mol Sci. 2017 Jan 22;18(1). pii: E217. doi: 10.3390/ijms18010217.
- In vitro CYP1A activity in the zebrafish: temporal but low metabolite levels during organogenesis and lack of gender differences in the adult stage. Saad M, Verbueken E, Pype C, Casteleyn C, Van Ginneken C, Maes L, Cos P, Van Cruchten S. Reprod Toxicol. 2016 Sep;64:50-6. doi: 10.1016/j.reprotox.2016.03.049. Epub 2016 Apr 1.
- Xenobiotic metabolism in the zebrafish: a review of the spatiotemporal distribution, modulation and activity of Cytochrome P450 families 1 to 3. Saad M, Cavanaugh K, Verbueken E, Pype C, Casteleyn C, Van Ginneken C, Van Cruchten S. J Toxicol Sci. 2016 Feb;41(1):1-11. doi: 10.2131/jts.41.1. Review.
- Incubation at 32.5°C and above causes malformations in the zebrafish embryo. Pype C, Verbueken E, Saad MA, Casteleyn CR, Van Ginneken CJ, Knapen D, Van Cruchten SJ. Reprod Toxicol. 2015 Aug 15;56:56-63. doi: 10.1016/j.reprotox.2015.05.006. Epub 2015 May 22.
- Ontogeny of CYP3A and P-glycoprotein in the liver and the small intestine of the Göttingen minipig: an immunohistochemical evaluation. Van Peer E, Verbueken E, Saad M, Casteleyn C, Van Ginneken C, Van Cruchten S. Basic Clin Pharmacol Toxicol. 2014 May;114(5):387-94. doi: 10.1111/bcpt.12173. Epub 2013 Dec 13.

Oral presentations

- ETS (European Teratology Society)
- BSMS (The Belgian Society for Mass Spectrometry)

Poster presentations

- The Belgian Society for Mass Spectrometry (BSMS) meeting, 2016. Drug metabolism in the zebrafish: an in vitro study using human CYP-specific substrates Saad Moayad, VerbuekenEvy , PypeCasper , CasteleynChristophe , Van GinnekenChris , MaesLouis2, Cos Paul2, Van Cruchten Steven
- The European Teratology Society meeting (42nd Annual meeting), 1-4/9/2014, Hamburg – Germany. EROD activity in microsomal protein fractions of zebrafish is gender- and developmental stage dependent. M. Saad, E. Verbueken , C. Pype , E. Van Peer , C. Casteleyn , C. Van Ginneken , P. Cos , S. Van Cruchten.
- Fish and amphibian embryos as alternative models in toxicology and teratology 1.- 2. December 2014, Aulnay-sous-Bois/Paris, France. The ontogeny of CYP1A activity in zebrafish. M. Saad, E. Verbueken , C. Pype , E. Van Peer , C. Casteleyn , C. Van Ginneken , P. Cos , S. Van Cruchten.
- The European Teratology Society meeting (43rd Annual meeting), 30th Aug to 3rd Sep2015, Amsterdam – the Netherland. Sex differences and ontogeny of CYP1A activity in the zebrafish: an in vitro investigation. Saad Moayad, Verbueken Evy, Pype Casper, Casteleyn Christophe, Van Ginneken Chris, Van Cruchten Steven.