CHARACTERIZATION OF ZEBRAFISH CYTOCHROME P450 1 AND 3A GENES

FUNCTIONAL CHARACTERIZATION OF ZEBRAFISH CYTOCHROME P450 1 AND 3A GENES USING HETEROLOGOUSLY EXPRESSED PROTEINS

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MARCUS SCORNAIENCHI, B.SC.

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AUTHOR: Marcus L. Scornaienchi, B.Sc. (McMaster University)

SUPERVISOR: Dr. J.Y. Wilson

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Abstract

Cytochrome P4501 (CYP1) and CYP3A enzymes are hepatic proteins critical for the metabolism of many endogenous and exogenous compounds. CYP1 and CYP3A proteins are well characterized in mammals, but aside from CYP1A, little is known of the function of the other CYP1s and CYP3A in non-mammalian vertebrates. In this study, zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1 and CYP3A65 have been heterologously cloned, expressed and purified. The catalytic activity of each CYP was tested using 11 synthetic mammalian CYP substrates. The catalytic activity and regioselectivity of each CYP to metabolize 17β-estradiol (E_2), an endogenous steroid, and benzo[a]pyrene (BaP), a model exogenous compound were also tested. The major CYPs that metabolized E_2 were CYP1A and CYP1C1, and to a lesser extent CYP1C2. Overall CYP1A has the broadest specificity and the highest activity for most substrates. CYP1C1 and CYP1C2 have similar specificity and rates of metabolism for most substrates. Zebrafish CYP1B1 is similar to mammalian CYP1B1 in both relative activity to CYP1A and substrate specificity. CYP1D1, the most recently identified vertebrate CYP1, poorly metabolized all substrates except for BaP. The role of CYP1D1 is currently unknown, but given the metabolic differences seen here compared to the other CYP1s, CYP1D1 may have a function unlike that of the other CYP1s. Zebrafish CYP3A65 had a low activity for typical mammalian CYP3A substrates suggesting it could also have a different substrate profile than mammalian CYP3As. This is the first study to analyze the

activity of the complete CYP1 and CYP3A profile of a non-mammalian vertebrate and will be important in determining the functions of these proteins.

Abbreviations

2-OH-E_2: 2-hydroxy-17β-estradiol
4-OH-E_2: 4-hydroxy-17β-estradiol
7-BR: 7-benzyloxyresorufin
7-ER: 7-ethyoxyresorufin
7-MR: 7-methoxyresorufin
7-PR: 7-pentoxyresorufin
AhR: arylhydrocarbon receptor
AMMC: 3-[2-(N,Ndiethyl-N-methylammonium)ethyl]-7-methoxy-4-methylcoumarin
ARNT: arylhydrocarbon receptor nuclear translocation protein
AROD: alkoxyresorufin O-dealkylase
BaP: benzo[a]pyrene
BFC: 7-benzyloxy-4-trifluoromethylcoumarin
BNF: β-naphthoflavone
BQ: 7-benzyloxyquinoline
BROD: 7-benxyloxyresorufin O-dealkylase
CEC: 3-cyano-7-ethoxycoumarin
COMT: catechol-*O*-methyltransferase
CYP (or P450): cytochrome P450
DBF: dibenzylfluorescein
E_2: 17β-estradiol
EE_2: ethynlestradiol
EH: epoxide hydrolase
ER: estrogen receptor
ERE: estrogen response element
EROD: 7-ethoxyresorufin O- dealkylase
Ala: δ-aminolevulinic acid
K_m: Michaelis-Menten constant
MAMC: 7-methoxy-4-(aminomethyl)coumarin
MFC: 7-methoxy-4-trifluoromethylcoumarin
MROD: 7-methoxyresorufin O- dealkylase
OmpA: outer membrane protein A
P450 (or CYP): cytochrome P450
PAH: polyaromatic hydrocarbon
PCB: polychlorinated biphenyl
PROD: 7-pentoxyresorufin O- dealkylase
XRE: xenobiotic response element

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Chapter 1: General Introduction

Cytochrome P450 Enzymes

Cytochrome P450 enzymes (CYPs) are a large family of enzymes named so because they maximally absorb at 450 nm when reduced with carbon monoxide. CYPs primarily catalyze a mixed-function oxidase reaction (Eq 1).

$$NADPH + H^+ + O_2 + RH \rightarrow NADP^+ + H_2O + ROH \text{ (Eq 1; Guengerich, 2001)}$$

The liver is a major site of CYP expression, particularly for those in the first four CYP families. CYPs are capable of metabolizing a wide range of substances including steroids (Zhang *et al.*, 2007), pharmaceuticals (McGinnity *et al.*, 1999), and xenobiotic compounds (Blom *et al.*, 2001), and they are critical for steroid hormone production (Loose *et al.*, 1983). CYP mediated metabolism can yield different metabolites for the same substrate as a result of regioselectivity by different CYP isoforms, or multiple metabolites produced by the same CYP (Lee *et al.*, 2003).

CYP proteins all recruit an iron heme ring at the catalytic center of the protein to be fully functional. For this to occur, the CYP protein must be properly folded. Eukaryotic CYPs are all membrane bound proteins and will not be active if they do not properly target and fold within a membrane. Most eukaryotic CYPs target to the endoplasmic reticulum, while some target to the mitochondria (Waterman *et al.*, 1995). The heme iron molecule is responsible for binding of a molecule of O_2, of which a single oxygen atom is used to hydroxylate compounds that are brought in close proximity to the heme centre (Guengerich, 2001). Two electrons are required for this to occur. For

most CYPs, both electrons are donated by NADPH through the cytochrome P450 reductase cofactor. Some CYPs, such as the CYP2s and CYP3As, have a preference for cytochrome b_5 as the second electron donor (Shimada *et al.*, 2005). This requirement for cytochrome b_5 is especially important for human CYP3A4 as it can increase the activity of the enzyme 10-fold (Lee *et al.*, 2003). Briefly, upon coming in contact with a substrate, the CYP catalytic cycle (Figure 1, Guengerich, 2001) starts with iron in its ferric state and recruitment of the first electron reduces the iron to the ferrous state. A molecule of O_2 is then recruited followed by the second electron donation. A proton, then water, are released and the substrate is hydroxylated. Following hydroxylation, the metabolite is released from the enzyme leaving the heme iron back in its ferric state (Guengerich, 2001).

Cytochrome P450 Nomenclature

CYPs are found in every domain of life; currently there are over 8128 CYP sequences identified. This has created a need for a nomenclature able to handle such a large amount of sequences that is easily expandable as novel sequences are added (Nelson, 2007). A standard nomenclature for assigning CYP gene names has been developed based on amino acid similarity. CYP nomenclature is not based on function as the function may not be known particularly with the increased rate of CYP genes identified by whole genome sequencing (Nelson, 2006). CYP nomenclature is based on assignment to gene families and subfamilies; the first numeric represents the CYP

family, an alpha character represents the subfamily and the last numeric represents the specific gene. For example; CYP2D6 belongs in the CYP2 family, the CYP2D subfamily, and was the sixth gene found in the CYP2D subfamily (Nebert *et al.*, 1989). CYPs are assigned to a family and subfamily on the basis of amino acid sequence identity; genes with ≥40% identity are placed in the same family and those genes with ≥ 55% identity are placed in the same subfamily (Nelson, 2006). However, phylogenetic analyses of sequences can be used to assign genes to appropriate families or subfamilies when sequence identity is near to the aforementioned cutoff values as can occur between more distant species (Nelson, 2006). The CYP1 family has members with lower than 40% identity (Godard *et al.*, 2005), but the genes form a monophyletic cluster in an evolutionary tree, confirming their correct nomenclature (Nelson, 2006). The CYP nomenclature system ensures an efficient mechanism to name novel CYP sequences as they are identified.

CYP1 Gene Family

The CYP1 genes are a well studied gene family, and the CYP1A subfamily genes are the most well studied CYP genes in vertebrate species. Outside of mammals, relatively recent identification of novel subfamilies in the CYP1 family has raise questions about the function of the CYP1 genes in non-mammalian vertebrates. All vertebrates contain CYP1A and 1B genes, while all non-mammalian vertebrates contain recently found CYP1C and 1D genes (Goldstone *et al.*, 2009). Phylogenetically, CYP1

subfamilies are monophyletic yet the CYP1A and 1D clades cluster together, as do the CYP1B and 1C clades, suggesting that two gene lineages were formed by an initial gene duplication event, followed by two subsequent duplications to form the 4 vertebrate CYP1 subfamilies (Figure 2, Goldstone *et al.*, 2009).

Mammals have two CYP1A genes (CYP1A1 and CYP1A2) while most fish have a single CYP1A gene (CYP1A), with the exception of some polyploid lineages (Goldstone *et al.*, 2007). Mammalian CYP1A1 metabolizes many xenobiotics, such as polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and dioxins, and endogenous compounds such as estrogens and eicosanoids (Nebert and Dalton, 2006). A common metabolic marker for CYP1A1 activity is 7-ethoxyresorufin (Murray *et al.*, 2001). Mammalian CYP1A2 is primarily responsible for metabolizing aromatic amines (Nebert *et al.*, 2004) and a common marker for specific CYP1A2 activity is caffeine N3-demethylation (Ghosal *et al.*, 2003) although 7-methoxyresorufin and, to a lesser extent 7-ethoxyresorufin, can also be used (Murray *et al.*, 2001). Fish CYP1A metabolizes similar compounds to mammalian CYP1A1, and 7-ethoxyresorufin also is used as a biomarker for fish CYP1A activity (Klotz *et al.*, 1984; Miranda *et al.*, 2006). All vertebrates have a single CYP1B gene, CYP1B1. In mammals CYP1B1 metabolizes PAHs, N-heterocyclic amines, and endogenous compounds such as estrogens (Nebert *et al.*, 2004). The CYP1C lineage has been lost in mammals. In fish, there has been a duplication resulting in two CYP1C genes, CYP1C1 and CYP1C2, while all other non-mammalian vertebrates have only a single CYP1C1 (Goldstone *et al.*, 2007). The most

recent vertebrate CYP1 subfamily to be identified is CYP1D (Goldstone and Stegeman, 2008). Non-mammalian vertebrates have a single CYP1D gene, CYP1D1. Mammals have a non-functional CYP1D pseudogene in their genome, previously referred to as CYP1A8P (the P designates pseudogene), that is now known as CYP1D1P (Goldstone and Stegeman, 2008).

Aryl Hydrocarbon Receptor

All CYP1 genes, except for CYP1D1, are inducible through the aryl hydrocarbon receptor (AHR) (Nebert *et al.*, 2004; Godard *et al.*, 2005; Goldstone *et al.*, 2009). The AHR pathway has been implicated in neuron development, oxygen dependent feeding behaviour, homeotic gene function, photoreceptor development, cardiovascular development, and reproduction (McMillan and Bradfield, 2007). The AHR is a ligand activated transcription factor. AHR agonists are planar compounds that include endogenous substrates such as indoles, tetrapyroles including bilirubin and biliverdin, arachidonic acid metabolites and eicosanoids, and exogenous substrates such as polyaromatic hydrocarbons (PAHs), dioxins, and polychlorinated biphenyls (PCBs) (Denison and Nagy, 2003; Nebert and Karp, 2008). AHR agonists act by binding the AHR, which causes a cascade involving translocation to the nucleus, dimerization with the AHR nuclear translocation protein (ARNT), binding to a xenobiotic response element (XRE) and upregulation of downstream genes including the CYP1s (Figure 3,

Androutsopoulos *et al.*, 2009). Often, the upregulation of genes results in increased metabolism of the AHR agonist as many AHR ligands are also CYP1 substrates.

CYP3A Gene Subfamily

The CYP3A gene subfamily is a critical liver enzyme that is responsible for metabolism of a wide variety of structurally unrelated substrates. CYP3A genes are the highest expressed CYPs in the liver, and are responsible for over half of all drugs metabolized in mammals (Qiu *et al.*, 2008). All vertebrates contain CYP3A genes and mammals have multiple CYP3A genes; humans have CYP3A4, 3A5, and 3A7. CYP3A4 is the most prominent human CYP3A gene, and is the highest expressed in human liver (Williams *et al.*, 2004). CYP3A5 is expressed at much lower levels, while CYP3A7 is mostly expressed only in children (Qiu *et al.*, 2008). Fish have only one or two CYP3A genes, but have novel CYP3 subfamilies including CYP3B, 3C and 3D which are not found in any other vertebrate group (Qiu *et al.*, 2008). Evolutionary analyses suggest there was a single CYP3A ancestor prior to the split of the Actinopterygii, ray finned fishes, from the other vertebrates (Figure 4, Qiu *et al.*, 2008). This CYP3A ancestor in fish underwent diversification resulting in the other CYP3 subfamilies, resulting in a monophyletic clade that includes all fish CYP3 subfamilies. In other vertebrates, CYP3A genes form a distinct cluster suggesting that individual gene duplications gave rise to the multiple CYP3A genes found in each clade (Qiu *et al.*, 2008).

Estrogen Metabolism

The metabolism and clearance of estrogens is important for an organism to properly regulate estrogen levels. In humans, 17β-estradiol (E_2) is metabolized in the liver, predominantly by the CYP1 family and the CYP3A subfamily (Lee *et al.*, 2003). The most common metabolites formed by mammalian CYP mediated metabolism of E_2 are 2-hydroxy-estradiol (2-OH-E_2) and 4-OH-E_2 (Figure 1, Lee *et al.*, 2001; Takahashi *et al.*, 2004). Generally 2-OH-E_2 is formed by mammalian CYP1A1, 1A2 and 3A4 at a rate that is ten times greater than the formation of 4-OH-E_2 (Lee, 2003). Mammalian CYP1B1 metabolizes similar amounts of 4-OH-E_2 as CYP1A1 and 3A4, but very little 2-OH-E_2 so that three times more 4-OH-E_2 is formed in relation to 2-OH-E_2 (Lee, 2003) by the CYP1B1 enzyme.

Less is known of E_2 metabolism in non-mammalian vertebrates. Fish whole tissue microsomal preparations were able to metabolize E_2 to form 2-OH-E_2 (Snowberger and Stegeman, 1987; Stein *et al.*, 1991; Butala *et al.*, 2004), 4-OH-E_2 (Butala *et al.*, 2004), estrone, 16β-OH-E_2 and 7α-OH-E_2 (Hansson and Rafter, 1983). Similar to mammalian E_2 metabolism, the liver is the most prominent site of metabolism tested and there is a propensity for the formation of 2-OH-E_2 over 4-OH-E_2 metabolites (Butala *et al.*, 2004). The only purified CYP proteins from fish that have been tested for E_2 metabolic activity are Japanese eel CYP1A9 and CYP1C1. Eel CYP1C1 did not appear to metabolize E_2, while only 2-OH-E_2 was formed by CYP1A9 at a much lower rate than mammalian CYP1As (Uno *et al.*, 2008). Thus, there is an overall low level of

understanding of CYP-mediated E_2 metabolism in fish that is needed to compare to mammalian CYP-mediated E_2 metabolism and determine patterns across vertebrates.

Cytochrome P450 Expression Systems

In vivo functional studies of CYPs are difficult because of the complex CYP expression patterns in each species and individual organs. Functional characterization of a novel CYP or determination of CYPs responsible for metabolism of specific substrates requires heterologous protein expression. Preferably, a suitable expression system would include features such as the ability to produce large amounts of functional protein, ease of generating multiple constructs for each different CYP isoform, and the ability to concentrate and purify the expressed protein so that sufficient amounts of metabolites are formed to allow for accurate measurements. CYPs have successfully been cloned and expressed in a variety of systems, including bacterial (Pritchard, 1997), insect (Kashiwada, 2005), mammalian (Cloutier *et al.*, 1997) and yeast (Corley-Smith, 2006). Bacterial cultures are a very robust system, capable of a fast growth rate and can produce a higher relative amount of protein then other systems (Waterman, 1995). Various expression systems have been compared for the recombinant production of bovine CYPc17 and it was found that *E. coli* membranes contained 50%, 200%, 250% or 1200% more of the desired protein then Sf9 insect, bovine adrenal, yeast, or COS microsomes, respectively (Waterman, 1995). In terms of absolute concentrations of CYPs present, solubilized *E. coli* membranes contain between

0.58 (Sandhu, 1994) and 0.9 (Gillam, 1995) nmol P450 mg protein^{-1}. Bacterial expression systems are far more robust than the alternatives as they are easier to develop, maintain, and show higher reproducibility (Pritchard, 2006).

Bacterial expression of eukaryotic CYPs can be problematic, because eukaryotic genes lack a proper signal sequence for targeting to a membrane and CYPs must be targeted to a membrane for proper folding (Pritchard, 1997). Typically, this has been resolved by introducing amino acid alterations at the N-terminal portion of the protein to make the protein more lipophylic and provide targeting to the bacterial cell membrane (Gillam, 1995). However, alterations in sequence could lead to an alteration in the function of the expressed protein. Alternatively, eukaryotic CYPs can be targeted to bacterial membranes by an in frame fusion of a native bacterial signal sequence 5' to the CYP cDNA. Pritchard *et al.* (2006) have successfully introduced the native bacterial signal sequences from *pelB* and *ompA* to target mammalian CYPs to the cell membrane; sequences that are subsequently cleaved off by bacterial signal peptidases and result in the expression of a functional, full-length eukaryotic CYP. The bacterial signal sequences included only the first 20 (*pelB*) or 21 (*ompA*) amino acids. CYP genes expressed with *ompA* showed a greater then four-fold increase in membrane CYP content (Pritchard, 1997). The cleavage of the signal sequence is critical as there is a decrease in CYP activity from an inhibition of coupling between the CYP and CYP reductase when the signal sequence remains (Pritchard, 2006). The addition of alanine and proline to the

ompA signal sequence (*ompA*+2), allows for more efficient cleavage (Pritchard, 2006). This signal sequence allows bacterial expression of unaltered P450s.

Objectives of the current study

This study was undertaken to provide the functional characterization, for the first time, of the complete suite of CYP1 and CYP3A proteins in a non-mammalian vertebrate, zebrafish. We chose to clone, express and purify these CYP isoforms due to their predicted ability to metabolize E_2 and their known importance in metabolism of exogenous, environmentally relevant compounds. E_2 metabolism in mammals (Lee *et al.*, 2001) and fish (Butala *et al.*, 2004) predominantly occurs in the liver and the dominant hepatic CYP isoforms in the both mammals (Lee *et al.*, 2003) and fish (Jonsson *et al.*, 2007) are CYP1 and CYP3A. Therefore, it is reasonable to predict that since CYP1 and CYP3A isoforms are important in metabolizing E_2 in mammals (Lee *et al.*, 2003), the same would be true for fish.

To date, information on fish CYP1A has been well documented, but less is known about CYP1B1 and CYP3As, and there is almost no functional knowledge of CYP1C1, 1C2 or 1D1. To examine the specific functions of the CYP1 genes, we have used 11 synthetic fluoremetric CYP substrates (Murray *et al.*, 2001; Stresser *et al.*, 2002), and benzo[a]pyrene (BaP), a naturally occurring PAH that has been used to characterize mammalian CYP1s (Gelboin, 1980; Gautier *et al.*, 1996) to determine which compounds are substrates for each of the expressed CYP1 genes. This will help in understanding the

function of non-mammalian CYPs, and specifically whether CYP1Cs and 1Ds have overlapping or novel functions.

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Figures

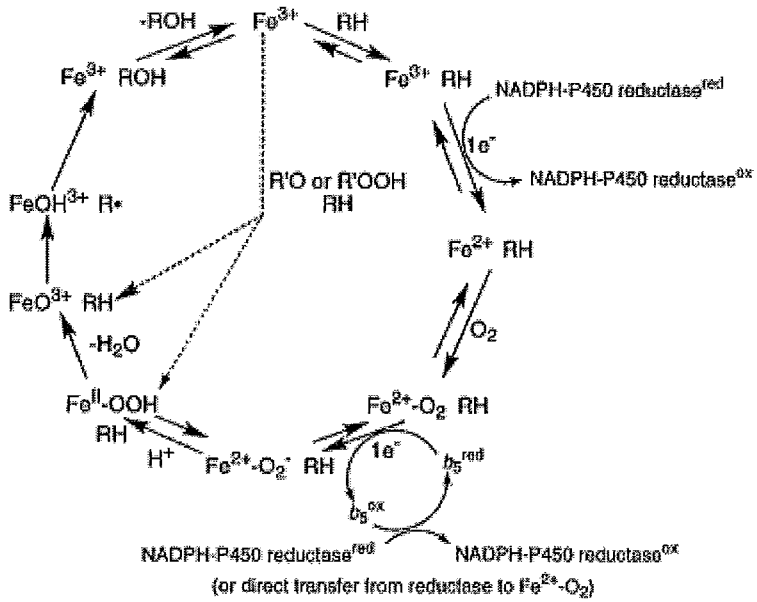
Figure 1. General Cytochrome P450 catalytic cycle. The iron molecule shown is from the heme-center of the enzyme as it catalyzes the substrate RH to the metabolite ROH. The second electron donation does not necessarily require cytochrome b_5, and may be donated directly by cytochrome P450 reductase. Taken from Guengerich *et al.*. (2001).

Figure 2. Representation of vertebrate CYP1 gene phylogeny. Each CYP subfamily is monophyletic, but the CYP1As and CYP1Ds form a subclade as do the CYP1Bs and CYP1Cs form a subclade. Taken from Goldstone *et al.* (2009).

Figure 3. Aryl hydrocarbon receptor (AHR) ligand-mediated induction pathway. A basic schematic of an AhR ligand, such as TCDD, binding to AhR, causing it to dissociate from a complex with XAP2, hsp90 and p53, and translocating to the nucleus. The aryl hydrocarbon receptor nuclear translocator (ARNT) dissociates from the aryl hydrocarbon receptor repressor (AHRR) and binds with the AHR. The AHR/ARNT complex then binds xenobiotic response elements (XREs) within promoter regions of AHR inducible genes to upregulate them. Taken from Androutsopolous *et al.* (2009).

Figure 4. Representation of vertebrate CYP3 gene diversification. All but the actinopterygii lineage contain only CYP3A genes. The actinopterygii contain CYP3A, 3B, 3C, and 3D genes. The grey triangle indicates incomplete sequences that were not used in construction of the tree. White triangles indicate genes expected, but not found. Taken from Qiu *et al.*, (2008).

Figure 5. Common metabolites of human cytochrome P450 mediated estradiol metabolism. Human CYP enzymes most prominent for the production of 2-hydroxy-estradiol and 4-hydroxy-estradiol are shown. Adapted from Takahashi *et al.* (2004)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RH
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Fe^{3+} RH
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RH
$FeOH^{3+}$ R•
Fe^{2+} RH
FeO^{3+} RH
O_2
$-H_2O$
Fe^{II}-OOH
RH
Fe^{2+}-O_2 RH
H^+
Fe^{2+}-O_2^- RH
$1e^-$
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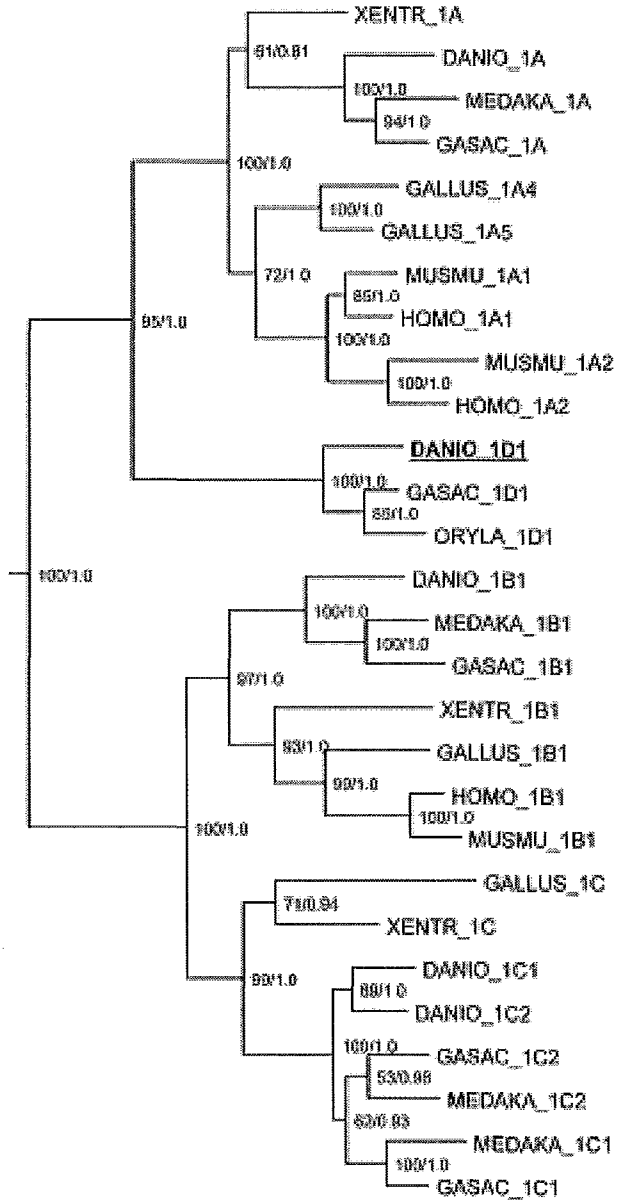
NADPH-P450 reductase^{ox}
(or direct transfer from reductase to Fe^{2+}-O_2)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GALLUS_1A5
72/1.0
MUSMU_1A1
85/1.0
HOMO_1A1
95/1.0
100/1.0
MUSMU_1A2
100/1.0
HOMO_1A2
DANIO_1D1
100/1.0
GASAC_1D1
85/1.0
ORYLA_1D1
100/1.0
DANIO_1B1
100/1.0
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GASAC_1B1
97/1.0
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93/1.0
GALLUS_1B1
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GALLUS_1C
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XENTR_1C
99/1.0
DANIO_1C1
88/1.0
DANIO_1C2
100/1.0
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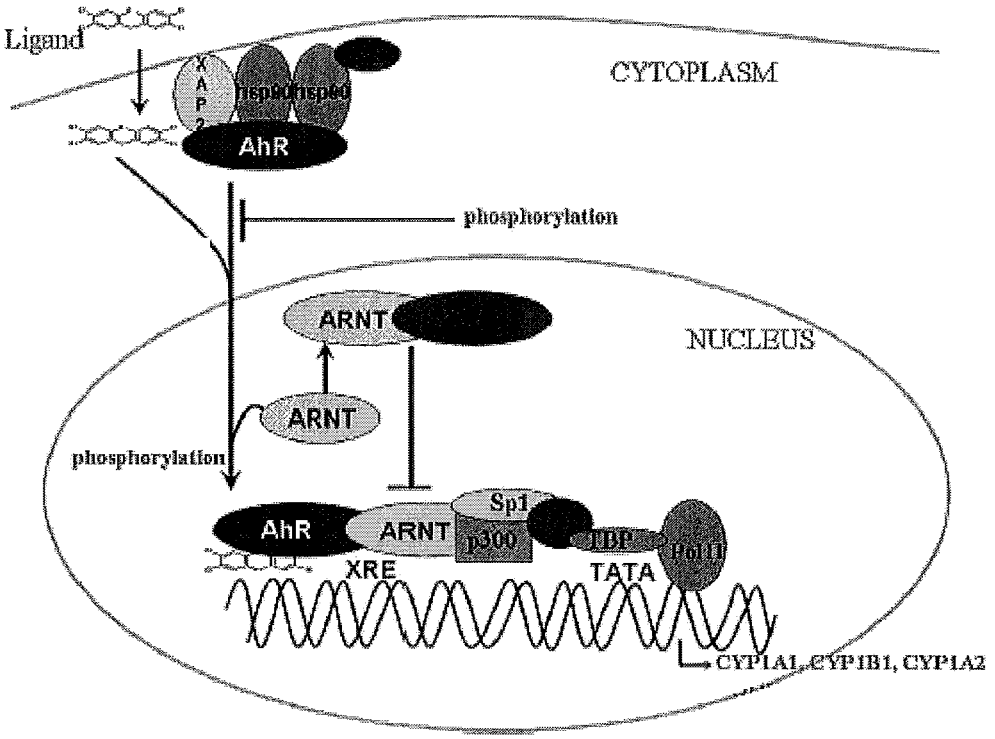
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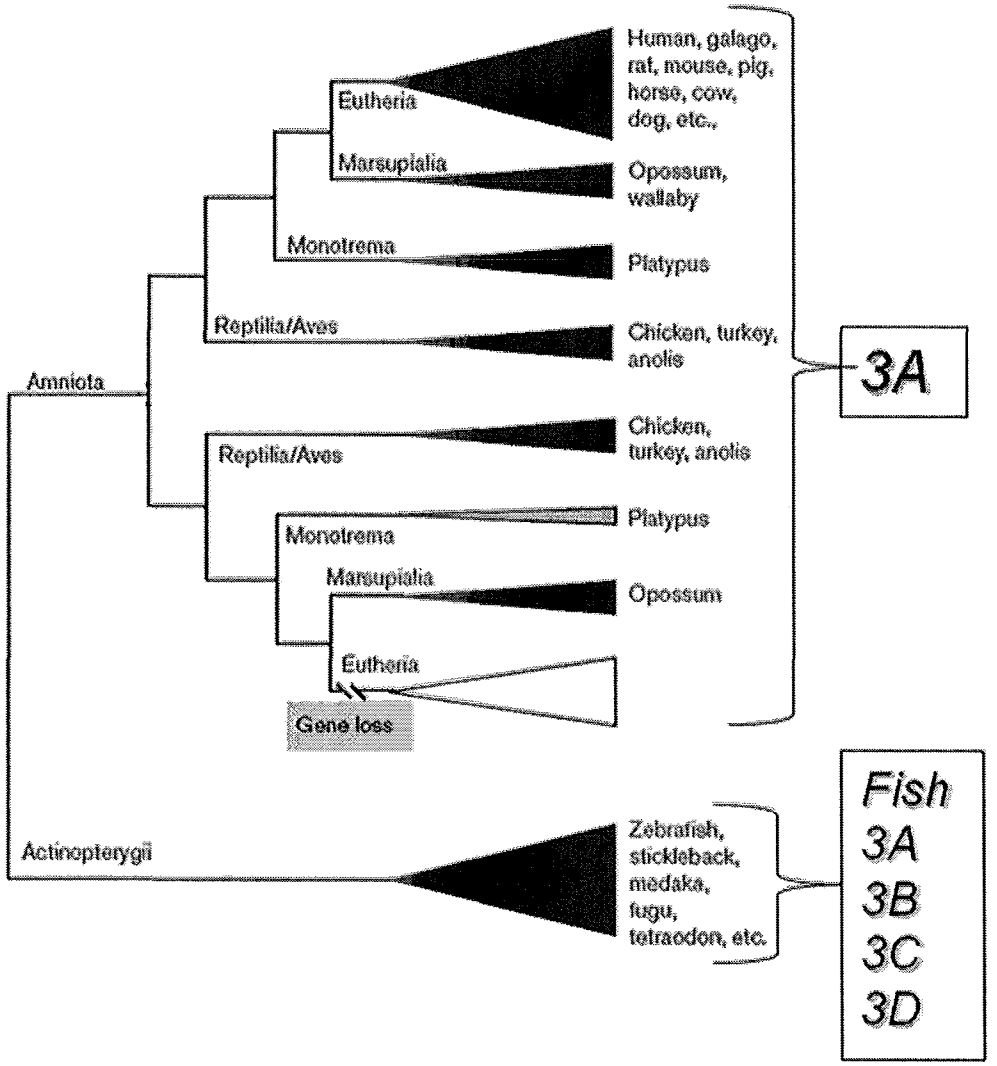
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Marsupialia
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3A
Amniota
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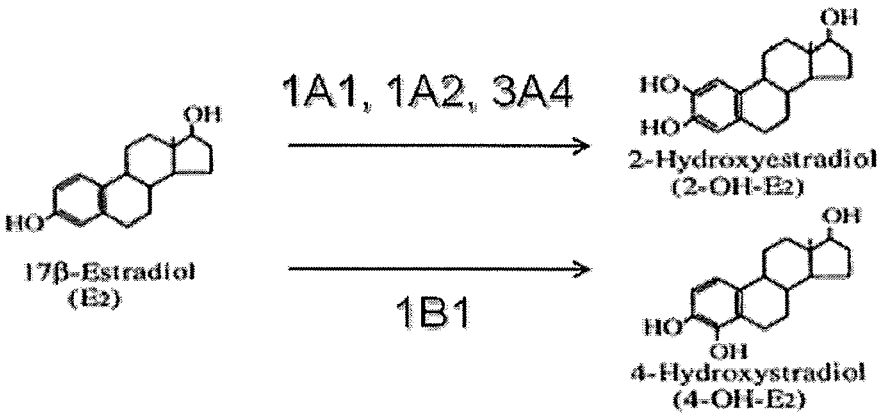
Chicken, turkey, anolis
Monotrema
Platypus
Marsupialia
Opossum
Eutheria
Gene loss
Actinopterygii
Zebrafish, stickleback, medaka, fugu, tetraodon, etc.
Fish
3A
3B
3C
3D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1B1
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HO
17β-Estradiol
(E2)
OH
HO
HO
2-Hydroxyestradiol
(2-OH-E2)
OH
HO
OH
4-Hydroxystradiol
(4-OH-E2)

Chapter 2: Cytochrome P450 mediated 17β-estradiol metabolism in zebrafish (*Danio rerio*) using a heterologous expression system

Author List:

Marcus L. Scornaienchi [a], Cammi Thornton [b], Kristine L. Willett [b], Joanna Y. Wilson [a, *]

[a] Department of Biology, McMaster University, Hamilton, ON, Canada, L8S4K1

[b] Department of Pharmacology and Environmental Toxicology Research Program, University of Mississippi, University, MS, USA, 38677-1848

Author's Contributions :

MLS – Planning and execution of the experiment, data analysis and preparing the manuscript

CT – Optimization, execution and gathering raw data for the GC/MS assay

KLW – Primary advisor for GC/MS assay

JYW – Primary Investigator, conceived of the study, experimental planning and manuscript editing

Keywords: cytochrome P450, zebrafish, estradiol metabolism, expression system, CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, CYP3A65

Abstract

Cytochrome P4501 (CYP1) and CYP3A proteins are primarily responsible for the metabolism of 17β-estradiol (E_2) in mammals. We have cloned and heterologously expressed CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1 and CYP3A65 from zebrafish to determine the cytochrome P450 mediated metabolism of E_2 in a non-mammalian species. Constructs of each CYP gene were created using a leader sequence from the bacterial *ompA*, to allow appropriate expression in *Escherichia coli* without 5' modification of the gene. Membrane vesicles were purified and functional CYP protein was verified using CO-difference spectra and fluorescent catalytic assays with the substrates 7-ethoxyresorufin (7-ER) and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC). *In vitro* metabolism of E_2 into 4-hydroxy-E_2 (4-OH-E_2), 2-hydroxy-E_2 (2-OH-E_2), and 16α-hydroxy-E_2 (16α-OH-E_2) metabolites was determined by GC/MS. The 2-OH-E_2 metabolite was produced by all CYPs tested, while 4-OH-E_2 was only found following incubation with CYP1A, CYP1B1, CYP1C1, and CYP1C2. The 16α-OH-E_2 metabolite was only produced by CYP1A. The highest rates of E_2 metabolism were from CYP1A and CYP1C1, followed by CYP1C2. CYP1B1, CYP1D1, and CYP3A65 all had low rates of E_2 metabolism. Zebrafish CYP1A, CYP1C1 and CYP1C2 E_2 metabolism produced similar ratios of 4-OH-E_2 to 2-OH-E_2 as mammalian CYP1As. CYP1B1 formed the highest ratio of 4-OH-E_2 to 2-OH-E_2 metabolites. These results suggest that contrary to mammals, fish CYP1A and CYP1C proteins are primarily responsible for E_2 metabolism, with only minor contributions from CYP3A65 and CYP1B1. Similar to mammals, 2-OH-E_2 is the

predominant metabolite from CYP mediated E_2 metabolism in fish which suggests this could occur in all vertebrate species.

Introduction

The first step in the metabolism of 17β-estradiol (E_2), the major estrogen in vertebrates, is NADPH-dependant oxidative metabolism catalyzed by hepatic cytochrome P450 (CYP) enzymes (Zhu and Conney, 1998). Mammalian CYP families are critical for E_2 metabolism including those in the CYP1A, 1B and 3A subfamilies. Yet, there are distinct differences in the CYP1 and 3 families across vertebrates. The CYP3A subfamily includes multiple genes in mammals; humans have four CYP3As (Qiu *et al.*, 2008). Most fish contain only one or two CYP3As; zebrafish have a single CYP3A gene, 3A65 (Qiu *et al.*, 2008). The CYP1 family contains more subfamilies in non-mammalian vertebrates. There are two CYP1A genes (CYP1A1 and 1A2) in mammals, and a single CYP1A gene in fish, with the exception of some polyploid species such as the salmonids (Goldstone *et al.*, 2007). CYP1B1 is the single CYP1B found in all vertebrate lineages. CYP1Cs are found in all non-mammalian vertebrate lineages (Goldstone *et al.*, 2007); fish have CYP1C1 and 1C2 paralogs arising from a gene duplication event (Godard *et al.*, 2005; Goldstone *et al.*, 2007). The CYP1D subfamily, a non-mammalian subfamily which has been recently identified, contains a single CYP1D1 gene (Goldstone *et al.*, 2009). All CYP1s and 3As are known to be expressed in the liver of mammals (Bieche *et al.*, 2007) and fishes (Jonsson *et al.*, 2007; Goldstone *et al.*, 2009), the major site of E_2 metabolism.

In mammals, the major product of E_2 oxidation is the 2-hydroxy-E_2 (2-OH-E_2) metabolite, with limited production of 4-OH-E_2 and small levels of metabolites hydroxylated at either the 6β, 16β, or 16α positions (Lee *et al.*, 2001). The regioselective hydroxylation of E_2 by purified CYPs has been well studied in mammals (Lee *et al.*, 2003). CYP1A1, CYP1A2 and CYP3A4 show the highest rates of metabolism in humans, mostly as a result of high 2-hydroxylation activity (Lee *et al.*, 2003). Consequently, these three CYPs have very low ratios of 4-OH-E_2 to 2-OH-E_2 formation (Lee *et al.*, 2003). On the other hand, E_2 metabolism by mammalian CYP1B1 produces similar amounts of 4-OH-E_2 to CYP1As and CYP3As, but very little 2-OH-E_2 such that three times more 4-OH-E_2 is formed in relation to 2-OH-E_2 (Lee *et al.*, 2003).

The two catecholestrogens, 2-OH-E_2 and 4-OH-E_2, are further metabolized by catechol-*O*-methyltransferase (COMT) into the major urinary estrogen metabolites 2-methoxy-E_2 (2-MeO-E_2) and 4-methoxy-E_2 (4-MeO-E_2) respectively (Creveling, 2003). However, the catecholestrogens can also be oxidized to form reactive semiquinones and quinones which can form adducts with purine bases (Dawling *et al.*, 2001; Belous *et al.*, 2007). COMT has a higher affinity for 2-OH-E_2 over 4-OH-E_2, resulting in an increased genotoxicity of the 4-OH-E_2 metabolite (Tsuchiya *et al.*, 2005). High levels of 4-OH-E_2 metabolites have been associated with human breast cancers (Rogan *et al.*, 2003) and elevated CYP1B1 mRNA transcripts have been identified in many cancerous tissues including breast, testis, and ovary (McKay *et al.*, 1995; Murray *et al.*, 1997) suggesting

that elevated levels of CYP1B1 mediated E_2 metabolism leads to carcinogenesis. 2-OH-E_2 metabolites appear to be tumour inhibitors (Tsuchiya *et al.*, 2005).

In fish liver microsomes, the presence of 2-OH activity was identified in channel catfish (Butala *et al.*, 2004), English sole (Stein *et al.*, 1991), scup and winter flounder (Snowberger and Stegeman, 1987) and recently 4-OH activity was detected in channel catfish (Butala *et al.*, 2004). The only reported E_2 metabolism by purified fish CYPs has been from Japanese eel showing 2-OH activity by CYP1A9 but not CYP1C1; neither produced 4-OH-E_2 metabolites (Uno *et al.*, 2008). Since E_2 metabolism predominantly occurs in the liver and the dominant metabolic products are 2-OH-E_2 with smaller amounts of 4-OH-E_2 for both mammals and fish, similar CYP families could be responsible for E_2 metabolism in mammalian and non-mammalian vertebrates.

Here, we describe our findings on the rates of formation of E_2 metabolites by purified zebrafish CYPs. Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1 and CYP3A65 each were cloned and expressed in bacteria to produce catalytically active proteins used for *in vitro* assessment of E_2 metabolism. As fish have similar CYP content in the CYP1 and CYP3 families as other non-mammalian vertebrates, our data will serve as a model for non-mammalian E_2 regulation.

Materials and Methods

Cloning of zebrafish CYPs

RNA was extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) from pools of liver or gill tissue taken from female WT zebrafish (n=8). cDNA was synthesized using cloned AMV RT (Invitrogen, Carlsbad, CA) and an oligo $(dT)_{20}$ primer. Zebrafish CYP1A, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 were amplified from liver cDNA, and CYP1B1 was amplified from gill cDNA using Platinum Taq Polymerase (Invitrogen, Carlsbad, CA) following manufacturer's protocol and using gene specific primers and annealing temperatures as listed in Table 1A. PCR products were gel purified, cloned into pGEM-T Easy vectors (Promega, Madison, WI) and transformed into competent *E. coli* JM109 cells (Promega, Madison, WI). Plasmids were purified by QIAprep Spin Miniprep Kits (Qiagen, Germantown MD) and sequenced by MobixLab (McMaster University, Hamilton, ON).

To facilitate proper membrane targeting of expressed CYPs in bacteria, an *ompA*(+2) sequence consisting of 69 nucleotides was attached in frame with the CYP start site by PCR using the methods of Pritchard *et al.* (2006). The source for the *ompA*(+2) was purified genomic JM109 DNA for CYP1A, CYP1B1, CYP1C1 and CYP3A65. The *ompA*-CYP1A and *ompA*-CYP1C1 constructs were used as *ompA* templates for attachment to CYP1C2 and CYP1D1 respectively. The *ompA*(+2) sequence was attached using two separate PCR reactions using a high fidelity polymerase, Accuprime *Pfx* (Invitrogen, Carlsbad, CA). The first PCR used a forward primer against the *ompA*

segment and a linker primer that primed against the first 21 bases of the CYP and the last 21 bases of the *ompA* segment (Table 1B). This generated an ~90 nucleotide fragment containing the first 21 bases of the appropriate CYP gene and was gel purified and used in a second PCR reaction with the same forward primer and a CYP specific reverse primer against the 3' end (Table 1B) and the zebrafish CYP cloned gene. The forward and reverse primers contained *NdeI* and *XbaI* restriction sites, respectively. PCR fragments containing the complete CYP gene and the *ompA*(+2) sequence were digested, gel purified, ligated into the pCW vector, and co-transfected with the pACYC vector containing human CYP reductase into JM109 cells. Final sequences were confirmed by sequencing prior to expression.

Expression and purification of zebrafish CYPs

Overnight cultures were diluted 1:100 into TB with ampicillin (50 μg mL^{-1}) and chloramphenicol (25 μg mL^{-1}) and shaken at 30°C and 200 rpm. Isopropyl β-D-1-thiogalactopyranoside (IPTG; 1 mM; Fisher Scientific, Pittsburgh, PA) was added when cultures reached an OD_{600} of between 0.7-1.0. Expression was optimized with the addition of 0-1 mM δ-aminolevulinic acid (Ala; MP Biomedicals, Solon, OH) based on maximizing the catalytic activity of purified membranes (see Catalytic Assays). Ala was added to a final concentration of 0.1 mM for all CYP constructs except CYP1A and CYP1B1 where Ala was added at 0.5 mM and 1.0 mM, respectively. Expression was allowed to proceed for another 20-24 hours. Cells were harvested and bacterial

membranes purified using previously published methods (Pritchard *et al.*, 2006). Total protein content was measured using a bicinchoninic acid (BCA) assay kit (Thermo Scientific, Rockford, IL) (Smith *et al.*, 1985).

Total P450 analysis and cytochrome c *activity*

Total P450 content was measured by diluting membranes into P450 spectrum buffer (Pritchard *et al.*, 1997) and measuring the CO-difference spectra using the peak absorbance difference between 450 nm and 490 nm and an extinction coefficient of 92 $mM^{-1}cm^{-1}$ (Omura and Sato, 1964). Cytochrome c reductase activity of membranes was determined by measuring the absorbance change at 450 nm of a reaction mixture containing 1 mg mL^{-1} of cytochrome c and 0.4 mM NADH in 0.2 M potassium phosphate buffer, pH 7.7 at 37°C using an extinction coefficient of 21.1 mM^{-1} cm^{-1} (Massey, 1959).

Catalytic Assays

Ethoxyresorufin-*O*-deethylase (EROD) activity was measured at 30°C using methods from Hahn *et al.* (1993). The rate of conversion of 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC, BD Biosciences, San Jose, CA) to 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) by purified membranes was measured in the presence of 1 mM BFC and 1.33 mg mL^{-1} NADPH in 0.375 M potassium phosphate buffer, pH 7.4 at 30°C, as optimized from Crespi and Stresser (2000). Catalytic assays containing cytochrome b_5 (Calbiochem) had 50 pmol added. All catalytic assays were normalized

for total P450 in the reaction mixture. Optimization of CYP expression was based on maximizing 7-ER (CYP1A, 1B1, 1C1, 1C2, 1D1) or BFC (CYP3A65) metabolism with assays normalized for total protein.

Zebrafish live microsomal preparation

Livers were collected from adult zebrafish that had been removed from our regular breeding stock and separated into male (n=57) and female (n=38) pools. Fish were approximately one year of age at collection but were still breeding. Microsomal fractions were collected according to Stegeman *et al.* (1979). Total protein levels were determined, and the remaining amount was used to determine E_2 metabolism.

E_2 metabolism

E_2 (Sigma-Aldrich, St. Louis, MO) was incubated with 0.5 mg membrane or microsomal protein at 28°C for 2 hours according to Spink *et al.* (1990). Reactions that contained cytochrome b_5 were supplemented with 100 pmol of cytochrome b_5. Reactions were terminated with 20 m*M* ascorbic acid (Sigma-Aldrich, St. Louis, MO), the deuterated estradiol internal standard was added, and metabolites extracted according to Butala *et al.* (2004). The samples were derivatized with *N,O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane (Sigma Aldrich, St. Louis, MO), evaporated under N_2 and resuspended in iso-octane (Sigma Aldrich, St. Louis, MO) prior to analysis. Samples were analyzed for the presence of estrone, estriol,

estradiol (E_2), 4-hydroxy-E_2, 2-hydroxy-E_2, 4-methoxy-E_2, 2-methoxy-E_2, 16α-hydroxy-E_2, and the deuterated standard by GC/MS according to Butala at el (2004). All samples were run in duplicate.

Materials

NADPH, leupeptin, aprotonin, phenylmethanesulphonylfluoride, and cytochrome c were purchased from Sigma-Aldrich (St. Louis, MO). Ampicillin, and chloramphenicol were purchased from Fisher Scientific (Pittsburgh, PA). The deuterated estradiol internal standard was prepared as described in Dehennin *et al.* (1980) and kindly provided by Dr. John Rimoldi (University of Mississippi). The pCW vector (Muchmore *et al.*, 1989) was a kind gift from Dr. F. W. Dahlquist (University of California Santa Barbara, Santa Barbara, CA) and the pACYC expression vector containing human Cytochrome P450 reductase was a kind gift from Dr. T. Friedberg (University of Dundee, Dundee, UK).

Results

Expression of zebrafish CYPs

The CO-difference spectra was analyzed for each expressed CYP (Figure 1) and peaks were seen at 420 and 450 nm, indicating the presence of significant amounts of both inactive and active P450. Total P450 content was between 0.139-0.655 nmol mg^{-1} protein (Table 2). Cytochrome c activity was measured to determine the levels of CYP

reductase in each sample. There were similar levels of reductase activity in each sample, except for cells expressing human CYP reductase only which showed approximately two to three fold higher activity than those co-expressed with a CYP gene (Table 2).

The catalytic activity of each recombinant protein was assessed using 7-ER and BFC to verify the proteins were functionally active. All CYP1s showed catalytic activity for 7-ER and BFC except for CYP1D1 and 3A65 which showed no activity for BFC and 7-ER respectively (Figure 2). CYP1D1 showed detectable but low catalytic activity for a broad range of other substrates tested (unpublished data). The addition of cytochrome b_5 significantly increased the ability of CYP1A, 1B1, 1C1 and 1C2 to metabolize 7-ER, but did not affect CYP1D1 or CYP3A65 in metabolizing 7-ER or BFC respectively (data not shown).

E_2 metabolism

There was no 2-MeO-E_2 or 4-MeO-E_2 detected in any of the samples, and no E_2 metabolites were detected in the control CYP reductase only sample. 16α-OH-E_2 was only detected in reactions with CYP1A at a rate of 6.8 ± 1.6 pmol nmol $P450^{-1}$ min^{-1}.

CYP1A and CYP1C1 showed the highest rates of E_2 metabolism and a strong preference for 2-OH-E_2 over 4-OH-E_2 (Table 3). CYP1C2 metabolized moderately less 2-OH-E_2 and 4-OH-E_2 then CYP1A and CYP1C1, but metabolism at the 2 position was highest (Table 3). CYP1A, CYP1C1, and CYP1C2 all had percent ratios of 4-OH-E_2/2-OH-E_2

between 3 and 8. CYP1B1, CYP1D1 and CYP3A65 had the lowest rates of E_2 metabolism in general (less than 40 pmol nmol $P450^{-1}$ min^{-1}) and 2-OH-E_2 formation predominated. CYP1B1 had the highest proportion of 4-OH-E_2 production (4-OH-E_2/2-OH-E_2 = 32%) of all the CYPs tested, but still formed twice as much 2-OH-E_2 than 4-OH-E_2. There was no 4-OH-E_2 produced by CYP1D1 or CYP3A65 without cytochrome b_5.

Addition of cytochrome b_5 approximately doubled the rate of formation of 2-OH-E_2 by CYP1A and CYP1C1, but reduced the amount of 4-OH-E_2 produced (Table 3). Cytochrome b_5 decreased the rate of E_2 metabolism by CYP1B1 and 1C2 while metabolism by CYP3A65 was not affected. Small amounts of estrone were produced, at rates below 3.0 pmol nmol $P450^{-1}$ min^{-1}, by CYP1A, 1B1, 1C1 and 1C2 with cytochrome b_5, but CYP3A65 with cytochrome b_5 produced estrone at a rate of 8.2 ± 5.3 pmol nmol $P450^{-1}$ min^{-1}. Estriol was detected only in samples with CYP1C1 or 1C2 that included cytochrome b_5. Estriol was produced at a rate of 223 ± 19 pmol nmol $P450^{-1}$ min^{-1} by CYP1C1 and 7 ± 0.24 pmol nmol $P450^{-1}$ min^{-1} by CYP1C2.

Zebrafish liver microsomes produced low, but detectable amounts of 2-OH-E_2 (Table 4) and estrone. Only one replicate reaction of each produced 2-OH-E_2, while both replicates produced estrone at 0.62 ± 0.03 pmol mg^{-1} min^{-1} from female liver and 0.23 ± 0.02 pmol mg^{-1} min^{-1} from male liver.

Discussion

E_2 metabolism in mammals is primarily catalyzed by CYP1 and CYP3A proteins (Lee *et al.*, 2003), important CYP enzymes that are highly expressed in mammalian liver, the primary site of E_2 metabolism (Lee *et al.*, 2001). Because E_2 metabolism also occurs predominantly in the liver of fish (Butala *et al.*, 2004), we chose to express zebrafish CYP1s and CYP3A65 to investigate their capacity for E_2 metabolism *in vitro*.

Bacterial expression of recombinant eukaryotic CYP proteins is possible, but often involves modification of the 5' end to allow appropriate targeting to membranes (Gillam *et al.*, 1995; Waterman *et al.*, 1995). Instead of 5' modification, we used an *ompA* strategy that involves the attachment of a leader sequence to the desired CYP gene, to target the native CYP to the bacterial outer membrane (Pritchard *et al.*, 1997). The leader sequence is cleaved off after targeting, allowing expression of unmodified, native CYP protein in bacteria. The expression conditions were optimized for each CYP, by expressing the proteins in cultures with a range of δ-aminolevulinic acid (Ala) concentrations (0.1-1 mM). CYP1B1 was the only CYP to show a large increase in activity with an increase in Ala to 1 mM (data not shown), which was also found to occur with bacterial expressed human CYP1B1 (Jansson *et al.*, 2000). All other CYPs required less, but some, Ala for optimal function. CO-difference spectra confirmed that a peak around 450 nm was present indicating that active CYP heme-protein was present in the preparations (See Figure 1 for a representative CO-difference spectra). Another major peak at 420 nm represented other heme-containing proteins or inactive CYP at levels

which are quite similar to other CYPs over-expressed in the same system (Pritchard *et al.*, 2006).

In addition to CO-difference spectra, functional protein was confirmed by fluorescent catalytic assays using 7-ER and BFC. 7-ER is widely used as a specific marker for CYP1 activity in mammals (CYP1A1, 1A2, 1B1, Shimada, 1998) and fish (CYP1A, 1B1, Hegelund *et al.*, 2004). BFC is specific for CYP3A activity in mammals (Crespi and Stresser, 2000) and was considered a suitable substrate for fish CYP3A proteins based on studies with fish hepatic microsomes (Hegelund *et al.*, 2004) and expressed fish CYP3A genes (Kashiwada *et al.*, 2005). All CYP1s metabolized 7-ER to some degree. CYP1A metabolized 7-ER about thirty times greater than CYP1B1 (Figure 2), a ratio comparable to that reported for human CYP1A1 and CYP1B1 (Murray *et al.*, 2001). Unlike the CYP1As and CYP1Bs, there is no catalytic data available for CYP1Cs or CYP1Ds, but clearly the expressed CYP1C1, 1C2 and 1D1 are all functional proteins as each were able to metabolize at least 7-ER (Figure 2). While CYP1D1 had low activity for 7-ER and did not metabolize BFC, the function of the protein is completely unknown and it is likely that we did not use optimal or specific substrates. CYP3A65 did not metabolize 7-ER but did metabolize BFC (Figure 2), similar to that reported for expressed medaka CYP3A proteins (Kashiwada *et al.*, 2005). However, unlike medaka (Kashiwada *et al.*, 2005), zebrafish CYP3A65 metabolism of BFC was not affected by cytochrome b_5. Collectively, the presence of P450 peaks in the CO-difference spectrum and the ability of these proteins to metabolize 7-ER and/or BFC confirm that the expressed proteins are

functional. Overall, three points favour the differences seen between these expressed CYP proteins for E_2 metabolism as representative of true *in vivo* functional differences: (1) the expressed proteins are functional, (2) the CYP3A65, CYP1A and CYP1B1 expressed proteins function as expected, and (3) there were similar activity levels of CYP reductase present for each expressed protein.

CYP1A and CYP1C1 demonstrated the highest overall activity for E_2 compared to the other zebrafish CYP1s and CYP3A65. They were also the only two zebrafish CYPs to show increased metabolism of E_2 when supplemented with cytochrome b_5. The rate of overall E_2 metabolism of CYP1B1 compared to CYP1A was similar to that of the metabolism of human CYP1B1 to CYP1A1 (Lee *et al.*, 2003). Surprisingly, CYP1C1 and CYP1C2 both metabolize E_2 to a higher degree then CYP1B1 and CYP3A65. Phylogenetically, vertebrate CYP1Cs are clustered in a clade with the CYP1Bs, which is distinct from the CYP1A/CYP1D clade (Goldstone *et al.*, 2009). That mammalian CYP1B1 is capable of E_2 metabolism but in fish the CYP1Cs have much higher, and likely, more biologically relevant E_2 metabolism than CYP1B1, suggests that E2 metabolism was present in the ancestor to the CYP1B and 1C clade, and that the fish CYP1Cs have retained this ancestral function while fish CYP1B1 has acquired novel function. This might suggest that both the tunicate CYP1Es and CYP1Fs, homologs to the vertebrate CYP1B/1C and CYP1A clade, respectively (Goldstone *et al.*, 2007), may be functionally capable of E2 metabolism.

Mammalian CYP genes show distinct regioselectivity in E2 metabolism. Like mammals, fish CYPs produced 2-OH-, 4-OH- and 16α-OH- E_2 metabolites. In mammals, CYP1A1 is the only CYP1 able to form 16α-OH-E_2, and does so in very low concentrations (Lee *et al.*, 2003) similar to what was seen by zebrafish CYP1A. 2-OH-E_2 was the predominant metabolite formed in both zebrafish (this study) and mammalian studies (Lee *et al.*, 2003) and the 4-OH-E_2 metabolite was produced to a much lower extent in both groups. In addition, Japanese eel CYP1A9 (one of two CYP1As in this species) also predominantly formed 2-OH-E_2 (Uno *et al.*, 2008), though at a lower rate than seen here, possibly due to an eight hour reaction time in that study.

Zebrafish CYP1Cs and CYP1A have a similar 4-OH-E_2 to 2-OH-E_2 ratio compared to mammalian CYP1As (Lee *et al.*, 2003). Zebrafish CYP1B1 was similar to mammalian CYP1B1 in its regioselectivity, as it metabolized the largest proportion of 4-OH-E_2 relative to 2-OH-E_2 formation compared to any other tested zebrafish CYP. However, unlike human CYP1B1 (Lee *et al.*, 2003), zebrafish CYP1B1 still metabolized more 2-OH-E_2 than 4-OH-E_2 and overall the rates of metabolism were much less than the mammalian ortholog. Distinct from the other CYP1s, CYP1D1 did not produce any 4-OH-E_2 metabolites. The lower ratio of 4-OH-E_2 to 2-OH-E_2 metabolism seen here from zebrafish CYP1B1 compared to human CYP1B1, and the preference of all other CYPs tested for 2-OH regioselectivity, agrees with the lower *in vivo* proportion of 4-OH-E_2 seen in channel catfish (Butala *et al.*, 2004) compared to human (Lee *et al.*, 2001) and rat (Dannan *et al.*, 1986) (Table 4). Our zebrafish microsomal protein had only low

activity for E_2, and we did not detect any 4-OH-E_2 metabolites. We also detected the production of estrone, which along with the parent compound, E_2, are the primary estrogens in fish that are either glucuronidated or sulfated for elimination (Stein *et al.*, 1991). Due to the small size of zebrafish, a large sample size was required to obtain sufficient microsomal material for the present study. Our microsomes were derived from one year old zebrafish and their reproduction was in decline. This may account for the lower levels of E_2 metabolism that were seen from our zebrafish compared to other fish (Figure 4). Additionally, there could be hormonal differences between species that could be responsible for the lower E_2 metabolism by zebrafish. The relatively low amounts of 4-OH-E_2 detected in our samples suggest that though fish are susceptible to genotoxic damage by increased levels of E_2 (Teles *et al.*, 2006), they may be less sensitive than mammals.

Similar to mammalian CYP1A1, zebrafish CYP1A is the dominant CYP1 expressed in the liver (Jonsson *et al.*, 2007) and would be responsible for the majority of CYP1 mediated hepatic E_2 metabolism. CYP1C1, 1C2 and CYP1B1 are all constitutively expressed at similar low levels in the liver (Jonsson *et al.*, 2007). CYP1A, 1B1, 1C1 and 1C2 are inducible through the AHR pathway (Jonsson *et al.*, 2007) and their role in E_2 metabolism suggests a potential mechanism for AHR control of E_2 concentrations.

CYP1D1 is constitutively expressed in the liver but, unlike other CYP1s, is not inducible through the AhR pathway (Goldstone *et al.*, 2009). It is possible that the low activities seen from CYP1D1 were due to the presence of predominantly non-functional

protein in the preparation, because we did not see high catalytic capacity in the fluorescent assays. However, all CYPs were similarly prepared, a P450 peak was present for this preparation and functional reductase activity was determined similar to the other CYP preparations. As we do not know the function of this protein, it is more likely, given the different metabolic capacity documented in this study, the distinct expression profile and lack of inducibility of CYP1D1 by typical AhR ligands in other studies (Goldstone *et al.*, 2009), that CYP1D1 has a function that is different and distinct from the other CYP1 genes, including E_2 metabolism.

Mammalian species have more CYP3A genes than fish, as a result of more recent local gene duplications (Thomas, 2007; Qiu *et al.*, 2008). Zebrafish CYP3A65 (Tseng *et al.*, 2005) and human CYP3A4, CYP3A5 and CYP3A7 (Maruyama *et al.*, 2007) show similar induction patterns in the liver. In humans, CYP3A4 efficiently metabolizes E_2 similar to the CYP1As (Lee *et al.*, 2003), while zebrafish CYP3A65 poorly metabolizes E_2 at rates that are similar to human CYP3A5 and CYP3A7 (Lee *et al.*, 2003). Zebrafish CYP3A65 only produced 4-OH-E_2 metabolites, when supplemented with cytochrome b_5, while mammalian CYP3As do produce 4-OH-E_2 regardless of whether cytochrome b_5 is present (Lee *et al.*, 2003). Although the mammalian CYP3A subfamily is phylogenetically clustered together with other vertebrate CYP3As, the CYP3A genes from the Actinopterygii class of ray-finned fishes, of which zebrafish are a member, cluster with fish CYP3B, CYP3C, and CYP3D genes (Qiu *et al.*, 2008). As such, fish CYP3As share less of a common evolutionary history with mammalian CYP3As than they do with fish

CYP3Bs, CYP3Cs, and CYP3Ds. In the case of fish and mammalian CYP3As, common nomenclature may not be reflective of common function. Fish CYP3As have some similar function to mammalian CYP3As; zebrafish CYP3A65 can metabolize E_2, and both zebrafish (this study) and medaka (Kashiwada *et al.*, 2005) CYP3A proteins can metabolize mammalian CYP3A fluorescent substrates such as BFC, however, there are clear differences in the total activity of fish CYP3As as compared to mammalian CYP3As.

Ours is the first study involving the cloning, heterologous expression, and metabolic analysis of purified membrane preparations of both CYP1 and CYP3A isoforms from a non-mammalian vertebrate species. Purified membrane preparations resulted in concentrated levels of catalytically active CYP proteins. Using these preparations, we found that CYP1A and CYP1C1 have the highest rate of E_2 metabolism, followed by more modest E_2 metabolism by CYP1C2. The dominant metabolite formed was 2-OH-E_2, similar to studies seen in other fish (Uno *et al.*, 2008) and humans (Lee *et al.*, 2003). Similar to human E_2 metabolism by CYP1B1 (Lee *et al.*, 2003), the ratio of 4-OH-E_2 to 2-OH-E_2 was highest in zebrafish CYP1B1, yet the overall rates of metabolism by zebrafish CYP1B1 were lower than expected. CYP3A65 had low E_2 metabolism comparable to human CYP3A5 and CYP3A7 (Lee *et al.*, 2003), formed no 4-OH-E_2 and may not contribute greatly to E_2 metabolism *in vivo*. CYP1D1 continued (Goldstone *et al.*, 2009) to display functional differences from other CYP1s. While overall E_2 metabolism in fish and other vertebrates is similar to mammalian E_2 metabolism, in non-mammalian

vertebrates the primary E_2 metabolizers *in vivo* are likely CYP1A and CYP1C enzymes. The contributions by CYP1B1 and CYP3A are likely less than in mammalian species.

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Tables

Table1. Primers and annealing temperatures used for (A) cloning of zebrafish CYPs and (B) fusion of the *ompA*(+2) segment to the zebrafish CYPs. All primers are listed in the 5' to 3' direction.

A

Construct	Forward Primer	Reverse Primer	Annealing Temperature (°C)	GenBank Accession Number
CYP1A	TGGAGCTAATTGGCACTGGT	CCTGGATTTCAGGAGCTCAA	53	**NM_131879**
CYP1B1	CCACCCGAACTCTGAAACTC	GTTTTCTTAGCCGCCTTCATTT	53	**NM_001013267**
CYP1C1	GGAGGCTGAGTTTGGACTGA	CCCATTCGACTGGATGTTTT	53	**NM_001020610**
CYP1C2	TGAGCCATCCTCCGGTAA	GCAGTGGGTTAGACAGCACA	53	**XM_686678**
CYP1D1	GCGATTTGCCAACACTGATA	TGCCAACATTAGCTTGATGC	54	**NM_001007310**
CYP3A65	GGAGCTTCATCATCTTCAGCA	CCTCCTCCTCCTCCTCAGAC	53	**NM_001020797**

B

Construct[a]	Reverse Primer	Linker Primer	Annealing Temperature (°C)[d]
ompA-CYP1A	[b]TCTAGAGCGGCCGCGAATTCACTA	TGGAAGAATAGCCAGAGCCATXXX[c]	50, 55
ompA-CYP1B1	TCTAGAGCGGCCGCGAATTCACTA	AGCCAGCAGGACATCCATCATXXX	50, 55
ompA-CYP1C1	TCTAGAGCGGCCGCGAATTCACTA	CTGTCCGCTCCATTCCCGCATXXX	50, 55
ompA-CYP1C2	TCTAGAGCAGTGGGTTAGACAGCACA	CTCTGAATCCGACTGCGCCATXXX	50, 55
ompA-CYP1D1	TCTAGATGGAACACTGAGAGAATGATGG	GGAGATATTCTCAAGATTCATXXX	55, 53
ompA-CYP3A65	TCTAGAGCGGCCGCGAATTCACTA	TGTTTCTGCCGAGAAGAACATXXX	50, 55

[a] – The forward primer used for *ompA*(+2)-CYP fusions was always 5'-GGAATTCCATATGAAAAAGACAGCTATCGCG-3' with an *NdeI* restriction site underlined

[b] – The *XbaI* restriction site is underlined

[c] – XXX represents 5'-CGGGACGGCCTGCGCTACGGTAGCGA-3' which primes against the 3' end of the *ompA*(+2) segment

[d] – The first temperature was used to anneal the forward and linker primer to the *ompA*(+2) template during step 1 of the PCR reaction. The second annealing temperature is used during the second step of PCR to attach the *ompA*(+2) segment to the full length CYP using the forward and reverse primers. See materials and methods section for details.

Table 2. Total protein, P450 content and cytochrome c activity in membrane vesicles purified from bacteria co-expressing zebrafish cytochrome P450 genes and human cytochrome P450 reductase, or expressing reductase alone.

Bacterially Expressed CYP	Total Protein (mg ml^{-1})	Total P450 Content (nmol mg^{-1})	Cytochrome c Activity (nmol mg^{-1} min^{-1})
CYPred[a]	11.65	N.D.	29.68 ± 3.2
CYP1A	16.42	0.655	16.77 ± 2.1
CYP1B1	19.20	0.278	16.27 ± 0.68
CYP1C1	22.25	0.395	13.93 ± 0.37
CYP1C2	20.50	0.473	17.52 ± 1.0
CYP1D1	22.77	0.139	15.50 ± 0.11
CYP3A65	17.04	0.282	18.74 ± 0.43

N.D. = not determined
[a] = Human cytochrome P450 reductase

Table 3. Rate of formation of 2-hydroxy-estradiol (2-OH-E_2) and 4-hydroxy-estradiol (4-OH-E_2) of heterologously expressed zebrafish, human or Japanese eel CYPs.

Species	CYP	2-OH-E_2 (pmol nmol $P450^{-1}$ min^{-1})	4-OH-E_2 (pmol nmol $P450^{-1}$ min^{-1})	ratio of 4-OH-E_2/2-OH-E_2 (%)
Zebrafish[a]	CYP1A	425.2 ± 85.0	21.8 ± 5.4	5
	CYP1A +b5	892.6 ± 81.1	N.D.[d]	n/a[e]
	CYP1B1	27.1 ± 10.7	8.8 ± 5.7	32
	CYP1B1 +b5	18.0 ± 0.1	4.7 ± 0.1	26
	CYP1C1	510.0 ± 1.1	40.9 ± 3.9	8
	CYP1C1 +b5	1083 ± 33	35.2 ± 0.7	3
	CYP1C2	155.4 ± 1.5	4.2 ± 0.2	3
	CYP1C2 +b5	57.0 ± 1.4	1.8 ± 0.3	3
	CYP1D1	31.4 ± 1.9	N.D.	n/a
	CYP3A65	25.6 ± 5.3	N.D.	n/a
	CYP3A65 +b5	13.4 ± 9.6	1.8 ± 1.4	13
Human[b]	CYP1A1	2523 ± 208	163 ± 22	7
	CYP1A2	4065 ± 156	343 ± 24	9
	CYP1B1	108 ± 3	371 ± 16	344
	CYP3A4	355 ± 41	78 ± 11	22
	CYP3A4 + b5	3093 ± 91	497 ± 27	16
	CYP3A5	125 ± 22	67 ± 5	53
	CYP3A7 + b5	146 ± 17	55 ± 3	58
Japanese Eel[c]	CYP1A9	177	N.D.	n/a
	CYP1C1	N.D.	N.D.	n/a

[a] Data from this study, using 50 µM E_2 at 28C
[b] Data from Lee *et al.* (2003), using 20 µM E_2 at 37C
[c] Data from Uno *et al.* (2008), using 100 µM E_2 assuming constant rate over 8 hour reaction at 37C
[d] N.D. = Not Detected
[e] n/a = not applicable because both metabolites not detected

Table 4. Rate of formation of 2-hydroxy-estradiol and 4-hydroxy-estradiol by liver microsomes from fish and mammalian species.

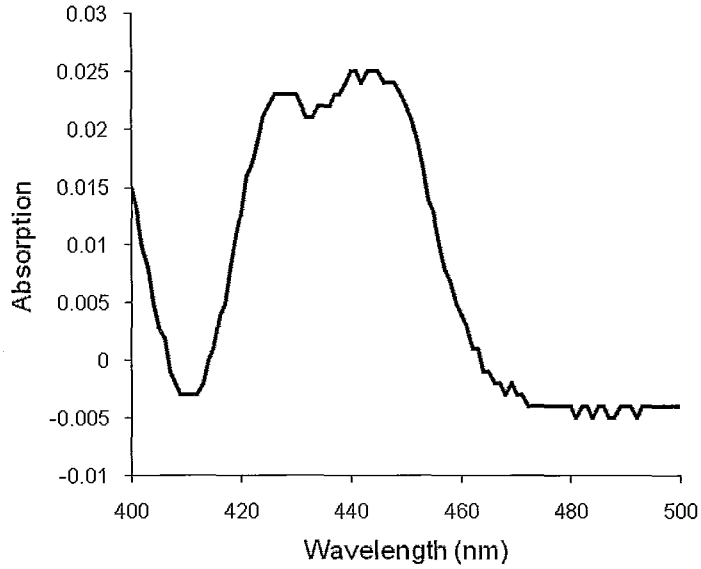
Species	2-OH-E_2 (pmol mg^{-1} min^{-1})	4-OH-E_2 (pmol mg^{-1} min^{-1})	ratio of 4-OH-E_2/2-OH-E_2 (%)
Zebrafish Male[a]	0.068	N.D.[g]	n/a[h]
Zebrafish Female[a]	0.269	N.D.	n/a
Channel Catfish Male[b]	20.3 ± 1.1	0.748 ± 0.032	3.7
Scup Male[c]	362 ± 141	-[i]	n/a
Scup Female[c]	157 ± 37	-	n/a
Winter Flounder Male[c]	296 ± 79	-	n/a
Winter Flounder Female[c]	89 ± 39	-	n/a
English Sole Juvenile[d]	260 ± 17	-	n/a
Human Male[e]	46.6 ± 25.7	7.1 ± 3.4	15
Human Female[e]	47.7 ± 28.2	8.2 ± 4.4	17
Rat Male[f]	1710 ± 180	390 ± 50	23
Rat Female[f]	240 ± 20	70 ± 20	29

[a] Data from this study, using 50 µM E_2 at 28C
[b] Data from Butala *et al.* (2004), using 50 µM E_2 at 30C
[c] Data from Snowberger and Stegeman (1987), using 25 µM E_2 at 25C for flounder, and 30C for scup
[d] Data from Stein *et al.* (1991), using 100 µM E_2 at 25C
[e] Data from Lee at al (2001), using 20 µM E_2 at 37C
[f] Data from Dannan *et al.* (1986), using 200 µM E_2 at 37C
[g] N.D. = not detected in this study
[h] n/a = not applicable because both metabolites not detected
[i] not determined in this study

Figures

Figure 1. Representative CO-difference spectrum of recombinant zebrafish CYP purified membrane vesicles. Example shown is for membrane vesicles containing CYP1C2.

Figure 2. Catalytic activity of recombinant zebrafish CYPs coexpressed with human CYP reductase. Data are shown for the metabolism of 7-ethoxyresorufin (7-ER) and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), typical CYP1 and CYP3A substrates in mammals (Lake *et al.*, 2009) and fish (Kashiwada *et al.*, 2005). 7-ER and BFC are metabolized to resorufin and HFC, respectively. Results are mean ± SEM, for duplicate reactions. Catalytic activity was below limits of detection for CYP3A65 (7-ER) and CYP1D1 (BFC). * Resorufin was detected in CYP1D1 at a rate of 24 ± 5 pmol nmol $P450^{-1}$ min^{-1}.


0.03
0.025
0.02
0.015
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-0.005
-0.01
Absorption
400
420
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460
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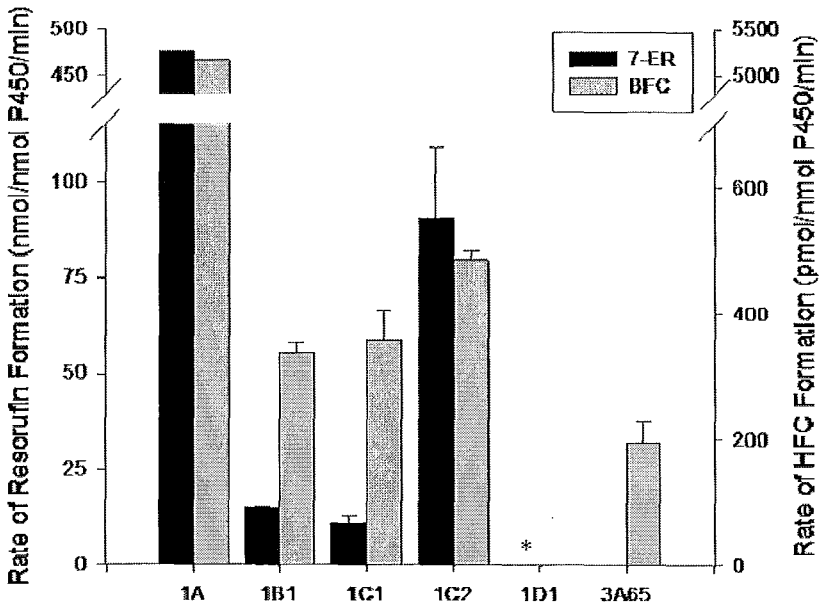
500
Wavelength (nm)


Rate of Resorufin Formation (nmol/nmol P450/min)
Rate of HFC Formation (pmol/nmol P450/min)
7-ER
BFC
500
450
100
75
50
25
0
5500
5000
600
400
200
0
*
1A
1B1
1C1
1C2
1D1
3A65

Chapter 3: Functional differences in the cytochrome P450 1 family enzymes from Zebrafish (*Danio rerio*) using heterologously expressed proteins

Author List:

Marcus L. Scornaienchi[a], Cammi Thornton[b], Kristine L. Willett[b], Joanna Y. Wilson[a, *]

[a] Department of Biology, McMaster University, Hamilton, ON, Canada.

[b] Department of Pharmacology, University of Mississippi, University, MS, USA.

Author's Contributions:

MLS – Planning and execution of the experiment, data analysis and preparing the manuscript

CT – Optimization, execution and gathering raw data for the LC/MS assay

KLW – Primary advisor for LC/MS assay

JYW – Primary Investigator, conceived of the study, experimental planning and manuscript editing

Keywords: cytochrome P450, zebrafish, substrate specificity, expression system, CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1

Abstract

Cytochrome P4501 (CYP1) proteins are important in metabolizing many exogenous compounds. Mammalian CYP1 genes are well characterized, but in other vertebrates only the function of CYP1As has been well studied. Here we have tested the catalytic activity of heterologously expressed zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2 and CYP1D1 proteins using 11 synthetic fluorometric substrates and benzo[a]pyrene. 7-ethoxyresorufin (7-ER) and 7-methoxyresorufin (7-MR) were most strongly metabolized by CYP1A and CYP1C2 to a lesser extent. 7-benzyloxyresorufin (7-BR) was moderately metabolized by CYP1A and CYP1B1 and 7-pentoxyresorufin (7-PR) had the lowest metabolism of any resorufin based substrate. 7-methoxy-trifluoromethylcoumarin (MFC), 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) and 3-cyano-7-ethoxycoumarin (CEC) were all metabolized at the highest rate by CYP1A. Dibenzylfluorescein (DBF) was only metabolized by CYP1A, CYP1B1 and CYP1D1. None of the CYPs tested were able to metabolize 7-benzyloxyquinoline (BQ), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC) or 7-Methoxy-4-(aminomethyl)-coumarin (MAMC). CYP1B1 and CYP1D1 showed the highest rate of BaP metabolism, followed by CYP1A and CYP1C1 while CYP1C2 did not show a significant relationship between metabolism and substrate. 3-hydroxy-BaP (3-OH-BaP) was the most prominent metabolite formed by CYP1B1, CYP1C2 and CYP1D1. 9-hydroxy-BaP (9-OH-BaP) was the most prominent metabolite formed by CYP1C1, and 3,6-dione-BaP was formed most from CYP1A mediated metabolism. Overall, CYP1A showed the broadest specificity and

had the highest metabolic rates for all substrates metabolized except for 7-BR, DBF and BaP. CYP1C1 and CYP1C2 had similar substrate specific with slightly different rates of metabolism. CYP1D1 only metabolized 7-ER, 7-BR, 7-PR, DBF and BaP, and had very low activities for all substrates but BaP suggesting the function of CYP1D1 may be different from other CYP1s.

Introduction

Cytochrome P450s (CYPs) are a large superfamily of enzymes that primarily catalyze mixed-function oxidation reactions (Guengerich, 2001). CYPs are capable of metabolizing a wide range of substances including steroids (Zhang *et al.*, 2007), pharmaceuticals (McGinnity *et al.*, 1999), and xenobiotic compounds (Blom *et al.*, 2001). The CYP1 family is important for the metabolism of cyclic structures including polyaromatic hydrocarbons (PAHs) and aromatic amines (Nebert *et al.*, 2004). CYP1 catalytic activity (ethoxyresorufin-*O*-deethylase activity; EROD) and expression (protein and transcript) have been widely used as a biomarker of exposure and effects to planar halogenated aromatic hydrocarbons.

Vertebrate CYP1 subfamilies include the CYP1A, CYP1B, CYP1C and CYP1D subfamilies, but not all vertebrate lineages contain all subfamiles or an orthologous CYP1 gene complement. Mammals have 3 CYP1 isoforms: CYP1A1, CYP1A2, and CYP1B1. Non-mammalian vertebrates have CYP1C and CYP1D subfamilies in addition to the CYP1A and 1B gene subfamilies (Goldstone *et al.*, 2007). Fish have a single CYP1A

gene, except for some polyploid species including the salmonids which have two CYP1A genes (Goldstone *et al.*, 2007). Like their mammalian counterparts, non-mammalian vertebrates have a single CYP1B gene, CYP1B1. The CYP1C genes are present as the paralogous CYP1C1 and CYP1C2 genes in fish (Goldstone *et al.*, 2007); a single CYP1C gene is thought to be present in birds, amphibians and reptiles (Goldstone *et al.*, 2007). The CYP1D gene family has only recently been identified in non-mammalian vertebrates, and contains a single CYP1D1 gene (Goldstone *et al.*, 2009).

Mammalian CYP1 enzymes are highly inducible by the aryl hydrocarbon receptor (AHR, Nakajima *et al.*, 2003). CYP1A1 is constitutively expressed at very low levels, but has the highest induction profile by AhR ligands (Nebert *et al.*, 2000). CYP1A2 and CYP1B1 have substantial constitutive expression and are induced by AhR ligands, but to lower levels then CYP1A1 (Kim *et al.*, 1998; Nebert *et al.*, 2004). In zebrafish, CYP1A is the highest constitutively expressed CYP1 in liver (Jonsson *et al.*, 2007). Zebrafish have lower levels of constitutively expressed CYP1B1, 1C1, 1C2, and 1D1 in the liver, gill and kidney compared to CYP1A (Jonsson *et al.*, 2007; Goldstone *et al.*, 2009). All zebrafish CYP1s, with the exception of CYP1D1, are upregulated through the AHR (Jonsson *et al.*, 2007; Goldstone *et al.*, 2009).

The function of mammalian CYP1s has been well documented, but less is known of other vertebrate CYP1s, outside CYP1A. Mammalian CYP1A1 and CYP1B1 are both able to metabolize PAHs (Nebert *et al.*, 2004) and estrogens (Lee *et al.*, 2003); CYP1B1, but not CYP1A1, can metabolize some aromatic amines (Nebert *et al.*, 2004). CYP1A2

can metabolize estrogens (Lee *et al.*, 2003) and many aromatic amines (Nebert *et al.*, 2004). Less overall metabolism is seen from CYP1B1, but the metabolites formed tend to be more highly reactive (Shimada *et al.*, 1999; Lee *et al.*, 2003; Nebert *et al.*, 2004). Previously, we have determined estradiol metabolism by zebrafish CYP1s and CYP3A65 (Scornaienchi *et al.*, In Submission) in a study that was, to our knowledge, the first to examine functional differences between non-mammalian vertebrate CYP1s. Zebrafish CYP1A metabolized estradiol with similar regioselectively, (Scornaienchi *et al.*, In Submission) and BaP at rates (Chung *et al.*, 2004) similar to mammalian CYP1As. Zebrafish CYP1B1 showed similar estradiol regioselectivity as mammalian CYP1B1 (Scornaienchi *et al.*, In Submission). Zebrafish CYP1A, 1C1, and 1C2 metabolized estradiol with a similar regioselectivity but CYP1C2 metabolized estradiol at a lower rate that CYP1A and 1C1 (Scornaienchi *et al.*, In Submission). Heterologous expression of the CYP1s was optimized for our research using the prototypical CYP1 substrate 7-ethoxyresorufin (7-ER). Zebrafish CYP1A, CYP1B1, CYP1C1, and 1C2 were all able to metabolize 7-ER (Scornaienchi *et al.*, In Submission). CYP1D1 did not metabolize estradiol or 7-ER at a high catalytic rate (Scornaienchi *et al.*, In Submission).

Catalytic activity and substrate specificity of individual CYP isoforms can be determined (Gautier *et al.*, 1996; Shimada *et al.*, 1999) using synthetic substrates that produce fluorescent metabolites (Crespi and Stresser, 2000) or analytical detection of metabolites produced from model compounds. For CYP1s, model compounds such as benzo(a)pyrene (BaP) (Yang *et al.*, 1975) and fluorescent assays based on the

alkoxyresorufin compounds, including 7-ER, are appropriate as they are known mammalian CYP1 substrates (Murray *et al.*, 2001). As the function of the non-mammalian CYP1 genes CYP1C1, 1C2 and 1D1, are not clear, inclusion of atypical mammalian CYP1 substrates may be appropriate to discern novel function not seen in mammalian species where these genes are lacking. Here, we present the first detailed catalytic assessment of the substrate specificity of CYP1s from a non-mammalian vertebrate. Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2 and CYP1D1 have previously been cloned and coexpressed with human CYP reductase (Scornaienchi *et al.*, In Submission). We have tested the metabolic capabilities of CYP1s using BaP and 11 synthetic fluorescent-based CYP substrates. The fluorescent substrates are comprised of 4 resorufin based compounds, 5 coumarin compounds, a fluorescein substrate, and a quinoline. This data will help to determine the functional roles of non-mammalian CYP1s and suggest whether the novel CYP1Cs and 1D proteins have overlapping or novel functions.

Methods

Cloning, expression and purification of zebrafish CYPs

Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1 and CYP3A65 were cloned and co-expressed with human cytochrome P450 reductase in *E. coli* JM109 cells, and purified bacterial membrane fractions were isolated as previously reported

(Scornaienchi *et al.*, In Submission). Expression was optimized for each cloned CYP and functional proteins confirmed with CO-difference spectra and catalytic assays.

Fluorescent based catalytic assays

All substrates and metabolites analyzed, as well as fluorescent wavelengths and concentrations used are shown in Table 1. Reactions involving the resorufin based substrates 7-ethoxyresorufin (7-ER), 7-methoxyresorufin (7-MR), 7-pentoxyresorufin (7-PR), and 7-benzyloxyresorufin (7-BR) were in 50 mM Tris, 0.1 M NaCl, pH 7.8. Reactions involving 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), 7-methoxy-trifluoromethylcoumarin (MFC), 7-benzyloxyquinoline (BQ), dibenzylfluorescein (DBF), 3-cyano-7-ethoxycoumarin (CEC), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), and 7-methoxy-4-(aminomethyl)-coumarin (MAMC) were in 0.5 M KPO_4 buffer, pH 7.5. Reactions were initiated with 1.33 nM NADPH (Sigma, St. Louis, MO) and all were assayed at 30°C. All resorufin based substrates (7- ER, MR, PR, BR) were from Sigma (St. Louis, MO) and BFC, MFC, BQ, DFB, CEC, AMMC and MAMC were from BD Gentest (Woburn, MA).

Benzo[a]pyrene metabolism

Incubations contained 0.1 M sodium phosphate, pH 7.4; 0.25 mg ml^{-1} human microsomal epoxide hydrolase (BD Biosciences, San Jose, CA); 1 mM NADPH, membrane fractions containing 100 mg total protein of recombinant zebrafish CYPs coexpressing

human CYP reductase; and BaP (Accustandard, New Haven, CT) at a final concentration of 0, 5, 10, 20, 40, 80, or 120 μM. BaP was dissolved in DMSO such that the final concentration of DMSO was 0.5%. Reactions without epoxide hydrolase were assayed at 40 μM BaP only.

Reactions were allowed to proceed at 28°C for 30 mins until terminated with 250 μl of cold acetone and incubation on ice for 10 minutes. The internal standard 6-hydroxychrysene (Accustandard, New Haven, CT) was added, metabolites extracted and the sample was dried down according to Kim *et al.* (1998). Evaporated samples were resuspended by sonication in 100 μl acetonitrile (Caledon, Georgetown, Canada) and stored under argon until analysis. Samples were analyzed by UPLC/MS for the presence of BaP-7,8,9,10-tetrol; BaP-9,10-dihydrodriol; BaP-7,8-dihydrodiol; BaP-1,6-dione; BaP-3,6-dione; BaP-6,12-dione; 9-OH-BaP; 3-OH-BaP; the parent compound BaP; and the internal standard, 6-hydroxychrysene, following methods from Zhu *et al.* (2008).

Results

The catalytic activity of each expressed CYP for 11 synthetic substrates (Table 1) and the model PAH, benzo[a]pyrene (BaP), were each assessed. None of the CYPs tested were able to metabolize the quinoline BQ, or the methylcoumarins AMMC and MAMC. All of the CYP1s metabolized 7-ER and 7-BR (Figure 1). CYP1A had the highest rate of 7-ER metabolism; catalytic activity was 1-4 orders of magnitude larger for this protein than the other CYP1s. CYP1C1, 1C2 and 1B1 all had rates of resorufin

production greater than 10 nmol nmol $P450^{-1}$ min^{-1}, although CYP1C2 had significantly more catalytic activity for 7-ER than CYP1C1 and 1B1. CYP1D1 had the lowest rate of metabolism for this, and all substrates tested.

The catalytic profiles for 7-ER and 7-MR were similar (Figure 1). CYP1A had the highest rate of resorufin formation and CYP1B1, 1C1 and 1C2 all had similar and more moderate rates of catalytic activity. Metabolism of 7-BR was similar across all CYP1s, except CYP1D1, which had much lower rates of 7-BR metabolism. 7-PR was metabolized by all CYP1s except CYP1B1. In general, 7-PR was metabolized the least of any alkoxyresorufin (Figure 1).

The trifluoromethylcoumarin substrates displayed similar metabolic profiles (Fig. 2). MFC and BFC were each metabolized the strongest by CYP1A, and moderately by each of the CYP1Cs. CYP1B1 was unable to metabolize MFC, and CYP1D1 did not metabolize either MFC or BFC (Fig. 2).

DBF was only metabolized by CYP1A, CYP1B1 and CYP1D1 (Fig. 3), but at rates that were much less than that seen for the alkoxyresorufins. CEC was metabolized the most by CYP1A and not at all by CYP1D1 (Fig. 3). CYP1C1 metabolized CEC at a 4-fold higher rate than CYP1C2. The catalytic rates of CYP1s for DBF and CEC were significantly less than those seen for the alkoxyresorufins (nmol compared to pmol).

Total metabolism of BaP was calculated as the sum of all detectable BaP metabolites for each CYP and was used to construct Lineweaver-Burk plots (Figure 4). CYP1A and CYP1C1 (Figure 4A) had lower rates of BaP metabolism then CYP1B1 and

CYP1D1 (Figure 4B). Reactions involving CYP1C2 did not show a significant relationship between metabolism and substrate (R^2<0.01, data not shown). CYP1C1 had the highest Km for BaP at 147.7 μM, and CYP1A, 1B1 and 1D1 all had similar Km values of between 25 and 70 μM (Table 2). Vmax values for CYP1A, 1C1 and 1D1 were similar at 121-203 pmol nmol $P450^{-1}$ min^{-1} while CYP1B1 had the highest Vmax (567 pmol nmol $P450^{-1}$ min^{-1}, Table 2). All metabolites were detected in at least one of the samples and quantifiable amounts of 9,10-diol, 1,6-dione, and 3,6-dione BaP metabolites were formed by all CYP1s (Table 3). The 3-OH BaP metabolite was the most prominent metabolite of CYP1B1, 1C2 and 1D1. CYP1C1 predominantly produced the 9-OH BaP metabolite while the 3,6-dione BaP metabolite was the major metabolite produced by CYP1A. The least formed BaP metabolite was BaP-7,8,9,10-tetrol (Table 3).

Discussion

Though the metabolic profile of the CYP1 family has been extensively investigated in mammals (Guo *et al.*, 1994; Gautier *et al.*, 1996; Lee *et al.*, 2003), less is known about the metabolic profile of CYP1s in fish, and especially the more recently discovered CYP1Cs and CYP1D1. We began by investigating the catalytic activity of each CYP using a variety of specific fluorescent substrates, some of which have been used in non-mammalian vertebrates (Stegeman *et al.*, 1997; Kashiwada *et al.*, 2005) and here we have begun to show differences in substrate specificity between zebrafish CYP1 isoforms. The alkoxyresorufins 7-ER and 7-MR are widely used as biomarkers in fish and

mammals for CYP1 activity. 7-ER and 7-MR are widely believed to be predominantly metabolized by CYP1A in fish (Klotz *et al.*, 1986; Schlezinger and Stegeman, 2001).

Though mammalian CYP1A1 is most responsible for 7-ER metabolism, CYP1A2 and CYP1B1 can also metabolize 7-ER (Shimada *et al.*, 1998). Zebrafish CYP1A was the dominant CYP responsible for 7-ER metabolism (Figure 1) with at least an order of magnitude higher activity for this substrate than the other CYP1s, except CYP1C2. CYP1B1 had activity that was about 30-fold less then CYP1A, similar to the difference seen between mammalian CYP1A and CYP1B1 (Murray *et al.*, 2001). The CYP1Cs were able to metabolize 7-ER to significant levels; CYP1C1 had activity similar to CYP1B1, and CYP1C2 had only a five-fold lower catalytic rate than CYP1A, suggesting that 7-ER is a broad CYP1 substrate in fish. The rates of 7-ER metabolism among the CYP1s suggests that levels of 7-ER activity in fish and possibly other non-mammalian vertebrates are the result of overall CYP1 activity, particularly from CYP1A and CYP1C2, and not solely CYP1A activity. CYP1D1 appears to be the sole CYP1 in fish with little capacity for 7-ER metabolism.

Mammalian CYP1A2 activity is commonly measured by 7-MR activity, though it is also strongly metabolized by CYP1A1 (Murray *et al.*, 2001). Zebrafish CYP1A does have the highest activity for 7-MR, with CYP1B1 metabolizing 7-MR at a 100-fold lower rate (Figure 1). Mammalian CYP1B1 had a low rate of 7-MR metabolism compared to either mammalian CYP1A1 or 1A2 (Murray *et al.*, 2001). A linear relationship between metabolism of 7-ER and 7-MR has been reported in scup liver microsomes (Schlezinger

and Stegeman, 2001), tilapia liver S9 preparations (Parente *et al.*, 2004) and AHR induced fish hepatocytes from multiple fish species (Smeets *et al.*, 2002), suggesting that both 7-ER and 7-MR activity were catalyzed by CYP1A (Smeets *et al.*, 2002). 7-ER and 7-MR are also the most structurally similar of any of the fluorescent compounds used and it is not surprising that they have similar enzyme metabolic profiles. Our data shows that CYP1A is likely the dominant CYP1 responsible *in vivo* for both 7-ER and 7-MR metabolism, but that significant contributions by other CYP1s, particularly CYP1C2, may be biologically relevant. 7-ER was metabolized three times faster than 7-MR by both zebrafish CYP1A and CYP1C2, similar to the ratios of 2.5 to 4 seen in fish hepatocytes (Smeets *et al.*, 2002), which supports the application of our *in vitro* data to *in vivo* functional differences.

7-PR activity, a marker of CYP2B in mammals, has been detected in fish, though the specific CYPs responsible for 7-PR metabolism have not been identified. In mammals, a major CYP2B inducer is phenobarbitol, while it inhibits PROD activity in fish (Addison *et al.*, 1987). 7-PR metabolism was not correlated with either 7-ER or 7-MR metabolism in fish liver S9 preparations (Parente *et al.*, 2004) suggesting that it is not a substrate for CYP1A. Our data does show that CYP1A, 1C1 and 1C2 are all able to metabolize 7-PR at similar rates, but much lower than any other alkoxyresorufin, suggesting some but not all 7-PR metabolism may be a result of CYP1 related activity. However, 7-PR was metabolized at a rate that was 100 times less than 7-ER by CYP1C2, and 250 times less than 7-ER by CYP1A (Figure 1). Tilapia S9 preparations had from 3 to

60 fold less PROD activity (Parente *et al.*, 2004) and turbot S9 preparations had 20 fold less 7-PR (Hartl *et al.*, 2007) than 7-ER metabolism. Since our rates of 7-PR metabolism compared to 7-ER for purified CYP1s are much lower than those reported for total hepatic preparations, it is likely that the major CYPs responsible for PROD activity in fish are not a CYP1.

7-BR is believed to have a broader enzyme pattern than the other alkoxyresorufins as seen in mammals where the substrate is metabolized by CYP1A, CYP2B and CYP3A (Hartl *et al.*, 2007). 7-BR and 7-ER were the only two fluorometric substrates to be metabolized by each zebrafish CYP1. Like our zebrafish CYP1A and 1B1 proteins, mammalian CYP1A1 and CYP1B1 metabolize 7-BR at similar rates (Murray *et al.*, 2001). Fish exposed to CYP1 inducers have increased 7-BR metabolism, but not to the extent that is seen with 7-ER (Addison *et al.*, 1987; Hartl *et al.*, 2007) or 7-MR (Hartl *et al.*, 2007). Our data suggests that the CYP1s, mostly CYP1A and CYP1B1, may be responsible for some 7-BR metabolism in fish, but supports the notion that other CYPs are responsible for most of the *in vivo* 7-BR activity in fish (Addison *et al.*, 1987; Hartl *et al.*, 2007).

MFC (Figure 2) and CEC (Figure 3), both substrates for human CYP2C9, 2C19 and 1A2, are metabolized by zebrafish CYP1A, and by a lesser extent from CYP1C1, and CYP1C2. Since both MFC and CEC are metabolized by mammalian CYP1A2, that they are metabolized by zebrafish CYP1A is not surprising. However, mammalian CYP1B1 can metabolize both CEC and MFC (Stresser *et al.*, 2002), yet zebrafish CYP1B1 was only able

to metabolize CEC and not MFC. Overall the CYP1s had a slightly higher preference for CEC over MFC, but neither is likely a specific CYP1 substrate and these compounds are likely broader CYP substrates in fish as they are in mammals.

BFC, a vertebrate CYP3A substrate not usually associated with CYP1 activity, was metabolized by CYP1A at an order of magnitude higher than the other CYP1s (Figure 2). CYP1A also metabolizes BFC at a faster rate than zebrafish CYP3A65 (Scornaienchi *et al.*, In Submission) suggesting that the use of BFC metabolism as a marker of CYP3A activity in fish (Hegelund *et al.*, 2004) may be problematic. DBF, another mammalian CYP3A4 substrate, was only minimally metabolized by zebrafish CYP1s (Figure 3). DBF is also a mammalian CYP2C8 and 2C9 substrate as well as a CYP3A substrate. Overall, CYP1A has the broadest substrate profile of any zebrafish CYP1 tested, and broader then would be predicted based on mammalian CYP1A enzymes.

Hepatic CYP1 enzymes catalyze the first step in the metabolism of BaP, a model polycyclic aromatic hydrocarbon (PAH), that can result in the activation to mutagenic and carcinogenic intermediates (Gelboin, 1980). The largest proportion of BaP metabolites found in mammals is usually 3-hydroxy-BaP (3-OH-BaP) (Gelboin, 1980), which was the most common zebrafish metabolite found from CYP1B1, 1C2 and 1D1. Interestingly, our zebrafish CYP1A had a higher proportion of 9-hydroxy-BaP (9-OH-BaP) then 3-OH-BaP, although heterologous zebrafish CYP1A expressed in yeast has previously been shown to form more 3-OH-BaP (Miranda *et al.*, 2006). Overall, our zebrafish CP1A produced the most BaP-3,6-dione, a metabolite with very little toxicity

(Gelboin, 1980). Both the Vmax and Km from our bacterial expressed CYP1A were about 10-fold lower than what has been reported for yeast expressed CYP1A (Miranda *et al.*, 2006). Different expression systems can have an effect on rates of BaP metabolism (Kim *et al.*, 1998), so whether BaP-3,6-dione, 3-OH-, or 9-OH-BaP is the primary metabolite formed by CYP1A in fish *in vivo* is unclear at this time. In our expressed proteins, 3-OH was formed less than all other BaP metabolites except the 6, 12-dione BaP metabolite (Table 3).

The diol epoxide metabolites are particularly genotoxic, of which 7,8-dihydroxy-9,10-epoxy-7,8,9,10-tetrahydrobenzo(a)pyrene (BaPDE) can most easily form DNA adducts (Peltonen and Dipple, 1995). BaPDE is formed from BaP through a series of reactions starting with the CYP catalyzed formation of BaP-7,8-epoxide, followed by epoxide hydrolase (EH) catalyzed formation of BaP-7,8-dihydrodiol, and then CYP formation of BaPDE (Peltonen and Dipple, 1995). Mammalian CYP1A1 forms the most BaP-7,8-dihydrodiol, the first step in production of the diol epoxide (Kim *et al.*, 1998). CYP1A2 has very low levels of BaP metabolism, and forms almost no BaP-7,8-dihydrodiol. Zebrafish CYP1A formed the most BaP-7,8-dihydriol and BaP-7,8,9,10-tetrol, similar to mammalian CYP1A1. The 7,8-diol was found in quantifiable amounts in reactions with zebrafish CYP1A and CYP1C2 while the tetrol was quantifiable only in reactions with zebrafish CYP1A, suggesting that CYP1A may the sole CYP1 enzyme responsible for the production of BaPDE in fish.

Contrary to all fluorescent substrates tested, CYP1D1 was very active in metabolizing BaP. CYP1D1 had the lowest Km for BaP and the second largest Vmax (Table 2). Surprisingly, the regioselectivity of CYP1D1 is not largely different for BaP compared to other fish CYP1s. CYP1D1 had higher 9,10-diol, 1,6-dione, and 6,12-dione metabolite formation suggesting that CYP1D1 may produce a distinct spectrum of BaP metabolites *in vivo*. This is the only substrate tested so far that is not poorly metabolized by CYP1D1, compared to the other CYP1s.

This is the first report of the catalytic profile of purified protein from the entire CYP1 family of a fish species. We have demonstrated that 7-ER and 7-MR, known mammalian CYP1A1 and CYP1A2 substrates respectively, and commonly used in fish as a marker of CYP1A activity, are highly metabolized by CYP1A, but CYP1C1 likely also contributes to this activity *in vivo*. Two other alkoxyresorufins, 7-PR and 7-BR, are likely not specific indicators of CYP1 activity *in vivo*. BFC, a mammalian CYP3A substrate, is strongly metabolized by CYP1A, and more than CYP3A65, such that it could contribute to BFC activity *in vivo*. Fish CYP1A was the broadest and most active CYP for the substrates tested. Both zebrafish CYP1A and CYP1B1 metabolized 7-ER, 7-BR, and 7-MR similar to mammalian CYP1A1 and CYP1B1 respectively. CYP1C1 and 1C2 had similar substrate specificity, though with differences in activity for most substrates. Overall, CYP1D1 seems to have very minimal, if any, activity for all of the substrates tested, suggesting the function of CYP1D1 is different from other CYP1s.

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Tables

Table 1. Synthetic cytochrome P450 substrates and the conditions for their associated fluorescent assays. Concentrations used and metabolites detected are given for each substrate. Bolded lettering in brackets represents the abbreviations used. The excitation (Ex) and emission (Em) conditions are shown as the wavelength/bandwidth of filters used. The central wavelength used was always within 10 nm of the manufacturer's recommended wavelength.

Substrate	Concentration	Metabolite	Ex (nm)	Em (nm)	Human CYP Specificity
7-ethoxyresorufin (**7-ER**)	2 mM	resorufin	540/35	590/20	1A1>1A2=1B1
7-methoxyresorufin (**7-MR**)	5 mM	resorufin	540/35	590/20	1A2
7-benzyloxyresorufin (**7-BR**)	5 mM	resorufin	540/35	590/20	1A, 2B, 3A
7-pentoxyresorufin (**7-PR**)	5 mM	resorufin	540/35	590/20	2B
7-benzyloxy-4-(trifluoromethyl)coumarin (**BFC**)	1 mM	7-hydroxy-4-(trifluoromethyl)coumarin (**HFC**)	400/30	528/20	3A4
7-methoxy-trifluoromethylcoumarin (**MFC**)	1 mM	7-hydroxy-4-(trifluoromethyl)coumarin (**HFC**)	400/30	528/20	2C9, 2C19, 1A2
7-benzyloxyquinoline (**BQ**)	1 mM	7-hydroxyquinoline (**HQ**)	400/30	528/20	3A4
dibenzylfluorescein (**DBF**)	10 μM	fluorescein	485/20	528/20	2C8, 2C9, 3A4
3-cyano-7-ethoxycoumarin (**CEC**)	10 μM	3-cyano-7-hydroxycoumarin (**CHC**)	400/30	460/40	2C9, 2C19, 1A2
3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (**AMMC**)	50 μM	3-[2-(N,N-diethylamino)ethyl]-7-hydroxy-4-methylcoumarin hydrochloride (**AHMC**)	400/30	460/40	2D6
7-Methoxy-4-(aminomethyl)-coumarin (**MAMC**)	50 μM	7-hydroxy-4-(aminomethyl)-coumarin (**HAMC**)	400/30	460/40	2D6

Table 2. Km and Vmax calculated for benzo[a]pyrene metabolism by heterologously expressed zebrafish CYP1 proteins. The sum of all detectable metabolites was used to construct Lineweaver-Burk plots (Figure 4) from which the values were determined.

	Km (μM)	Vmax (pmol nmol $P450^{-1}$ min^{-1})
CYP1A	49.9	121
CYP1B1	68.0	567
CYP1C1	147.7	134
CYP1C2	n/a[a]	n/a
CYP1D1	26.9	203

[a] n/a = no significant correlation (R^2<0.01) to determine Km or Vmax from Lineweaver-Burk

Table 3. Benzo[a]pyrene metabolite spectrum by heterologously expressed zebrafish CYP1s. Rate of formation of each metabolite is given as pmol nmol $P450^{-1}$ min^{-1} in reactions with 120 μM BaP and human epoxide hydrolase at 28°C.

CYP Isoform	Tetrol	9,10-diol	7,8-diol	1,6-dione	3,6-dione	6,12-dione	9-OH	3-OH	Total Metabolites
CYP1A	28.85	31.73	34.55	22.34	54.00	ND	38.82	*	210.29
CYP1B1	*	5.46	*	25.58	30.22	12.09	16.29	382.11	471.75
CYP1C1	*	9.25	*	6.60	9.46	*	98.48	32.14	155.92
CYP1C2	*	3.30	27.24	10.98	18.96	*	*	280.88	341.37
CYP1D1	*	74.80	ND	52.53	33.47	41.40	27.88	142.17	372.24

* - Metabolite formed at smaller BaP concentrations
ND – Metabolite was not detected

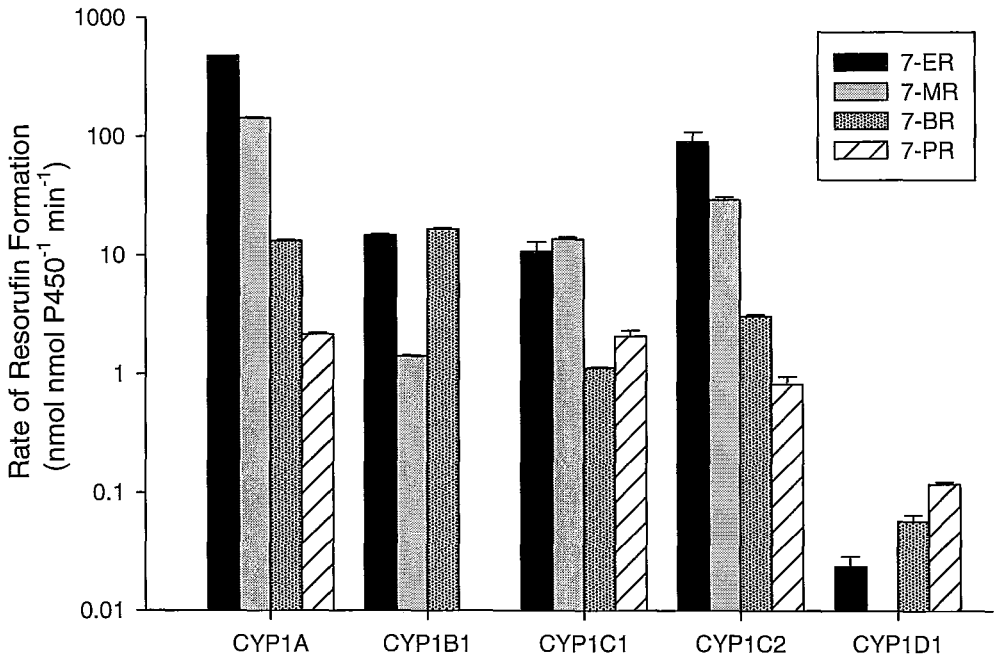
Figures

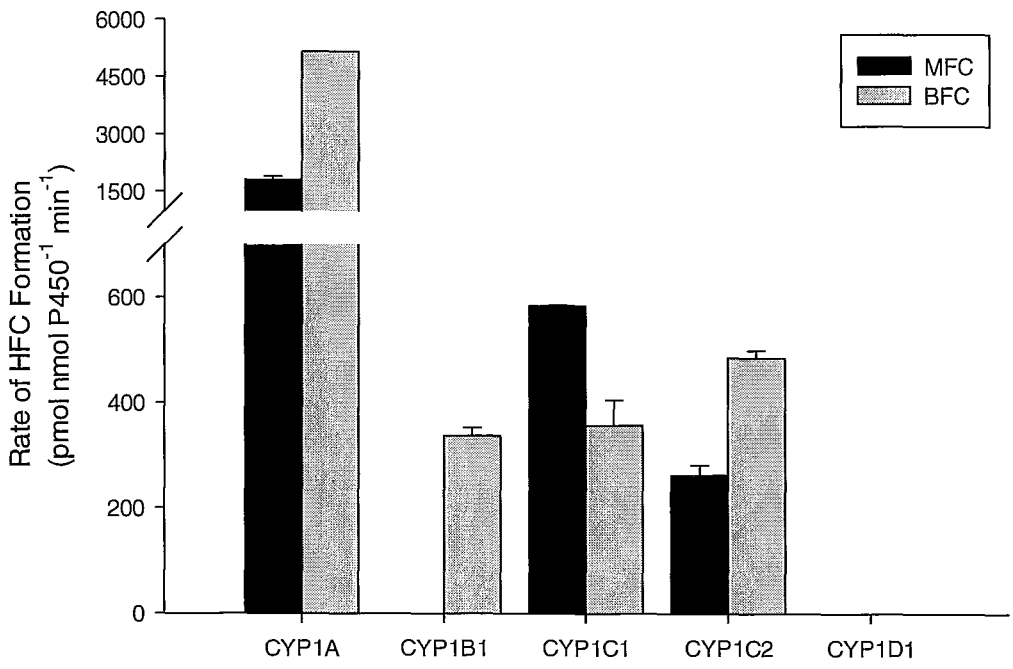
Figure 1. Metabolism of alkoxyresorufins by zebrafish CYPs coexpressed with human CYP reductase. Dealkylation was detected by quantification of the fluorescent resorufin metabolite (see materials and methods, and Table 1 for full details). Results are Mean ± SEM, n=2. Resorufin from the metabolism of 7-PR by CYP1B1 and 7-MR by CYP1D1 was below the limits of detection.

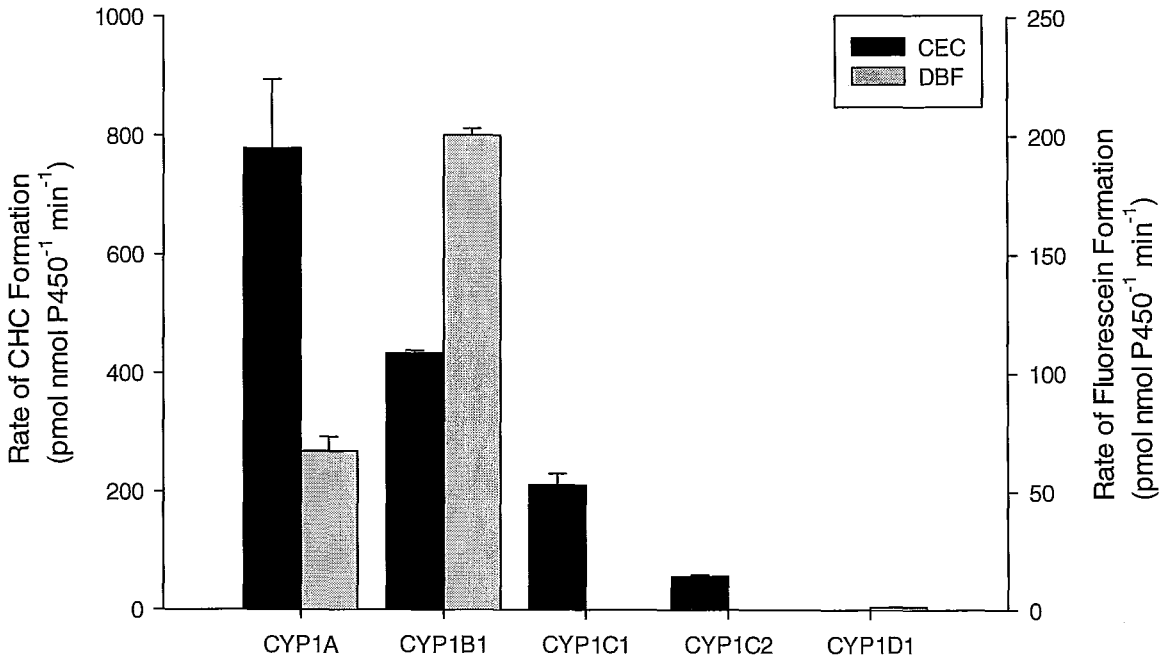
Figune 2. Metabolism of trifluoromethylcoumarins by zebrafish CYPs coexpressed with human CYP reductase. Dealkylation was detected by quantification of the fluorescent metabolite HFC from either BFC or MFC (see materials and methods, and Table 1 for full details). Results are Mean ± SEM, n=2. HFC formation from MFC by CYP1B1, and MFC and BFC by CYP1D1 was below the limits of detection.

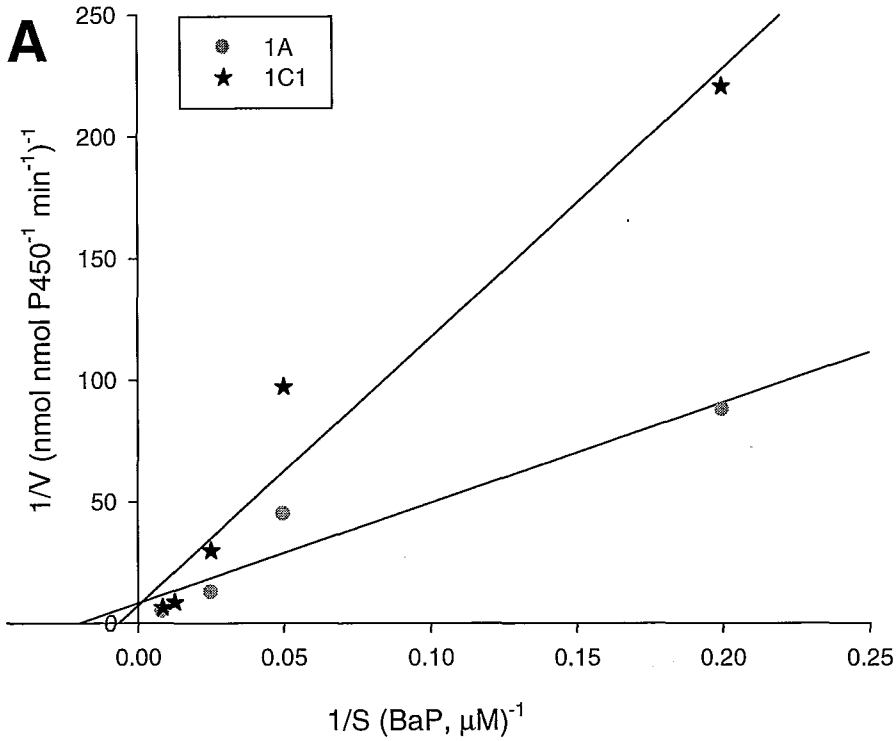
Figure 3. Metabolism of CEC and DBF by zebrafish CYPs coexpressed with human CYP reductase. Metabolism was quantified by detection of the fluorescent metabolite CHC or fluorescein for CEC and DBF, respectively (see materials and methods, and Table 1 for full details). Results are Mean ± SEM, n=2. Fluorescein formation was below the limits of detection in the CYP1C1 and 1C2 reactions, and CHC formation was below the limits of detection for CYP1D1.

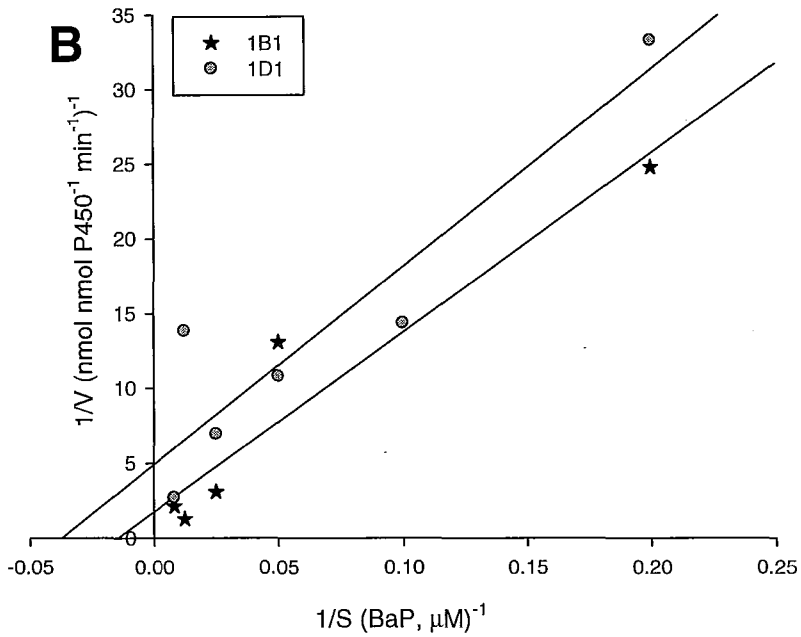
Figure 4. Lineweaver-Burke plots of total benzo[a]pyrene metabolites formed by zebrafish (A) CYP1A and CYP1C1 and (B) CYP1B1 and CYP1D1. Purified membranes were incubated with 5, 10, 20, 40, 80, or 120 μM BaP.


1000
100
10
1
0.1
0.01
Rate of Resorufin Formation
(nmol nmol P450^{-1} min^{-1})
7-ER
7-MR
7-BR
7-PR
CYP1A
CYP1B1
CYP1C1
CYP1C2
CYP1D1


6000
4500
3000
1500
600
400
200
0
Rate of HFC Formation
(pmol nmol P450-1 min-1)
MFC
BFC
CYP1A
CYP1B1
CYP1C1
CYP1C2
CYP1D1


Rate of CHC Formation
(pmol nmol P450-1 min-1)
Rate of Fluorescein Formation
(pmol nmol P450-1 min-1)
CEC
DBF
1000
800
600
400
200
0
250
200
150
100
50
0
CYP1A
CYP1B1
CYP1C1
CYP1C2
CYP1D1


A
1A
1C1
250
200
150
100
50
0
0.00
0.05
0.10
0.15
0.20
0.25
1/V (nmol nmol P450-1 min-1)-1
1/S (BaP, µM)-1


B
1B1
1D1
35
30
25
20
15
10
5
0
-0.05
0.00
0.05
0.10
0.15
0.20
0.25
1/V (nmol nmol P450-1 min-1)-1
1/S (BaP, µM)-1

Chapter 4: General Discussion

In this study, the function of the complete profile of cytochrome P4501 (CYP1) and CYP3A enzymes in a non-mammalian vertebrate, zebrafish, has been investigated for the first time. Zebrafish CYP1A, 1B1, 1C1, 1C2, 1D1 and 3A65 have been cloned and co-expressed with human CYP reductase. After membrane purification, CO-difference spectra were analyzed to confirm proper membrane targeting and folding (Chapter 2, Figure 1) and functional protein was confirmed using the substrates 7-ER (mammalian CYP1) or BFC (mammalian CYP3A; Chapter 2, Figure 2). Each CYP protein was characterized by their ability to metabolize 11 synthetic fluorometric substrates, 17β-estradiol (E_2), and benzo[a]pyrene (BaP). The metabolism of E_2 and BaP was used to assess the regioselectivity of the CYP enzymes as the major potential metabolites were determined for each reaction. This is the first reported functional characterization for CYP1D1. Only limited functional testing has been previously completed for CYP1C1, CYP1C2 and CYP3A proteins in fish.

Estrogen signaling

Estrogen is an ancient hormone, important for many processes including gonad development, sexual differentiation of the brain, regulating sexual behaviour and the reproductive endocrine axis (Forlano *et al.*, 2006). Estrogens are formed from androgens by the aromatization of CYP19, also known as aromatase. Most mammals have a single CYP19 but fish have two forms, CYP19A which is highly expressed in

ovaries, and CYP19B which is highly expressed in the brain (Forlano *et al.*, 2006). Estrogen produced in the brain, by CYP19B, is considered to act locally, but may be regulated by circulating levels of estrogens (Forlano *et al.*, 2006). Estrogens act by binding estrogen receptors (ERs) in the cytosol, of which two types exist, ERα and ERβ. Bound ERs will hetero- or homo-dimerize, enter the nucleus and bind to estrogen response elements (EREs) in promoter regions of genes which are then upregulated (Matthews and Gustafsson, 2003).

The promoter region of CYP19B contains EREs, and CYP19B expression levels are increased by increasing levels of E_2 causing a positive feedback loop for E_2 synthesis in the brain of teleosts (Callard *et al.*, 2001; Cheshenko *et al.*, 2007). CYP19A does not have any EREs in its promoter region (Callard *et al.*, 2001) and has differing responses to estrogens depending on model, tissue and life stage assayed (Cheshenko *et al.*, 2007). CYP19A is not upregulated by increased E_2 levels in zebrafish larva, but is in CHO cell reporter assays (Cheshenko *et al.*, 2007); After E_2 exposure, CYP19A mRNA levels and aromatase activity are downregulated in adult zebrafish ovaries (Hinfray *et al.*, 2006).

Estradiol metabolism

CYP1A and CYP1C1 were found to be the predominant CYPs that metabolized E_2 *in vitro*. The tissues that will be most affected by alterations in CYP mediated E_2 regulation will likely be the liver, gonads, gut, and brain which are the primary sites of ER expression (Menuet *et al.*, 2002). Upregulation of CYP1A or 1C1 in the gill may also

increase first pass metabolism of exogenous estrogens as they enter the fish from the aquatic environment. CYP1A and 1C1 are highly inducible through the AhR in the liver, gut, and gill, while only CYP1A, and not 1C1, is highly inducible in the brain and gonads (Jonsson *et al.*, 2007). Upregulation of CYPs involved in estradiol metabolism could result in reductions in tissue concentrations and ultimately tissue effects of E_2. As the brain and gonads are the primary sites of E_2 synthesis by CYP19B and CYP19A respectively (Sawyer *et al.*, 2006), induction of CYP1A in these organs has the largest potential to affect circulating levels of endogenous E_2. Upregulation of CYP1A through the AhR occurs through exposure to planar aromatic compounds such as beta-naphthoflavone (BNF). BNF increases the rate of E_2 metabolism in mammalian liver microsomes (Sepkovic *et al.*, 1994) but does not increase metabolism in English sole (Stein *et al.*, 1991) or scup (Snowberger and Stegeman, 1987).

AhR-ER crosstalk

CYP19B does not have any xenobiotic response elements (XREs) in its promoter, yet is affected by AhR ligands. Exposure of zebrafish to the AhR agonists TCDD or BaP alone had a very slight induction of CYP19B levels, but when co-incubated with E_2 reduced the level of induction of CYP19B compared to induction by E_2 alone (Cheshenko *et al.*, 2007). This has been proposed to be the result of the AhR/ARNT/ligand complex interacting with unligated ER, which occurs in mammals (Ohtake *et al.*, 2003). This "ER-hijacking" mechanism has also been seen in fish where the estrogenic effect of AhR

ligands are nullified upon the addition of the specific ER antagonist ICI182,780 (Cheshenko *et al.*, 2007; Mortensen and Arukwe, 2008). Further increasing AhR-ER crosstalk is the induction of ERα mRNA and protein levels by AhR ligands (Mortensen and Arukwe, 2008). This leads to higher estrogenicity of AhR ligand target cells.

Inhibition of AhR ligands by estrogens

There is an inhibitory AHR-ER crosstalk that may affect CYP protein levels. E_2 exposure has been shown to reduce both total protein and specific CYP content in brook trout hepatic microsomes (Stegeman *et al.*, 1982). After exposure to E_2, rainbow trout showed decreased 7-ER metabolism, suggesting a decrease in CYP1 enzyme activity (Vodicnik and Lech, 1983), and medaka had decreased CYP3A38 and 3A40 protein levels (Kashiwada *et al.*, 2007). In addition to these decreases in constitutive hepatic CYP1 and CYP3A proteins, numerous ER ligands are also able to decrease CYP1 protein levels induced by TCDD (Safe *et al.*, 2000). Any increases in activated ERs can lead to decreased CYP protein levels further potentiating and prolonging estrogenic effects, which has been suggested to act during reproduction to keep hormone levels elevated (Kashiwada *et al.*, 2005). One mechanism of this inhibition could be the result of a downregulation of ARNT. Nonylphenol reduces PCB126 induced ARNT mRNA levels in salmon (Mortensen and Arukwe, 2008). The CYP-mediated metabolism of E_2 is critical for maintaining proper estrogen levels. However, too much E_2 will inhibit CYP protein levels and this has the potential to further increase estrogen levels.

CYP1A, CYP1C1 and CYP1C2 activity

The major zebrafish CYPs found to metabolize E_2 *in vitro* were CYP1A, 1C1 and to a lesser extent, CYP1C2. CYP1A and 1C2 are likely also responsible for the majority of 7-ethoxyresorufin (7-ER) and 7-methoxyresorufin (7-MR) metabolism *in vivo*. 7-ER and 7-MR metabolism, which are correlated in fish (Schlezinger and Stegeman, 2001; Smeets *et al.*, 2002; Parente *et al.*, 2004), could function as potential biomarkers of tissue specific CYP mediated metabolism of E_2 *in vivo*. However, β-napthoflavone treated scup had rates of 2-OH-E_2 formation decrease while 7-ER metabolism was greatly increased (Snowberger and Stegeman, 1987). Channel catfish were found to have a small increase in the rate of 2-OH-E_2 formation and a moderate increase 4-OH-E_2 after BaP induction, but a large increase in 7-ER metabolism (Butala *et al.*, 2004). The difference between what would be predicted from our *in vitro* results and actual *in vivo* results could be that more CYPs responsible for metabolizing E_2 than 7-ER are upregulated, such as the CYP3s. Another factor could be that typical mammalian CYP1 inducers do not induce fish CYP1s to the same extent, or induce other CYPs. Trout exposed to BNF show induction of 7-ER, but a higher fold induction of MFC, suggesting that CYPs other than CYP1 are induced after exposure to AHR ligands (Smith, 2009).

The metabolism of a specific catalytic substrate or formation of a specific metabolite is a method to quickly and efficiently determine the activity of a specific CYP isoform in a heterogenous sample. Aside from the formation of 16α-OH-E_2 by CYP1A,

there was no substrate or metabolic product that was unique to any other CYP protein tested in this study. We did determine that both 7-ER and 7-MR, considered mammalian CYP1A1 and fish CYP1A substrates (Schlezinger and Stegeman, 2001; Smeets *et al.*, 2002; Parente *et al.*, 2004), should also be considered markers of CYP1Cs in fish. The catalytic profiles of CYP1C1 and CYP1C2 were found to be very similar. Both CYP1Cs metabolized the same fluorometric substrates, and produced the same E_2 and BaP metabolites. Unique to the other CYPs tested, zebrafish CYP1Cs produced estriol from E_2 when supplemented with cytochrome b_5, which could be a specific marker for CYP1C activity in fish. There were no specific markers for CYP1B1, 1D1 or CYP3A65.

CYP1B1 activity

Human CYP1B1 has shown a high affinity for 7-benzyloxyresorufin (7-BR) that is similar to CYP1A1 (Murray *et al.*, 2001; Stresser *et al.*, 2002). Zebrafish CYP1B1 was found to metabolize 7-BR at comparable rates to zebrafish CYP1A. Zebrafish CYP1B1 also metabolized 7-ER and 7-MR at around 100-fold lower rates than zebrafish CYP1A, which is similar to what is seen between mammalian CYP1A1 and CYP1B1 (Murray *et al.*, 2001; Stresser *et al.*, 2002). Similar to mammalian CYP1B1, we did not find a distinct probe for zebrafish CYP1B1.

CYP1D1 activity

CYP1D1 is the most recent CYP1 identified, and the most functionally different from the other CYP1s. It either did not metabolize, or weakly metabolized all substrates tested except for BaP. Since CYP1D1 was as active metabolizing BaP as the other expressed CYP1s in this study, the low activity seen by CYP1D1 for other substrates is likely not a result of the preparation used here, but intrinsic to the protein. CYP1D1 is also the only CYP1 to not be upregulated through the AhR pathway (Goldstone *et al.*, 2009). CYP1D1 likely plays some role in fish, as it has persisted as a functional CYP1 protein, unlike mammals which have lost a functional CYP1D protein (Goldstone *et al.*, 2009). This suggests that CYP1D1, compared to other CYP1s, may have a different substrate profile than CYP1s. For the identification of distinct CYP1D1 activity *in vivo*, it will be required to find a compound that is strongly metabolized by CYP1D1, and only minimally by other CYPs. Based on the differences of CYP1D1 from other CYP1s, this substrate is not likely to be a typical CYP1 substrate and the discovery of a suitable compound may require extensive testing.

CYP3A65 activity

CYP3A65 only metabolized a few compounds, with moderate to really low activity. CYP3A65 did metabolize BFC, which has been used as a measure of CYP3A activity in fish (Kashiwada *et al.*, 2005) but at a rate less than that of CYP1A suggesting that BFC metabolism is not a specific marker of CYP3A activity *in vivo*. The metabolism

of testosterone has been shown to be a marker of CYP3A activity in fish, and the two CYP3A paralogs in medaka have different metabolic profiles for testosterone (Kashiwada *et al.*, 2005). Testosterone was not used in this study, but is an excellent candidate to determine overall CYP3A activity in a sample. The low level of activity by zebrafish CYP3A is somewhat surprising since mammalian CYP3A proteins have high activity for E_2 (Lee *et al.*, 2003) and various catalytic substrates including BFC, BQ, and DBF (Stresser *et al.*, 2002). Fish CYP3A proteins may have a different suite of substrates that they preferentially metabolize compared to their mammalian counterparts. Medaka CYP3A38 and 3A40 (Kashiwada *et al.*, 2005) had similar rates of BFC metabolism as was found here from zebrafish CYP3A65. However, medaka CYP3A activity was increased by the addition of cytochrome b_5, which did not occur with zebrafish CYP3A65. Of interest is the functional role of the other CYP3 genes in fish. Zebrafish have multiple CYP3C genes, while CYP3B and 3D genes have been identified in fugu and stickleback (Qiu *et al.*, 2008). CYP3C1 was expressed in zebrafish, and does produce functional protein (Corley-Smith *et al.*, 2006). Whether each of the CYP3B, 3C and 3D genes are expressed and produce functional protein is unknown as they are the result of genomic assemblies (Qiu *et al.*, 2008). These proteins may have an overlapping function to CYP3A, and thus increase the low activity for typical CYP3A substrates seen so far in fish compared to mammals. CYP3A substrates would provide a suitable starting point to begin to characterize CYP3B, 3C and 3D proteins.

Future Directions

The creation of zebrafish CYP1 and CYP3A constructs has allowed for the catalytic profile of the full complement of zebrafish CYP1 and 3A proteins to be assessed for the first time. Although we were not able to find any CYP1B1, 1C1, 1C2, 1D1 or 3A65 specific substrates, this process can be continued to search for other potential CYP substrates. Clearly, the CYP1s have strong overlapping substrate specificity, except for the CYP1D1, suggesting that these proteins have some similar functions and specific substrates are less likely to be identified.

In our studies, zebrafish CYP3A65 was found to not be as active as mammalian CYP3As. Fish contain other CYP3 subfamilies that are unique to fish and not found in any other vertebrates. The function of fish CYP3Bs, 3Cs and 3Ds are unknown presently, though expression data indicates that, at least zebrafish CYP3C1, may play an important role in the liver, intestine and ovary (Corley-Smith *et al.*, 2006). The liver and intestine are important sites for metabolism of many endogenous and exogenous compounds suggesting that CYP3C1 may be important for these processes. The investigation of zebrafish CYP3 functions has been started by the *in vitro* study of CYP3As here and elsewhere (Kashiwada *et al.*, 2005), but should be continued to include all CYP3 subfamilies.

The purified zebrafish CYPs can also be utilized to test antibody specifity. So far, antibodies against zebrafish CYP1A and CYP1D1 have been developed (Goldstone and Stegeman, 2008), and our purified CYPs can be used to confirm that those antibodies,

and future CYP1 antibodies developed, do not cross-react to any other CYP1s. In addition, antibodies can be tested for the ability to inhibit CYP enzyme activity. If they are able to inhibit *in vitro* activity using our recombinant proteins, they could be used on whole tissue microsome samples to inhibit specific CYP isoforms to determine the *in vivo* metabolic contribution of each.

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Appendix I: Catalytic profile of a heterologously expressed Zebrafish (*Danio rerio*) Cytochrome P450 3A65 protein

Introduction

Cytochrome P450 3A (CYP3A) proteins are critical liver enzymes responsible for metabolism of a wide variety of structurally unrelated substrates. CYP3A genes are the highest expressed hepatic CYPs, and are responsible for over half of all drugs metabolized in mammals (Tseng *et al.*, 2005; Qiu *et al.*, 2008). All vertebrates contain CYP3A genes; fish have one or two CYP3A genes, but have novel CYP3 subfamilies including CYP3B, 3C and 3D which are not found in any other vertebrate group (Qiu *et al.*, 2008). Evolutionary analyses suggest there was a single CYP3A ancestor prior to the split of the Actinopterygii, ray finned fishes, from the other vertebrates (Qiu *et al.*, 2008). This CYP3A ancestor in fish underwent diversification giving rise to the other fish CYP3 subfamilies, resulting in a monophyletic clade that includes all fish CYP3 subfamilies. In other vertebrates, CYP3A genes form a distinct cluster suggesting that individual gene duplications gave rise to the multiple CYP3A genes found in each species (Qiu *et al.*, 2008).

Generally, CYP3A activity is determined, rather than activity from specific CYP3A isoforms, as individual CYP3As usually share similar substrates (Stresser *et al.*, 2002), making detection of the expression of individual CYP3A isoforms impossible with

catalytic assays. CYP3A activity is typically determined by the rate of testosterone 6β-hydroxylation (Ghosal *et al.*, 2003) or by metabolism of substrates such as dibenzylfluorescein (DBF) or 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) in fluorimetric assays (Crespi and Stresser, 2000). Here, the catalytic activity of zebrafish CYP3A65 was investigated using heterologous expressed protein and assays for the metabolism of benzo[a]pyrene (BaP) and 11 synthetic fluorescent-based CYP substrates. The fluorescent assays include the following substrates: 4 resorufin based compounds, 5 coumarin compounds, a fluorescein compound, and a quinoline.

Methods

Cloning, expression and purification of zebrafish cytochrome P450 3A

Zebrafish CYP3A was cloned, co-expressed with human Cytochrome P450 reductase in *E. coli* JM109 cells, and bacterial membranes were purified as reported in Chapter 2. Expression was optimized, and functional proteins confirmed with CO-difference spectra.

Fluorescent based catalytic assays

All substrates and metabolites analyzed, as well as fluorescent wavelengths and concentrations used are shown in Table 1 of Chapter 2. Reactions involving the resorufin based substrates 7-ethoxyresorufin (7-ER), 7-methoxyresorufin (7-MR), 7-pentoxyresorufin (7-PR), and 7-benzyloxyresorufin (7-BR) were done using methods

from Hahn *et al.* (1993). Reactions involving 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC), 7-methoxy-trifluoromethylcoumarin (MFC), 7-benzyloxyquinoline (BQ), dibenzylfluorescein (DBF), 3-cyano-7-ethoxycoumarin (CEC), 3-[2-(N,N-diethyl-N-methylamino)ethyl]-7-methoxy-4-methylcoumarin (AMMC), and 7-methoxy-4-(aminomethyl)-coumarin (MAMC) were in 0.5 M KPO_4 buffer, pH 7.5 and adapted from work with mammalian CYP proteins (Crespi and Stresser, 2000). Reactions were initiated with 1.33 nM NADPH (Sigma, St. Louis, MO) and all were assayed at 30°C. All resorufin based substrates (7- ER, MR, PR, BR) were from Sigma (St. Louis, MO) and BFC, MFC, BQ, DBF, CEC, AMMC and MAMC were from BD Gentest (Woburn, MA).

Benzo[a]pyrene metabolism

Zebrafish CYP3A65 mediated metabolism of BaP was assayed as reported in Chapter 3. Samples were analyzed by UPLC/MS for the presence of BaP-7,8,9,10-tetrol; BaP-9,10-dihydrodriol; BaP-7,8-dihydrodiol; BaP-1,6-dione; BaP-3,6-dione; BaP-6,12-dione; 9-OH-BaP; 3-OH-BaP; the parent compound BaP; and the internal standard, 6-hydroxy chrysene.

Results and Discussion

Zebrafish CYP3A65 was previously shown to be functional and catalytically active after heterologous expression and purification (Chapter 2). CYP3A65 was unable to metabolize any of 7-ER, 7-MR, 7-PR, 7-BR, MFC, BQ, CEC, AMMC, or MAMC. It

metabolized both DBF and BFC at similar rates (Figure 1) but these rates were much lower than those of zebrafish CYP1A (Chapter 1). BFC is a commonly used indicator of CYP3A activity in mammals (Crespi and Stresser, 2000), and has been used in killifish (Hegelund *et al.*, 2004) and medaka (Kashiwada *et al.*, 2005). The rate of zebrafish CYP3A65 mediated metabolism of BFC, 200 pmol nmol $P450^{-1}$ min^{-1} (Figure 1), is similar to that reported for medaka CYP3A38 and 3A40 (Kashiwada *et al.*, 2005). Though BFC is very specific in humans for CYP3A4, in rat it is also largely metabolized by CYP2s (Crespi and Stresser, 2000) and so may not necessarily be a specific CYP3A indicator in all species. In humans, CYP3A4 largely metabolizes BFC; CYP3A5 does not metabolize BFC, and CYP3A7 metabolizes BFC at a rate that is 50-fold less than CYP3A4 (Crespi and Stresser, 2000) suggesting that BFC may not be metabolized by all CYP3As. Zebrafish CYP1B1, 1C1 and 1C2 all metabolized BFC at similar rates to CYP3A65, while zebrafish CYP1A metabolized BFC at a 10-fold higher rate (Chapter 3), suggesting that BFC is not a specific CYP3A substrate in fish and should be applied to hepatic studies with caution. Based on our results and previous results (Hegelund *et al.*, 2004; Kashiwada *et al.*, 2007), BFC is a substrate for fish CYP3A activity, though induction of CYP1s, and especially CYP1A, will affect the rate of BFC metabolism and make the use of BFC in microsomal studies more difficult.

DBF, a mammalian CYP1A1 and 3A substrate (Crespi and Stresser, 2000), was metabolized by zebrafish CYP3A65 at a rate of 225 pmol nmol $P450^{-1}$ min^{-1} (Figure 1). CYP3A65 metabolized DBF at a similar rate as CYP1A, and at a 3-fold lower rate than

CYP1B1 (Chapter 2). In human liver microsomes, DBF metabolism is correlated with testosterone 6β-hydroxylase, which is specific for human CYP3A4 and 3A5 (Ghosal *et al.*, 2003). DBF may be a slightly better CYP3A substrate than BFC for fish, in particular because fish CYP1B1 is expressed and induced by AhR ligands significantly less than CYP1A, although neither BFC nor DBF are specific for the determination of CYP3A activity in fish. Zebrafish CYP1A for example, metabolized 7ER, a specific CYP1 substrate, at rates that were at least ten-fold higher than any other CYP making it a good compound for CYP1A activity (Chapter 3, Figure 1).

CYP3A65 poorly metabolized BaP compared to zebrafish CYP1s (Chapter 2). BaP-3,6-dione, and BaP-6,12-dione were formed in nearly all substrate concentrations, with BaP-7,8,9,10-tetrol, BaP-7,8-diol, BaP-1,6-dione, 3-OH-BaP and 9-OH-BaP only formed in one or two smaller substrate concentrations (Table 1). A Lineweaver-Burk plot using the summed rate of metabolite formation for each concentration of BaP resulted in no significant ($R^2 = 0.25$) relationship for CYP3A65. The overall rates for CYP3A65 mediated metabolism of BaP are much lower than those for the CYP1s, predominantly due to only low formation of either 3-OH-BaP or 9-OH-Bap, which were major metabolites from CYP1 metabolism (Chapter 2). Similar to zebrafish CYP3A65, human CYP3A4 does not have a high rate of BaP metabolism (Gautier *et al.*, 1996).

Zebrafish CYP3A65 shows similarities to mammalian CYP3As in its ability to metabolize both BFC and DBF, to poorly metabolize BaP, and to not metabolize any of the resorufins. However, another mammalian CYP3A substrate, BQ, was not

metabolized by zebrafish CYP3A65 and both DBF and BFC are metabolized as well or better with zebrafish CYP1 proteins suggesting that typical mammalian CYP3A substrates are not specific to fish CYP3A proteins. Fish are the only vertebrates to contain multiple CYP3 subfamilies. The functional role that these extra subfamilies, CYP3B, 3C, and 3D, have in fish has not been investigated, though their function could be related to mammalian CYP3A function, and thus play a role in *in vivo* metabolism of mammalian CYP3A substrates. Currently, there is no specific assay for catalytic function of fish CYP3As and caution is needed to interpret BFC, BQ or DBF metabolism in fish microsomal studies.

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Tables

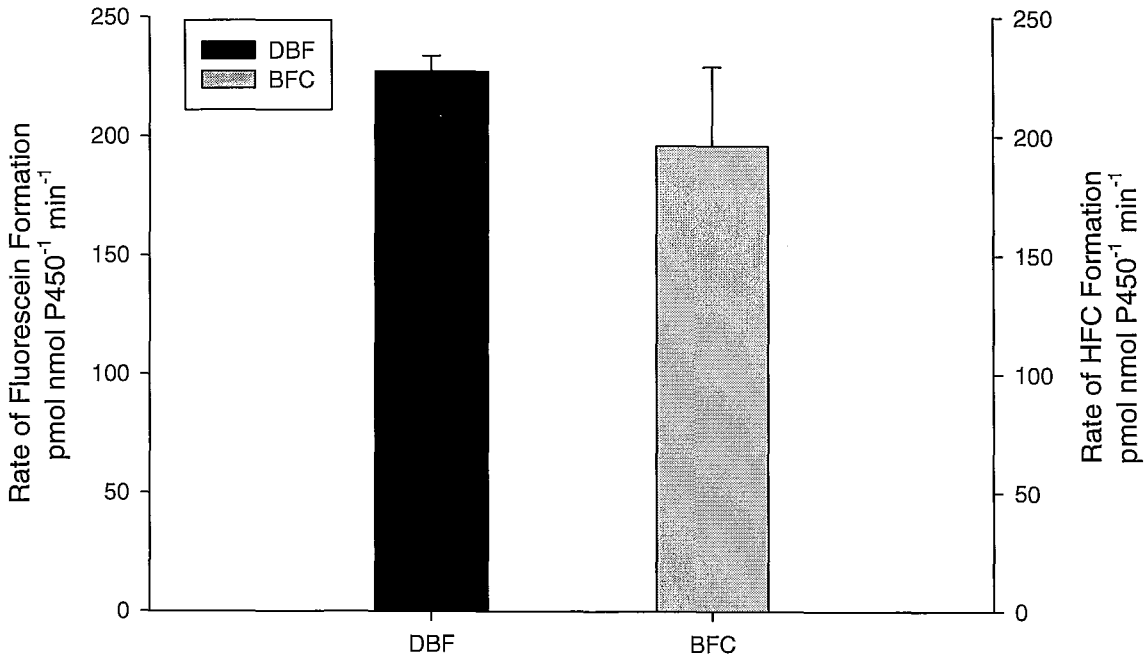
Table 1. Metabolism of benzo[a]pyrene by heterologous zebrafish CYP3A65 coexpressed with human CYP reductase. Rate of formation for each metabolite is given as pmol nmol $P450^{-1}$ min^{-1}, using 120 µM BaP, and incubated with human epoxide hydrolase at 28°C.

CYP Isoform	Tetrol	9,10-diol	7,8-diol	1,6-dione	3,6-dione	6,12-dione	9-OH	3-OH
CYP3A65	*	ND	*	*	13.61	18.97	*	*

* - Metabolite formed at smaller BaP concentrations
ND – Not detected

Figures

Figure 1. Metabolism of dibenzylfluorescein (DBF) and 7-benzyloxy-4-(trifluoromethyl)coumarin (BFC) by zebrafish CYP3A65 coexpressed with human CYP reductase. Metabolism was quantified by detection of the fluorescent metabolite fluorescein or HFC for DBF and BFC respectively (see materials and methods for full details). Results are Mean ± SEM, n=2.


DBF
BFC
Rate of Fluorescein Formation
pmol nmol P450-1 min-1
Rate of HFC Formation
pmol nmol P450-1 min-1
250
200
150
100
50
0
DBF
BFC