Review

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

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ABSTRACT — The zebrafish (*Danio rerio*) has been increasingly explored in pharmaceutical research as a promising alternative model for toxicological screens. This necessitates a thorough knowledge on the biotransformation processes for a correct interpretation of pharmacological and toxicological data. Physiologically, cytochrome P450 (CYP) enzymes, specifically CYP families 1-3, play a pivotal role in drug metabolism. And yet, information regarding activity of CYP, its isoforms, and conjugation enzymes in zebrafish is either scarce or conflicting. To account for this discrepancy, the available spatiotemporal, modulation and activity data on zebrafish CYP 1-3 families are reviewed in this paper and compared with human CYP data. The CYP genetic features and synteny are well characterized, as is their expression in different organ systems. Moreover, several substrates metabolized by humans also show metabolism in zebrafish, with other CYP isoforms possibly involved. Altogether, the five CYP1 members, 41 CYP2 members and five CYP3 members in zebrafish show distinct evolutionary and orthological similarities with humans.

Key words: Zebrafish, Cytochrome P450, Biotransformation, Xenobiotics, Ontogeny, Synteny

INTRODUCTION

The zebrafish (*Danio rerio*) is a vertebrate organism that has become ubiquitous in modeling development and toxicology. This laboratory animal offers the power of whole-animal investigations paired with the convenience of cell culture techniques that are cost- and time-effective, as well as requiring minimal infrastructure. From a morphological standpoint, the zebrafish is a convenient model compared to its higher vertebrate counterparts. The transparency of the embryo, in particular, affords simple, non-invasive visual analysis of developmental processes *in vivo*. Marked by high fecundity, the zebrafish allows for large sample sizes with fast organismal development and maturation. Therefore, this organism has become a highly desired alternative model for early selection of compounds during the drug development process. Since the toxic effects of compounds depend on the achieved exposure within the organism, a thorough knowledge of the biotransformation processes is necessary for proper

safety assessment. However, biotransformation capacity in zebrafish adults and embryos still needs more elucidation (Beker van Woudenberg *et al.*, 2013; Brannen *et al.*, 2010; Weigt *et al.*, 2012)

Drug metabolism is largely achieved by an oxidative reaction catalyzed through cytochrome P450 enzymes (CYPs). Originally found in rat liver microsomes, CYPs are in fact present in all eukaryotic organisms (such as animals, plants, and fungi) and even in some prokaryotes. CYPs 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). 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

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Table 1. Zebrafish CYP families 1, 2 and 3.

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

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).

In the zebrafish, the full complement of *CYP* genes and their developmental expression have 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 are also affected. In humans, constitutive androstane receptor (CAR), pregnane X-receptor (PXR) and aryl hydrocarbon receptor (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 phys-

iological 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).

The following review 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).

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) (Jönsson *et al.*, 2007a, 2007b; Goldstone *et al.*, 2009). 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

CYPs in zebrafish

Table 2. Synteny between zebrafish and human CYPs.

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	-	2C(8,9,18,19)
	D	-	2D6
	E	-	2E1
	K	2K6,2K8,2K16-22, 2K31	2W1
	P,AD,V,N	2AD(2,3,6), 2J20, 2N13,2P(6,7,10), 2V1	2J2
	U	2U1	2U1
	R	2R1	2R1
	X	2X(6-10)	-
CYP3	Y	2Y3	2(A6,A13,B6,F1,S1)
	A	3A65	Inactive CYP3A genes
	C	3C(1-4)	3(A3,A4,A7)

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, 7-ethoxyresorufin (ER), 7-methoxyresorufin (MR), 17 β -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, 2010b). 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 (Jönsson *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 hours post-fertilization (hpf) (Goldstone *et al.*, 2009). Because of this gap in expression, Mattingly and Toscano (2001) suggested that a post-transcriptional 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, 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-pentoxoresorufin (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 (Jönsson *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* independ-

ent 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 (Jönsson *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. 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 *CYP1Cs* is higher than all other CYP1s at 80 hpf (Jönsson *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 (Jönsson *et al.*, 2007a, 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

(Jönsson *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 ovaries. In particular, *CYP1C1* and *CYP1C2* also have high basal expressions in the gills and the kidneys, respectively (Jönsson *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 6 β -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 CYP1s, *CYP1D1* is not induced by AHR agonists such as 6-formylindolo[3,2-b]carbazole (Jönsson *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 6 β -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 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 (Mosadeghi *et al.*, 2007; Yuan *et al.*, 2013; Kubota *et al.*, 2013, 2015). In contrast, several substrates tend to induce CYP2s 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 CYP2s (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 16 α -carbonitrile (PCN), but not with phenobarbital (PB), 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP), or PCB126 (Kubota *et al.*, 2013, 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 ovaries of adult zebrafish with a late onset of expression in the embryos. It is poorly expressed at 3 days post fertilization (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. 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* (Goldstone *et al.*, 2010; Genome Reference Consortium, 2015), 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 activ-

ity 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, 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 Ca²⁺ 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 suppres-

sors 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 metabolism profiles are reported, e.g. for testosterone (Chng *et al.*, 2012). The metabolism 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 CYP3As in human beings and mammals in general, which can be induced by several xenobiotics such as rifampicin, clotrimazole and nifedipine in man and dexamethasone and pregnenolone 16 α -carbonitrile (PCN) in mice (Coumoul *et al.*, 2002; Liddle and Goodwin, 2002; Moore *et al.*, 2002; Guengerich, 1999). In zebrafish larvae, CYP3A65 is also induced by pregnenolone (PN), rifampicin and dexamethasone (Tseng *et al.*, 2005; Kubota *et al.*, 2015). 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 24 hr 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 48 hr instead of 24 hr. 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.

Conclusion

Zebrafish CYPs, as the most important enzymes in biotransformation, show a strong evolutionary and ortho-logical 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 that a distinct paucity in information regarding xenobiotic CYP activity within family 1-3 exists. Uncovering these elusive mechanisms will aid in enhancing developmental toxicity tests, which contain contradicting information regarding CYP activity and function. With increasing knowledge on the biotransformation processes in zebrafish at different developmental stages compared to humans, the zebrafish can be further validated and strengthened as a vertebrate model in drug discovery and toxicology.

Conflict of interest---- The authors declare that there is no conflict of interest.

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