Cytochrome P450-mediated 17 β -estradiol metabolism in zebrafish (*Danio rerio*)

Marcus L Scornaienchi, Cammi Thornton¹, Kristine L Willett¹ and Joanna Y Wilson

Department of Biology, McMaster University, 1280 Main Street West, Hamilton, Ontario, Canada L8S4K1

¹Department of Pharmacology and Environmental Toxicology Research Program, University of Mississippi, University, Mississippi 38677-1848, USA

(Correspondence should be addressed to J Y Wilson; Email: joanna.wilson@mcmaster.ca)

Abstract

Cytochrome P4501 (CYP1) and CYP3A proteins are primarily responsible for the metabolism of 17 β -estradiol (E₂) in mammals. We have cloned and heterologously expressed CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 from zebrafish (*Danio rerio*) to determine the CYP-mediated metabolism of E₂ in a non-mammalian species. Constructs of each CYP cDNA were created using a leader sequence from the bacterial *ompA* gene to allow appropriate expression in *Escherichia coli* without 5' modification of the gene. Membrane vesicles were purified, and functional CYP protein was verified using carbon monoxide difference spectra and fluorescent catalytic assays with the substrates 7-ethoxyresorufin and 7-benzyloxy-4-(trifluoromethyl)-coumarin. Rates of *in vitro* E₂ metabolism into 4-hydroxyE₂ (4-OHE₂), 2-hydroxyE₂ (2-OHE₂), and 16 α -hydroxyE₁ (16 α -OHE₁) metabolites were determined by gas chromatography/mass spectrometry. The 2-OHE₂ metabolite was produced by all

CYPs tested, while 4-OHE₂ was only detected following incubation with CYP1A, CYP1B1, CYP1C1, and CYP1C2. The 16 α -OHE₁ metabolite was only produced by CYP1A. The highest rates of E₂ metabolism were from CYP1A and CYP1C1, followed by CYP1C2. CYP1B1, CYP1D1, and CYP3A65 had low rates of E₂ metabolism. E₂ metabolism by zebrafish CYP1A, CYP1C1, and CYP1C2 produced similar ratios of 4-OHE₂ to 2-OHE₂ as previous studies with mammalian CYP1As. CYP1B1 formed the highest ratio of 4-OHE₂ to 2-OHE₂ metabolites. Contrary to mammals, these results suggest that fish CYP1A and CYP1C proteins are primarily responsible for E₂ metabolism, with only minor contributions from CYP3A65 and CYP1B1. Similar to mammals, 2-OHE₂ is the predominant metabolite from CYP-mediated E₂ metabolism in fish, suggesting that all vertebrate species produce the same major E₂ metabolite.

Journal of Endocrinology (2010) **206**, 317–325

Introduction

The major estrogen in vertebrates is 17 β -estradiol (E₂). The first step in the metabolism of E₂ is NADPH-dependant oxidative metabolism catalyzed by hepatic cytochrome P450 (CYP) enzymes (Zhu & Conney 1998). Mammalian CYP enzymes are critical for E₂ metabolism, including those belonging to the CYP1A, CYP1B, and CYP3A subfamilies; yet, there are distinct differences in the CYP1 and CYP3 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 (*Danio rerio*) have a single CYP3A gene, CYP3A65 (Qiu *et al.* 2008). The CYP1 family contains more subfamilies in non-mammalian vertebrates. There are two CYP1A genes (CYP1A1 and CYP1A2) 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 gene found in all vertebrate lineages. CYP1Cs are found in all non-mammalian vertebrate lineages (Goldstone *et al.* 2007). Fish have CYP1C1 and CYP1C2

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). The liver is the major site of E₂ metabolism; all CYP1s and CYP3As are known to be expressed in the liver of mammals (Bieche *et al.* 2007) and fish (Jonsson *et al.* 2007, Goldstone *et al.* 2009).

In mammals, the major product of E₂ oxidation is the 2-hydroxyE₂ (2-OHE₂) metabolite, with limited production of 4-hydroxyE₂ (4-OHE₂) and small levels of metabolites hydroxylated at either the 6 β , 16 β , or 16 α positions (Lee *et al.* 2001). The regioselective hydroxylation of E₂ 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-OHE₂ to 2-OHE₂ formation (Lee *et al.* 2003). On the other hand, E₂ metabolism by mammalian CYP1B1 produces similar amounts of 4-OHE₂ to CYP1As and CYP3As, but very

little 2-OHE₂, such that three times more 4-OHE₂ is formed in relation to 2-OHE₂ (Lee *et al.* 2003).

The two catecholestrogens, 2-OHE₂ and 4-OHE₂, are further metabolized by catechol-*O*-methyltransferase (COMT) into the major urinary estrogen metabolites: 2-methoxyE₂ (2-MeOE₂) and 4-methoxyE₂ (4-MeOE₂) respectively (Creveling 2003). However, the catecholestrogens can also be oxidized to form reactive semiquinones and quinines, which can form adducts with purine bases (Dawling *et al.* 2001, Belous *et al.* 2007). COMT has a higher affinity for 2-OHE₂ over 4-OHE₂, resulting in an increased genotoxicity of the 4-OHE₂ metabolite (Tsuchiya *et al.* 2005). High concentrations of 4-OHE₂ 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 E₂ metabolism can lead to carcinogenesis. 2-OHE₂ metabolites appear to be tumor inhibitors (Tsuchiya *et al.* 2005).

In fish liver microsomes, the presence of E₂ 2-hydroxylase activity was identified in channel catfish (Butala *et al.* 2004), English sole (Stein *et al.* 1991), scup, and winter flounder (Snowberger & Stegeman 1987), and recently, 4-OH activity was detected in channel catfish (Butala *et al.* 2004). The only reported E₂ metabolism by purified fish CYPs has been from Japanese eel showing 2-OH activity by CYP1A9 but not by CYP1C1; however, neither CYP produced 4-OHE₂ metabolites (Uno *et al.* 2008). Since E₂ metabolism predominantly occurs in the liver, and the dominant metabolic products are 2-OHE₂ with smaller amounts of 4-OHE₂ for both mammals and fish, similar CYP families could be responsible for E₂ metabolism in mammalian and non-mammalian vertebrates.

In the present study, we describe our findings on the rates of formation of E₂ metabolites by bacterially expressed zebrafish CYPs. Zebrafish CYP1A, CYP1B1, CYP1C1, CYP1C2, CYP1D1, and CYP3A65 were each cloned and expressed in bacteria to produce catalytically active proteins used for *in vitro* assessment of E₂ 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 vertebrate CYP-mediated E₂ metabolism.

Materials and Methods

Cloning of zebrafish CYPs

RNA was extracted with TRIzol reagent (Invitrogen) from pools of liver or gill tissue taken from female wild type zebrafish (*D. rerio*; *n* = 8). cDNA was synthesized using cloned AMV RT (Invitrogen) 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) following the manufacturer's protocol and using gene-specific primers and annealing temperatures as listed in Table 1. PCR products were gel purified, cloned into pGEM-T Easy vectors (Promega), and transformed into competent *Escherichia coli* JM109 cells (Promega). Plasmids were purified by QIAprep Spin Miniprep Kits (Qiagen) and sequenced by MobixLab (McMaster University, Hamilton, ON, USA).

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 PCRs using a high fidelity polymerase, Accuprime Pfx (Invitrogen). 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 1). This generated a ~90 nucleotide fragment containing the first 21 bases of the appropriate CYP gene, and was gel purified and used in a second PCR with the same forward primer and a CYP-specific reverse primer against the 3' end (Table 1) of 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 NADPH-CYP reductase (CPR) into JM109 cells. Final sequences were confirmed by sequencing prior to expression.

Expression and purification of zebrafish CYPs

Overnight cultures of *E. coli* were diluted 1:100 into TB with ampicillin (50 µg/ml) and chloramphenicol (25 µg/ml) and shaken at 30 °C and 200 r.p.m. Isopropyl β-D-1-thiogalactopyranoside (1 mM; Fisher Scientific, Pittsburgh, PA, USA) was added when cultures reached an OD₆₀₀ of between 0.7 and 1.0. Expression was optimized with the addition of 0–1 mM δ-aminolevulinic acid (Ala; MP Biomedicals, Solon, OH, USA) 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 and 1.0 mM respectively. Expression was allowed to proceed for another 20–24 h. Cells were harvested, and bacterial membranes were purified using previously published methods (Pritchard *et al.* 2006). Total protein content was measured using a bicinchoninic acid assay kit (Pierce protein research products) according to the manufacturer's protocols (Smith *et al.* 1985).

Table 1 Primers and annealing temperatures used for (A) cloning of zebrafish cytochrome P450 (CYPs) and (B) fusion of the *ompA*(+2) segment to the zebrafish CYPs. All primers are listed in the 5'–3' direction

A	Forward primer	Reverse primer	Annealing temperature (°C)	GenBank accession number
Construct				
CYP1A	TGGAGCTAATTGGCACTGGT	CCTGGATTTCAGGAGCTCAA	53	NM_131879
CYP1B1	CCACCCGAACCTCTGAAACTC	GTTTTCTTAGCCGCCTTCATT	53	NM_001013267
CYP1C1	GGAGGCTGAGTTTGGACTGA	CCCATTGCACTGGATGTTTT	53	NM_001020610
CYP1C2	TGAGCCATCCTCCGGTAA	GCAGTGGGTTAGACAGCACA	53	XM_686678
CYP1D1	GCGATTTGCCAACTGATA	TGCCAACATTAGCTTGATGC	54	NM_001007310
CYP3A65	GGAGCTTCATCATCTTCAGCA	CCTCCTCCTCCTCCTCAGAC	53	NM_001020797
B	Reverse primer	Linker primer	Annealing temperature (°C) ^b	
Construct ^a				
<i>ompA</i> -CYP1A	<u>TCTAGAGCGGCCGCGAATTC</u> ACTA ^c	TGGAAGAATAGCCAGAGCCATXXX ^d	50, 55	
<i>ompA</i> -CYP1B1	<u>TCTAGAGCGGCCGCGAATTC</u> ACTA	AGCCAGCAGGACATCCATCATXXX	50, 55	
<i>ompA</i> -CYP1C1	<u>TCTAGAGCGGCCGCGAATTC</u> ACTA	CTGTCCGCTCCATTCCCGCATXXX	50, 55	
<i>ompA</i> -CYP1C2	<u>TCTAGAGCAGTGGGT</u> TAGACAGCACA	CTCTGAATCCGACTGCGCCATXXX	50, 55	
<i>ompA</i> -CYP1D1	<u>TCTAGATGGA</u> AACTGAGAGAATGATGG	GGAGATATTCTCAAGATTCATXXX	55, 53	
<i>ompA</i> -CYP3A65	<u>TCTAGAGCGGCCGCGAATTC</u> ACTA	TGTTTCTGCCGAGAAGAACATXXX	50, 55	

^aThe forward primer used for *ompA*(+2)-CYP fusions was always 5'-GGAATCCATATGAAAAGACAGCTATCGCG-3' with an NdeI restriction site underlined.

^bThe first temperature was used to anneal the forward and linker primer to the *ompA*(+2) template during step 1 of the PCR. 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.

^cThe XbaI restriction site is underlined.

^dXXX represents 5'-CGGGACGGCCTGCGCTACGGTAGCGA-3', which primes against the 3' end of the *ompA*(+2) segment.

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 carbon monoxide (CO) difference spectra using the peak absorbance difference between 450 and 490 nm and an extinction coefficient of 92/mM/cm (Omura & 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 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 per cm (Massey 1959).

Catalytic assays

Ethoxyresorufin-O-deethylase 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, USA) to 7-hydroxy-4-(trifluoromethyl)coumarin by purified membranes was measured in the presence of 1 mM BFC and 1.33 mM NADPH in 0.5 M potassium phosphate buffer (pH 7.4) at 30 °C, as optimized from Crespi & Stresser (2000). Catalytic assays containing cytochrome *b*₅ (Calbiochem, EMD Chemicals Inc., Gibbstown, NJ, USA) 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-ethoxyresorufin (7-ER; CYP1A, 1B1, 1C1, 1C2, 1D1) or BFC (CYP3A65) metabolism with assays normalized for total protein.

Zebrafish liver microsomal preparation

Zebrafish (*D. rerio*) were purchased from DAP International (Etobicoke, ON, Canada). Fish were kept in a semi-recirculating system at 28–30 °C and fed three times per day, alternating between tropical flake food and fresh brine shrimp (*Artemia* sp.). Adult zebrafish were removed from our regular breeding stock, and livers were collected and pooled (male *n*=57; female *n*=38) and flash frozen in liquid nitrogen. Fish were ~1 year of age at collection, and were still actively breeding. Microsomal fractions were collected according to Stegeman *et al.* (1979). All samples were kept at –80 °C until use. Total protein concentrations were determined, and the remaining microsomal pools were used to determine hepatic E₂ metabolism.

E₂ metabolism

E₂ (Sigma–Aldrich) was incubated with 0.5 mg membrane or microsomal protein at 28 °C for 2 h according to Spink *et al.* (1990) with and without cytochrome *b*₅ (100 pmol). The incubation time was chosen based on data collected during the optimization of the assay for studies with fish

(Butala *et al.* 2004). Reactions were terminated with 20 mM ascorbic acid (Sigma–Aldrich); the deuterated estradiol internal standard was added, and metabolites were extracted according to Butala *et al.* (2004). The samples were derivatized with *N,O*-bis(trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Sigma–Aldrich), evaporated under N₂ and resuspended in iso-octane (Sigma–Aldrich) prior to analysis. Samples were analyzed for the presence of estrone (E₁), estriol, E₂, 4-OHE₂, 2-OHE₂, 4-MeOE₂, 2-MeOE₂, 16 α -hydroxyE₁ (16 α -OHE₁), and the deuterated standard by GC/MS according to Butala *et al.* (2004). All samples were run in duplicate.

Materials

NADPH, leupeptin, aprotonin, phenylmethanesulphonyl-fluoride, and cytochrome *c* were purchased from Sigma–Aldrich. Ampicillin and chloramphenicol were purchased from Fisher Scientific. 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 FW Dahlquist (University of California Santa Barbara, Santa Barbara, CA, USA), and the pACYC expression vector containing human CPR was a kind gift from Dr T Friedberg (University of Dundee, Dundee, UK).

Results

Expression of zebrafish CYPs

The CO difference spectra were analyzed for each expressed CYP (Fig. 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 and 0.655 nmol/mg protein (Table 2). Cytochrome *c* activity was measured to determine the levels of NADPH–CPR in each sample. There were similar levels of reductase activity in each

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

Table 2 Total protein, P450 content, and cytochrome *c* activity in membrane vesicles purified from bacteria co-expressing zebrafish cytochrome P450 (CYP) genes and human NADPH–CYP reductase (CPR), or expressing CPR alone. Cytochrome *c* activity expressed as mean activity $\pm$ S.E.M. of reactions done in triplicate

	Total protein (mg/ml)	Total P450 content (nmol/mg)	Cytochrome <i>c</i> activity (nmol/mg per min)
Construct			
CPR ^a	11.65	– ^b	29.68 $\pm$ 3.2
CYP1A	16.42	0.655	16.77 $\pm$ 2.1
CYP1B1	19.20	0.278	16.27 $\pm$ 0.68
CYP1C1	22.25	0.395	13.93 $\pm$ 0.37
CYP1C2	20.50	0.473	17.52 $\pm$ 1.0
CYP1D1	22.77	0.139	15.50 $\pm$ 0.11
CYP3A65	17.04	0.282	18.74 $\pm$ 0.43

^aHuman NADPH–cytochrome P450 reductase.
^b– Total P450 content not determined.

sample, except for cells expressing human CPR 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 as substrates to verify that the proteins were functionally active. All CYP1s showed catalytic activity for 7-ER and BFC except for CYP1D1 and CYP3A65, which showed no metabolism of BFC and 7-ER respectively (Fig. 2). CYP1D1 showed detectable but low catalytic activity for a broad range of other substrates tested (unpublished data). The addition of cytochrome b₅ significantly increased the ability of CYP1A, CYP1B1, CYP1C1, and CYP1C2 to metabolize 7-ER. However, cytochrome b₅ did not affect the metabolism of 7-ER by CYP1D1 or the metabolism of BFC by CYP3A65 (data not shown).

E₂ metabolism

E₂ metabolism data are expressed as mean $\pm$ S.E.M. of reactions done in duplicate. There was no 2-MeOE₂ or 4-MeOE₂ detected in any of the samples, and no E₂ metabolites were detected in reactions with the control membranes (expressing CPR only). Since *E. coli* do not express COMT, the enzyme responsible for the methylation of hydroxylated E₂ metabolites, the measurement of 2-MeOE₂ and 4-MeOE₂ was additional negative controls for our assay. 16 α -OHE₁ was only detected in reactions with CYP1A at a rate of 6.8 $\pm$ 1.6 pmol/nmol P450 per min.

CYP1A and CYP1C1 showed the highest rates of E₂ metabolism and a strong preference for 2-OHE₂ over 4-OHE₂ (Table 3). CYP1C2 metabolized moderately less 2-OHE₂ and 4-OHE₂ than CYP1A and CYP1C1, but metabolism at the 2 position was highest (Table 3). All CYP1A, CYP1C1, and CYP1C2 had percent ratios of 4-OHE₂/2-OHE₂ between 3 and 8. CYP1B1, CYP1D1, and CYP3A65 had the lowest rates of E₂ metabolism in general (<40 pmol/nmol P450 per min), and 2-OHE₂

Figure 2 Catalytic activity of recombinant zebrafish CYPs co-expressed with human NADPH-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 $\pm$ S.E.M., for duplicate reactions. Catalytic activity was below the limits of detection for CYP3A65 (7-ER) and CYP1D1 (BFC). Values represent Mean $\pm$ S.E.M. of reactions done in duplicate. *Resorufin was detected in CYP1D1 at a rate of 24 ± 5 pmol/nmol P450/min.

formation predominated. CYP1B1 had the highest proportion of 4-OHE₂ production (4-OHE₂/2-OHE₂ = 32%) of all the CYPs tested, but still formed twice as much 2-OHE₂ than 4-OHE₂. There was no 4-OHE₂ produced by CYP1D1 or CYP3A65 without cytochrome b₅.

Addition of cytochrome b₅ approximately doubled the rate of formation of 2-OHE₂ by CYP1A and CYP1C1, but reduced the amount of 4-OHE₂ produced (Table 3). Cytochrome b₅ decreased the rate of E₂ metabolism by CYP1B1 and CYP1C2, while metabolism by CYP3A65 was not affected. Small amounts of estrone were produced, at rates below 3.0 pmol/nmol P450/min, by CYP1A, CYP1B1, CYP1C1, and CYP1C2 with cytochrome b₅, but CYP3A65 with cytochrome b₅ produced estrone at a rate of 8.2 ± 5.3 pmol/nmol P450/min. Estrinol was detected only in samples with CYP1C1 or CYP1C2 that included cytochrome b₅. Estrinol was produced at a rate of 223 ± 19 pmol/nmol P450/min by CYP1C1 and 7 ± 0.24 pmol/nmol P450/min by CYP1C2.

Zebrafish liver microsomes produced low, but detectable amounts of 2-OHE₂ (Table 4) and estrone. Only one replicate reaction of each produced 2-OHE₂, while both replicates produced estrone at 0.62 ± 0.03 pmol/mg/min from female liver and 0.23 ± 0.02 pmol/mg/min from male liver.

Discussion

E₂ 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 E₂ metabolism (Lee *et al.* 2001). Because E₂ 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 E₂ metabolism *in vitro*.

Bacterial expression of recombinant eukaryotic CYP proteins is possible, but often involves the 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 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); this was consistent with previous bacterial expressed human

Table 3 Rate of formation of 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂) by heterologously expressed zebrafish with and without cytochrome b₅. Data from human and eel are from published studies for comparison with the zebrafish data

	2-OHE ₂ (pmol/nmol P450/min)	4-OHE ₂ (pmol/nmol P450/min)	Ratio of 4-OHE ₂ / 2-OHE ₂ (%)
CYP			
Zebrafish^a			
CYP1A	425.2 $\pm$ 85.0	21.8 $\pm$ 5.4	5
CYP1A + b ₅	892.6 $\pm$ 81.1	ND ^b	NA ^c
CYP1B1	27.1 $\pm$ 10.7	8.8 $\pm$ 5.7	32
CYP1B1 + b ₅	18.0 $\pm$ 0.1	4.7 $\pm$ 0.1	26
CYP1C1	510.0 $\pm$ 1.1	40.9 $\pm$ 3.9	8
CYP1C1 + b ₅	1083 $\pm$ 33	35.2 $\pm$ 0.7	3
CYP1C2	155.4 $\pm$ 1.5	4.2 $\pm$ 0.2	3
CYP1C2 + b ₅	57.0 $\pm$ 1.4	1.8 $\pm$ 0.3	3
CYP1D1	31.4 $\pm$ 1.9	ND	NA
CYP3A65	25.6 $\pm$ 5.3	ND	NA
CYP3A65 + b ₅	23.0	3.2	14
Human^d			
CYP1A1	2523 $\pm$ 208	163 $\pm$ 22	7
CYP1A2	4065 $\pm$ 156	343 $\pm$ 24	9
CYP1B1	108 $\pm$ 3	371 $\pm$ 16	344
CYP3A4	355 $\pm$ 41	78 $\pm$ 11	22
CYP3A4 + b ₅	3093 $\pm$ 91	497 $\pm$ 27	16
CYP3A5	125 $\pm$ 22	67 $\pm$ 5	53
CYP3A7 + b ₅	146 $\pm$ 17	55 $\pm$ 3	58
Japanese eel^e			
CYP1A9	177	ND	NA
CYP1C1	ND	ND	NA

^aData from this study, using 50 μ M E₂ at 28 $^{\circ}$ C for 2 h; values represent mean $\pm$ S.E.M. of duplicate reactions.

^bND – metabolite not detected.

^cNA – not applicable; one or both metabolites not detected.

^dHuman data from Lee *et al.* (2003), using 20 μ M E₂ at 37 $^{\circ}$ C for 20 min; values represent mean $\pm$ S.D. of triplicate reactions.

^eJapanese eel data calculated based on Uno *et al.* (2008), using 100 μ M E₂, at 37 $^{\circ}$ C assuming constant rate over 8-h reaction.

Table 4 Rate of formation of 2-hydroxyestradiol (2-OHE₂) and 4-hydroxyestradiol (4-OHE₂) by liver microsomes from zebrafish. Data from other fish and mammalian species are from published studies for comparison to the zebrafish data

	Sex	N	2-OHE ₂ (pmol/mg/min)	4-OHE ₂ (pmol/mg/min)	Ratio of 4-OHE ₂ /2-OHE ₂ (%)
Species					
Zebrafish ^a	Male	1	0.068	ND ^b	NA ^c
	Female	1	0.269	ND	NA
Channel catfish ^d	Male	3	20.3 ± 1.1	0.748 ± 0.032	3.7
Scup ^e	Male	5	362 ± 141	— ^f	NA
	Female	6	157 ± 37	—	NA
Winter flounder ^e	Male	13	296 ± 79	—	NA
	Female	13	89 ± 39	—	NA
English sole ^g	Juvenile	5	260 ± 17	—	NA
Human ^h	Male	21	46.6 ± 25.7	7.1 ± 3.4	15
	Female	12	47.7 ± 28.2	8.2 ± 4.4	17
Rat ⁱ	Male	3–4	1710 ± 180	390 ± 50	23
	Female	3–4	240 ± 20	70 ± 20	29

^aData from this study, using 50 μM E₂ at 28 °C.
^bND – metabolite not detected.
^cNA – not applicable; one or both metabolites not detected.
^dChannel catfish data from Butala *et al.* (2004), using 50 μM E₂ at 30 °C; values represent mean ± s.e.m.
^eData from Snowberger & Stegeman (1987), using 25 μM E₂ at 25 °C for flounder, and 30 °C for scup; values represent mean ± s.d.
^f– not investigated in this study.
^gEnglish sole data from Stein *et al.* (1991), using 100 μM E₂ at 25 °C; values represent mean ± s.e.m.
^hHuman data from Lee *et al.* (2001), using 20 μM E₂ at 37 °C; values represent mean ± s.d.
ⁱRat data from Dannan *et al.* (1986), using 200 μM E₂ at 37 °C; values represent mean ± s.e.m.

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 Fig. 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 overexpressed 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 a widely used specific substrate for CYP1 activity in mammals (CYP1A1, 1A2, 1B1; Shimada *et al.* 1998) and fish (CYP1A, 1B1; Hegelund *et al.* 2004). BFC is selective for CYP3A activity in mammals (Crespi & Stresser 2000), and was considered as 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 catalyzed 7-ER metabolism at a rate 30 times greater than that of CYP1B1 (Fig. 2), a ratio comparable to that reported for human CYP1A1 and CYP1B1 (Murray *et al.* 2001). Unlike the CYP1As and CYP1Bs, there is no previous catalytic data available for CYP1Cs or CYP1Ds, but clearly the expressed CYP1C1, CYP1C2, and CYP1D1 are functional proteins as each were able to metabolize at least 7-ER (Fig. 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 the substrates used are not optimal or specific for CYP1D1 activity. CYP3A65 did not metabolize 7-ER

but did metabolize BFC (Fig. 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 b₅. 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 favor the differences seen between these expressed CYP proteins for E₂ 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 CPR present for each expressed protein.

CYP1A and CYP1C1 demonstrated the highest overall activity for E₂ compared with the other fish CYP1s. They were also the only two zebrafish CYPs to show increased metabolism of E₂ when supplemented with cytochrome b₅. The rate of overall E₂ metabolism of CYP1B1 compared with CYP1A was similar to that of the metabolism of human CYP1B1 to CYP1A1 (Lee *et al.* 2003). Surprisingly, both CYP1C1 and CYP1C2 metabolize E₂ to a higher degree than 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 E₂ metabolism but in fish the CYP1Cs have much higher, and likely, more biologically relevant E₂ metabolism than CYP1B1, suggests that E₂ metabolism was present in the ancestor to the CYP1B and CYP1C 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-OHE₂, 4-OHE₂, and 16 α -OHE₁ metabolites. In mammals, CYP1A1 was the only CYP1 able to hydroxylate at the 16 α position, and does so in very low concentrations (Lee *et al.* 2003) similar to what was seen by zebrafish CYP1A. 2-OHE₂ was the predominant metabolite formed in both zebrafish (this study) and mammalian studies (Lee *et al.* 2003), and the 4-OHE₂ 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-OHE₂ (Uno *et al.* 2008), though at a lower rate than seen here, possibly due to an 8 h reaction time in that study.

Zebrafish CYP1Cs and CYP1A have a similar 4-OHE₂ to 2-OHE₂ ratio compared with mammalian CYP1As (Lee *et al.* 2003). Zebrafish CYP1B1 was similar to mammalian CYP1B1 in its regioselectivity, as it metabolized the largest proportion of 4-OHE₂ relative to 2-OHE₂ formation compared with any other tested zebrafish CYP. However, unlike human CYP1B1 (Lee *et al.* 2003), zebrafish CYP1B1 still metabolized more 2-OHE₂ than 4-OHE₂, and overall, the rates of metabolism were much less than the mammalian ortholog. Distinct from the other CYP1s, CYP1D1 did not produce any 4-OHE₂ metabolites. The lower ratio of 4-OHE₂ to 2-OHE₂ metabolism seen here from zebrafish CYP1B1 compared with human CYP1B1, and the preference of all other CYPs tested for 2-OH regioselectivity, agrees with the lower *in vivo* proportion of 4-OHE₂ seen in channel catfish (Butala *et al.* 2004) compared with human (Lee *et al.* 2001) and rat (Dannan *et al.* 1986; Table 4). Our zebrafish microsomal protein had only low activity for E₂, and we did not detect any 4-OHE₂ metabolites. We also detected the production of estrone, which along with the parent compound, E₂, are the primary estrogens in fish that are either glucuronidated or sulfated for elimination (Stein *et al.* 1991). Owing to the small size of zebrafish, a large sample size was required to obtain sufficient microsomal material for the present study; in fact, our microsomal pools required ~40–60 animals each and precluded a large number of replicates. To obtain sufficient numbers of animals, we collected livers from 1-year-old animals that were slated for removal from our breeding stock, and were already in reproductive decline. This likely led to an under-representation of the activity of zebrafish microsomal E₂ activity, and may account for the lower levels of E₂ metabolism that were seen from our zebrafish compared with other fish (Table 4). Additionally, there may be hormonal differences between species that could be responsible for the lower E₂ metabolism by zebrafish. The relatively low amounts of 4-OHE₂ detected in our samples suggest that though fish are susceptible to genotoxic damage by increased levels of E₂ (Teles *et al.* 2006), they may be less sensitive than mammals.

Unlike mammalian liver where CYP1A2 is the dominant CYP1, fish lack the CYP1A2 gene and CYP1A is the dominant hepatic CYP1 (Jonsson *et al.* 2007). All CYP1C1, CYP1C2, and CYP1B1 are constitutively expressed at similar low levels in the liver (Jonsson *et al.* 2007). CYP1A, CYP1B1, CYP1C1, and CYP1C2 are inducible through the AHR pathway (Jonsson *et al.* 2007), and their role in E₂ metabolism suggests a potential mechanism for AHR control of E₂ concentrations. Our data suggests that CYP1A would be responsible for the majority of CYP1-mediated hepatic E₂ metabolism in fish. First, CYP1A has higher *in vitro* activity for E₂ metabolism compared with all other CYP1s and CYP3A65 (Table 3). Secondly, liver expression of CYP1A is significantly higher than all other CYP1s (Jonsson *et al.* 2007). Thirdly, CYP1A has a much higher induction via AHR agonists (Jonsson *et al.* 2007) suggesting that even when induced, CYP1A-mediated E₂ metabolism is likely to predominate *in vivo*. The relatively high production of estrone compared with 2-OHE₂ using zebrafish (this study), channel catfish (Butala *et al.* 2004), English sole (Stein *et al.* 1991), winter flounder, and scup (Snowberger & Stegeman 1987) microsomes suggests that CYP3A65, a very highly expressed hepatic CYP with the highest estrone production of our expressed proteins, may also be important for *in vivo* E₂ metabolism in spite of its lower overall enzymatic activity *in vitro*.

CYP1D1 is constitutively expressed in the liver but, unlike other CYP1s, is not inducible through the AhR pathway (Goldstone *et al.* 2009). CYP1D1 expression was significantly less than CYP1A in liver, gut, gill, and kidney but higher in brain of zebrafish (Goldstone *et al.* 2009). Coupled with the lack of AHR-mediated induction of CYP1D1 (Goldstone *et al.* 2009), compared with all other CYP1 genes (Jonsson *et al.* 2007), this data suggest that CYP1D1 does not share a primary functional role with CYP1A1 in xenobiotic metabolism. Basal expression of CYP1A was threefold higher than CYP1D1 in liver (Goldstone *et al.* 2009); the low overall activity of CYP1D1 *in vitro* for E₂ metabolism, and the much lower expression in liver compared with CYP1A, strongly suggests that CYP1D1 is unlikely to play an important *in vivo* role in E₂ metabolism. 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 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 induction 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 E₂ 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 E₂ similar to the CYP1As (Lee *et al.* 2003), while zebrafish CYP3A65 poorly metabolizes E₂ at rates that are similar to human CYP3A5 and CYP3A7 (Lee *et al.* 2003). Zebrafish CYP3A65 only produced 4-OHE₂ metabolites, when supplemented with cytochrome b₅, while mammalian CYP3As do produce 4-OHE₂ regardless of whether cytochrome b₅ 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 functions compared with mammalian CYP3As; zebrafish CYP3A65 can metabolize E₂, 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 with 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 E₂ metabolism, followed by more modest E₂ metabolism by CYP1C2. The dominant metabolite formed was 2-OHE₂, similar to studies seen in other fish (Uno *et al.* 2008) and humans (Lee *et al.* 2003). Similar to human E₂ metabolism by CYP1B1 (Lee *et al.* 2003), the ratio of 4-OHE₂ to 2-OHE₂ was highest in zebrafish CYP1B1, yet the overall rates of metabolism by zebrafish CYP1B1 were lower than expected. CYP3A65 had low E₂ metabolism comparable with human CYP3A5 and CYP3A7 (Lee *et al.* 2003), formed no 4-OHE₂, and may not contribute greatly to E₂ metabolism *in vivo*. CYP1D1, which had a different developmental and tissue expression pattern from CYP1A and was not inducible by AhR agonists in zebrafish (Goldstone *et al.* 2009), had functional differences compared with other CYP1s. CYP1D1 has been suggested to have an endogenous function (Goldstone *et al.* 2009); our study suggests that E₂ metabolism is not a likely function of this protein. While overall E₂ metabolism in fish and other vertebrates is similar to mammalian E₂ metabolism, in non-mammalian vertebrates, the primary E₂ metabolizers *in vivo* are likely CYP1A and CYP1C enzymes. The contributions by CYP1B1 and CYP3A are likely less than in mammalian species.

Declaration of interest

The authors declare that there is no conflict of interest that can be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Sciences and Engineering Research Council of Canada (NSERC) Discovery (grant number 328204) to JYW and the National Institute of Environmental Health Sciences (NIH; grant number R01ES012710) to KLW. Funding for MLS was partially provided by the Department of Biology, McMaster University.

Acknowledgements

We would like to thank Dr Thomas Friedberg for supplying the human NADPH-cytochrome P450 reductase construct, Dr Frederick W Dahlquist for supplying the pCW vector, and Dr John Rimoldi for supplying the deuterated estradiol standard. We thank Emily Smith and Eric Hirsch for the optimization of fluorescent assays. This research was conducted in accordance with the accepted standards of humane animal care and approved under the McMaster University Animal Ethics Research Board, Animal Use Protocol #06-08-47.

References

- Belous AR, Hachey DL, Dawling S, Roodi N & Parl FF 2007 Cytochrome P450 1B1-mediated estrogen metabolism results in estrogen-deoxyribonucleoside adduct formation. *Cancer Research* **67** 812–817.
- Bieche I, Narjoz C, Asselah T, Vacher S, Marcellin P, Lidereau R, Beaune P & de Waziers I 2007 Reverse transcriptase-PCR quantification of mRNA levels from cytochrome (CYP)1, CYP2 and CYP3 families in 22 different human tissues. *Pharmacogenetics and Genomics* **17** 731–742.
- Butala H, Metzger C, Rimoldi J & Willett KL 2004 Microsomal estrogen metabolism in channel catfish. *Marine Environmental Research* **58** 489–494.
- Crespi CL & Stresser DM 2000 Fluorometric screening for metabolism-based drug–drug interactions. *Journal of Pharmacological and Toxicological Methods* **44** 325–331.
- Creveling CR 2003 The role of catechol-O-methyltransferase in the inactivation of catecholestrogen. *Cellular and Molecular Neurobiology* **23** 289–291.
- Dannan GA, Porubek DJ, Nelson SD, Waxman DJ & Guengerich FP 1986 17β-estradiol 2- and 4-hydroxylation catalyzed by rat hepatic cytochrome P-450: roles of individual forms, inductive effects, developmental patterns, and alterations by gonadectomy and hormone replacement. *Endocrinology* **118** 1952–1960.
- Dawling S, Roodi N, Mernaugh RL, Wang X & Parl FF 2001 Catechol-O-methyltransferase (COMT)-mediated metabolism of catechol estrogens: comparison of wild-type and variant COMT isoforms. *Cancer Research* **61** 6716–6722.
- Dehennin L, Reiffsteck A & Scholler R 1980 Simple methods for the synthesis of 20 different, highly enriched deuterium labeled steroids, suitable as internal standards for isotope-dilution mass-spectrometry. *Biomedical Mass Spectrometry* **7** 493–499.
- Gillam EM, Guo Z, Ueng YF, Yamazaki H, Cock I, Reilly PE, Hooper WD & Guengerich FP 1995 Expression of cytochrome P450 3A5 in *Escherichia coli*: effects of 5' modification, purification, spectral characterization, reconstitution conditions, and catalytic activities. *Archives of Biochemistry and Biophysics* **317** 374–384.
- Godard CA, Goldstone JV, Said MR, Dickerson RL, Woodin BR & Stegeman JJ 2005 The new vertebrate CYP1C family: cloning of new subfamily members and phylogenetic analysis. *Biochemical and Biophysical Research Communications* **331** 1016–1024.

- Goldstone JV, Goldstone HM, Morrison AM, Tarrant A, Kern SE, Woodin BR & Stegeman JJ 2007 Cytochrome P450 1 genes in early deuterostomes (tunicates and sea urchins) and vertebrates (chicken and frog): origin and diversification of the CYP1 gene family. *Molecular Biology and Evolution* **24** 2619–2631.
- Goldstone JV, Jonsson ME, Behrendt L, Woodin BR, Jenny MJ, Nelson DR & Stegeman JJ 2009 Cytochrome P450 1D1: a novel CYP1A-related gene that is not transcriptionally activated by PCB126 or TCDD. *Archives of Biochemistry and Biophysics* **482** 7–16.
- Hahn ME, Lamb TM, Schultz ME, Smolowitz RM & Stegeman JJ 1993 Cytochrome-P4501a induction and inhibition by 3,3',4,4'-tetrachlorobiphenyl in an Ah receptor-containing fish hepatoma-cell line (Plhc-1). *Aquatic Toxicology* **26** 185–208.
- Hegelund T, Ottosson K, Radinger M, Tomberg P & Celander MC 2004 Effects of the antifungal imidazole ketoconazole on CYP1A and CYP3A in rainbow trout and killifish. *Environmental Toxicology and Chemistry/SETAC* **23** 1326–1334.
- Jansson I, Stoilov I, Sarfarazi M & Schenkman JB 2000 Enhanced expression of CYP1B1 in *Escherichia coli*. *Toxicology* **144** 211–219.
- Jonsson ME, Orrego R, Woodin BR, Goldstone JV & Stegeman JJ 2007 Basal and 3,3',4,4',5-pentachlorobiphenyl-induced expression of cytochrome P450 1A, 1B and 1C genes in zebrafish. *Toxicology and Applied Pharmacology* **221** 29–41.
- Kashiwada S, Hinton DE & Kullman SW 2005 Functional characterization of medaka CYP3A38 and CYP3A40: kinetics and catalysis by expression in a recombinant baculovirus system. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology* **141** 338–348.
- Lake BG, Price RJ, Giddings AM & Walters DG 2009 *In vitro* assays for induction of drug metabolism. *Methods in Molecular Biology* **481** 47–58.
- Lee AJ, Kosh JW, Conney AH & Zhu BT 2001 Characterization of the NADPH-dependent metabolism of 17beta-estradiol to multiple metabolites by human liver microsomes and selectively expressed human cytochrome P450 3A4 and 3A5. *Journal of Pharmacology and Experimental Therapeutics* **298** 420–432.
- Lee AJ, Cai MX, Thomas PE, Conney AH & Zhu BT 2003 Characterization of the oxidative metabolites of 17beta-estradiol and estrone formed by 15 selectively expressed human cytochrome p450 isoforms. *Endocrinology* **144** 3382–3398.
- Maruyama M, Matsunaga T, Harada E & Ohmori S 2007 Comparison of basal gene expression and induction of CYP3As in HepG2 and human fetal liver cells. *Biological and Pharmaceutical Bulletin* **30** 2091–2097.
- Massey V 1959 The microestimation of succinate and the extinction coefficient of cytochrome *c*. *Biochimica et Biophysica Acta* **34** 255–256.
- McKay JA, Melvin WT, Ah-See AK, Ewen SW, Greenlee WF, Marcus CB, Burke MD & Murray GI 1995 Expression of cytochrome P450 CYP1B1 in breast cancer. *FEBS Letters* **374** 270–272.
- Muchmore DC, McIntosh LP, Russell CB, Anderson DE & Dahlquist FW 1989 Expression and nitrogen-15 labeling of proteins for proton and nitrogen-15 nuclear magnetic resonance. *Methods in Enzymology* **177** 44–73.
- Murray GI, Taylor MC, McFadyen MC, McKay JA, Greenlee WF, Burke MD & Melvin WT 1997 Tumor-specific expression of cytochrome P450 CYP1B1. *Cancer Research* **57** 3026–3031.
- Murray GI, Melvin WT, Greenlee WF & Burke MD 2001 Regulation, function, and tissue-specific expression of cytochrome P450 CYP1B1. *Annual Review of Pharmacology and Toxicology* **41** 297–316.
- Omura T & Sato R 1964 The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties. *Journal of Biological Chemistry* **239** 2379–2385.
- Pritchard MP, Ossetian R, Li DN, Henderson CJ, Burchell B, Wolf CR & Friedberg T 1997 A general strategy for the expression of recombinant human cytochrome P450s in *Escherichia coli* using bacterial signal peptides: expression of CYP3A4, CYP2A6, and CYP2E1. *Archives of Biochemistry and Biophysics* **345** 342–354.
- Pritchard MP, McLaughlin L & Friedberg T 2006 Establishment of functional human cytochrome P450 monooxygenase systems in *Escherichia coli*. *Methods in Molecular Biology* **320** 19–29.
- Qiu H, Taudien S, Herlyn H, Schmitz J, Zhou Y, Chen G, Roberto R, Rocchi M, Platzer M & Wojnowski L 2008 CYP3 phylogenomics: evidence for positive selection of CYP3A4 and CYP3A7. *Pharmacogenetics and Genomics* **18** 53–66.
- Rogan EG, Badawi AF, Devanesan PD, Meza JL, Edney JA, West WW, Higginbotham SM & Cavalieri EL 2003 Relative imbalances in estrogen metabolism and conjugation in breast tissue of women with carcinoma: potential biomarkers of susceptibility to cancer. *Carcinogenesis* **24** 697–702.
- Shimada T, Yamakazi H, Foroozesh M, Hopkins NE, Alworth WL & Guengerich FP 1998 Selectivity of polycyclic inhibitors for human cytochrome P450s 1A1, 1A2, and 1B1. *Chemical Research in Toxicology* **11** 1048–1056.
- Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ & Klenk DC 1985 Measurement of protein using bicinchoninic acid. *Analytical Biochemistry* **150** 76–85.
- Snowberger EA & Stegeman JJ 1987 Patterns and regulation of estradiol metabolism by hepatic microsomes from two species of marine teleosts. *General and Comparative Endocrinology* **66** 256–265.
- Spink DC, Lincoln DW II, Dickerman HW & Gierthy JF 1990 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes an extensive alteration of 17 beta-estradiol metabolism in MCF-7 breast tumor cells. *PNAS* **87** 6917–6921.
- Stegeman JJ, Binder RL & Orren A 1979 Hepatic and extrahepatic microsomal electron transport components and mixed-function oxygenases in the marine fish *Stenotomus versicolor*. *Biochemical Pharmacology* **28** 3431–3439.
- Stein JE, Hom T, Sanborn HR & Varanasi U 1991 Effects of exposure to a contaminated-sediment extract on the metabolism and disposition of 17-beta-estradiol in English sole (*Parophrys-Vetulus*). *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology* **99** 231–240.
- Teles M, Pacheco M & Santos MA 2006 Biotransformation, stress and genotoxic effects of 17beta-estradiol in juvenile sea bass (*Dicentrarchus labrax* L.). *Environment International* **32** 470–477.
- Thomas JH 2007 Rapid birth-death evolution specific to xenobiotic cytochrome P450 genes in vertebrates. *PLoS Genetics* **3** e67.
- Tseng HP, Hsue TH, Buhler DR, Wang WD & Hu CH 2005 Constitutive and xenobiotics-induced expression of a novel CYP3A gene from zebrafish larva. *Toxicology and Applied Pharmacology* **205** 247–258.
- Tsuchiya Y, Nakajima M & Yokoi T 2005 Cytochrome P450-mediated metabolism of estrogens and its regulation in human. *Cancer Letters* **227** 115–124.
- Uno T, Okamoto S, Masuda S, Imaishi H, Nakamura M, Kanamaru K, Yamagata H, El-Kady MA, Kaminishi Y & Itakura T 2008 Bioconversion by functional P450 1A9 and P450 1C1 of *Anguilla japonica*. *Comparative Biochemistry and Physiology. Part C, Pharmacology, Toxicology & Endocrinology* **147** 278–285.
- Waterman MR, Jenkins CM & Pikuleva I 1995 Genetically engineered bacterial cells and applications. *Toxicology Letters* **82–83** 807–813.
- Zhu BT & Conney AH 1998 Functional role of estrogen metabolism in target cells: review and perspectives. *Carcinogenesis* **19** 1–27.

Received in final form 3 May 2010

Accepted 3 June 2010

Made available online as an Accepted Preprint
3 June 2010