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## The cytochrome P450 2AA gene cluster in zebrafish (*Danio rerio*); expression of *CYP2AA1* and *CYP2AA2* and response to phenobarbital-type inducers

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### Abstract

The cytochrome P450 (CYP) 2 gene family is the largest and most diverse CYP gene family in vertebrates. In zebrafish, we have identified 10 genes in a new subfamily *CYP2AA*, which does not show orthology to any human or other mammalian *CYP* genes. Here we report evolutionary and structural relationships of the 10 *CYP2AA* genes and expression of the first two genes, *CYP2AA1* and *CYP2AA2*. Parsimony reconstruction of the tandem duplication pattern for the *CYP2AA* cluster suggests that *CYP2AA1*, *CYP2AA2* and *CYP2AA3* likely arose in the earlier duplication events and thus are most diverged in function from the other *CYP2AAs*. On the other hand, *CYP2AA8* and *CYP2AA9* are genes that arose in the latest duplication event, implying functional similarity between these two CYPs. A molecular model of *CYP2AA1* showing the sequence conservation across the *CYP2AA* cluster reveals that the regions with the highest variability within the cluster map into *CYP2AA1* near the substrate access channels, suggesting differing substrate specificity. Zebrafish *CYP2AA1* transcript was expressed predominantly in intestine, while *CYP2AA2* was most highly expressed in kidney, suggesting differing roles in physiology. In liver *CYP2AA2* expression but not that of *CYP2AA1*, was increased by 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and, to a lesser extent, by phenobarbital (PB). In contrast, pregnenolone 16 $\alpha$ -carbonitrile (PCN) increased *CYP2AA1*, but not *CYP2AA2* in liver. The results identify a *CYP2* subfamily in zebrafish that includes genes apparently induced by PB-type chemicals and PXR agonists, the first concrete *in vivo* evidence for a PB-type response in fish.

### Keywords

Cytochrome P450; CYP2; Zebrafish; Molecular evolution; Homology modeling; Phenobarbital

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## Introduction

Cytochrome P450 (CYP) enzymes play key roles in the synthesis and degradation of physiologically important endogenous substrates such as steroids, cytokines and fatty acids, and in the biotransformation of xenobiotics. The *CYP2* gene family is the largest and most complex of the 18 *CYP* gene families in vertebrates. Currently there are over 40 *CYP2* subfamilies and hundreds of genes, with differing numbers of genes even between closely related species, arising from differences in deletion or expansion in the number of genes (Kirischian et al., 2011; Kubota et al., 2011a; Nelson et al., 2013). Understanding the regulation and function of the CYP2s is important because of the broad range of pharmacological, toxicological, and endogenous substrates transformed by these proteins.

An intriguing question involving the CYP2s in fish concerns the long known yet not understood difference between mammals and fish in their response to phenobarbital (PB) type inducers. In mammals, PB and a number of structurally diverse xenobiotics, including the highly potent 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and *ortho*-substituted polychlorinated biphenyl (PCB) congeners, strongly induce the expression of members of the CYP2B subfamily (Kopec et al., 2010; Nims et al., 1993; Smith et al., 1993a, 1993b; Vezina et al., 2004; Waxman, 1999). There is a long history of efforts to identify and demonstrate a CYP2B-like induction by PB or related compounds in fish (Bainy et al., 1999; Buhler and Wang-Buhler, 1998; Celander et al., 1996; Elskus and Stegeman, 1989; Förlin, 1980; Goksøyr, 1985; Iwata et al., 2002; Kleinow et al., 1990; Klotz et al., 1986; Stegeman et al., 1990). However, studies seeking PB-type induction involving analysis of microsomal enzymes or of RNA expression for CYPs related to CYP2B have been inconclusive. Some studies have shown increased expression of CYP1A in trout treated by PB, but this does not appear to involve mechanisms like those involved in CYP2B induction in mammals (Sadar et al., 1996).

Failure to detect a PB-type induction of *CYP* genes in fish as compared to mammals could reflect a difference in regulatory mechanisms, lack of *CYP* genes that are responsive to PB, or the use of the wrong endpoints. In mammals the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) are involved in the induction of *CYP* genes by PB (Waxman, 1999). Teleost fish possess a *PXR* but apparently lack a *CAR* gene (Handschin et al., 2004; Maglich et al., 2003; Reschly and Krasowski, 2006). *CAR* (*NR1I3*) either arose from *PXR* (*NR1I2*) after the divergence of ray-finned fish and lobe-finned fish (the line leading to mammals), or was lost in teleosts (Mathas et al., 2012). *In vitro* reporter gene assays with a *PXR* ligand-binding domain have shown that a zebrafish *PXR* can be activated by structurally diverse chemicals, with differing potencies among chemicals and among species (Ekins et al., 2008; Milnes et al., 2008; Moore et al., 2002). Some *in vivo* studies with fish including zebrafish also revealed that known agonists for human *PXR*, including pregnenolone 16 $\alpha$ -carbonitrile (PCN), increased expression levels of *PXR* itself and *CYP3A* (Bresolin et al., 2005). However, whether fish *CYP2s* are responsive to potential agonists for the *PXR* remains to be understood.

Our initial approach to find fish homologues of *CYP2* genes that are responsive to PB and PB-type inducers was to amplify sequences with primers designed from conserved regions in mammalian *CYP2B* genes. The first efforts resulted in identification of the *CYP2Ns* and *CYP2Ps* (Oleksiak et al., 2000, 2003). Subsequent efforts to find fish *CYP2B*-like genes and study their regulation have employed zebrafish and resulted in cloning of sequences defining a novel subfamily, *2AA*. More recently we identified and annotated the full suite of *CYP* genes in zebrafish (Goldstone et al., 2010), including 10 *CYP2AA* genes. The *CYP2AA* genes are excellent examples of the evolution of sequence diversity within tandemly-duplicated gene clusters, and provide an important opportunity for understanding the evolution of

functional diversity in toxicologically and pharmacologically relevant gene subfamilies. Here we report on cloning of the full-length *CYP2AA1* and *CYP2AA2*, evolutionary and structural relationships of the 10 *CYP2AAs*, and the expression of the first two genes *in vivo*, including response to PB, TCPOBOP and PCN.

## Materials and methods

### Fish Husbandry

Sexually mature zebrafish (*Danio rerio*) of the Tupfel/long (TL) fin wild-type strain were used in all experiments. Fish were maintained under standard light and temperature conditions as described previously (Jönsson et al., 2007). The experimental procedures were approved by the Animal Care and Use Committee of the Woods Hole Oceanographic Institution.

### Cloning strategy

Prior to the genomic sequence of zebrafish being available, a BLAST search using human *CYP2B* against the zebrafish ESTs database was carried out and matching regions were used for designing primers (in 2001). Adult specimens ( $n = 8$ ) killed by cervical scission were used for cloning of a *CYP2* subfamily in zebrafish. Messenger RNA (mRNA) was prepared from the liver of individual fish using the MicroPoly(A)Pure™ kit (Ambion). Oligo d(T)-primed cDNA synthesis from mRNA was carried out by RT using the SMART™ PCR cDNA synthesis kit (Clontech). Using the cDNA synthesized, an initial fragment was yielded with the forward primer 5'-CATTATCAAGCATTTCCCTGGTCC-3' and the reverse primer 5'-CGCTGAATCTCGTGAACAGTGG-3', after which the resulting sequences suggested two distinct *CYP2* genes. Forward, reverse and nested primers distinct to each of these genes were designed to obtain PCR products that were used as templates for rapid amplification of cDNA ends (RACE). Primers to clone the full-length coding sequence of *CYP* genes were designed to the untranslated region of 5' and 3' ends.

### Sequence and structural analyses of *CYP2AA*

*CYP2AA1* and *2AA2* were cloned in 2002 prior to the sequencing of the zebrafish genome. Subsequently, additional *CYP2AA* genes were identified in a cluster in the zebrafish genome (Goldstone et al., 2010). Phylogenetic trees were constructed by analyzing inferred or confirmed amino acid sequences using maximum likelihood (RAxML 7.0.4) (Stamatakis, 2006) using the WAG model of amino acid substitution (Whelan and Goldman, 2001). Synteny (gene order) was examined using the Ensembl browser. Parsimony tandem duplication analysis was performed using DILTAG (Lajoie et al., 2010). Molecular models were constructed using Modeller [v9.8; (Eswar et al., 2007)]. Multiple models were built for each *CYP2AA* isoform based on structures of mammalian *CYP2s* (*CYP2C5*: 1DT6, *CYP2B4*: 1PO5, *CYP2A6*: 3EBS, *CYP2C19*: 4GQS). The best model was chosen on the basis of the discrete optimized potential energy (DOPE) score. Alignment scoring was determined using Scorecons (Valdar, 2002). Substrate access channels were determined using the Caver 3.0.1 plugin for PyMol (Chovancova et al., 2012).

### Tissue-specific gene expression

Adult zebrafish were anesthetized by MS-222 and were euthanized by decapitation. The organs including intestine, liver, testes, ovary, heart, kidney, brain, and eye were collected by dissection. Three replicates were collected for both males and females, resulting from four individuals pooled per replicate for each organ. The dissected organs were flash frozen in liquid nitrogen and were stored at  $-70$  °C until RNA isolation.

## Chemical exposure

To study response of zebrafish to archetypal inducers of *CYP2B* genes, male specimens ( $n = 8$ ) were injected *i.p.* with TCPOBOP (1.5  $\mu\text{g/g}$ ) or PB (70  $\mu\text{g/g}$ ) dissolved in a solution of 15% dimethylsulphoxide (DMSO) and 85% saline. Fish from the vehicle control group was injected with the DMSO/saline solution. As a separate experiment, male specimens ( $n = 6$ ) were injected *i.p.* with PCN (20  $\mu\text{g/g}$ ) dissolved in DMSO. Fish from the vehicle control group was injected with DMSO. Stock solutions were prepared in order to inject 2  $\mu\text{l/g}$  fish for each chemical. The fish were kept in system water (Jönsson et al., 2007) in 3 l tanks with constant aeration, and were fed with live brine shrimp (*Artemia salina*) twice a day for the first two days. Three days after the injection, fish were killed by cervical section and liver was excised immediately and frozen in liquid nitrogen. Total RNA was isolated from the liver of individual fish using RNA STAT-60 (Tel-Test B, Inc., Friendswood, TX). The quantity of RNA was determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE, USA). cDNA was synthesized from 1  $\mu\text{g}$  total RNA using iScript kit (Bio-Rad).

## Quantitative real time PCR

Quantitative real time PCR was performed using the iQ SYBR Green Supermix (Bio-Rad) in a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad), according to the manufacture's instruction. Gene-specific primers for real time PCR were synthesized by Eurofins MWG Operon (Huntsville, AL, USA), and are shown in Supplemental Table S1. In each sample, the genes were analyzed in duplicate with the following protocol: 95 °C for 3 min and 95 °C for 15 s/62 °C for 1 min (45 cycles). A melt curve analysis was performed at the end of each PCR run to ensure that a single product was amplified. The preliminary study confirmed that the right product was amplified by qPCR. Relative mRNA expression of each target gene was normalized to that of *ARNT2* and *EF1 $\alpha$*  ( $E^{-\Delta\text{Ct}}$ ; where  $\Delta\text{Ct} = [\text{Ct}(\text{target genes}) - \text{Ct}(\text{geometric mean of } ARNT2 \text{ and } EF1\alpha)]$ ). Relative changes due to treatment (TCPOBOP, PB, or PCN) were determined by  $E^{-\Delta\Delta\text{Ct}}$  ( $E^{-\Delta\text{Ct}[\text{sample}]} / \text{mean } E^{-\Delta\text{Ct}[\text{control}]}$ ). PCR efficiencies ( $E$ ) for within-experiment amplicon groups were determined by the LinRegPCR program (Ramakers et al., 2003; Ruijter et al., 2009).

## Statistics

Messenger RNA expression levels of *CYP2AA* genes are presented as mean  $\pm$  SD for tissue distribution analysis and as range with 25th to 75th percentile for chemical effect analysis. Significant differences in the mRNA levels among 7 tissues were determined for each gene by one-way ANOVA which if significant was followed by Tukey-Kramer test. Significance of differences in the mRNA expression levels between control and treatment groups (i.e., TCPOBOP or PB) was determined for each gene by one-way ANOVA which if significant was followed by Dunnett test. In a separate experiment, significance of differences in the mRNA expression levels between control and PCN-treated group was determined by Student *t*-test. Spearman's rank correlation test was conducted for relationships of expression levels of *CYP2AA* genes with *PXR* gene in each treatment group. The significant level was set at 0.05. All statistical analyses were performed with GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

## Results

### Molecular cloning of CYP2AAs

The initial identification of zebrafish *CYP2AA* genes was accomplished prior to sequencing of the genome and thus before it was possible to search the genome to annotate all CYPs. Initially, a fragment of 458 bp was amplified and the sequence analysis indicated the

presence of consistent nucleotide differences in different clones, suggesting that there were at least two different sequences with 89% identity, indicating the presence of two genes or possible allelic variation. RACE analysis with primers designed to amplify the 5' and 3' cDNA ends of both genes resulted in full-length sequences of the coding regions. Alignment and comparison with known *CYP2* family sequences resulted in a new subfamily designated *CYP2AA*, with the first sequences termed *CYP2AA1* (AF497969) and *CYP2AA2* (#####; see Supplemental Fig. S1). Open reading frames for *CYP2AA1* and *CYP2AA2* are 1494 bp each, coding for proteins of 498 amino acids.

The *CYP2AA1* and *CYP2AA2* nucleotide sequences share 88% identity, while the deduced amino acid sequences share 84% identity. The amino acid sequence of the cloned *CYP2AA2* is identical to the most recent Ensembl *CYP2AA2* sequence entry (ENSDARP00000021614), while the cloned *CYP2AA1* showed a 14 amino acid difference from its Ensembl entry (ENSDARP00000093164), suggesting an allelic variation of *CYP2AA1*. The *CYP2AA1* and *CYP2AA2* predicted protein sequences were 38-41% identical to mammalian *CYP2Bs* and killifish *CYP2Ns* and *CYP2Ps*. Several functional motifs (i.e., a putative heme-binding motif, conserved threonine residue, proline rich region, etc.) between zebrafish *CYP2AAs* and other vertebrate *CYP2s* were well conserved (Supplemental Fig. S1).

### Evolutionary and structural relationships of the *CYP2AAs*

The zebrafish genome has 10 *CYP2AA* genes located on chromosome 23 in a duplicated array (Goldstone et al., 2010). The *CYP2AAs* are most closely related to the *CYP2X* subfamily, found in zebrafish and other fishes, and somewhat less closely related to the fish *CYP2K*, and mammalian *CYP2A/B/F/G/S/T* clusters (Goldstone et al., 2010; Kirischian et al., 2011). In order to understand evolutionary relationships within the *CYP2AA* gene cluster, we constructed a maximum likelihood phylogenetic tree (Fig. 1A) and conducted a parsimony reconstruction of the *CYP2AA* gene duplication pattern (Fig. 1B). There are likely eight independent tandem duplication events in this cluster (Fig. 1B). Multiple duplication patterns exhibit the same lowest parsimony score (Supplemental Fig. S2). Four alternative patterns with identical parsimony scores are possible in the second duplication. However, in all cases the last three rounds of duplication are identical. Parsimony reconstruction suggests that *CYP2AA1*, *CYP2AA2*, and *CYP2AA3* are genes that likely arose in the earlier duplication events and thus are most diverged from the other *CYP2AAs*. In contrast, *CYP2AA8* and *CYP2AA9* seem to occur in the latest duplication event. The genomic arrangement of the *CYP2AA* cluster genes (Fig. 1B) shows that *CYP2AA8* and *CYP2AA9* are located next to each other, while *CYP2AA1*, *CYP2AA2*, and *CYP2AA3* are present sequentially in the cluster.

Although the *CYP2AAs* are all closely related, the existence of such a large tandemly duplicated array of genes implies some functional differences between the expressed proteins (Bergthorsson et al., 2007). The amino acid sequence identity between the duplicated genes ranges from 65-85%. The regions of highest amino acid variability were mapped onto a molecular model of *CYP2AA1* based on the crystal structures of closely related mammalian *CYP2s* (37-44% identity over the length of available crystal structures). This mapping (Fig. 2) reveals that these variable regions occur primarily at the entrances to the substrate access channels, as well as a few residues at the N and C-termini of the structure. These substrate access channels are created by tertiary structure, which brings together amino acids that are distant in the primary sequence (e.g., the highly variable residues 105 and 372, both red in Fig 2). Notably, much of the variability is located with the substrate recognition sites (SRSs), identified from the analysis of primary sequence but which form the substrate access channels and active site (Gotoh, 1992). Separate model

construction and analysis of other CYP2AAs showed that the position of these computed substrate access channels does not appear to be significantly altered in any of the CYP2AA models (data not shown).

### Tissue distribution of CYP2AA1 and CYP2AA2 transcripts

To determine the tissue distribution of the *CYP2AA1* and *CYP2AA2* expression, total RNA isolated from various tissues was reverse transcribed and internal primers designed to specific regions from both genes were used for quantitative real time PCR analysis. *CYP2AA1* transcripts were more highly expressed in intestine than the other tissues analyzed (Fig. 3A, 3B), in which *CYP2AA1* was evenly expressed. In contrast, *CYP2AA2* transcripts were particularly abundant in kidney, while they were detected at very low levels in gonad and heart (Fig. 3C, 3D). No clear sex-difference in the tissue distribution profile was observed for either *CYP2AA1* or *CYP2AA2*.

### Response to select xenobiotics in adults

We first examined responses of *CYP2AA1* and *CYP2AA2* to PB and TCPOBOP in liver of adult zebrafish (Fig. 4). No significant differences in *CYP2AA1* transcript levels were observed among treatments. In contrast, treatment with TCPOBOP caused significant up-regulation of *CYP2AA2*, with a 3.2-fold increase in the transcript levels relative to the vehicle control group. A smaller increase (2.1-fold) in *CYP2AA2* transcript levels was also observed for PB-treated fish, although this was not statistically significant. In order to examine possible mechanisms by which these chemicals showed inter-individual variability of *CYP2AA* expression in each treatment group and by which TCPOBOP and, to a lesser extent, PB increased transcript levels of *CYP2AA2*, we performed correlation analyses between *PXR* and each of *CYP2AA1* and *CYP2AA2*. Spearman's rank correlation tests showed a significant positive correlation between *PXR* and *CYP2AA1* in the PB-treated group (Fig. 5). None of the other correlations examined between *PXR* and *CYP2AA1* or *CYP2AA2* was statistically significant (Supplemental Fig. S3). We also tested the response of the *CYP2AA* genes to PCN, another potential agonist for zebrafish *PXR*. Injection of adult zebrafish with PCN significantly increased transcript levels of *CYP2AA1* in liver, but not *CYP2AA2* (Fig. 6A, 6B). Yet, neither *CYP2AA1* nor *CYP2AA2* showed significant correlation with *PXR* in these samples (data not shown), although the number of samples was small for this analysis ( $n = 6$ ).

## Discussion

The CYP2 gene family is the largest CYP gene family in vertebrates. The diversity of *CYP2* genes in teleost fishes is as great as that in mammals, but the function and regulation of CYP2s in fish is, with some exceptions, unknown. Establishing similarities and differences between CYP2s in different taxa is made more difficult by evolutionary distance, which can obscure homologous relationships and result in distinct classification for orthologs. For example, based on sequence, the *CYP2N* and *CYP2P* subfamilies found in fish do not occur in mammals, yet molecular phylogeny and syntenic arrangement of the genes indicate that the *CYP2Ns* and *CYP2Ps* all are co-orthologs of mammalian *CYP2Js* (Goldstone et al., 2010; Kirischian et al., 2011). Moreover, nearly identical regio- and enantio-selectivity for oxidation of arachidonic acid indicates that at least one *CYP2P* (*CYP2P3*) and human *CYP2J2* are closely related functionally (Oleksiak et al., 2000; Oleksiak et al., 2003).

Understanding the roles of CYPs in zebrafish is important to the use of this premier non-mammalian species in toxicological, pharmacological and carcinogenesis research (e.g., (Kubota et al., 2011b; Teraoka et al., 2009). In zebrafish there are 44 *CYP2* genes in 11 subfamilies (Goldstone et al., 2010), most of which do not show obvious orthology to

human or other mammalian genes. These include genes in the new *CYP2AA* subfamily. The *CYP2AA* subfamily consists of 10 genes that are tandemly duplicated in a locus on chromosome 23 but that locus does not share synteny with any mammalian *CYP2* genes. This lack of shared synteny appears to reflect lineage-specific gene subfamily expansion and translocation, occurring as well with CYPs in other vertebrate species (Kubota et al., 2011a; Nelson et al., 2013; Nelson et al., 2004). The *CYP2AA* subfamily is phylogenetically closely related to the fish *CYP2X* subfamily, another *CYP2* gene subfamily that does not appear to share synteny with any mammalian *CYP2*s.

While the biological significance of these tandemly duplicated *CYP2AA* genes is not known, we suggest here that analysis of the pattern of tandem duplication as well as the genomic arrangement may help to predict functional and evolutionary diversity within a particular cluster of *CYP* genes (Fig. 1). We examined evolutionary relationships within the set of *CYP2AA* genes, with the objective of inferring functional similarity and difference within the subfamily. Parsimony reconstruction of the tandem duplication pattern for the *CYP2AA* cluster suggests that *CYP2AA8* and *CYP2AA9* likely are similar in original function, while *CYP2AA1*, *CYP2AA2* and *CYP2AA3* are likely to be most divergent in function from the other *CYP2AAs*. Although *CYP2AA1* and *CYP2AA9* are located next to each other, these two genes appear to have arisen from an earlier duplication event, implying that functional diversity could arise despite their proximity to one another.

Sub- and neo-functionalization within the *CYP2AA* cluster may proceed in both expression patterns (as observed in *CYP19A1/CYP19A2* expression in ovary versus brain) and in protein function [as in the *CYP17A1/CYP17A2* functional divergence (Zhou et al., 2007)]. Detailed expression patterns of the complete *CYP2AA* set will provide further insight into possible spatiotemporal divergence. Regarding functional divergence, effort was made to express *CYP2AA1* and *CYP2AA2* proteins in COS-7 cells, yet there was no detectable activity with potential substrates, including alkoxyresorufins. Although the analysis of substrate differences requires detailed functional studies, interesting details can be gleaned from a closer examination of the primary protein sequence and molecular modeling. Mapping of the observed sequence variability within the *CYP2AA* cluster onto a three dimensional protein model provides insight into the spatial location of the protein regions that determine substrate specificity (Fig. 2). As noted above, the regions with the highest variability within the *CYP2AA* cluster occur near the substrate access channels, suggesting that there are indeed differences in substrate specificity, and that these differences could be controlled by the (relatively few) variable residues near the entrances to the substrate channels.

In a recent study of the complement of *CYP*s in Atlantic cod (*Gadus morhua*) neither EST- nor genome-assemblies contained *CYP* genes that clustered in the *CYP2AA* clade (Karlsen et al., 2012). Our survey of fish genomes of seven other species currently available on Ensembl (i.e., coelacanth, fugu, medaka, platyfish, stickleback, tetraodon, and tilapia) also failed to identify any *CYP* genes that are readily annotated as members of the *CYP2AA* subfamily. Whether the genes in *CYP2AA* subfamily are specific to *Cypriniformes* to which zebrafish belong, or common to a broader lineage of fish requires further investigation as more fish genomes being available.

We, and others, have long sought some fish *CYP* genes that, like mammalian *CYP2Bs*, are responsive to PB and PB-type chemicals (Bainy et al., 1999; Buhler and Wang-Buhler, 1998; Celander et al., 1996; Elskus and Stegeman, 1989; Förlin, 1980; Goksøyr, 1985; Iwata et al., 2002; Kleinow et al., 1990; Klotz et al., 1986; Stegeman et al., 1990). In mammals, PB-type induction of *CYP2B* genes occurs via both CAR and PXR (Waxman, 1999). While zebrafish lack CAR, they do possess PXR (Maglich et al., 2003; Reschly and Krasowski,

2006), and several lines of evidence indicate that a variety of structurally diverse chemicals can activate PXR of zebrafish (Ekins et al., 2008; Milnes et al., 2008; Moore et al., 2002). These prior studies led us to hypothesize that fish respond to PB and related chemicals through PXR.

The present study showed evidence for the induction of transcripts of *CYP2AA1* and *CYP2AA2* in liver of zebrafish treated with the mammalian PXR agonist PCN and with a PB-type inducer TCPOBOP. An earlier study also reported that PCN induces the mRNA expression level of *PXR* in liver of zebrafish, suggesting a positive self-regulation (Bresolin et al., 2005). Indeed, we also found a significant up-regulation of the *PXR* mRNA levels in liver of zebrafish treated with PCN (Kubota et al., unpublished results). However, we failed to find coordinate induction of *PXR* and *CYP2AA1*, as no significant correlation was found between levels of transcripts for these two genes in the PCN-treated group, although with a limited number of samples ( $n = 6$ ). There also was no significant up-regulation of *PXR* mRNA expression in the TCPOBOP-treated group ( $n = 8$ ) (data not shown), nor significant correlation between expression levels of *PXR* and *CYP2AA2*. TCPOBOP is known to activate human PXR, but not zebrafish PXR *in vitro*, although under differing conditions of the reporter assay (e.g., the expression plasmids differed, consisting of a full-length for human PXR and a ligand binding domain for zebrafish PXR) (Ekins et al., 2008; Milnes et al., 2008; Moore et al., 2002). Overall, there still is an unresolved question about mechanisms underlying up-regulation of *CYP2AA* genes in response to PCN and TCPOBOP. Whether the PCN- and TCPOBOP-induced transcript expression of *CYP2AA1* and *CYP2AA2* observed in the present study depends upon the PXR signaling pathway requires further investigation.

There was no significant difference in the transcript level of *CYP2AA1* between the vehicle control group and the PB-treated group. Nevertheless, the significant positive correlation between *PXR* and *CYP2AA1* in fish treated with PB is pronounced and presumably represents a shared mechanism of the transcript regulation of these two genes in response to PB. *In vitro* studies have shown that PB has a potency to activate zebrafish PXR (Moore et al., 2002; Reschly et al., 2007), yet the efficiency of PB for the activation was the lowest among the chemicals tested, including androstanol, 5 $\alpha$ -cyprinol 27-sulfate and, and TCDD (Reschly et al., 2007). It is likely that since PB has a weak potency to activate zebrafish PXR *in vitro*, the induction of its potential target genes, perhaps including *CYP2AA1*, may be weak *in vivo*. Alternatively, there is a possibility that *PXR* alleles identified in zebrafish (Bainy et al., unpublished results) contribute to the inter-individual differences in the *PXR* and *CYP2AA1* responses, as some fish showed no or very weak response to PB but others showed substantial response in the present study.

While the results here show a *CYP2* transcript induction by PB-type compounds, there are still important questions about this observation, and about PB response(s) in fish in general. Is the magnitude of the response biologically significant, and are protein levels induced as well? Does the mechanism of induction involve ligand activation of PXR or some other transcription factor? How might cellular context influence the response of the *CYP2AAs*? Are increased rates of metabolism of substrates, such as observed with PCBs *in vivo* in fish exposed to congeners that are mammalian *CYP2B*-like inducers (Buckman et al., 2007), linked to *CYP2AAs* or to other *CYPs* yet to be described but that also respond to PB-type inducers? Inference from substrate metabolism alone is weak, without linkage of the activity to a specific catalyst, but can suggest directions for research. The *CYP2AA* genes offer subjects for further addressing the question of PB responsiveness in fish. However, the indications of *CYP2AA* response do not preclude the possibility of other *CYPs* that respond to PB-type inducers, or involvement of *CYPs* that are not PB responsive in catalytic



functions with substrates that in mammals are metabolized by CYP2Bs. Identification of responsive genes may be forthcoming, as full transcriptome analyses are completed.

At present, as described above, there still is uncertainty about the breadth of occurrence of the *CYP2AA* subfamily in the fishes. Whether the PB-type induction of *CYP2AA* genes observed in zebrafish is conserved in other fish awaits the complete annotation of more fish genomes and subsequent studies on the chemical effect on the gene expression. It also will be interesting to determine whether fish have *CYP2* genes other than *CYP2AAs* that are responsive to PB and related chemicals. For example, zebrafish *CYP2Y3* and *CYP2Y4* are syntenic and therefore orthologous to genes in human *CYP2A/B/F/G/S/T* cluster, which includes PB responsive genes (Zhang et al., 2010)

In summary, on zebrafish chromosome 23 there are 10 *CYP2AA* genes that presumably arose from eight independent tandem duplication events. We cloned the cDNAs of the first two genes, *CYP2AA1* and *CYP2AA2*, and found that they are expressed in extrahepatic tissues. Our finding of the prominent expression of zebrafish *CYP2AA1* in intestine and *CYP2AA2* in kidney opens the possibility that these enzymes have distinct physiological roles in intestinal and renal functions. We provided evidence showing a PB-type response of *CYP2* genes, including *CYP2AA1* and *CYP2AA2*, with differing responses between the two genes. However, further studies are necessary to address regulatory mechanisms of these *CYP2* genes upon exposure to PB-type inducers, PXR agonists, and other xenobiotic inducers, including AHR ligands. Mapping of the regulatory elements upstream of each of these tandemly duplicated genes could indicate shared or divergent regulation. Knocking down of PXR expression in zebrafish embryos using morpholino antisense oligonucleotides and subsequent exposure of the embryos to PXR agonists also is one possible approach currently underway to address this question.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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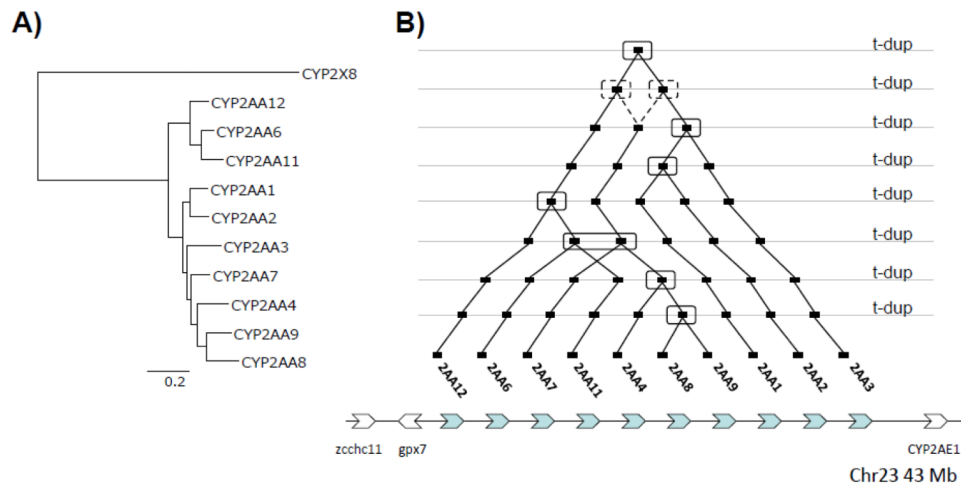
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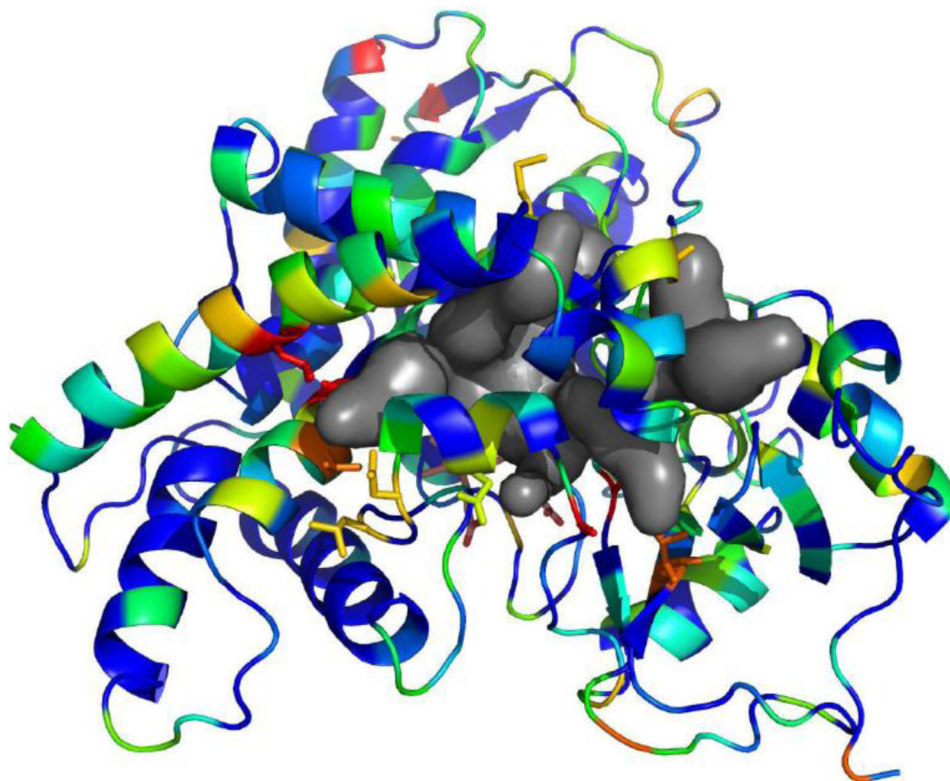
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### Highlights

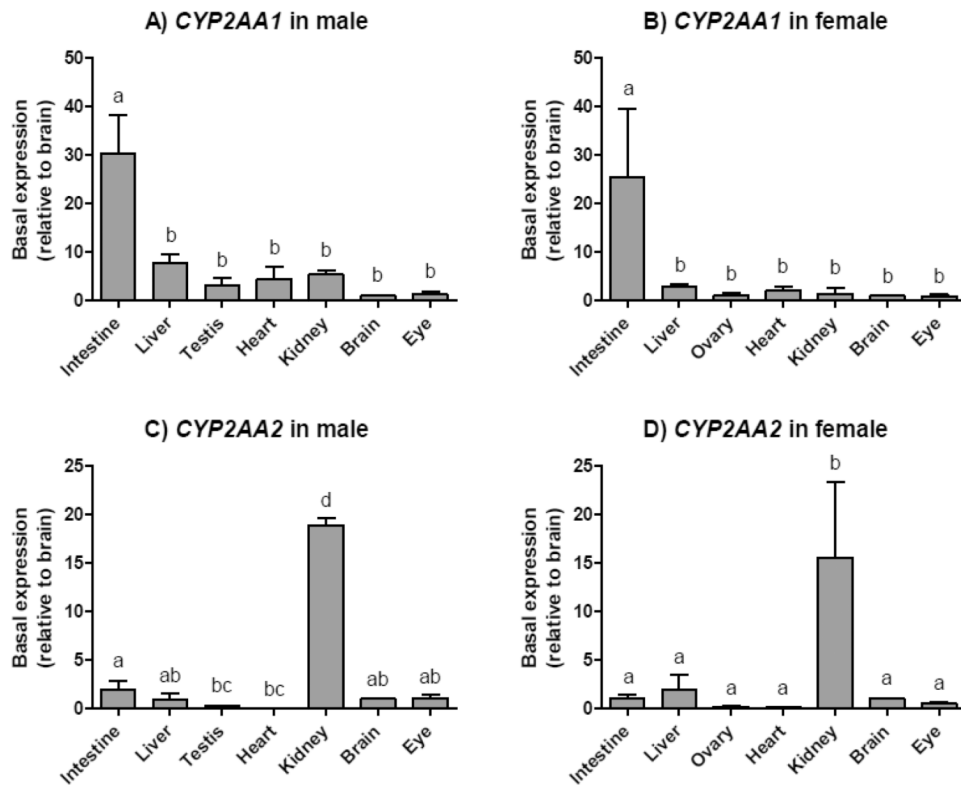
- A tandemly duplicated cluster of ten CYP2AA genes was described in zebrafish.
- Parsimony and duplication analyses suggest pathways to CYP2AA diversity.
- Homology models reveal amino acid positions possibly related to functional diversity.
- The CYP2AA locus does not share synteny with any CYP2 subfamily in mammals.
- Induction of *CYP2AA1* and *CYP2AA2* indicates a phenobarbital-type response in fish.



**Fig. 1.** Maximum likelihood phylogeny, parsimony reconstruction of tandem duplication, and syntenic relationships for the *CYP2AA* cluster. The maximum likelihood phylogeny (A) was constructed using RA×ML, with zebrafish *CYP2X8* as the outgroup. The tandem duplication (t-dup) pattern (B) for the *CYP2AA* cluster located on Zv9 Chromosome 23 was constructed using DILTAG. Duplication events are noted with a box. Note that four alternative patterns with identical parsimony scores are possible, illustrated for two cases by the dashed lines in the second duplication. In all cases the last three rounds of duplication are identical.

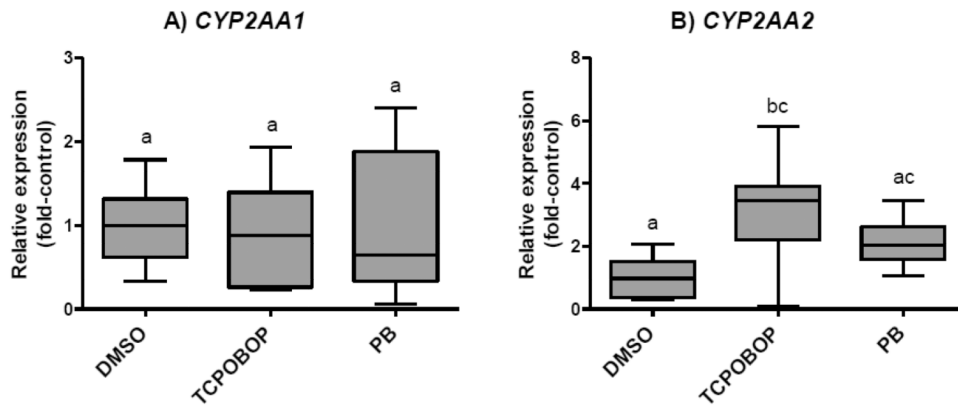


**Fig. 2.** Homology model of CYP2AA1 showing the sequence conservation across the CYP2AA cluster and computed substrate access channels. The model was constructed using Modeller 9v8 based on four mammalian CYP2 crystal structures. Colors indicate the range of BLOSUM80-based sequence conservation scores across the CYP2AA cluster, with red indicating the regions of highest variability and dark blue the regions without any substitutions. Also shown are the top substrate access channels computed with Caver 3.0. Note that the two primary regions with low conservation are located at the entrance to the channels with the highest computed throughput.

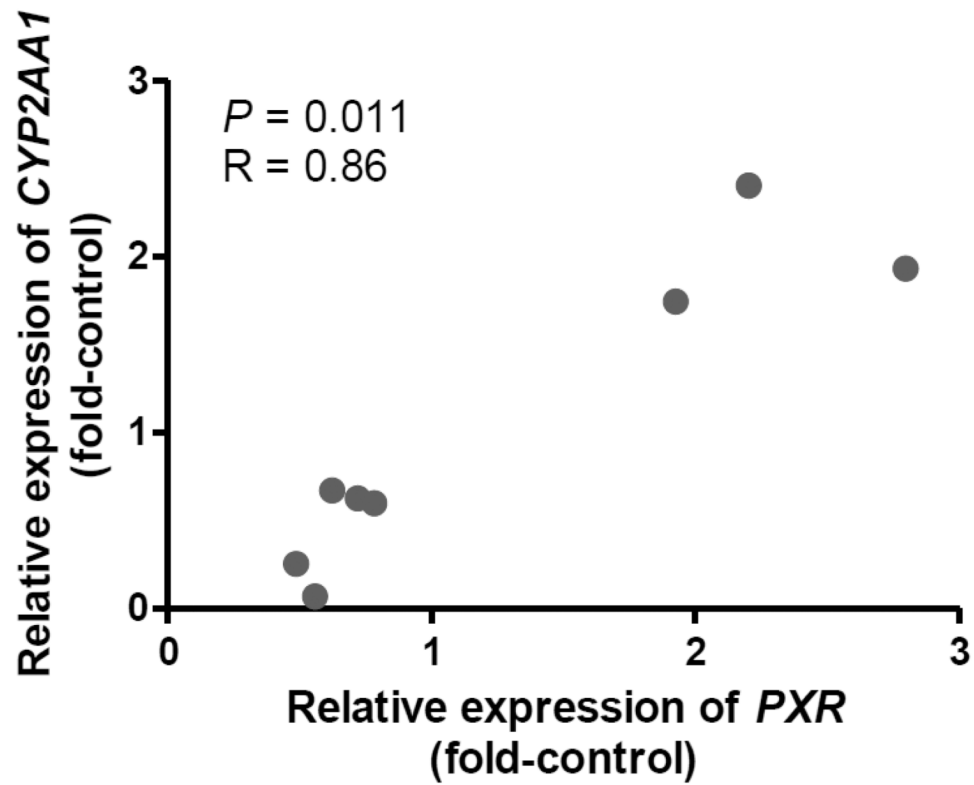
**Fig. 3.**

Tissue distribution of *CYP2AA1* (A, B) and *CYP2AA2* (C, D) in adult zebrafish. Transcript levels of *CYP2AA1* and *CYP2AA2* were determined by qPCR in seven tissues from males and females. Data were normalized by geometric mean of two separate reference genes, *Ef1a* and *ARNT2*. Results are shown as values in each tissue relative to the average value in brain (mean + SD;  $n = 3$  for each sex), as this organ showed one of the least inter-individual variability and sex-differences in the *CYP2AA* expression levels. Statistical differences in transcript levels among tissues were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ( $p < 0.05$ ). Outliers were excluded based on the Grubbs test.

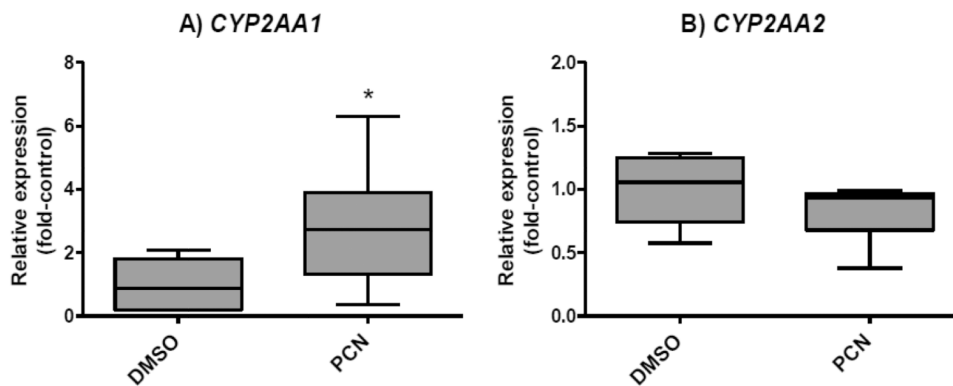




**Fig.4.** Effects of TCPOBOP and PB on mRNA expression levels of *CYP2AA1* (A) and *CYP2AA2* (B). *CYP2AA* transcript levels were determined by qPCR in liver of adult male zebrafish treated with vehicle DMSO, TCPOBOP (1.5  $\mu\text{g/g}$ ) or PB (70  $\mu\text{g/g}$ ). Data were normalized by geometric mean of two separate reference genes, *EF1a* and *ARNT2*. Results are shown as values in each treatment relative to the average value in DMSO control (fold-control). The whiskers show data range, while the box extends from the 25<sup>th</sup> to 75<sup>th</sup> percentile. Statistical differences in transcript levels among treatments were determined by one-way ANOVA followed by Tukey's multiple comparisons test and are shown by different letters ( $p < 0.05$ ).  $N = 8$ .



**Fig. 5.** Spearman's rank correlation of gene expression between *PXR* and *CYP2AA1* in liver of adult zebrafish treated with PB ( $n = 8$ ). A significant positive correlation was only seen in this relationship and none of the other correlations examined between *PXR* and *CYP2AA1* or *CYP2AA2* was statistically significant (see also Supplemental Fig. S3).



**Fig. 6.** Effects of PCN on mRNA expression levels of *CYP2AA1* (A) and *CYP2AA2* (B). *CYP2AA* transcript levels were determined by qPCR in liver of adult zebrafish treated with vehicle DMSO or PCN ( $\mu\text{g/g}$ ). Data were normalized by geometric mean of two separate reference genes, *EF1a* and *ARNT2*. Results are shown as values in each treatment relative to the average value in DMSO control (fold-control). The whiskers show data range, while the box extends from the 25<sup>th</sup> to 75<sup>th</sup> percentile. Statistical differences in transcript levels between vehicle control and PCN-treated group were determined by Student *t*-test and are shown by an asterisk ( $p < 0.05$ ).  $N = 6$ .