XENOBIOTIC METABOLISM AND ZEBRAFISH (*DANIO RERIO*) LARVAE

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Abstract

There is a requirement for the characterisation of the metabolism of xenobiotics in zebrafish larvae, due to the application of this organism to toxicity testing and ecotoxicology. Genes similar to mammalian cytochrome P450 (CYP) 1A1, CYP2B6, CYP3A5 and UDP-glucuronosyl-transferase (UGT) 1A1 were demonstrated to be expressed during normal embryonic development, with increased expression post hatching in Wik strain zebrafish larvae (72 hours post fertilisation, hpf). Activities towards ethoxy-resorufin, 7-ethoxy-coumarin and octyloxymethylresorufin, using an *in vivo* larval assay, were also detected in 96 hpf Wik strain zebrafish larvae, indicative of oxidative and conjugative metabolism. The expression of the identified genes was modulated upon exposure to Aroclor 1254, and the metabolic activities towards ethoxy-resorufin, 7-ethoxy-coumarin and octyloxymethylresorufin were observed to be inducible by exposure to *in vitro* inhibitors of CYP activities. Wik strain zebrafish larvae (72 hpf) were also demonstrated to metabolise the pharmaceuticals acetaminophen and ibuprofen by oxidative and conjugative processes using liquid chromatography mass spectrometry and scintillation counting. Finally, the organic solvents dimethyl-sulfoxide and methanol were observed to reduce the expression of CYP and UGT genes, and the metabolism of ethoxy-resorufin, after 24 hours exposure to ≤0.1% volume/volume concentrations of the two solvents. It is suggested that these inhibitory effects are in part due to a reduction in the expression of the aryl hydrocarbon receptors, which are known regulators of drug metabolism genes. Overall the expression of genes and enzymatic activities similar to the mammalian drug metabolism genes have been demonstrated in Wik strain zebrafish larvae (96 hpf), and the first examples of the metabolism of pharmaceuticals by zebrafish larvae are also demonstrated. The modulation of the metabolism of xenobiotics by organic solvents suggests that caution must be exercised when interpreting data from toxicity tests when high solvent concentrations are applied to zebrafish larvae. The continued use of zebrafish larvae as a toxicity testing model is strengthened by the findings of this work.

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List of Abbreviations

ABC	Adenosine triphosphate binding cassette
AhR	Aryl hydrocarbon receptor
AhRr	Aryl hydrocarbon receptor repressors
AIP	AhR interacting protein
ARNT	AhR nuclear translocator protein
β-NF	Beta-naphthoflavone
Bq	Bequerels
CAR	Constitutively activated receptor
cDNA	Copy deoxyribonucleic acid
CDNB	1-chloro-2,4-dinitrobenzene
CYP	Cytochromes P450
C_t	Cycle threshold
DHEA	Dehydroepiandrosterone
DMF	Dimethyl-formamide
DMSO	Dimethyl sulfoxide
dpf	Days post fertilisation
dpm	Disintegrations per minute
E. coli	*Escherichia coli*
ECOD	Ethoxy-coumarin-*O*-deethylase
EF1α	Elongation factor 1 alpha
EROD	Ethoxy-resorufin-*O*-deethylase
ESI	Electrospray ionisation
FMO	Flavin-containing mono-oxygenases
GFP	Green fluorescent protein

GSH	Glutathione
GST	Glutathione-*S*-transferase
hpf	Hours post fertilisation
HPLC	High performance liquid chromatography
Kv	Kilo volts
LC	Liquid chromatography
LC-MS(MS)	Liquid chromatography-mass spectrometry with ion fragmentation
MDR1	Multidrug resistance protein 1
mRNA	Messenger ribonucleic acid
ml	Millilitres
MS	Mass spectrometry
MSMS	Ion mass transition
MS^n	Multiple levels of ion fragmentation
m/z	mass to charge ratio
NADPH	Nicotinamide adenine dinucleotide phosphate
NAT	N-acetyl transferase
NMR	Nuclear magnetic resonance imaging
NPBQI	N-acetyl-p-benzoquinoneimine
NNAL	Nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol
NRF2	NF-E2p45-related protein
NSAID	Non-steroidal anti-inflammatory drug
OATP	Organic anion transporter protein
OECD	Organisation for economic co-operation and development
OOMR	Octyloxymethylresorufin
PAH	Polyaromatic hydrocarbons
PAPS	5'-phosphoadenosine-3'-pyrophosphate
PCB	Polychlorinated biphenyls
PCR	Polymerase chain reaction

PGH_2	Prostaglandin H_2
PGI_2	Prostacyclin
PHAH	Polyhalogenated aromatic hydrocarbons
PhiP	2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine
PPAR	Peroxisome proliferator activated receptor
PROD	Pentoxyresorufin-*O*-dependentylase
PXR	Pregnane X receptor
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RPl13α	Ribosomal protein l13 alpha
RTPCR	Reverse transcriptase PCR
RXR	Retinoid X receptor
SEM	Standard error of the mean
SULT	Sulfotransferase
TCDD	2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin
UDP	Uridine diphosphate
UGT	UDP-glucuronosyl transferase

Chapter 1

General Introduction

1.0 General introduction

There is increasing interest in the use of zebrafish *(Danio rerio)* larvae as a model organism for toxicity testing, ecotoxicology screening and disease modelling, with a particular focus on drug screening assays (Berghmans et al 2008, Richards et al 2008, Winter et al 2008). The importance of metabolism upon the toxicity and pharmacology of chemicals is well documented (see below), and it is important that the extent to which such processes occur in zebrafish larvae is assessed if these organisms are to be used effectively for chemical screening. The detection of pharmaceutical residues in the aquatic environment has also resulted in an interest in the fate of these chemicals in aquatic organisms such as fish (Dorne et al 2007). The potential for the metabolism of xenobiotics has not been fully characterised in zebrafish, and with the application of zebrafish to ecotoxicology and toxicity testing, it is essential that these processes are understood. This project focuses upon the characterisation of the potential for the metabolism of xenobiotics by zebrafish larvae, in relation to the application of these fish in drug screening and ecotoxicology testing. As an introduction, this chapter reviews the principles of drug metabolism, summarising the expression of relevant genes with examples of substrates for the different enzymatic systems involved in the metabolism of xenobiotics, and comparisons between different mammalian and fish species are drawn, with a focus upon zebrafish.

1.1.1 Drug metabolism

There are several different processes that can occur to metabolise a compound, including oxidation, reduction and conjugation reactions. Generally, oxidative and reductive processes expose functional moieties on a compound (known as phase I metabolism), which can then undergo conjugation (known as phase II metabolism) with sugars (e.g. glucuronic acid), a

peptide (glutathione), amino acids (e.g. glycine) or other moieties (e.g. sulfonate) resulting in a more readily excreted product (Nebbia 2001). If an appropriate functional group is already present in a molecule then conjugation reactions can occur without prior phase I metabolism. For example, acetaminophen can be directly conjugated to both sulfate or glucuronic acid moieties without any prior oxidation due to the presence of a functional hydroxyl group, whereas benzene requires oxidative hydroxylation via an epoxide intermediate (resulting in the formation of a functional group) before it can undergo conjugation (see Figure 1.1, Snyder and Hedli 1996, Pirmohamed 2008). Generally, the exposure of a functional group (through phase I metabolism) is required before subsequent conjugation reactions (phase II metabolism) followed by excretion, which can be mediated by drug transporter proteins such as P-glycoprotein, multi-drug resistance protein and organic anion transporter proteins (Xu et al 2005). These transporter proteins play a critical role in the influx, efflux and distribution of xenobiotic and endobiotic compounds in many tissues including the brain, kidney, liver and intestine in humans (Xu et al 2005). There is a variety of enzymatic systems that catalyse the oxidation, reduction and conjugation processes involved in drug metabolism, and these have been extensively characterised and reviewed for mammalian species and are discussed below (Hayes and Pulford 1995, Guengerich 2001, Gamage et al 2001, Guillemette 2003, Hayes et al 2005, Guengerich 2006, Brown et al 2008, Testa and Krämer 2008).

The toxicity of xenobiotics can be modulated by metabolic processes such as oxidation and conjugation reactions. Thus there is the potential for the conversion of a toxic compound into a non-toxic derivative and *vice versa*. It is therefore clear that the metabolism of xenobiotics is of great importance in terms of the safety assessment of drug candidate molecules, and in the environmental fate of xenobiotics, and the effects of such compounds upon non-human species including fish and aquatic invertebrates. The processes of xenobiotic metabolism are

aimed at converting a compound into a more readily excreted, and usually less toxic (although this is not always the case), form so that the compound can be cleared from the body.

1.1.2 Cytochromes P450 and drug metabolism

The cytochromes P450 (CYP) are a group of heme-containing (heme-thiolate) enzymes present in a wide variety of species, ranging from plants and animals to micro-organisms (Anzenbacher and Anzenbacherova 2001). The CYP enzymes have been implicated in a wide variety of processes such as pigment synthesis, defence against chemical insult and the synthesis of steroids, prostaglandins and retinoic acid (Nebert and Dalton 2006). There have been 57 CYP genes identified in humans to date, with similar genes grouped into families and sub-families based on amino acid sequence similarity derived from the gene sequence (>40% for the same family and >55% for the same sub-family, Brown et al 2008).

There are a wide variety of reactions that can be catalysed by CYP enzymes, ranging from mono-oxidation of compounds to the dehydration, dehydrogenation, isomerisation and reduction of substrates (Mansuy 1998). Mono-oxidation reactions involve the insertion of an oxygen atom into the carbon-hydrogen (C-H) bond resulting in the hydroxylation of the substrate (Mansuy 1998). Depending on the nature of the substrate this type of reaction can result in oxidative de-alkylations (the insertion of oxygen at a C-H bond next to a heteroatom such as oxygen, nitrogen or sulfur can result in the cleavage of the heteroatom bond and de-alkylation, Mansuy 1998). The hydroxylation of amines (primary and secondary), carbon double bonds, aromatic rings and lone pairs of heteroatoms can also be catalysed by CYP enzymes resulting in the formation species such as epoxides, arene oxides, *N*-oxides and

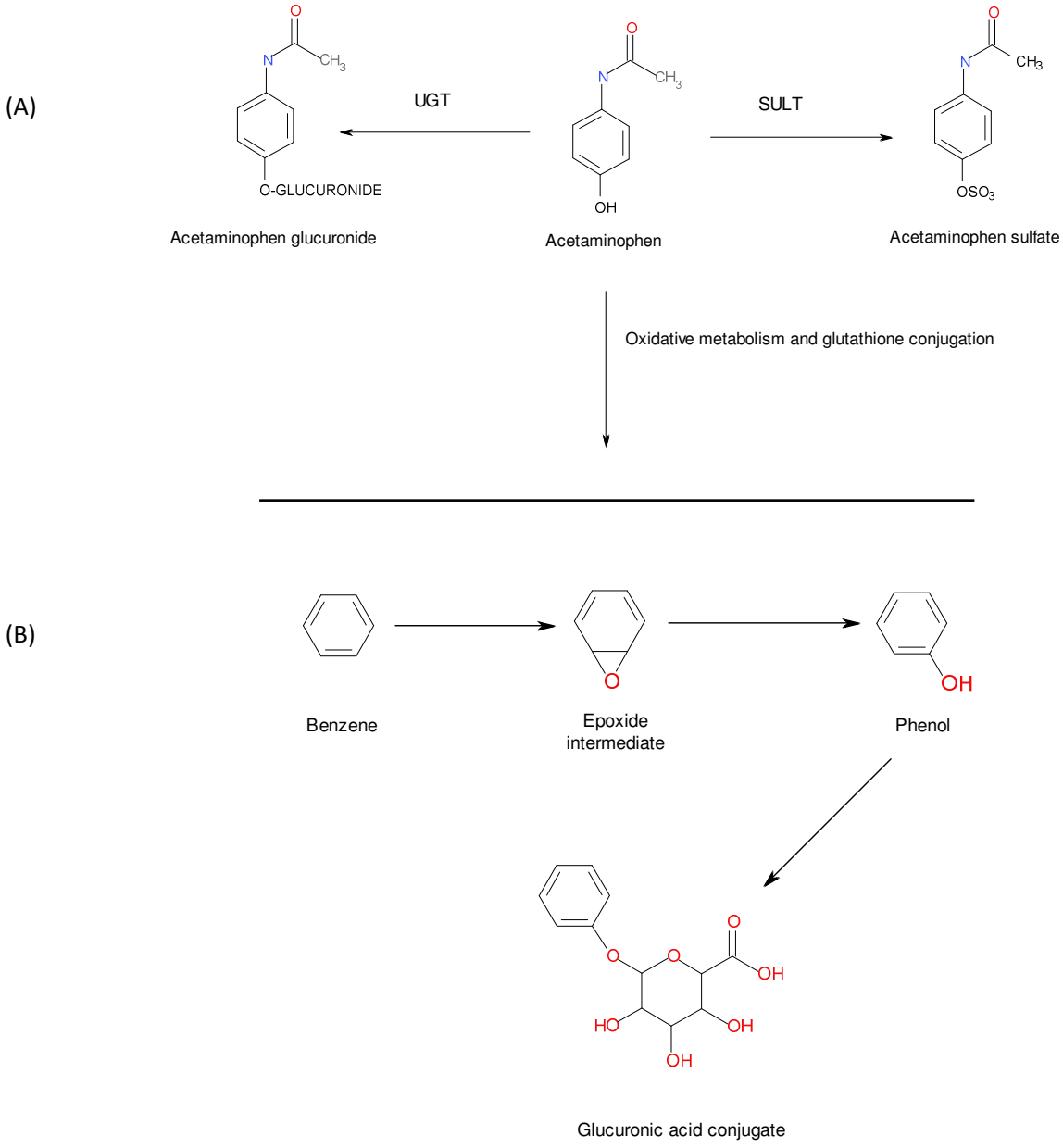


Figure 1.1: The metabolism of acetaminophen (A) and a simplified schematic of benzene metabolism (B). Acetaminophen can undergo conjugation with glucuronic acid or sulfate moieties without prior activation by oxidative metabolism, whereas benzene requires oxidation to phenol (via an epoxide intermediate) before it can undergo conjugation reactions. SULT (Sulfotransferases) and UGT (UDP-glucuronosyl transferases)

sulfoxides, depending on the substrate (Mansuy 1998). Other oxidation reactions catalysed by CYP enzymes include peroxidase-like activities and dehydrogenation reactions (formation of carbon double bonds from CH-CH in substrates such as testosterone, Mansuy 1998).

A generalised reaction mechanism for CYP-catalysed oxidation reactions is shown in Figure 1.2, adapted from Guengerich 2001. Briefly, this mechanism involves the binding of the substrate (1), followed by the reduction of the iron centre (with the electron donated by NADPH-P450-reductase, 2) and binding of molecular oxygen (3). The complex formed by the binding of oxygen is very unstable and can result in the production of superoxide anions (Guengerich 2001). A second electron, donated either by cytochrome B_5 or NADPH-P450-reductase (4), further reduces the iron-oxygen complex, and this is followed by the addition of a proton and the cleavage of the oxygen-oxygen (O-O) bond, releasing water (5 and 6 respectively). Finally, the resulting electron-deficient complex abstracts either a hydrogen or an electron from the substrate (7), and the resulting intermediate collapses, producing the oxidised substrate and regenerates the iron centre (8 and 9).

There are also several non-oxidation reactions catalysed by CYP enzymes. Prostaglandin synthesis involves the isomerisation of prostaglandin H_2 (PGH_2) to form prostacyclin (PGI_2) or thromboxane, reactions that are catalysed by two CYP enzymes (prostacyclin synthase and thromboxane synthase, Mansuy 1998). The reduction of polyhalogenated substrates, nitoraromatic compounds, tertiary amines and arene oxides has been well reported (Mansuy 1998). These reduction reactions occur under anaerobic conditions, as the P450 is required to be in a reduced state in order to catalyse these reactions (Mansuy 1998).

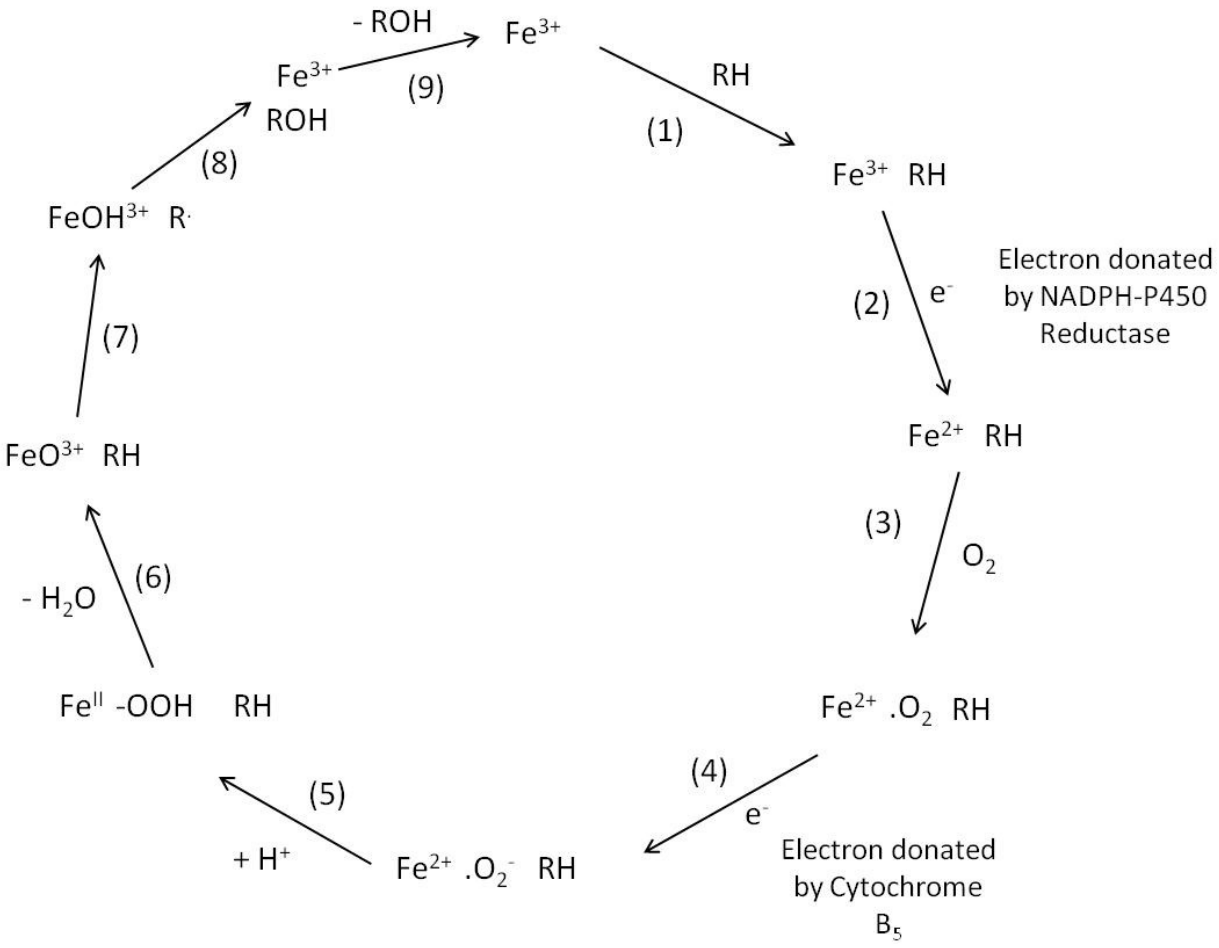


Figure 1.2: The proposed catalytic cycle of cytochromes P450 for oxidation reactions (Adapted from Guengerich 2001). The enzyme substrate is designated RH and the product is designated ROH. The iron atom in the heme of the cytochrome P450 is designated Fe.

The metabolism of the majority of xenobiotics and foreign compounds is catalysed by CYP family 1, 2 and 3 isoforms. Although there is considerable overlap of specificity towards substrates between different families and individual isoforms, each family tends to have a (if broad) specificity towards substrates of certain physiochemical properties (Pelkonen et al 2008). The potential for a substrate to be metabolised by multiple CYP isoforms is of great mechanistic importance when considering the potential for drug-drug interactions (where multiple compounds requiring metabolism by the same enzyme isoform compete for enzymatic metabolism, or where one compound modulates the activity/expression of an isoform that metabolises another compound), as a compound metabolised by a single isoform will be considerably more susceptible to these kinds of interactions than a compound metabolised by multiple enzymes (Pelkonen et al 2008).

The CYP1 family are responsible for the metabolism of polyaromatic hydrocarbons (PAH), aromatic amines and some pharmaceuticals such as acetaminophen and caffeine (Brown et al 2008). There are currently 3 identified members of CYP1 enzymes, CYP1A1, CYP1A2 and CYP1B1. The CYP1A1 isoform has been demonstrated to be expressed in a variety of different tissues but frequently is only detected after induction of this gene by ligands of the aryl hydrocarbon receptor (AhR), whereas CYP1A2 is expressed mainly in the liver of humans at high levels and has been shown to be inducible by AhR ligands (Omiescinski et al 1999). It has been reported that CYP1B1 is expressed in a variety of extrahepatic human tissues, such as kidney, liver, brain, breast and lung, albeit at a very low level (Murray et al 2001). Compounds such as PAH and polyhalogenated aromatic hydrocarbons (PHAH, such as polychlorinated biphenyls and dioxin-like compounds) are generally metabolised by CYP1A1 and CYP1B1, whereas the metabolism of aromatic amines is catalysed largely by CYP1A2 (Nebert and Dalton 2006). The metabolism of these compounds can result in the

formation of reactive epoxides or quinone derivatives, and such metabolites have been implicated in carcinogenesis (Shimada and Guengerich 2006). The *N*-hydroxylation of 2-amino-1-methyl-6-phenylimidazo [4,5-b] pyridine (PhIP), a reaction catalysed by CYP1A2, followed by sulfation or acetylation has been demonstrated to result in a carcinogenic species that can modify guanine bases in DNA (Malfatti et al 2005, Metry et al 2007). Indeed, the importance of CYP1A2 in the bioactivation of PhIP has been demonstrated *in vivo* using CYP1A2 null mice (Kim and Guengerich 2005). The CYP1 family enzyme CYP1B1 has also been reported to have a role in the bioactivation of heterocyclic arylamines using recombinant CYP1B1 expressed in *Escherichia.coli* (*E. coli*, Shimada et al 2001). The CYP1A2 and CYP1B1 enzymes have been reported to metabolise oestrogen to its 2- and 4-hydroxy-derivatives, as well as being involved in the metabolism of xenobiotics, unlike CYP1A1 (Hasler et al 1999). For example, the anti-androgenic drug flutamide undergoes hydroxylation, catalyzed by CYP1A2, and both CYP1A2 and CYP1B1 have been demonstrated, using recombinant enzymes, to catalyze the 4-hydroxylation of tamoxifen (CYP1A2) and the isomerisation of trans-4-hydroxy-tamoxifen to its cis-derivative (CYP1B1, Crewe et al 2002). Other CYP1B1 substrates identified, using recombinant human CYP1B1, include 7-ethoxy-resorufin, bufuralol, theophylline and 7-ethoxy-coumarin (Shimada et al 1997). The 3-*N*-demethylation of caffeine to paraxanthine is considered as a marker activity for CYP1A2 in humans, whereas the 8-*C*-hydroxylation of caffeine identified as a CYP1A2 marker in rats (Kot and Daniel 2008). The 4-hydroxylation of oestrogen is used as a specific indicator of CYP1B1 activity (Shimada et al 1997).

The CYP2 family comprises of several important drug metabolising enzymes including CYP2B6, CYP2C8, CYP2C9, CYP2C19 and CYP2D6 (Guengerich 2006). The CYP2 family genes are expressed in a variety of human tissues including lung, kidney, small intestine and

brain (Nishimura et al 2003). However, the CYP2 genes are most highly expressed (based upon mRNA levels) in the liver, with CYP2E1 having the highest level of mRNA present in the liver out of all the CYP genes in humans (Nishimura et al 2003, Biéche et al 2007). There is no common pattern of regulation or specificity towards substrates for members of the CYP2 family, for example CYP2B6 metabolises non-planar compounds that are weakly basic or neutral whereas the CYP2C enzymes are reported to metabolise a wide range of drugs (up to 20% of all prescribed drugs) that contain at one or more aromatic ring moieties and are weakly acidic in nature (Totah and Rettie 2005, Brown et al 2008). The CYP2D6 isoform has been implicated in the metabolism of compounds that contain a basic nitrogen atom approximately 5 angstroms for sites of oxidation, and CYP2E1 substrates are small molecules that can be halogenated, hydrophobic or hydrophilic (Brown et al 2008). The CYP2D6 enzyme is of great clinical relevance, as it can metabolise compounds from a wide variety of different therapeutic classes, including: analgesics, anti-arrhythmics, anti-depressants, anti-hypertensives, anti-emetics, anti-histamines, anti-psychotics, beta-adrenergic blocking compounds as well as anti-diabetic compounds (Zanger et al 2004). Thus substrates of this family of enzymes span a wide variety of pharmaceutical groups (examples of CYP2 substrates are given in Table 1.1). The CYP2B6 and CYP2E1 isoforms have also been implicated in the activation of pro-carcinogens and toxic species such as diethylnitrosamine, and CYP2E1 is a well-described source of reactive oxygen species due to poor coupling of its redox cycle (Omiecinski et al 1999, Kang et al 2007, Miller 2008, Pelkoven et al 2008).

Table 1.1: Examples of substrates metabolised by CYP2 enzyme family.

Isoform	Example Substrates	Reference
CYP2B6	Bupropion, Diazepam, Efavirenz, Ketamine, Lidocaine, Methadone Midazolam, Tamoxifen	Ekins et al 2008
CYP2C8	Cerivastatin, Chloroquine, Diclofenac, Ibuprofen, Methadone, Morphine, Rosiglitazone, Warfarin	Tolah and Rettie 2005
CYP2C9	Celecoxib, Diclofenac, Ibuprofen, Phenytonin, Naproxen, Tamoxifen, Tolbutamide, Warfarin	Hersh and Moore 2004
CYP2C19	Chlopyrifos, Diazepam, Diclofenac, Fluoxetine, Omeprozole, *S*-mephenytoin, Tolbutamide, Warfarin	Wang et al 2004, Brown et al 2008
CYP2D6	Codeine, Debrisoquine, Propranolol, Nicergoline, Tamoxifen	Zagner et al 2004
CYP2E1	Acetaminophen, Acetone, Ethanol, Halogenated anesthetics, Benzene	Brown et al 2008

These compounds are from a wide variety of different chemical and pharmacological classes of compounds with a broad range of different physiochemical properties. It should be noted that this is not an exhaustive list of substrates for these enzymes, and that these examples have been selected to highlight the wide variety of therapeutic classes metabolised by the CYP2 enzymes.

The CYP3 family consists of 5 isoforms, CYP3A3, CYP3A4, CYP3A5, CYP3A7 and CYP3A43 in humans (McArthur et al 2003, Nelson 2009). The CYP3 isoforms are expressed in a range of human tissues including the liver, lung, kidney, intestine and brain, with the highest expression found in the liver, particularly the CYP3A4 isoform, and CYP3A7 is mainly expressed in foetal tissues with minimal expression in human adult tissues (Nishimura et al 2003). It has been reported by Williams et al (2002), using a variety of CYP3 substrates, that CYP3A5 generally has an equivalent or lower metabolic capacity than CYP3A4, with CYP3A7 having the lowest capacity of all the isoforms. The CYP3A5 isoform has been reported to only be expressed in a relatively small percentage of the Caucasian population (<25%), resulting in some dispute regarding the relative importance of this isoform to the metabolism of xenobiotics (Brown et al 2008). The CYP3A enzymes have broad substrate specificities, metabolising endogenous steroids, such as testosterone progesterone and oestradiol, as well as a wide range of pharmaceutical compounds (de Wildt et al 1999). These pharmaceuticals tend to be large molecular weight compounds from a diverse range of therapeutic classes including, diazepam (benzodiazepine), diclofenac (non-steroidal anti-inflammatory drug, NSAID), erythromycin (antibiotic) midazolam (benzodiazepine), nifedipine (anti-hypertensive) and tamoxifen (chemotherapeutic drug, de Wildt et al 1999, Brown et al 2008).

1.1.3 Glutathione-*S*-Transferases

The Glutathione-*S*-Transferase (GST) enzymes are a group of broad-specificity enzymes that catalyse the addition of the tripeptide glutathione (glutamate-cysteine-glycine, GSH) to a large variety of substrates including pharmaceuticals, endogenous metabolites and oxidised species that contain an electrophilic centre (Hayes and Pulford 1995). There are 3 different

types of GST enzymes, cytosolic, mitochondrial and microsomal (also known as membrane associated proteins in eicosanoid and glutathione – MAPEG, Hayes et al 2005). All of the members of these families metabolise the GST marker substrate 1-chloro-2,4-dinitrobenzene (CDNB, Hayes et al 2005). The GSTs are divided into the families alpha, mu, pi, sigma and theta, with classification based on predicted amino acid sequence identity (40% or greater for same family, although this is not a definitive classification and there can be some difficulty assigning some genes to a given family, Hayes and Pulford 1995). The cytosolic GST enzymes are divided into 5 alpha family enzymes, 5 mu family, 1 pi, 1 sigma, 2 theta, 1 zeta and 2 kappa isoforms (Hayes et al 2005), whereas there is only single identified mitochondrial GST identified in humans (a kappa family enzyme, Hayes et al 2005). Soluble GST proteins have been detected in a variety of human tissues, including brain, heart, kidney, liver, lung, pancreas and the small intestine (Sherratt et al 1997). Different isoforms within and between families have different tissue patterns of expression, with (for example) GST theta detected in all tissues, whereas GST alpha-1 is mainly found in kidney and liver and testis tissues (Sherratt et al 1997). Examples of GST substrates include pharmaceuticals such as acetaminophen (after oxidation by CYP2E1 and CYP3A4 to the reactive intermediate *N*-acetyl-p-benzoquinoneimine), cyclophosphamide (chemotherapeutic agent), chlorabucil (chemotherapeutic agent) and fosfomycin (antibiotic), as well as epoxide derivatives of xenobiotic compounds such as PAH and halogenated aliphatic compounds (Dirven et al 1994, Hayes et al 2005, Parker et al 2008, Pirmohamed 2008, Pushparajah et al 2008). As the electrophilic centre of GST substrates can be centred upon a carbon, nitrogen or sulfur atom there are a vast amount of substrates for these enzymes, and this promiscuity is facilitated by a hydrophobic substrate binding site that has a broad specificity for a wide range of compounds (Hayes and Pulford 1995). Although the majority of GSH-conjugates are less toxic and more readily excreted that the parent compound there are examples of

GSH-conjugation resulting in the production of toxic species as a result of unstable and reversible conjugates, resulting in cleavage back to the un-conjugated species, or the production of a GSH-conjugate that is more reactive than the parent compound (Hayes and Pulford 1995). Examples of compounds that undergo these processes are p-nitrophenol acetate, fluorodifen, benzyl, phenyl and allyl isothiocyanate and alkyldihalides (Hayes and Pulford 1995). In addition to the conjugation of GSH to electrophilic substrates GST enzymes can also catalyse the reduction of products of oxidative stress (such as DNA and phospholipid hydroperoxides, Hayes and Pulford 1995). The GST enzymes have also been shown to function as peroxidases and isomerases and thus are involved in phenylalanine degradation, testosterone and progesterone synthesis, and arachidonic acid metabolism (Hayes and Pulford 1995, Hayes et al 2006). It should be noted that glutathione conjugates can undergo further metabolism to form mercapturic acid derivatives (Hinchman et al 1991). This process involves the sequential removal of the glutamate and glycine residues (catalysed by γ-glutamyl-transpepidase and dipeptidases respectively) to form a cysteine-*S*-conjugate, which can then undergo acetylation catalysed by *N*-acetyl transferases (Hinchman et al 1991).

1.1.4 UDP-glucuronosyl transferases

The UDP-glucuronosyl-transferases (UGT) catalyse the addition of a glucuronic acid moiety, from the endogenous precursor UDP-glucuronic acid, to a wide range of different nucleophilic functional groups of endogenous and exogenous compounds, including hydroxyl, carboxylic acid, amines (primary, secondary and tertiary), sulfhydryl and ester moieties (Testa and Krämer 2008). The UGT enzymes are described as low affinity but high capacity systems, with high molecular weight products of glucuronidation (molecular weight >450) frequently

excreted in bile (Testa and Krämer 2008). There are 4 distinct families of UGT enzymes (1, 2, 3 and 8) currently known in humans, with family 1 comprising 10 isoforms (UGT1A1 – UGT1A10), family 2 divided into subfamilies UGT2A (UGT2A1 – UGT2A3) and UGT2B (UGT2B4, UGT2B7, UGT2B10, UGT2B11, UGT2B15, UGT2B17 and UGT2B18), UGT3 comprised of 2 isoforms (UGT3A1 and UGT3A2) and a single UGT8 enzyme (Testa and Krämer 2008). The UGT enzymes are found in a wide range of different organs in humans, especially the liver and intestine, but also the nasal mucosa, skin, lung, brain, kidneys, prostate, uterus, breast and placenta (Guillemette 2003). Due to the variety of chemical entities that can be conjugated by UGT isoforms, there is a wide range of environmental chemicals, pharmaceuticals, xenobiotics and endogenous compounds that can undergo conjugation to glucuronic acid, and thus UGT enzymes have been described as having broad and overlapping substrate specificities (Guillemette 2003). The benzodiazepine midazolam has been reported to undergo *N*-glucuronidation (with UGT1A4 implicated as the major isoforms responsible for catalysing this reaction), as well as *O*-glucuronidation of the hydroxy-derivatives of midazolam, and the β-blocker propanolol has been reported to undergo stereoselective *N*-glucuronidation by a variety of different UGT isoforms including UGT1A9, UGT2B4 and UGT2B7 (Hanioka et al 2008, Klieber et al 2008, Hyland et al 2009). Bisphenol A is conjugated to glucuronic acid using human liver microsomes, and *in vitro* experiments suggest that UGT2B15 is the major isoforms responsible for this conjugation (Hanioka et al 2008). Caricinogens such as the nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) can undergo *N*-glucuronidation, resulting in the formation of non-tumorigenic glucuronide derivatives (Chen et al 2008). Other examples of UGT substrates are morphine and acetaminophen (*O*-glucuronidation), malotliate (*S*-glucuronidation) and sulfinpyrazone (*C*-glucuronidation (Testa and Krämer 2008). Endogenous substrates of the UGT enzymes include steroid hormones (e.g. testosterone, androsterone and β-oestradiol), bile acids (e.g.

lithocholic acid, deoxycholic acid and chenodeoxycholic acid) and billirubin (Testa and Krämer 2008).

1.1.5 Sulfotransferases

The conjugation of xenobiotics and endogenous compounds to sulfonate moieties, derived from the endogenous sulphate donor 5'-phosphoadenosine-3'-phosphosulphate (PAPS) is catalysed by a group of enzymes termed Sulfotransferases (SULT), and generally functions to yield a more readily-excreted product, although the production of electrophilic (and thus chemically-reactive) species have been reported (Glatt 2000, Glatt et al 2001, Gamage et al 2005). The conjugation of a substrate to a sulfonate moiety occurs at nucleophilic atoms, such as oxygen, nitrogen or sulphur, and it is due to this that the term sulfonation, rather than sulfation (the process of the conjugation of a sulfonate moiety to an oxygen atom), is used for this process. The formation of *O*-sulfate derivatives of compounds such as 4-aminoazobenzene has been reported to result in the formation of carcinogenic species (Glatt 2000). As with the previously discussed CYP, GST and UGT genes and enzymes, members of the SULT superfamily are classified into distinct families and subfamilies, based upon percentage identity of the predicted amino acid sequence. There are currently 3 families of SULT (family 1, family 2 and family 4), and each family, broadly speaking, has different substrate specificities (Glatt et al 2001). The SULT1 family isoforms are known as phenolic SULTs, and metabolise a broad range of substrates including xenobiotics (e.g. acetaminophen, 1-naphthol, 4-nitrophenol, 4-hydroxy-tamoxifen and vanillin and naproxen derivatives) and endogenous compounds such as neurotransmitters (e.g. dopamine), whereas SULT2 family members are also known as hydroxy-steroid metabolising SULTs, with substrates including testosterone, dehydroepiandrosterone, 17-ethinyl-oestradiol and various bile acids (Glatt et al 2001, Falany et al 2005). It should be noted that the SULT2

isoforms can also metabolise some pharmaceuticals, for example 4-hydroxy-tamoxifen is a substrate for several SULT1 isoforms as well as for SULT2A1 (Falany et al 2006). There are currently no identified substrates of the SULT4 family enzymes (Gamage et al 2005). The SULT1A isoforms are found in a variety of different tissues, with SULT1A detected in the liver (high levels of SULT1A1), brain, breast, intestine (high levels of SULT1A3 detected), endometrium, adrenal gland, platelets and the lungs (both SULT1A1 and SULT1A3), whereas SULT1A2 protein has not been detected in any tissues screened despite the detection of corresponding mRNA (Gamage et al 2005). Other SULT1 enzymes (SULT1B, SULT1C and SULT1E) have been detected in several tissues such as intestine, colon, stomach, kidney and thyroids and liver, and these enzymes have been implicated in thyroid metabolism (SULT1B1), oestrogen metabolism (SULT1E1) or have unknown physiological roles (Gamage et al 2005). The SULT2 genes are localised in hormone-responsive tissues, in keeping with the role of these enzymes in the metabolism of hydroxy-steroids, although the different isoforms do have slightly different patterns of tissue expression (Gamage et al 2005).

1.1.6 Other xenobiotic-metabolism systems

Although the systems described above can be thought of as the “major” pathways for the metabolism of xenobiotics, there are other potential metabolic pathways present, and some of these “minor” pathways can be, for certain substrates, a significant route of metabolism. In addition to CYP-dependent oxidation of substrates, the flavin mono-oxygenases (FMO) can also catalyse the *N*- and *S*-oxygenation of a variety of xenobiotics (Krueger and Williams 2005). The FMO differ from CYP enzymes in that they do not contain a heme moiety (Kreuger and Williams 2005). Alcohol dehydrogenases, aldehyde dehydrogenases and

epoxide hydrolases have also been reported to function in the metabolism of xenobiotics (Lash 1994). Additional conjugative systems catalysing the acetylation (*N*-acetyl-transferases, NAT), methylation (methyl-transferases) and conjugation with amino acids, such as glycine, are also present (Lohr et al 1998).

1.1.7 Polymorphisms and drug metabolism

The metabolism of endogenous and xenobiotic compounds (as described above) can occur by a variety of different pathways. The tissue localisation, the broad and overlapping substrate specificity of different isoforms and systems, the concentration of substrate, and status of the organism or tissue (in terms of the levels and availability of co-factors such as PAPS or UDP-glucuronic acid) all determine which metabolic pathways occur for a particular substrate. The above systems are also inducible, and can be regulated at the transcriptional, translational and post-translational levels by a wide variety of xenobiotics, which can result in a substrate increasing the rate of its own clearance by inducing the enzyme that metabolises it (Xu et al 2005). The induction of genes involved in xenobiotic metabolism is mediated via nuclear receptors such as the Aryl hydrocarbon receptor (AhR), Pregnane X receptor (PXR) and Constitutive activated receptor (CAR), and is discussed further in Chapter 6 (Xu et al 2005). A further complicating factor in the metabolism of endogenous and exogenous compounds is that a large proportion of the enzymes involved in these pathways are polymorphic, which results in alternative forms of certain isoforms, resulting in alterations in the metabolic capabilities of different enzymes (Božina et al 2009). There have been polymorphic variants identified of a wide variety of drug metabolism enzymes including CYP, UGT, SULT, GST and NAT isoforms, and due to the interactions between these different systems, there is substantial inter-individual variation in terms of the metabolism of a wide

variety of xenobiotic and endogenous compounds (Labuda et al 1999, van der Weide and Steijns 1999, Coughtrie 2002, Ehmer et al 2004, Sparks et al 2004, Blevins-Primeau et al 2009). The most clinically relevant examples of the affects of these polymorphic systems are CYP2D6 and NAT (Božina et al 2009). The use of xenobiotics such as debrisoquine, dextromethorphan and sparteine for *in vivo* phenotyping of CYP2D6 has allowed to identification of ultra-rapid, extensive, intermediate and poor metaboliser phenotypes by determining the ratio of urinary metabolite and unchanged compound over a given time (Zanger et al 2004). The identification of different variants of CYP2D6 that encode for a range of non-functional proteins (mainly premature terminations, but not exclusively, of the coding sequence), functional proteins that are somewhat compromised in enzyme activity, and altered coding sequences that result in excess production of the protein have been reported (Zanger et al 2004). The existence of these different polymorphisms of the drug metabolism systems enzymes, in addition to the induction and inhibition of gene expression and enzyme activities by xenobiotics, contributes to the potential for a variety of different responses to xenobiotic exposures within a population.

1.2 Zebrafish as a model organism

Zebrafish are a well characterised model vertebrate in the fields of molecular genetics and developmental biology (Kimmel et al 1995). Zebrafish embryos and larvae have been used to investigate molecular processes including angiogenesis, lipid metabolism, cardiac function and homeostasis (Langheinrich 2003). A model of steatohepatitis in zebrafish treated with thioacetamide has also been reported, and the zebrafish has emerged as an alternative model organism in the field of Parkinsons' Disease research and other neurodegenerative disorders (Amali et al 2006, Best and Alderton 2008, Flinn et al 2008, Flinn et al 2009).

Zebrafish are also being applied to modelling of the immune system (as they have both adaptive and innate immune systems) and to the field of oncology (Stern and Zon 2003, Berghmans et al 2005, Ceol et al 2008, Meeker and Trede 2008, Payne and Look 2009). The zebrafish is also becoming a popular model organism for toxicological screening, and zebrafish are a standard ecotoxicology testing species recommended by the Organisation for Economic Co-operation and Development (OECD) for fish toxicity testing as part of environmental risk assessments and hazard classification (Langheinrich 2003, OECD 2006). There have been a number of studies assessing a variety of different compounds for developmental toxicity including the widespread contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which has not only been characterized for its effects upon the morphology and physiology of zebrafish embryos throughout development, but has also undergone extensive investigations into the molecular mechanisms of this toxicity (Carney et al 2006). The advantages of zebrafish are listed in Table 1.2, with a particular focus upon zebrafish larvae. Adult zebrafish are also a useful model organism, especially now with the availability of the *casper* strain of zebrafish, which is a transparent adult fish, allowing *in vivo* imaging of fluorescent labels within the adult fish (White et al 2008). Zebrafish larvae (>3 days post fertilization, dpf) are well-suited to drug discovery and toxicity testing assays, as these fish are hatched from the chorion, small in size, and the majority of major organs, such as liver, gills, heart, digestive system and locomotor responses have partially or fully formed, suggesting that the functions of these organs are in place (Kimmel et al 1995).

Table 1.2: The advantages of zebrafish embryos and larvae as a model organism

Characteristics	Advantages and applications
Rapid generation of large numbers of embryos	100-200 embryos per female can be produced in a single spawning per week, allowing enough embryos for medium to high throughput screens.
Transparent embryos and larvae	Allows observation of organ development and morphology. Fluorescent tags (e.g. green fluorescent protein (GFP)), dyes and compounds can also be observed *in vivo*.
Similarities in anatomy and physiology	Vertebrate body plan and similarities in nervous, renal, cardiovascular, respiratory, gastrointestinal, endocrine, immune and reproductive systems to mammalian species.
Similarities in sequence identity at protein level and pharmacology	Sequence similarity approaching 100% in conserved regions of some proteins. High level of functional conservation.
Embryo and larval size	Embryos and larvae ($\leq$ 5 dpf) can be cultured in 96-well plates and in as little as 50 µl of water. Applications to high throughput screening and reduces amounts of test compounds required thus reducing costs
Husbandry costs	Substantially lower than that for rodents. Reported to be 1/100 to 1/1000th of that for mice
Genetic toolbox available	Widespread use of transient gene knock-down and genetically-modified fish (e.g. expressing GFP), sequenced genome which is becoming increasingly annotated. Microarrays commercially available.
Absorption of oxygen and compounds from water across the skin at early life-stages	Zebrafish can survive for several days without a functioning circulatory system. Compounds can be administered directly into the water allowing easy exposure of embryos and larvae to compounds

Information collated from: Langheirich 2003, Goldsmith 2004, Lieschke and Currie 2007.

As a result of these characteristics of zebrafish larvae, there has been increasing interest in the application of these organisms as a pre-clinical testing species, with a view to functioning as an *in vivo* screen to reduce the number of compounds reaching mammalian testing and therefore drug development costs (McGrath and Li 2008). The application of zebrafish larvae to the assessment of the effects of compounds upon cardiac output, locomotor activity, convulsant liability, gut motility, and the impairment of visual and auditory functions has been proposed, and some validation of these assays has been reported (Barros et al 2008, Berghmans et al 2008, Richards et al 2008, Winter et al 2008). The assessment of the effect of 35 compounds upon visual response, assessed by the optomotor response (monitoring the swimming behaviour of zebrafish larvae in response to moving black and white stripes below the tank), reported 71% correct classification of compounds using data from *in vitro* and *in vivo* clinical models of retinal toxicity for comparison (Berghmans et al 2008, Richards et al 2008). A zebrafish assay to determine the seizure liability of compounds (by monitoring the swimming patterns of larval zebrafish) has been validated using a set of 25 compounds, with the correct classification (compared to *in vitro* and *in vivo* clinical models) of 72% of compounds reported (Winter et al 2008). The validation of assays to assess cardiac toxicity, by measuring atrial and ventricular rates (78% correct classification using a set of 9 compounds) and perturbation of gut motility, by measuring the frequency of gut contractions (correct classification of 60% using a set of 10 compounds) has also begun (Berghmans et al 2008). Although further validation of these types of assays is required, due to the low number of compounds used, the potential benefits of using such systems as pre-clinical *in vivo* toxicity screens is illustrated by these examples.

1.3 Comparison of drug metabolism genes and activities between mammals and fish

Xenobiotic metabolism has substantial implications for disease modelling, ecotoxicology, toxicity testing and drug screening, due to the potential for the metabolic activation and deactivation of compounds. All of these testing strategies utilise both *in vitro* (e.g. microsomal fractions from tissues, cultured cells or over-expressed proteins in yeast and bacteria) and *in vivo* systems (e.g. rodents species including mouse and rat, non-rodent mammals such as dogs, and environmentally-relevant species including fish, freshwater and marine invertebrates, and molluscs). The comparison of drug metabolism systems between these model species and humans is a complex process, with substantial problems identifying direct homologues of human enzymes in non-mammalian (and indeed non-human) species due to the use of amino acid sequence identity as a determinant of similarity, rather than metabolic activity towards characteristic substrates. This is illustrated in Appendix 1, by the alignments of the CYP1A1 amino acid sequence with the predicted zebrafish CYP1A sequence, and the alignment of the CYP2B6 and CYP2J26 (a gene identified as similar to human CYP2B6 – see chapter 3) from zebrafish (see appendix A1 and A2). This is further complicated by the broad and overlapping substrate specificities of CYP, UGT, GST and SULT enzymes, resulting in some difficulty in identifying enzyme isoform specific marker reactions that can be used for screening.

The CYP1, CYP2 and CYP3 family isoforms identified in the human (*Homo sapiens*), rat (*Rattus norvegicus*), mouse (*Mus musculus*), dog (*Canis familiaris*), zebrafish and Atlantic salmon (*Salmo salar*) genomes are listed in Table 1.3. In addition to this, cytochrome P450 genes have been identified in the freshwater invertebrate *Daphnia pulex*, other fish species including the European flounder (*Platichthys flesus*) and rainbow trout (*Onchorynchus*

mykiss), and a variety of insects and plants (Buhler and Wang-Buhler 1998, Williams et al 2000, Baldwin et al 2009, Nelson 2009). Although some comparisons can be drawn between species based upon gene sequences identified within the genomes of organisms, Table 1.3 highlights that enzyme identification based upon predicted amino acid sequence is not sufficient to identify orthologous and homologous enzymes across different species. This results in a requirement for the comparison of enzymatic activities in order to compare metabolic capabilities across different species.

There has been substantial investigations using *in vitro* preparations such as cultured cells, microsomal and cellular fractions from a wide variety of different tissues and fish species including Atlantic salmon, carp (*Cyprinus carpio*), channel catfish (*Ictalurus punctatus*), cod (*Gadus mohua*), dab (*Limanda limanda*), fathead minnow (*Pimephales promelas*) and rainbow trout (Varanasi et al 1987, Buhler and Wang-Buhler 1998, Morcillo et al 2004, Stuchal et al 2006, Fitzsimmons et al 2007, Matsuo et al 2008, González et al 2009). These studies tend to use substrates such as ethoxy-resorufin, 7-ethoxy-coumarin, 7-pentoxy-resorufin, carcinogenic compounds (e.g. benzo-[a]-pyrene), and endogenous hormones and steroids (e.g. oestradiol and testosterone) which are indicative of CYP-mediated metabolism, albeit with a lack of isoform specificity (Kern et al 1997, Kobayashi et al 2002). Conjugative metabolism has also been assessed in a variety of different fish species, using substrates including 1-chloro-2,4-dinitrobenzene (GST), 2-trifluoromethyl-4-nitrophenol (UGT), p-nitrophenol (UGT and SULT) and 1-naphthol (SULT, Fitzsimmons et al 2007). Overall, these studies demonstrate that a variety of fish species possess similar types of metabolic capability as that observed for mammalian species however, there is a lack of isoforms specific characterisation, or identification of the isoforms(s) responsible for catalysing these processes, resulting in a lack of comparative data with mammalian systems.

Species	CYP1	CYP2	CYP3
Human (*Homosapiens*)	1A1, 1A2, 1B1	2A6, 2A7, 2A13, 2B6, 2B7, 2C8, 2C9, 2C18, 2C19, 2D6, 2D7, 2D8, 2E1, 2F1, 2G1, 2G2, 2J2, 2R1, 2S1, 2T2, 2T3, 2U1, 2W1	3A4, 3A5, 3A73, 3A43
Rat (*Rattus norvegicus*)	1A1, 1A2, 1B1	2A1, 2A2, 2A3, 2B1, 2B2, 2B3, 2B12, 2B15, 2B21, 2B31, 2C7, 2C11, 2C12, 2C13, 2C22, 2C23, 2C24, 2C79, 2C80, 2C81, 2D1, 2D2, 2D3, 2D4, 2D5, 2E1,	3A1, 3A2, 3A9, 3A18, 3A62, 3A73
		2F4, 2G1, 2J3, 2J4, 2J10, 2J13, 2J16, 2R1, 2S1, 2T1, 2U1, 2W1, 2AB1, 2AC1	
Mouse (*Mus musculus*)	1A1, 1A2, 1B1	2A4, 2A5, 2A12, 2A22, 2B9, 2B10, 2B13, 2B19, 2B23, 2C29, 2C37, 2C38, 2C39, 2C40, 2C44, 2C50, 2C54, 2C55, 2C65, 2C66, 2C67, 2C68, 2C69, ,	3A11, 3A13, 3A16, 3A25, 3A41, 3A44, 3A57, 3A59
		2C70, 2D9, 2D10, 2D11, 2D12, 2D13, 2D22, 2D26, 2D34, 2D40, 2E1, 2F2, 2G1, 2J5, 2J6, 2J7, 2J8, 2J9, 2J11, 2J12, 2J13, 2R1, 2S1, 2T4, 2U1, 2W1, 2AB1, 2AC1	
Dog (*Canis familiaris*)	1A2	2A, 2A13, 2A6/7, 2B11, 2C21, 2C, 2C41, 2D15, 2E1, 2F, 2G1, 2J, 2R1, 2S1, 2T, 2U1, 2W1, 2AB1, 2AC1,	3A12, 3A26, 3A, 3A?
Zebrafish (*Danio rerio*)	1A, 1B1, 1C1, 1C2, 1D1	2J20, 2J21, 2J22, 2J23, 2J24, 2J25, 2J26, 2J27, 2J28, 2J29, 2J30, 2K6, 2K7, 2K8, 2K16, 2K17 2K19, 2K20, 2K21, 2K22, 2N13, 2P6, 2P7, 2P8, 2P9, 2P10,	3A65, 3C1/1, 3C1/2, 3C1/3
		2P12, 2R1, 2U1, 2X6, 2X7, 2X8, 2X9, 2X10, 2X11, 2Y3, 2Y4, 2AA1, 2AA2, 2AA3, 2AA4, 2AA5, 2AA6, 2AA7, 2AA8, 2AD2, 2AD3, 2AD6, 2AE1,	
Atlantic salmon (*Salmo salar*)	1A, 1B, 1C, 1D	2K, 2M1, 2M2, 2P, 2R1, 2U1, 2X, 2Y, 2AD, 2AE	3A

Table 1.3: A comparison of CYP1, CYP2 and CYP3 family isoforms identified in human (*Homo sapiens*), rat (*Rattus novegicus*), mouse (*Mus musculus*), dog (*Canis familiaris*), zebrafish (*Danio rerio*) and Atlantic salmon (*Salmo salar*). The above table only lists annotated genes and does not include pseudogenes. Genes were identified using the gene database at www.zfin.org and at the P450 homepage (Nelson 2009), accessed December 2009.

Unlike the majority of the fish species listed above and reviewed by Fitzsimmons et al (2007), zebrafish are well suited to the laboratory (see Section 1.2). There are 89 CYP genes currently identified in zebrafish (compared to 57 in human, 102 in mouse and 54 in dog) , with 42 of these being members of the CYP2 family (Goldstone et al 2009, Nelson 2009). The metabolism of a variety of different CYP substrates by zebrafish homogenates (adult and larval tissues) is listed in Appendix 1 (Figure A3), and compared with both mammalian and non-mammalian species. It is clear that although the metabolism of several non-specific CYP substrates has been demonstrated, these are mainly using adult and juvenile tissue homogenates (Troxel et al 1997, Arukwe et al 2008). A CYP3A activity, identified by the detection of a 6β-hydroxylation activity towards testosterone, has also been reported using primary hepatocytes from zebrafish (Reschly et al 2008). It is clear from this list that zebrafish have not undergone exhaustive characterisation for CYP-mediated metabolism but that there is enough evidence to suggest, in adult fish, that similar metabolic systems to those found in mammalian species are present. There is however a distinct lack of characterisation of such systems in zebrafish larvae (<7 dpf). As it is these early life-stages of zebrafish that are being applied to toxicity testing, it is necessary that larvae at these stages of development are assessed for CYP activities. A variety of CYP genes have been demonstrated to be expressed during mouse embryonic development and CYP3A7 is a human CYP isoform that is mainly detected in foetal tissues, therefore the expression of CYP isoforms during zebrafish development is not surprising (Choudhary et al 2003, Nishimura et al 2003). It is currently thought that these expressed CYP isoforms have some role in normal embryonic development. The expression of zebrafish CYP and UGT isoforms throughout development is discussed further in Chapter 3. Only EROD activity has been assessed in zebrafish larvae < 7 dpf, with Mattingly and Toscano (2001) reporting that EROD activity is only detected post-hatching, and Noury et al (2006) demonstrating a low activity towards ethoxy-resorufin using live larvae from a variety of different ages below 8 dpf.

Conjugative processes have also been assessed using zebrafish as a model species. The sulfotransferases have been extensively characterised for zebrafish, with 16 SULT isoforms (SULT1st1-8, SULT2st1-3, SULT3st1 and st2, SULT4a1, SULT6b1 and SULTX) currently identified in the zebrafish genome (www.zfin.org accessed December 2009, Liu et al 2005). The SULT1, SULT2 and SULT3 genes have all been over-expressed *in vitro*, and characterised for substrate affinities towards endogenous and exogenous substrates, including oestrogens, flavenoids, thyroid hormone derivatives, hydroxychlorobiphenyls and dopamine (Ohkimoto et al 2003, Sugahara et al 2003, Liu et al 2005, Yasuda et al 2005a, Yasuda et al 2005b, Yasuda et al 2006, Liu et al 2008, Yasuda et al 2008, Table 1.4). The expression of all of these identified SULT genes throughout development has been assessed by reverse transcriptase PCR (RTPCR), and has demonstrated that the different isoforms have distinct temporal patterns of expression throughout development (Ohkimoto et al 2003, Sugahara et al 2003, Liu et al 2005, Yasuda et al 2005a, Yasuda et al 2005b, Yasuda et al 2006, Liu et al 2008, Yasuda et al 2008). It has also been reported that there is activity towards 1-chloro-2,4-dinitrobenze (a model substrate for GST activities) from both microsomal and soluble fractions of zebrafish embryos and larvae throughout development, with an increase in the activity of the soluble fraction towards this substrate post-hatching (Wiegand et al 2000). Indeed, the glutathione conjugate of atrazine has been identified using enzyme extracts from zebrafish embryos (Wiegand et al 2001). Although there have been 10 UGT sequences identified in the zebrafish genome, there is currently no evidence demonstrating the presence of UGT activities in embryonic or larval zebrafish (George and Taylor 2002). However, Lindholst et al (2003) identified using adult zebrafish whole-body homogenates both the sulphate and glucuronic acid conjugates of bisphenol A.

It is apparent that although there is evidence to suggest that fish species in general, and indeed zebrafish, possess similar xenobiotic metabolism pathways as those observed in

mammals, that particularly at embryonic and larval developmental stages (<7 dpf), further characterisation is required. The majority of substrates that have been used in different fish species, such as ethoxy-resorufin, polycyclic aromatic hydrocarbons and aryl-amines, are promiscuous substrates or environmentally relevant carcinogens, and generally substrates of the CYP1 family enzymes. There has been little investigation regarding the metabolism of pharmaceutical compounds by fish species, and with the application of zebrafish and other small fish species to toxicity testing this lack of knowledge needs to be addressed. Although conjugative metabolism has been assessed in zebrafish, mainly the SULT enzymes and demonstration of GST activity towards the marker substrate CDNB, there is a lack of characterisation regarding the extent of UGT activity at embryonic and larval stages, and of activities towards pharmaceuticals.

Table 1.4: The characterisation of zebrafish SULT isoforms *in vitro* using over-expressed enzymes

Enzyme	pH dependency	Substrates	Modulation by metal ions?	Temp. Tolerance (℃)
SULT1 ST1	6-9	Hydroxychlorobiphenyls	Y	40
SULT1 ST2	4.5 and 10.5	Hydroxychlorobiphenyls Esterone Dopamine 17β-Oestradiol Phytoestrogens	Y	40
SULT1 ST4	7	Thyroid hormones Esterone Flavenoids Isoflavenoids Phenolic compounds	Y	<43
SULT ST5	6	Phenolic compounds Thyroid hormone derivatives	Y	
SULT ST6	6.5 and 10.5	Phenolic compounds Esterone 17β-Oestradiol		
SULT2 ST1	9.5	Steroids Neurosteroids	Y	20 - 43
SULT2 ST2	6.5	DHEA Hydroxysteroids		
SULT2 ST3	6.5	Corticosterone Hydroxysteroids		
SULT3 ST1	6.5	17β-Oestradiol DHEA Diethylstilbestrol Bisphenol A		

SULT3 ST2	5.5	DHEA Pregnenolone 17β-Oestradiol 17α-Oestradiol		
SULT X	7.5 - 9.0	Flavenoids phenolic compounds Isoflavenoids Dopamine	Y	40

The biochemical assessment of different SULT isoforms in zebrafish. Purified protein extracts for each isoform were assessed for pH dependency, substrate specificity, metal ion inhibition and temperature tolerance of activity. Examples of phenolic compounds include β-naphthol, p-nitrophenol and n-propyl-gallate. Metal ions include Fe^{2+}, Zn^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+} and Hg^{2+}. Data from Ohkimoto et al 2003, Sugahara et al 2003, Liu et al 2005, Yasuda et al 2005a, Yasuda et al 2005b, Yasuda et al 2006, Liu et al 2008, Yasuda et al 2008.

1.4 Aims of Project

In order to characterise further xenobiotic metabolism in zebrafish at early life-stages (<7 dpf), it was determined that, based upon the observations of Mattingly and Toscano (2001) and Wiegand et al (2000), larvae post hatching would be the most useful stage of development to undergo characterisation – as both Ethoxy-resorufin-*O*-deethylase (EROD) activity (as a marker of oxidative metabolism) and 1-Chloro-2,4-dinitrobenzene (CDNB) activity (a marker of conjugative metabolism) have been detected at these stages. The zebrafish genes CYP1A, CYP2J26, zgc:153269 (CYP3A) and UGT1A1 were selected as representative genes of the CYP1, CYP2, CYP3 and UGT families and due to the sequence similarities with the human CYP1A1, CYP2D6, CYP3A5 and UGT1A1 genes. The broad CYP substrates ethoxy-resorufin, ethoxy-coumarin and octyloxymethylresorufin were selected to assess CYP activities in zebrafish because these substrates broadly reflect CYP1, CYP2 and CYP3 family activities in mammalian systems respectively, unlike pentoxy-resorufin which undergoes dealkylation catalysed by CYP1A2, CYP2B1 and CYP2C13 in rat (Kobayashi et al 2002). The expression of SULT and GST genes and activities was not assessed in this study due to the extensive characterisation of these systems as discussed in chapter 1 (pages 27-31 and Weigand et al 2000). Wik strain fish only were utilised in this project, as this strain is a widely used wild-type strain in the field of toxicology. These fish were generously provided by Brixham Environmental Laboratory (AstraZeneca) and the University of Exeter to set up the zebrafish facility at The University of Birmingham.

The aims of this project were as follows.

1. To identify and assess the expression of genes similar to mammalian CYP1, CYP2, CYP3 and UGT1A1 throughout development.

2. Using an *in vivo* zebrafish larvae assay (96 hpf), assess the metabolism of the CYP and UGT substrates ethoxy-resorufin, ethoxy-coumarin and octyloxymethylresorufin by Wik strain hatched zebrafish larvae.

3. Assess the modulation of the above activities by known inducers of drug metabolism genes (e.g. polychlorinated byphenyls) and *in vitro* inhibitors (e.g. α-naphthoflavone and SKF525A).

4. It is hypothesised that as zebrafish larvae have been demonstrated to posess some xenobiotic metabolism genes, larval zebrafish would be able to metabolise the NSAIDs acetaminophen and ibuprofen by oxidative and conjugative mechanisms similar to those described for mammals. This hypothesis was tested *in vivo*, using a combination of liquid-chromatography-mass spectrometry and scintillation counting. This study would also provide quantitative information.

5. Determine the effects of the solvents dimethyl-sulfoxide and methanol exposures on the expression and activity of CYP enzymes in zebrafish larvae, since these are commonly used vehicles for substrates.

Chapter 2

Materials and Methods

2.1 Maintenance of zebrafish and group spawning

Mixed sex adult Wik strain zebrafish, approximately 9 months of age, were maintained at 26 ± 1°C in groups of 20 fish in the husbandry unit at The University of Birmingham, with a light/dark cycle of 14/10 hours (290 – 300 Lux), respectively. Fish were held in a Techniplast aquarium in dechlorinated town's water, after filtration to 50 µm (mesh filter, biological filtration using Siprorax Grav ring biological filtration and mechanical filtration to 50 µm – paper, Techniplast, Northampton, UK), and sterilisation (ultra violet – 30000 µW/s/cm^2). Water was maintained at a conductance of 480 – 500 µS and at pH 7.4. These groups were fed *Artemia nauplii* and zm200 (150 – 300 µm) –zm400 (500 - 800 µm) granulated feed (54% protein adult feed, ZMsystems Ltd, Winchester, UK). The groups of fish were spawned and fertilised eggs were collected and maintained under the same conditions as adult fish, but at 28 ± 1°C, in accordance with the developmental staging of Kimmel et al (1995), until of a suitable age. Zebrafish embryos and larvae were not deemed to be free-feeding until 5 dpf. Under these conditions zebrafish embryos hatched from the chorion between 2 and 3 dpf.

2.2 Exposure of zebrafish larvae (96 hpf) to Aroclor 1254 (CAS Number 11097-69-1), α-naphthoflavone (CAS Number 604-59-1), SKF525A (CAS Number 62-68-0), dimethyl sulfoxide only or methanol only

Zebrafish larvae (72 hours post fertilization, hpf) were incubated with 100 µg/l Aroclor 1254 (dissolved in methanol – final solvent concentration of 0.05% v/v, Supelco lot # LA39006, Sigma-Aldrich, Schnelldorf, Germany), methanol only (0.05%, 0.01% or 0.001% v/v, HPLC grade, Fisher Scientific, UK) or dimethyl sulfoxide (DMSO) only (0.1% or 0.01% v/v, analytical grade, Fisher Scientific, UK) for 24 hours at 28 ± 1°C in dilution water (obtained from the Techniplast aquarium, The University of Birmingham) in 50 ml Falcon tubes. After these exposures the larvae were utilized for RNA extraction, or for use in the assays for

xenobiotic metabolism as described in sections 2.3 and 2.5. Zebrafish larvae (96 hpf) were incubated with α-naphthoflavone (1, 5 or 10 μM, dissolved in DMSO – final solvent concentration of 0.1% v/v), SKF525A (5 μM, dissolved in DMSO, final solvent concentration of 0.1% v/v) or 0.1% v/v DMSO only in dilution water, for the duration of the EROD, ethoxy-coumarin-*O*-deethylase (ECOD) and octyloxymethylresorufin-*O*-dealkylase (OOMR) assays in 10 ml Falcon tubes (see section 2.6).

2.3 RNA extraction from whole zebrafish embryos and larvae

Total RNA was extracted from approximately 100 zebrafish larvae (24, 48, 72 and 96 hpf) using a QIAGEN RNeasy mini kit as directed in the manufacturer's guidelines for tissue extraction (Qiagen, Crawley, UK). Briefly, zebrafish larvae were homogenised in RLT buffer (containing β-mercaptoethanol) and centrifuged at maximum speed (16000g, 3 minutes, room temperature) before the addition of 70% ethanol to the supernatant. The supernatant was then added to an RNeasy spin column and centrifuged (8000g, 30 seconds, room temperature), before washes with RW1 buffer and RPE buffer (8000g, 30 seconds, room temperature). The RNA was eluted in 100 μl of RNase-free water. The isolated RNA was then electrophoresed on a 2.5% agarose gel to determine purity and quantified by spectrophotometry using a Nanodrop ND1000 (Thermo Scientific, LabTech, East Sussex, UK).

2.4 The synthesis of cDNA, the Polymerase Chain Reaction (PCR), and Quantitative PCR (qPCR)

From the isolated RNA, 500 ng of RNA was used as a template for cDNA synthesis. A solution of RNA template, random hexamers (1 μl, Biolabs, Hertfordshire, UK) and nuclease-free water was heated to 65°C for 5 minutes. To this solution 0.1 M DTT (2 μl),

First strand buffer (5 µl) and Superscript II reverse transcriptase (0.5 µl) from the Invitrogen Superscript II kit (Invitrogen, Paisley, UK) together with 25 mM dNTP mix (0.4 µl, Bioline, London, UK). This was then incubated at 25°C for 10 minutes, 42°C for 90 minutes and 70°C for 15 minutes in a thermocycler, before storage at -20°C. The cDNA was quantified by spectrophotometry using a Nanodrop ND1000.

The human sequences corresponding to CYP2B6, CYP3A5 and UGT1A1, as well as the zebrafish CYP1A, Aryl Hydrocarbon Receptor 1 and 2 sequences (Accession Numbers: NM_000767.4, NM_000777, NM_000463 and NM_131879), were used in a BLAST search of the zebrafish genome using the BLAST search at www.zfin.org (accessed February 2008). Primers were designed using the PRIMER3 software against sequences from zebrafish that had high similarity with the human genes (Table 2.1). The primers for zebrafish RPL13α and EF1α were identified from the literature (Tang et al 2007).

The synthesised cDNA was used as a template for PCR, using the primers designed against the target genes (Table 2.1). The Bioline PCR kit was used for the PCR mix with a final volume of 50 µl (Bioline, London, UK). The primers were diluted to a 10 µM stock solution for use with this reaction. The genes of interest were amplified using a thermocycler program of 95°C for 5 minutes; 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes. Products were electrophoresed on a 2.5% agarose gel and visualised by ethidium bromide staining. Fragment size was approximated using a 100 base pair marker ladder (New England Biolabs, Ipswich, UK).

The PCR products were sequenced on a capillary sequencer ABI 3700 (The University of Birmingham Genomics Laboratory). Fragments of the sequenced products were BLAST searched at www.zfin.org and genes with high similarity were identified. This was used to confirm that the primers amplified the target genes of interest.

The validated primers were utilised in quantitative polymerase chain reaction (qPCR) assays using a Sensimix dT Sybr Green kit (Quantace, Finchley, UK). A mastermix of 50x Sybr green dye, 2x Sensimix and deionised water was added to 1 µl of each primer (10 mM) and 250 ng/µl of cDNA template, with a final volume of 25 µl per well, in a 96 well plate format. Products were amplified and detected, following a dissociation protocol, with a program of 95°C for 30 seconds, 60°C for 30 seconds, using an Abiprism 7000 sequence detection system. The melt curves for all samples were analysed and Ct values were recorded for each gene in the linear phase of amplification. Analysis of the data followed the ΔΔCt method (Livak and Schmittgen 2001). The efficiencies of the qPCR reactions were determined using the LinRegPCR software, as described by Ramakers et al (2003), in order to meet the requirements of the ΔΔCt method of analysis. Three different cDNA samples, each synthesised from total RNA extracted from separate pools of 100 larvae, were analysed for each gene.

2.5 *In vivo* fluorescence assays of xenobiotic metabolism

The following assays were adapted and optimised from the ethoxyresorufin-*O*-deethylase assay protocol used in cell culture models as described by Green et al (2008). Thirty zebrafish larvae (96 hpf) per time-point, were exposed in 10 ml Falcon tubes to 7-ethoxyresorufin (8 µM, Sigma-Aldrich, Poole, UK), 7-ethoxycoumarin (100 µM, Sigma-Aldrich, Poole, UK) or Vivid substrate octyloxymethylresorufin (OOMR, 8 µM, Invitrogen, Paisley, UK) dissolved in DMSO (0.1% v/v) for up to 10 hours in triplicate (five time-points per assay) in dilution water at 28 ± 1°C. Dicumarol (10 µM, Sigma-Aldrich, Poole, UK) was added at each time-point, for the EROD and OOMR assays, to prevent degradation of the resorufin product by DT Diaphorase. After incubation of the substrates, 750 µl of the water was added to 250 µl of either 100 mM sodium acetate buffer (pH 4.5) only or 250 µl of β-glucuronidase/arylsulphatase extract (Roche, West Sussex, UK) diluted 666-fold with 100

mM sodium acetate buffer (pH 4.5) and incubated for 2 hours at 37°C. The incubated mixture was transferred to a fluorescence curvette and 1 ml of 100% ethanol was added. The fluorescence of each sample was measured at the wavelengths detailed below using a Perkins Elmer LS50B fluorimeter. Quantification of the fluorescence of each sample was determined using a standard curve of each product (resorufin and 7-hydroxycoumarin obtained from Sigma-Aldrich, Poole, UK), with the fluorescence determined under the appropriate conditions (resorufin λ_{ex} = 530 nm, λ_{em} = 590 nm; 7-hydroxycoumarin λ_{ex} = 368 nm, λ_{em} = 456 nm). No spontaneous formation of fluorescent product was observed for these assays.

2.6 Fluorescence assays of xenobiotic metabolism with co-exposure to α-naphthoflavone or SKF525A

Zebrafish larvae were assessed for EROD, ECOD and OOMR activities, as described above (section 2.5) with the following modifications. Zebrafish larvae (30 per replicate) were co-incubated with the substrate and inhibitor or 0.1% DMSO (solvent control) for 2 hours (ECOD assay), 4 hours (EROD assay) or 10 hours (OOMR assay) before measurement of activity, as previously described (sections 2.2 and 2.5). Activities were compared to the solvent control for calculation of the mean percentage inhibition. Both α-naphthoflavone and SKF 525A were obtained from Sigma-Aldrich (Poole, UK).

2.7 Exposure of zebrafish larvae to ibuprofen and ^{14}C-Ibuprofen

Zebrafish larvae (72 hpf, 10 per replicate) were exposed to 100 µg ^{14}C-ibuprofen/l (American Radiolabels Company, USA), or an ethanol solvent control. Fish were exposed in triplicate for 0, 3, 6, 9, 12, 18 or 24 hours, at 28 ± 1°C, in 10 mls of dilution water (water

obtained from zebrafish aquarium system) in glass beakers. These fish and water samples were then used for the assessment of ^{14}C incorporation into larval tissues.

Zebrafish larvae (72 hpf, 100 per replicate) were also exposed to 100 µg/l of ibuprofen (CAS Number 31121-93-4, Sigma, Poole, UK) or ^{14}C-ibuprofen in triplicate, or an ethanol solvent control for 24 hours, at 28 ± 1°C. Larvae were exposed in 10 ml volumes. All stock solutions were made in ethanol and the solvent concentration was the same in all treatments (0.0015% v/v) which did not exceed that recommended by international testing guidelines (OECD, 1992). These larvae and water samples were used for the assessment of metabolism using LC-MS(MS).

2.8 Assessment of ^{14}C-incorporation in zebrafish larvae by scintillation counting

After exposure, samples of the dilution water (1.5 ml) were collected using a pasteur pipette and stored at -80 ± 1°C in 2 ml microcentrifuge tubes until required for analysis. Zebrafish larvae were removed from the dilution water and rinsed twice in HPLC grade water (Fisher Scientific, Loughborough, UK) before being homogenised manually between two microscope slides coated in scintillation fluid (Optiphase hisafe 3, Fisher Scientific, uk). The homogenate was then collected into an microcentrifuge using a cell scraper to collect all liquid from the microscope slides. These slides were then rinsed using a small volume of scintillation fluid and 100 µl of 100% Triton X-100 (Sigma-Aldrich, Poole, UK) was added to the microcentrifuge tube, and made up to a final volume of 2 ml. This was then vortexed thoroughly and incubated at room temperature overnight, followed by further vortexing. The water sample aliquots were defrosted on ice and added to scintillation fluid in a 1:7 (sample:scintillation fluid ratio) up to a final volume of 2 ml. Larval and water samples were then assessed for ^{14}C content using a 5 minute count for ^{14}C detection using a Packard

Tricarb 1600 TA Liquid Scintillation Analyser. Results were recorded as disintegrations per minute (dpm).

2.9 Extraction of larval samples exposed to ibuprofen for LC-MS(MS) analysis

Zebrafish larvae were removed from dilution water and rinsed twice in HPLC grade water (Fisher Scientific). The washed larvae were then homogenised in 500 µl HPLC grade dichloromethane (Fisher Scientific) using a Precellys 24 bead homogeniser (Precellys, Leicestershire, UK). Homogenates were transferred into 1.5 ml micro-centrifuge tubes and centrifuged (9000 g, 30 mins, 4 ± 1°C). The supernatant was retained and evaporated under nitrogen until dry. Residues were then re-suspended in 100 µl methanol:acetic acid (12.5:0.1) in water (all reagents were HPLC grade, Fisher Scientific). Extracts were then stored at -80 ±1°C, until required for analysis. Samples of the dilution water used for exposures were retained and stored at -80 ±1°C until required for analysis.

2.10 LC-MS(MS) analysis of larval extracts and water samples exposed to ibuprofen

Water samples and larval extracts were separated by liquid chromatography, as detailed in Table 2.2. Elutents were detected using a Finnigan LXQ-orbitrap (Thermo Electron Corporation) as detailed in Table 2.2. The *m/z* for ibuprofen and its metabolites were detected in negative ion mode, and identified based on known metabolism in mammalian systems. Accurate ion mass measurements and MS-MS fragmentation were recorded for the parent compound and any potential metabolites for ibuprofen exposed samples.

2.11 Exposure of zebrafish larvae to acetaminophen, and the extraction of these larvae for LC-MS analysis

Zebrafish larvae (72 hpf, 100 larvae per replicate) were exposed in glass beakers to 60 µg/l or 500 µg/l acetaminophen dissolved in DMSO (Sigma-Aldrich, Poole, UK) or a solvent only (< 0.1% v/v DMSO), for 6 hours and 24 hours in triplicate.

After these exposures, the zebrafish larvae were rinsed twice in HPLC grade water (Fisher Scientific), and homogenised in 500 µl of methanol using a Precellys 24 bead homogeniser (Precellys, Leicestershire, UK). Homogenates were transferred into 1.5 ml micro-centrifuge tubes and centrifuged (9000 g, 30 mins, 4 ± 1°C). The supernatant was retained and evaporated to dryness using a SPDIIIV concentrator with a RVT4104 refrigerated vapour trap (Thermo Electron Corporation). Residues were re-suspended in 100 µl methanol:acetic acid (12.5% :0.1%) in water (all reagents were HPLC grade, Fisher Scientific). Extracts were then stored at -80 ± 1°C, until required for analysis. Samples of the dilution water used for exposures were retained and stored at -80 ± 1°C until required for analysis.

2.12 LC-MS analysis of zebrafish larval extracts and water samples from the acetaminophen exposures

Water samples and larval extracts were separated by liquid chromatography, as detailed in Table 2.3. Elutents were detected using a Finnigan LXQ-orbitrap (Thermo Electron Corporation) as detailed in Table 2.3. The *m/z* for acetaminophen and its metabolites were detected in positive and negative ion modes, and identified based on known metabolism in mammalian systems in addition to comparisons with acetaminophen sulfate and glucuronide analytical standards (Sigma-Aldrich, Poole, UK).

2.13 Scintillation counting of liquid chromatography fractions from larval extracts and water samples

Larval extracts and water samples were separated into fractions (1 minute per fraction) using the LC method described above (Table 2.2) for the duration of the LC program. These fractions were added to 5 ml of scintillation fluid and assessed for ^{14}C using a 20 minute counting program on a Packard Tricarb 1600TA Liquid Scintillation Analyser. Radioactivity was expressed in Bequerels (Bq), and was quantified into nanomoles or picomoles based upon the specific activity of the radiolabel (1.85GBq / millimole) and corrected for a ratio of ^{14}C:^{12}C of 5.8:1 as determined using LC-MS(MS). Any ^{14}C detected by scintillation counting were compared to the retention times of metabolites identified using LC-MS(MS) to attempt to identify the nature of the ^{14}C.

2.14 Assessment of ibuprofen conjugation by scintillation counting

Zebrafish larvae (15 per replicate) were exposed in glass beakers to 100 µg/l ^{14}C-ibuprofen for 24 hours or solvent only as described in 2.11. Larvae were rinsed twice in dilution water before homogenisation in 25 mM sodium acetate buffer (pH 4.5). The homogenates were centrifuged (9000g, 30 minutes, -1°C) and the supernatant was retained. Samples were divided into two groups (n=3 per group), with one group incubated with β-glucuronidase/arylsulphatase mix (diluted 2664-fold, Roche, West Sussex, UK) and the other incubated without the addition of the β-glucuronidase/arylsulphatase enzyme mix for 2 hours at 37°C. Water samples (750 µl) from the exposures were added to 100 mM sodium acetate buffer (pH 4.5, 250 µl) and were incubated with or without the addition of the β-glucuronidase/arylsulphatase mix as for the larvae homogenates. Both water and larval samples were extracted three times with dichloromethane (2 volumes of dichloromethane to every volume of sample) by vortexing (HPLC grade, Fisher Scientific),

with the dichloromethane extracts evaporated to dryness under a stream of nitrogen. The residues were re-suspended in 150 µl of sterile water, and scintillation fluid was added in a 1 : 7 ratio (Optiphase Hisafe 3, Fisher Scientific, West Sussex, UK). The amount of radioactivity present in both the polar and non-polar fractions for each sample was determined using a 20 minute count for ^{14}C detection using a Packard Tricarb 1600 TA Liquid Scintillation Analyser. Results were recorded as disintegrations per minute (dpm) and converted into Bequerels (Bq), and expressed as a percentage of the total detected radioactivity in both polar and non-polar fractions for each sample.

2.15 Statistical Analysis

The data was assessed for normal distribution and homogeneity of variance by Sharipo-Wilks' test and Levenes' test, respectively, using SPSS version 16 for Windows (SPSS, v.16). Data that did not meet the requirements for normal distribution or homogeneity of variance were analysed by non-parametric statistics, using a Kruskal-Wallis test and Mann-Whitney U test (SPSS, v.16). Data that was normally distributed and met the criteria for homogeneity of variance was analysed by one-way ANOVA with a Tukey post-hoc test in SPSS or by independent samples t-test (v.16). For analysis of real time PCR data, samples were analysed in triplicate using cycle threshold values (Ct) as described for fluorescence assays. All graphs display the mean ± the Standard Error of the Mean (SEM). Values of $p<0.05$ were deemed to be significant. A p-value of <0.05, <0.01 and <0.001 are indicated by *, ** and ***, respectively.

Table 2.1: Primers for use for the amplification of gene specific sequences of zebrafish CYP1A, CYP2J26, the CYP-gene zgc: 153269, UGT1A1, AhR1, AhR2, EF1α and RPL13α.

Gene	Left Primer	Right Primer
CYP1A	TCC TGG AAA TCG GAA ACA AC	CTG AAC GCC AGA CTC TTT CC
CYP2J26	AAG CCC ACA AAA ACC TCC CT	ATA TCA TTG GAT GGG CGG TA
zgc: 153269 (CYP3A)	GGT GGA GGA GAT CGA CAA AA	ACC GTT TTC TTA GCG GAC CT
UGT1A1	CTG CTG GTT GCA TTG AAG AA	CGA TGA CGT CCA GAG AGT GA
RPl13α	TCT GGA GGA CTG TAA GAG GTA TGC	AGA CGC ACA ATC TTG AGA GCA G
EF1α	CTG GAG GCC AGC TCA AAC AT	ATC AAG AAG AGT AGT ACC GCT AGC ATT AC
AhR1	GTG ACC TCC AGT GGG ACA GT	GGG GTA AAG CCA GGG TAG AG
AhR2	CCC TCA AGT GAC AGT GCT GA	CTT GTT TTG CCC ATG GAG AT

Table 2.2. Liquid Chromatography Conditions and Mass Spectrometer Operating Parameters for the analysis of ibuprofen exposed larval extracts and water samples

Column	Waters Xbridge $C_{18,}$ 5 µm, 2.1 mm internal diameter
Oven Temperature	30 ± 0.1 ℃, Dionex Ultimate 3000
Injection Volume	10 µl
Elutant A Elutant B	0.1% Ammonia in water Methanol
Gradient (A:B)	Time 0 min: 95%:5% Time 6 min: 0%:100% Time 8 min: 0%:100% Time 8.1 min: 95%:5% Time 11 min: 95%:5%
Flow Rate	200 µl/min
Autosampler	Dionex Ultimate 3000
Spray Voltage	3.0 kV
Sheath Gas	N_2 at 40 arbitrary units
Auxiliary Gas	N_2 at 10 arbitrary units
Sweep Gas	N_2 at 0 arbitrary units
Capillary Temperature	300℃

Table 2.3. Liquid Chromatography Conditions and Mass Spectrometer Operating Parameters for the analysis of acetaminophen exposed larval extracts and water samples

Column	Fortis Pace $C_{18,}$ 3 µm, 2.1 mm internal diameter
Oven Temperature	30 ± 0.1 ℃, Dionex Ultimate 3000
Injection Volume	10 µl
Elutant A Elutant B	0.1% Ammonia in water Methanol
Gradient (A:B)	Time 0 min: 95%:5% Time 6 min: 0%:100% Time 8 min: 0%:100% Time 8.1 min: 95%:5% Time 11 min: 95%:5%
Flow Rate	200 µl/min
Autosampler	Dionex Ultimate 3000
Spray Voltage	3.0 kV
Sheath Gas	N_2 at 40 arbitrary units
Auxiliary Gas	N_2 at 1 arbitrary units
Sweep Gas	N_2 at 0 arbitrary units
Capillary Temperature	300℃

Chapter 3

Oxidative and conjugative xenobiotic metabolism in zebrafish larvae *in vivo*

3.1 Introduction

The zebrafish is a well characterised model vertebrate in the fields of molecular genetics and developmental biology, and is becoming a popular model organism in toxicology (Kimmel et al 1995, Langheinrich 2003). There is also considerable interest in using zebrafish larvae as front-loading screens for drug discovery, with the assessment of toxic end-points including cardiac output, visual function and movement (Berghmans et al 2008, Richards et al 2008, Winter et al 2008). These studies have provided evidence that zebrafish larvae are a useful model system for predicting some human toxicity, albeit with a limited number of test compounds. Assays assessing seizure liability, cardiac, intestinal, visual and locomotor function were reported as having 72%, 78%, 60%, 78% and 70% predictive toxicity, respectively, known from mammalian studies (Berghmans et al 2007, Richards et al 2008, Winter et al 2008). A lack of bioavailability of compounds was implicated as a major reason for false negative predictions of toxicity, and this was demonstrated using quantitative Liquid Chromatography - Mass Spectrometry to measure uptake of the parent compound (Berghmans et al 2008). However, it was not determined if there was any metabolism of the bioavailable test compounds and thus not established if metabolic activation or inactivation, in relation to toxicity, occurred. In order for zebrafish larvae to be a useful test system for predicting potential human toxicity, it would be required that the larvae could metabolically activate compounds to toxic species. There is limited information regarding the presence of cytochrome (CYP) P450 isoforms or conjugation enzymes (these enzymes are responsible for the oxidative and conjugative metabolism of many pharmaceuticals, as well as endogenous compounds; Hersh and Moore 2004) in zebrafish, particularly in relation to expression at larval stages (summarised below, and extensively discussed in Chapter 1).

Although several different CYP genes have been identified and sequenced in the zebrafish, few of these enzymes have been tested for gene expression, induction and substrate specificity, to allow comparisons with mammalian CYP enzymes (Chung et al 2004, Wang-Buhler et al 2005, Tseng et al 2005). The identification of genes corresponding to CYP1, CYP2 and CYP3 mammalian genes is not surprising, as there have been several analogous genes and activities reported in different fish species (Williams et al 1998, Nabb et al 2006, Vaccaro et al 2007). There is also a small number of reports of P450 activities identified in adult and juvenile zebrafish using whole-body or tissue homogenates (Chung et al 2004, Nabb et al 2006, Reschly et al 2007, Arukwe et al 2008). However, there is a lack of information regarding the activities of P450 enzymes in embryos and larvae, and it has been reported that EROD activity is only detected post-hatch in zebrafish larvae (Mattingly and Tuscano 2001).

To strengthen the justification for the use of zebrafish larvae in drug discovery and toxicity testing, it is essential that the status of xenobiotic metabolism be understood in this model species throughout development. Therefore, the aims of this investigation were to identify genes homologous to mammalian CYP1, CYP2, CYP3 and UDP-Glucuronosyl Transferase (UGT) genes, and to assess the ability of zebrafish larvae *in vivo* to metabolise substrates used as markers for these enzymes in mammals. The modulation of the identified genes and activities by a broad-acting inducing agent (Aroclor 1254, a mixture of polychlorinated biphenyls, PCB) and by known *in vitro* inhibitors of CYP enzymes was also assessed.

3.2 Methods

Zebrafish larvae (72 hpf) were exposed to 100 µg/l of Aroclor 1254 (dissolved in methanol with a final volume of 0.05% v/v) or methanol only (0.05% v/v) for 24 hours in 50 ml of dilution water at 28 ±1°C where required (page 34, section 2.2). RNA was extracted from 100 embryos or larvae using a Qiagen RNeasy mini kit as directed by the manufacturer for extraction from tissues, and quantified by spectrophotometry using a Nanodrop ND1000 (page 35, section 2.3). The extracted RNA was used to synthesise cDNA using the Invitrogen superscript II kit, using random hexamers for inital priming, and synthesised cDNA was quantified by spectrophotometry using a nanodrop ND1000. Primers specific for the zebrafish genes CYP1A, CYP2J26, zgc:153269, RPl13α and EF1α were used to amplify these genes by PCR, with the identity of the products confirmed by sequencing, and also to quantify gene expression by qPCR using the Quantace sensimix dT kit and analysed by the ΔΔCt method (section 2.4 page 35). Zebrafish larvae were assessed for EROD, ECOD and OOMR activities by the addition of the appropriate substrate (8 µM ethoxy-resorufin, 100 µM ethoxy-coumarin, or 8 µM octyloxymethylresorufin, all dissolved in DMSO with a final volume of 0.1% v/v) to 30 larvae (96 hpf) for up to 10 hours in 1 ml of dilution water. Dicumarol (10 µM) was also added in the EROD and OOMR assays. After incubation with the substrate, 750 µl of the assay medium was removed and added to 250 µl of 666-fold diluted β-glucuronidase/arylsulfatase (in 100 mM sodium acetate buffer, pH 4.5) or just buffer only, and incubated for 2 hours at 37 ± 1°C. Ethanol was added to the the solution (1 ml) and fluorescence was measured at the appropriate wavelengths detailed in section 2.5, page 37. The EROD, ECOD and OOMR assays were also used to assess the effects of α-naphthoflavone and SKF525A by co-incubating the assay substrates with the appropriate inhibitor (dissolved in DMSO, final volume 0.1%) for 2 hours (ECOD assay), 4 hours (EROD assay) and 10 hours (OOMR assay, page 38, section 2.6). These methods are further detailed in chapter 2.

3.3 Results

3.3.1 The identification of cytochromes P450 and UDP-glucuronosyl transferase genes in the zebrafish

The human genes CYP2B6 (NM_000767.4), CYP3A5 (NM_000777) and UGT1A1 (NM_000463) were selected as representative genes of CYP2, CYP3 and UGT classes, respectively, and used as a template for the identification of similar genes in zebrafish. Using a BLAST search (www.zfin.org) of the zebrafish cDNA/RNA sequence database (accessed February 2008), the genes CYP2J26 (zgc:91790), the CYP-gene zgc:153269 and UGT1A1 (zgc:123097) were identified as being most homologous to the human genes. The zebrafish CYP1A gene (zgc:109747) has previously been identified and extensively characterised. Primers for each of the genes identified in the zebrafish were designed using the Primer3 software (http://frodo.wi.mit.edu/primer3/) and determined to be specific for each gene by sequencing of PCR products. The sequences of the PCR products were used as a template for BLAST searches of the zebrafish cDNA/RNA sequence database (www.zfin.org), and all identified the genes that the primers were designed against. All of these genes were found to be expressed by zebrafish larvae at 96 hpf.

3.3.2 Developmental expression of zebrafish CYP and UGT genes assessed by RTPCR

The expression of the CYP1A, CYP2J26, the CYP-gene zgc:153269 (CYP3 and UGT1A1 was assessed across development by RT-PCR at 24, 48, 72 and 96 hpf (Figure 3.1). The CYP1A gene was expressed at all timepoints that were assessed, and appeared to increase in expression throughout the timecourse. The CYP2J26 gene also showed a steadily increasing expression pattern throughout the developmental timecourse assessed. The gene zgc:153269 (CYP3) seemed to be more highly expressed at 24 hpf,

with lower expression detected by 48 hpf and increasing through to 96 hpf, whereas UGT1A1 was highly expressed from 48 hpf onwards. All these genes showed an increase in expression after 48 hpf, which coinsides with when embryos begin to hatch from their chorions, and would thus coincide with increased exposure to xenobiotics.

3.3.3 Activities towards ethoxyresorufin (EROD), ethoxycoumarin (ECOD) and octyloxymethylresorufin (OOMR) in vivo in zebrafish larvae

Having determined that zebrafish larvae express CYP1, CYP2, CYP3 and UGT genes throughout embryonic development, and due to reports that EROD activity is not detected until post-hatching (Mattingly and Tuscano 2001), it was determined that 96 hpf larvae would be used for the assessment of CYP activities *in vivo*. It should be noted that these assays reflect the processes of absorption, metabolism and excretion, as the larvae are intact and alive throughout the incubations. Also, the three substrates used in this study are metabolised by multiple different CYP isoforms in mammalian systems, therefore enzyme kinetic analyses could not be included within these assays. Correlations of the amount of product yielded per larvae over time have been generated to demonstrate the time-dependent increases in fluorescent product formation.

A time-dependent increase in fluorescence, corresponding to the detection of resorufin, was observed for larvae exposed to ethoxyresorufin in the EROD assay (Figure 3.2). Incubation of the surrounding medium with a β-glucuronidase/arylsulphatase preparation resulted in a significant increase in the detection of resorufin compared to media incubated with buffer alone ($p<0.001$, Kruskal-Wallis test). This demonstrated that zebrafish larvae (96 hpf) metabolise ethoxyresorufin to resorufin, a CYP1-dependent process in mammals, and also conjugate resorufin.

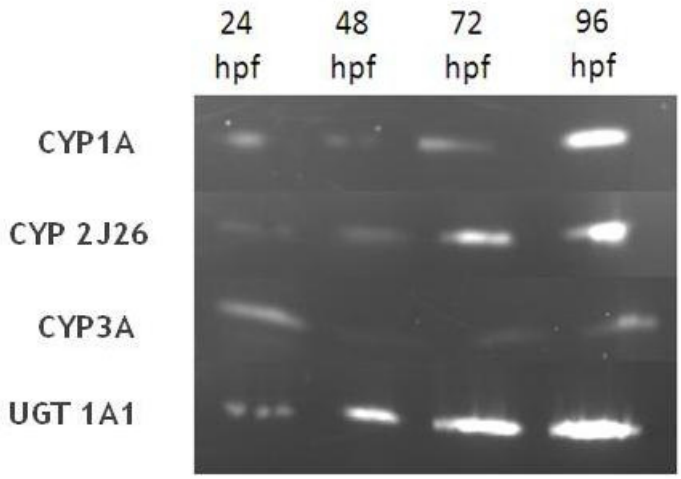


Figure 3.1: The developmental expression of zebrafish CYP1A, CYP2J26, zgc:153269 (CYP3A) and UGT1A1 in zebrafish larvae. Total RNA was extracted from a pool of 100 larvae, which was used for cDNA synthesis. Equal amounts of cDNA (1 µg per sample to ensure equal loading, thus a reference gene was not required) was used as a template for PCR amplification of the above genes across normal embryonic development. Three independent experiments were assessed, with the above image being representative of these experiments.

The metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin (ECOD assay) is a general marker for CYP2 activities in mammals. A time-dependent increase in the production of 7-hydroxycoumarin was observed with larvae incubated with 7-ethoxycoumarin (Figure 3.3A). The comparison of excreted product detected with and without the use of the deconjugation enzymes was statistically significant (Figure 3.3B, $p<0.05$, Kruskal-Wallis test), with significant differences between deconjugated and not deconjugated samples observed at 1, 2 and 4-hour time-points ($p<0.05$ for all comparisons, Mann Whitney U test). The conversion of octyloxymethylresorufin to resorufin was used as a substrate for CYP3-mediated metabolism. A time-dependent increase in resorufin production was observed (Figure 3.4).

3.3.4 The induction of CYP and UGT gene expression, and EROD activity by Aroclor 1254

After exposure to 100 µg/l Aroclor 1254 for 24 hours, the genes CYP1A, the CYP-gene zgc:153269 (CYP3) and UGT1A1 appeared to be induced based upon a 5-fold, 2-fold and 3-fold increase in expression compared to the methanol solvent control, respectively (Figure 3.5). However, these apparent increases were only supported in the case of CYP1A by statistical significance ($p<0.01$, independent samples t-test), and CYP2J26 was neither induced or repressed by Aroclor 1254 exposure. The two reference genes, elongation factor 1α and Ribosomal protein l13α, were both found to be modulated by either methanol or Aroclor 1254 exposure, and thus were not used during the analysis of the data, with CYP2J26 being used as a reference gene. It was also observed that, by comparison of the methanol solvent control to untreated samples, methanol exposure repressed the expression of CYP1A, the CYP-gene zgc:153269 (CYP3) and UGT1A1. This solvent effect is being investigated further in zebrafish and other species (see Chapter 5).

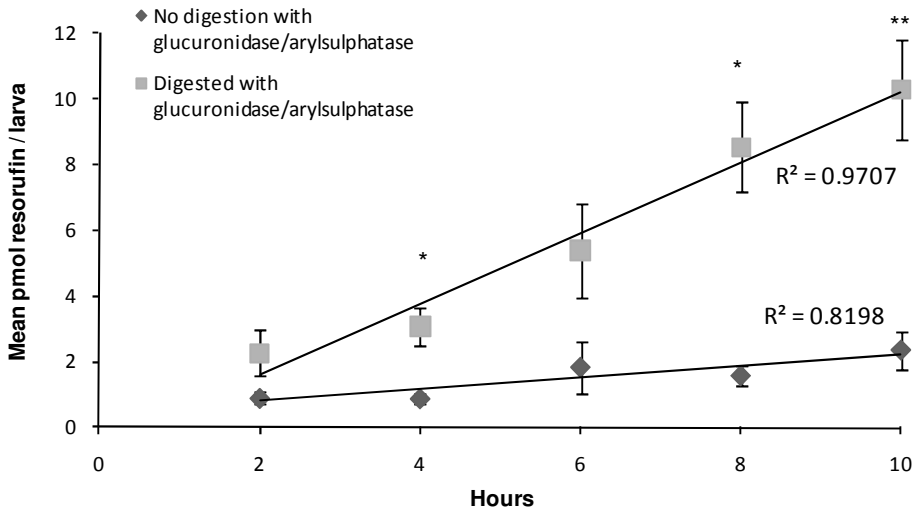


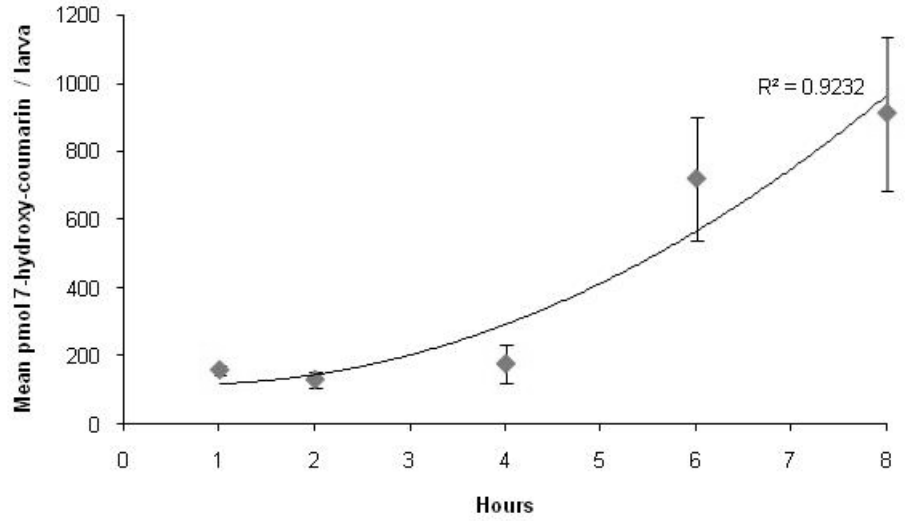
Figure 3.2: EROD assay. Thirty zebrafish larvae (96 hpf) per time-point were incubated with 8 µM 7-ethoxyresorufin over 10 hours at 28 ± 1˚C, ± β-glucuronidase/arylsulphatase digestion of excreted product for 2 hours at 37 ± 1˚C. Each point on the graph represents the mean ± SEM (n = 3 separate experiments). * and ** indicate $p<0.05$ and $p<0.01$, respectively.

The effect of Aroclor 1254 exposure on EROD activity was also assessed, and compared with a methanol solvent control. A significant induction of EROD activity was observed ($p<0.005$, Kruskal-Wallis test), although as observed for the expression of CYP genes exposure to methanol repressed EROD activity compared to untreated larvae (Figure 3.6).

3.3.5 The effects of CYP inhibitors α-naphthoflavone and SKF525A on EROD, ECOD and OOMR activity in vivo

Exposure of larvae to increasing concentrations of α-naphthoflavone for the duration of the EROD assay resulted in a dose-dependent induction in resorufin production compared to solvent controls (Figure 3.7A, $p<0.05$ for 5 and 10 μM exposures). Co-exposure of SKF525A with ethoxycoumarin did not cause any significant inhibition of ECOD activity (Figure 3.7B), however, OOMR activity was significantly increased with co-exposure of substrate and SKF525A ($p<0.05$, Mann Whitney U test, Figure 3.7B), indicating an inability to show CYP inhibition due to induction in the *in vivo* assay.

A



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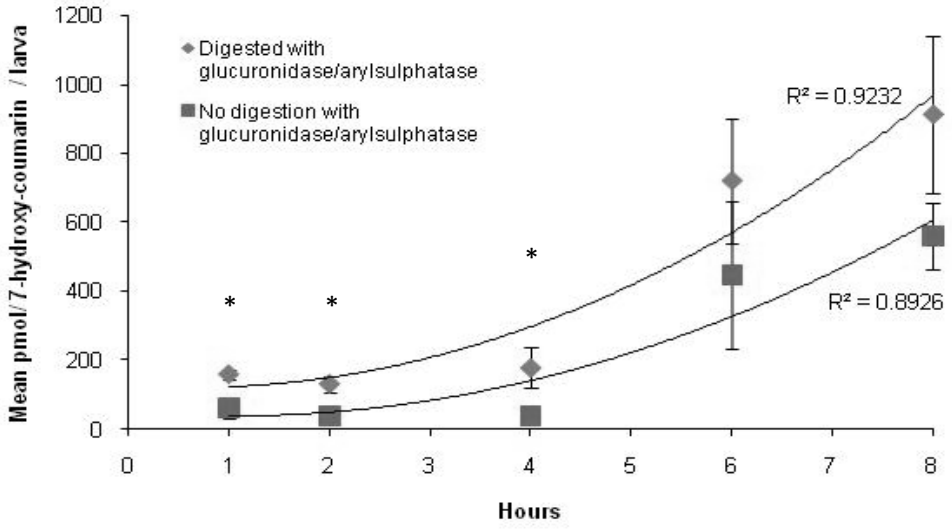


Figure 3.3: ECOD assay. Thirty zebrafish larvae per time-point were incubated over 8 hours with 100 μM 7-ethoxycoumarin before deconjugation by β-glucuronidase/arylsulphatase at 37 ± 1°C for 2 hours (A) and the comparison of ECOD activity with and without deconjugation (B). Each point on the graph represents mean ± SEM (n = 3 separate experiments). * indicates $p<0.05$.

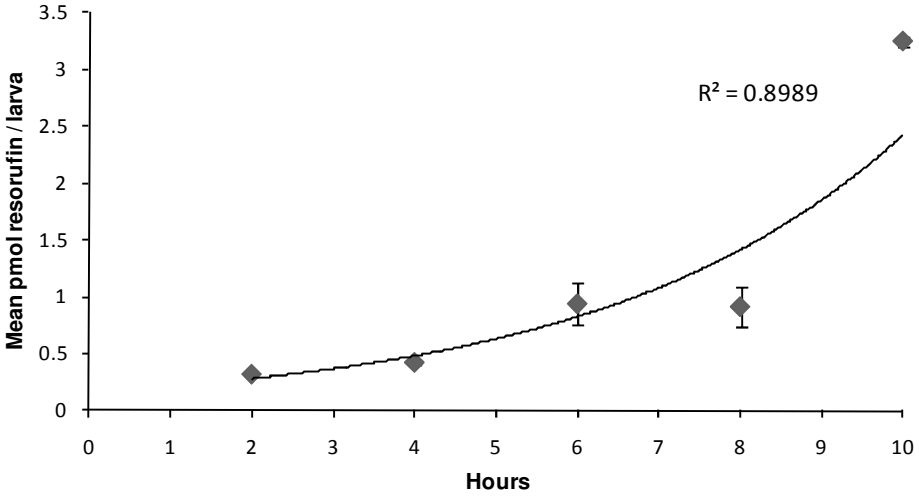


Figure 3.4: OOMR Assay. Thirty zebrafish per time-point were incubated with the OOMR substrate for up to 10 hours at 28 ± 1°C before deconjugation by β-glucuronidase/arylsulphatase at 37 ± 1°C for 2 hours. Each point on the graph represents the mean ± SEM (n = 3 separate experiments).

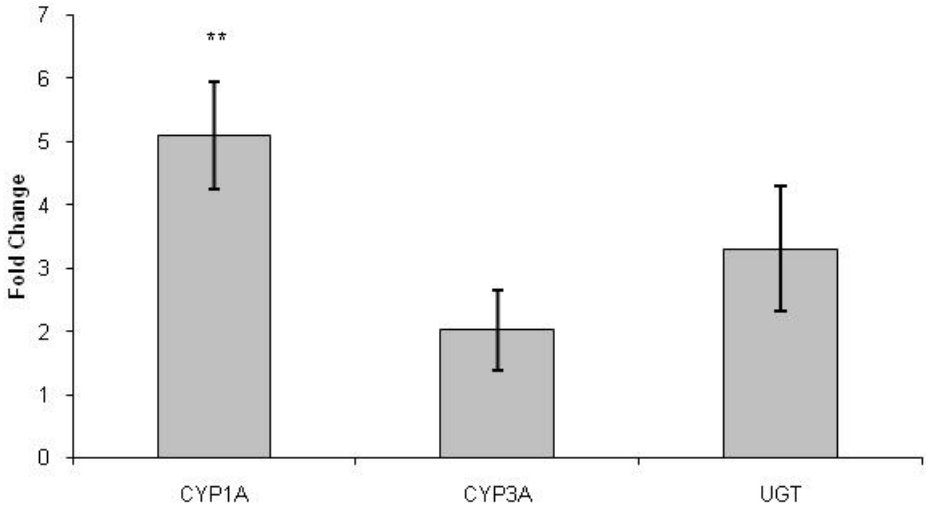


Figure 3.5: The expression of zebrafish genes CYP1A, zgc:153269 (CYP3A) and UGT1 was assessed following treatment with Aroclor 1254 (100 µg/l for 24 hours) compared to changes induced by 0.05% v/v methanol (solvent control) by qPCR. Gene expression was normalised to that of CYP2J26, as the expression of this gene was unaffected by either treatment (100 pooled larvae per sample; n = 3; mean ± SEM). ** Indicates $p<0.01$.

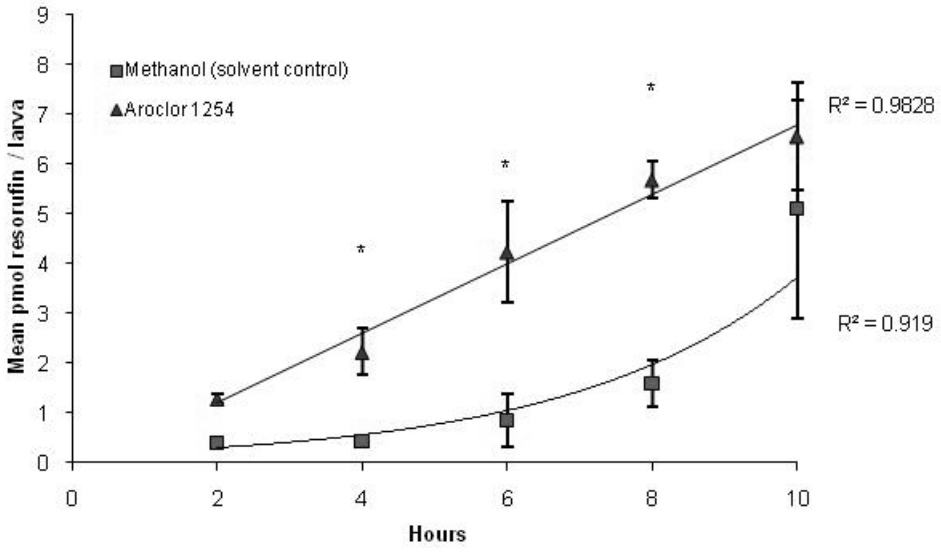


Figure 3.6: EROD assay for zebrafish larvae with deconjugation (as described in the text) after a 24-hour pre-treatment with methanol only (0.05% v/v) or Aroclor 1254 (100 µg/l dissolved in methanol) (30 pooled larvae per time-point; n = 3 separate experiments; mean ± SEM). * Indicates $p<0.05$.

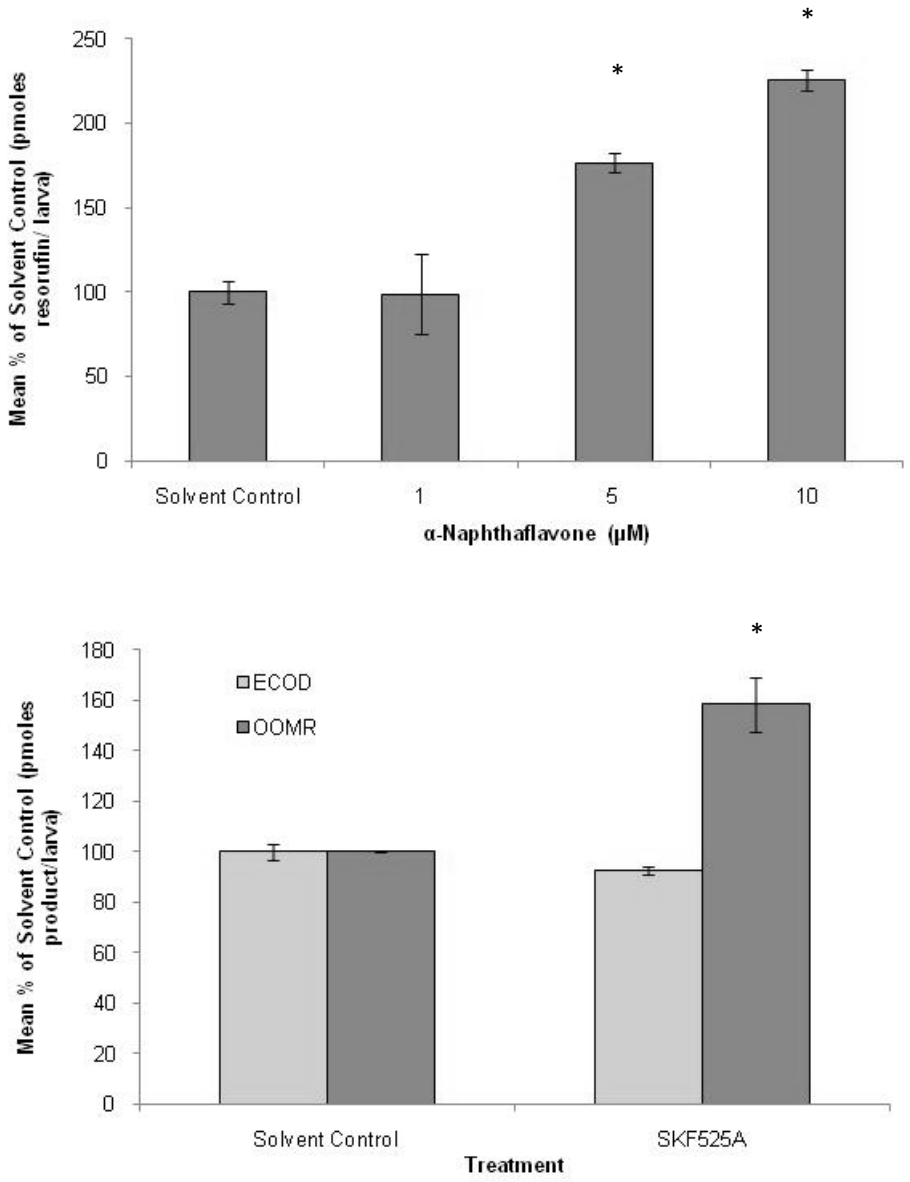


Figure 3.7: The effects of α-naphthoflavone and SKF525A on EROD, ECOD and OOMR activities in zebrafish larvae (96 hpf). Zebrafish larvae (30 larvae per replicate) were exposed to the appropriate substrate and inhibitor for 2 hours (ECOD assay), 4 hours (EROD assay) or 10 hours (OOMR assay) and assessed for product formation. Figure A shows the effect of α-naphthoflavone exposure upon EROD activity. Figure B shows the effect of SKF525A upon ECOD and OOMR activities. The graphs show mean percentage of the solvent control ± SEM (n = 3 separate experiments). * Indicates $p<0.05$.

3.4 Discussion

This study has assessed the expression of genes that have been identified as similar to mammalian CYP1A, CYP2B6, CYP3A5 and UGT1A1. There have been many CYP genes identified in the zebrafish genome, however the expression of genes similar to the mammalian genes responsible for xenobiotic metabolism is less well studied. There is also substantial difficulty identifying zebrafish orthologs to mammalian CYP genes, due in part to the criteria for CYP identification being based upon similarities in sequence identity to mammalian genes, and also in part to the larger number of CYP genes present in the zebrafish genome. Goldstone et al (2009) have reported identifying 89 CYP genes in the zebrafish genome compared with the 57 CYP genes in the human genome, http://drnelson.utmem.edu/CytochromeP450.html). As a result of these difficulties, based upon sequence identity, the genes CYP2J26 (zgc:91790), the CYP-gene zgc:153269 and UGT1A1 (zgc:123097) were identified as orthologs to the mammalian CYP2B6, CYP3A5 and UGT1A1, respectively. This identification has not considered any functional similarities or substrate specificity, and is based purely upon gene sequence similarity. These genes were selected to be representative of the key mammalian families responsible for the oxidative metabolism of a variety of pharmaceuticals, and expression was assessed throughout development. Gene expression appears to broadly increase throughout normal embryonic development, with higher expression observed in larvae post-hatching. Each of the different CYP genes assessed showed distinctive patterns of expression, with that observed for CYP1A gradually increasing throughout development, in agreement with previously published expression time-courses in zebrafish (Jőnsson et al 2007). The CYP-gene zgc:153269 was observed to have an expression pattern different to those observed for CYP3A65 and CYP3C1 in zebrafish, with higher expression of the gene at 24 hpf than at 48 or 72 hpf, with an increase in expression by 96 hpf (Corley-Smith et al 2005, Tseng et al 2005), whereas the expression of CYP2J26 and UGT1A1 has not previously been assessed throughout development in zebrafish. In

this study, both these genes gradually increase in expression throughout early development (24-96 hpf). The observation that CYP-genes are expressed in distinct patterns is consistent with the observation of distinct and specific patterns of expression in embryonic and newborn mice (Choudhary et al 2005, Hart et al 2009). Goldstone et al (2009) have also reported that the majority of CYP genes in zebrafish have distinct temporal patterns of expression throughout development. It has been proposed that although the CYP1-3 family enzymes are generally regarded as xenobiotic-metabolising enzymes, due to these distinct expression patterns seen throughout development, that these genes have a critical role in normal embryonic development (Choudhary et al 2004). Indeed, it has been shown that CYP1B1 is required for the normal development of the eye (Choudhary et al 2007). The CYP1-3 families have also been reported to metabolise a range of endogenous signalling molecules implicated in embryonic development, such as steroids, retinoid, fatty acids and vitamin D (Choudhary et al 2004). It is thus possible that the observed temporal patterns of expression for the identified CYP genes in zebrafish have a role in endogenous molecule signalling required for normal development. It has also been demonstrated that CYP1A was significantly induced. The CYP-gene zgc:153269 (CYP3) and UGT1A1 also had a higher level of expression, albeit not statistically significant, after exposure to Aroclor 1254, a known modulator of drug metabolism genes, whereas CYP2J26 levels were not altered.

Activities towards 7-ethoxyresorufin, 7-ethoxycoumarin and octyloxymethylresorufin were detected in an *in vivo* assay format. These assays required the uptake, metabolism and excretion of the substrates for any activity to be detected, and each substrate is a broad marker for CYP1, CYP2 and CYP3 families respectively. Due to these considerations, kinetic analysis of the enzyme activity *per se* could not be attempted. The excretion of the fluorescent products into the surrounding media is required for the detection of activity in these assays. Zebrafish have been reported to express ABC transporter proteins such as multidrug resistance protein 1 (MDR1) in adult livers, and the ABC transporter inhibitor

cyclosporine A causes a substantial increase in rhodamine B accumulation in zebrafish embryos (24 hpf, Bresolin et al 2005, Scholz et al 2008). In addition to the presence of drug transporters, organs important for the excretion of compounds such as the gills develop from 72 hpf onwards (Kimmel et al 1995). The use of 7-ethoxyresorufin as a substrate in a live larval zebrafish assay has been previously reported by Noury et al (2006), and EROD activity has also been assessed using fluorescence microscopy in Japanese medaka and zebrafish larvae (Carney et al 2004, Billard et al 2006, Carney et al 2008). The rate of product formation observed by Noury et al (2006) is similar to that calculated for a linear trend reported in this study (2.93 fmoles/larvae/minute) however, this does not take into account conjugative (phase II) metabolism, as resorufin has been reported to undergo glucuronidation which prevents the detection of the fluorescent product (Behrens et al 1998). The use of β-glucuronidase/arylsulphatase resulted in an approximately 6-fold increase in the amount of product formation over a period of 10 hours, and demonstrated that substantial phenolic conjugation was occurring. The metabolism of 7-ethoxycoumarin to 7-hydroxycoumarin has been associated with several mammalian CYP2 isoforms including CYP2B, CYP2C and CYP2E1 (Kern et al 1997). Significant conjugative (phase II) metabolism was also demonstrated in this assay. These demonstrations of phenolic conjugation are the first, to the knowledge of the author, of these processes functioning *in vivo* in zebrafish larvae. Octyloxymethylresorufin metabolism to resorufin is primarily catalysed by CYP3A4 and CYP3A5 isoforms, and the detection of activities towards both of these substrates for zebrafish larvae suggests that these larvae possess systems similar to CYP2 and CYP3 family enzymes. The non-linear increases in fluorescence observed in the ECOD and OOMR assays have been attributed to the metabolism of ethoxy-coumarin and octyloxymethylresorufin by multiple CYP isoforms. The demonstration of phenolic conjugation in the EROD and ECOD assays is the first *in vivo* demonstration of this process in zebrafish larvae known to the author. The observation that zebrafish larvae can metabolise broad specificity CYP substrates *in vivo*

agrees with the reported metabolism of 7-ethoxyresorufin by larval homogenates and the CYP3A-specific 6β-hydroxylation of testosterone in zebrafish primary hepatocytes (Mattingly and Toscano 2001, Reschly et al 2008). Whole-body homogenates of zebrafish larvae have been reported to have a basal EROD activity of 2.5 pmol/min/mg protein (3 dpf, post-hatching) and 5.5 pmol/min/mg protein (4 dpf) respectively (Mattingly and Toscano 2001). Other substrates with broad specificities such as methoxyresorufin, pentoxyresorufin and benzyloxyresorufin have been used to assess CYP activities in microsomal preparations from zebrafish fry that are free-feeding (>5 dpf, Aukwe et al 2008). Taken together, the work presented here demonstrates that zebrafish larvae (<5 dpf) express genes and have activities similar to those observed in mammalian systems for the metabolism of xenobiotics, as has been observed for zebrafish at later stages of fish development. The detection of these enzyme activities co-incides with increased expression of CYP and UGT genes during the development of these early life stages.

The modulation of EROD activity *in vivo* by α-naphthoflavone and Aroclor 1254 was also assessed. Pre-exposure to Aroclor 1254 for 24 hours resulted in the induction of EROD activity compared to a methanol solvent control. This observation is consistent with the observed induction of CYP1A gene expression, and the reported effects of polychlorinated biphenyls on fish species, although adult zebrafish have been reported to be insensitive to EROD induction by Aroclor 1254 exposure (Troxel et al 1997, Borlak and Thum 2001). Induction of CYP1A is mediated via the aryl-hydrocarbon receptor and this regulatory system has been extensively studied in zebrafish, with AhR agonists such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), β-naphthoflavone and a mixture of polychlorinated biphenyls inducing CYP1 gene expression and EROD activity (Hahn 1998, Örn et al 1998, Carney et al 2006, Jőnsson et al 2007). It should be noted that there was an observed inhibitory effect of methanol, which was used as a solvent for the

Aroclor 1254 (as supplied), at both the gene expression and enzyme activity levels. The basis of this modulation is further investigated and discussed in Chapter 5. It has also been demonstrated that the *in vitro* CYP1 inhibitor α-naphthoflavone also induces EROD activity in a dose-dependent manner using the *in vivo* EROD assay. Although it would be expected that α-naphthoflavone would inhibit EROD activity, it should be noted that α-naphthoflavone is a weak agonist for the aryl hydrocarbon receptor in zebrafish, and has been shown to induce CYP1 gene expression in larvae (Timme-Laragy et al 2007). Billard et al (2006) have also reported that exposure to α-naphthoflavone alone does not inhibit EROD activity *in vivo* using 96 hpf zebrafish larvae. The studies of Timme-Laragy et al (2007) and Billard et al (2006) used fluorescence microscopy to assess the accumulation of resorufin in the gastro-intestinal tract as a measure of EROD activity, and therefore may not account for the amount of conjugated resorufin present, whereas this study has observed an induction in the total amount of resorufin excreted (including conjugated resorufin) upon co-exposure to α-naphthoflavone. As a 4-hour co-exposure of α-naphthoflavone with the substrate was utilized in this work it also suggests that the observed induction of EROD activity is a rapid process.

The *in vitro* CYP inhibitor SKF525A had a very minor inhibitory effect upon ECOD activity in zebrafish larvae over 2 hours exposure. It is possible that this is because there was insufficient time for uptake of the inhibitor, however, it has also been demonstrated that SKF525A can induce CYP activities *in vivo*, such as OOMR activity, and thus the inhibitory effects of this compound could not be demonstrated. Although SKF525A is a general CYP inhibitor *in vitro*, it has been reported that SKF525A induces both pentoxyresorufin-*O*-dependentylase (PROD) and EROD activities in B6C3F1 mice (Jeong et al 2004). These observed increases in CYP activities highlight the differences in complexity between *in vitro* systems, such as tissue or whole organism homogenates, and *in vivo* systems where there is potential for inhibitory compounds to function as inducing agents via agonistic interaction with nuclear receptors. Overall, the modulation

of CYP activities in zebrafish by inhibitors of CYP enzymes reflects the complexity of combined inhibitory and inducing effects *in vivo*. The demonstration of the potential of compounds such as α-naphthoflavone to have a mix of inhibitory and inducing effects *in vivo* also has implications in environmental monitoring. EROD activity is routinely used as a biomarker in aquatic species for assessing exposure to polyaromatic hydrocarbons, polychlorinated biphenyls and other organic contaminants (Sarkar et al 2006). The potential for a chemical to both induce and inhibit EROD activity could result in a minimal or lack of response to exposure, and thus potentially make EROD activity an unreliable biomarker.

For zebrafish larvae to be a useful model organism in toxicity testing it is important that human metabolic processes are reflected by the zebrafish system. As discussed previously, it is difficult to identify orthologous genes to the mammalian CYP complement, and the identification of such genes provides no information regarding functional activities or substrate specificities. As discussed in both the general introduction and this chapter, EROD, ECOD and OOMR are substrates for a wide range of CYP isoforms and the demonstration of their metabolism in zebrafish larvae suggests that there are some similar activities between zebrafish and mammals. However, due to the lack of isoform specificity of these substrates, it is difficult to draw direct comparisons with mammalian systems. The use of isoform-characteristic reactions such as the 6α-hydroxylation of Paclitaxel (CYP2C8), tolbutamide hydroxylation (CYP2C9), the hydroxylation of coumarin (CYP2A6), and the 6β-hydroxylation of testosterone (CYP3A4), would further highlight similarities and differences between mammals and the zebrafish (Brown et al 2008). The metabolism of some common pharmaceuticals, such as ibuprofen (CYP2C8/9-catalysed hydroxylation) and the oxidation of paracetamol to *N*-acetyl-p-benzoquinoneimine (CYP2E1 and CYP3A4) have also been linked with a limited number of specific CYP

isoforms and could be used not only to demonstrate pharmaceutical metabolism, but to determine similarities in substrate specificities of mammalian and zebrafish xenobiotic metabolism systems (Kepp et al 1997, Tonge et al 1998). Overall, this study provides further evidence that zebrafish larvae express genes and activities similar to mammalian systems responsible for xenobiotic metabolism. Both oxidative and conjugative metabolism have been demonstrated, as has the inducibility of EROD activity by AhR agonists *in vivo*, supporting application of this model in toxicological testing and drug discovery and the use of zebrafish larvae for ecotoxicology testing.

Chapter 4

The assessment of non-steroidal anti-inflammatory drug (NSAID) metabolism by zebrafish larvae

4.1 Introduction

Chapter 3 demonstrated the presence of xenobiotic metabolism genes and enzyme activities similar to those identified in mammalian species in zebrafish larvae. However, further characterisation of these drug metabolism systems using more selective enzyme substrates and the demonstration of the metabolism of pharmaceuticals is required to further characterise the utility of these organisms in toxicity testing. The metabolism of pharmaceuticals by fish also has implications for the fate of these materials in the aquatic environment.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely used analgesics in both human and veterinary medicines. This class of pharmaceutical includes drugs such as diclofenac, acetaminophen and ibuprofen. The metabolic degradation of ibuprofen (2-[4-(2-methylpropyl)phenyl]propanoic acid) is well-described in mammals, involving oxidation of the parent compound to hydroxy-ibuprofen and carboxy-ibuprofen, and conjugation with glucuronic acid of both parent and metabolite compounds (Kepp et al 1997, Figure 4.1). The oxidation of ibuprofen is catalysed by the CYP2C8/9 isoforms in humans (Brown et al 2008). The metabolism of acetaminophen (*N*-(4-hydroxyphenyl)acetamide) can involve the direct conjugation of the parent compound to either glucuronic acid or sulfate moieties, as well as oxidative metabolism by CYP 2E1 and CYP2A6 (Graham et al 2005, Figure 4.2). It has also been suggested that CYP1A2 and CYP3A4 may have a role in the metabolism of acetaminophen into its toxic derivative *N*-acetyl-p-benzoquinoneimine (NPBQI), although the CYP2E1 isoform has been implicated *in vivo* as the major isoform for this metabolism in human volunteers administered therapeutic doses of acetaminophen (Chen et al 1998, Tonge et al 1998, Manyike et al 2000). This results in the metabolic activation of this compound to a toxic intermediate, that can cross-react with proteins, deplete cellular thiols, induce oxidative stress and result in damage to the liver (Pirmohamed 2008). Due to the relative specificity of these compounds in terms of metabolism by different CYP isoforms, and the potential for a variety of metabolic fates

(e.g. oxidative metabolism by CYPs, conjugation to glucuronic acid, sulfate and glutathione and subsequent degradation to a mercapturic acid), these compounds are good candidates as model substrates to assess metabolic capabilities in non-mammalian species.

Liquid Chromatography – Mass Spectrometry (with ion fragmentation, LC-MS(MS)) has emerged as a sensitive tool for the detection of pharmaceuticals and their derivatives. This technique has been used to detect pharmaceuticals in the aquatic environment, as well as in waste water treatment plant discharges (Xia et al 2005, Kasprzyk-Horden et al 2008, Palmer et al 2008). Typically, reverse phase liquid chromatography (LC) in combination with mass spectrometry (MS) use combinations of water, methanol and acetonitrile as a mobile phase, with the inclusion of mobile phase modifiers such as ammonium acetate, formic acid or acetic acid to improve chromatographic separation of the analytes (Korfmacher 2005). Ionisation of the column eluent is achieved using a combination of high voltage and heat at atmospheric pressure, with electrospray ionisation (ESI) utilizing the nebulisation of the eluent by high voltage resulting in the production of aerosol particles that allow individual ions to enter the mass spectrometer system (ion evaporation, Korfmacher 2005). There is a wide variety of different mass analysers available for application towards identifying pharmaceutical residues, including time of flight, quadropole and ion-trap instruments (Lim and Lord 2002). The use of multiple levels of ion fragmentation (MS^N) can be beneficial to the identification of pharmaceutical residues, where a precursor ion is fragmented into a variety of daughter ions which can be detected, and the process can be repeated multiple times, providing structural information for the precursor ions (Ermer and Vogel 2000).

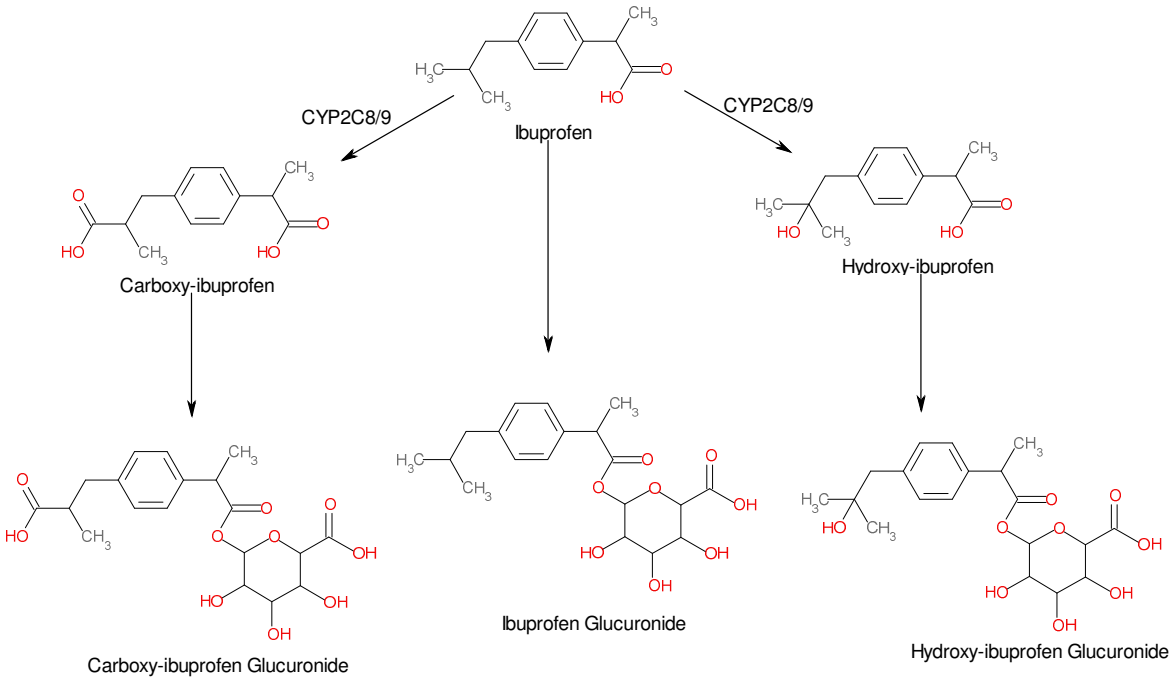


Figure 4.1. The metabolism of ibuprofen in mammals. Oxidative metabolism of ibuprofen to hydroxy-ibuprofen and carboxy-ibuprofen is mediated by CYP2C8/9. Conjugation of these metabolites, as well as ibuprofen, to glucuronic acid moieties also occurs.

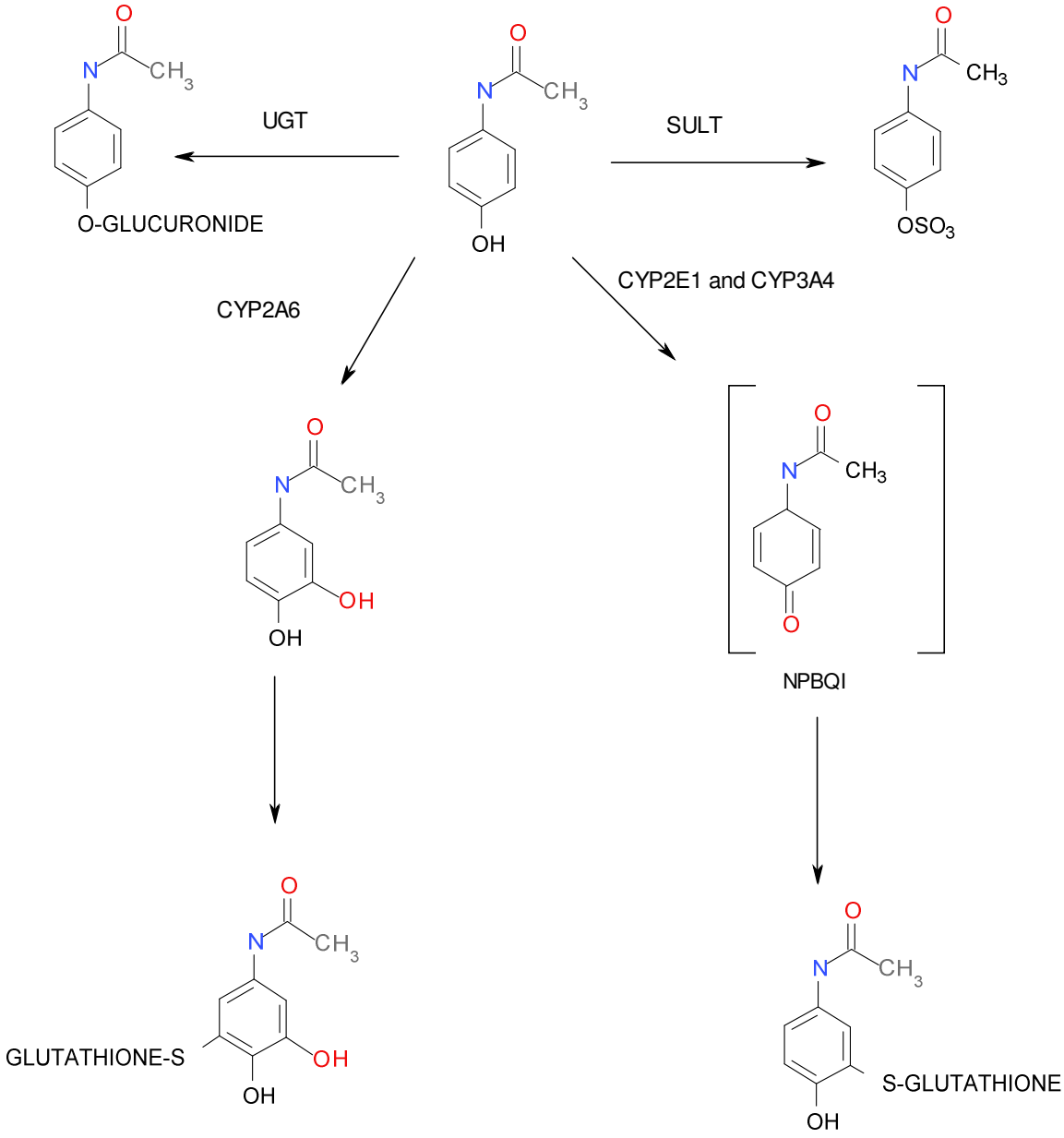


Figure 4.2. The metabolism of acetaminophen in mammals. The pathways of conjugative metabolism via the addition of sulfate or glucuronic acid moieties, as well oxidative metabolism, via cytochrome P450 isoforms, are represented. The formation of glutathione conjugates is also shown. These glutathione conjugates can undergo further metabolism to form mercapturic acid derivatives. UGT – UDP-glucuronosyl transferases. SULT – Sulfotransferases. NPBQI - *N*-acetyl-p-benzoquinoneimine.

In addition to this, the application of high resolution accurate ion mass measurements of both parent and daughter ions can allow the determination of the chemical composition of these species (Lim et al 2007).

In order to characterise further the extent of xenobiotic metabolism that occurs in zebrafish larvae (96 hpf) LC-MS(MS) and scintillation counting are used as sensitive methods of detecting and identifying derivatives of the NSAIDs acetaminophen and ibuprofen, selected due to the relative substrate specificities of these compounds for specific CYP isoforms, and range of potential metabolic fates. Due to limited access time on the machinery, the analysis of acetaminophen-exposed samples was assessed by LC-MS only.

4.2 Methods

To assess the incorporation of ^{14}C-ibuprofen zebrafish larvae (72 hpf, 10 larvae per replicate) were exposed to 100 μg/l of ^{14}C-ibuprofen or an ethanol solvent control for 0, 3, 6, 9, 12, 18 or 24 hours at 28 ± 1°C. These fish were then extracted by manual disruption between microscope slides coated in scintillation fluid, and collected in a microcentrifuge tube with scintillation fluid and Triton-X-100 added. These samples were incubated at room temperature overnight, before assessment by scintillation counting. For the assessment for ibuprofen metabolism using LC-MS(MS) zebrafish larvae (72 hpf, 100 per replicate) were exposed to 100 μg/l of ibuprofen, or ^{14}C-ibuprofen, or an ethanol solvent control (0.0015% v/v) for 24 hours. These samples were then extracted in dichloromethane, the solvent evaporated, and samples re-suspended in methanol:acetic acid:water. Water samples were also retained for analysis. These samples were then analysed by LC-MS(MS) as detailed in chapter 2. Fractions (1 minute fractions) from the LC separated fractions of ^{14}C-ibuprofen exposed samples (and solvent control samples) were collected and assessed by scintillation counting. To determine the extent of conjugation of ibuprofen and its metabolites, zebrafish larvae were exposed to ^{14}C-ibuprofen (100 μg/l, 72 hpf larvae, 15 larvae per replicate, n=3 per treatment) or ethanol (solvent control) for 24 hours, followed by the homogenisation of the larvae in 25 mM sodium acetate (pH 4.5) buffer. Samples were incubated at 37°C for 2 hours after the addition of β-glucuronidase/arylsulfatase (diluted 2664-fold) or no addition of enzyme mix to the samples. All larval homogenates and water samples were then extracted three times with dichloromethane, evaporated to dryness, and re-suspended in sterile water. Scintilation fluid was then added to the samples, which were then assessed for radioactivity. Both polar and non-polar fractions of the dichloromethane extractions were assessed by scintillation counting.

4.3 Results

4.3.1 Assessment of acetaminophen metabolism by zebrafish larvae

Zebrafish larvae were determined to have been exposed to nominal concentrations of 60 µg/l and 500 µg/l of acetaminophen respectively. An ion of *m/z* 152 and retention time 3.4 minutes (corresponding to acetaminophen) was identified in the water samples at all time-points and exposures sampled. No other acetaminophen derivatives were observed in the water samples. In larval extracts (500 µg/l, 24 hours exposure), an ion of *m/z* 230 (retention time 2.5 minutes) was detected. This ion was identified as acetaminophen sulfate based upon the *m/z* ratio and the comparison of the retention time of the ion with that of an analytical standard (Figure 4.3A and B). Two other ions of note were identified in larval extracts after 24 hours exposure at both concentrations used (*m/z* 455 and *m/z* 457, retention time 3.5 minutes, Figure 4.3C). These ions were tentatively identified as the negative and positive ion forms of the acetaminophen glutathione conjugate. Due to the lack of availability of an analytical standard it was not possible to use retention time to help identify these ions more thoroughly. Neither of the identified ions was observed in larval extracts or water samples from the solvent only exposures.

4.3.2 Larval uptake of ^{14}C-ibuprofen

Over a 24 hour time-course there was a steady increase in the incorporation of ^{14}C measured in the zebrafish larval extracts exposed to 100 µg ^{14}C-ibuprofen/l (500 nM, Figure 4.4). Although only approximately 2.5% of the total ibuprofen administered to the water was incorporated into the larvae by 24 hours, this measurement does not include any material that is uptaken, metabolised and excreted by the larvae. Water samples from each time-point throughout the time-course did not show any significant changes in the ^{14}C present in these samples, however, the qualitative context did change (see below).

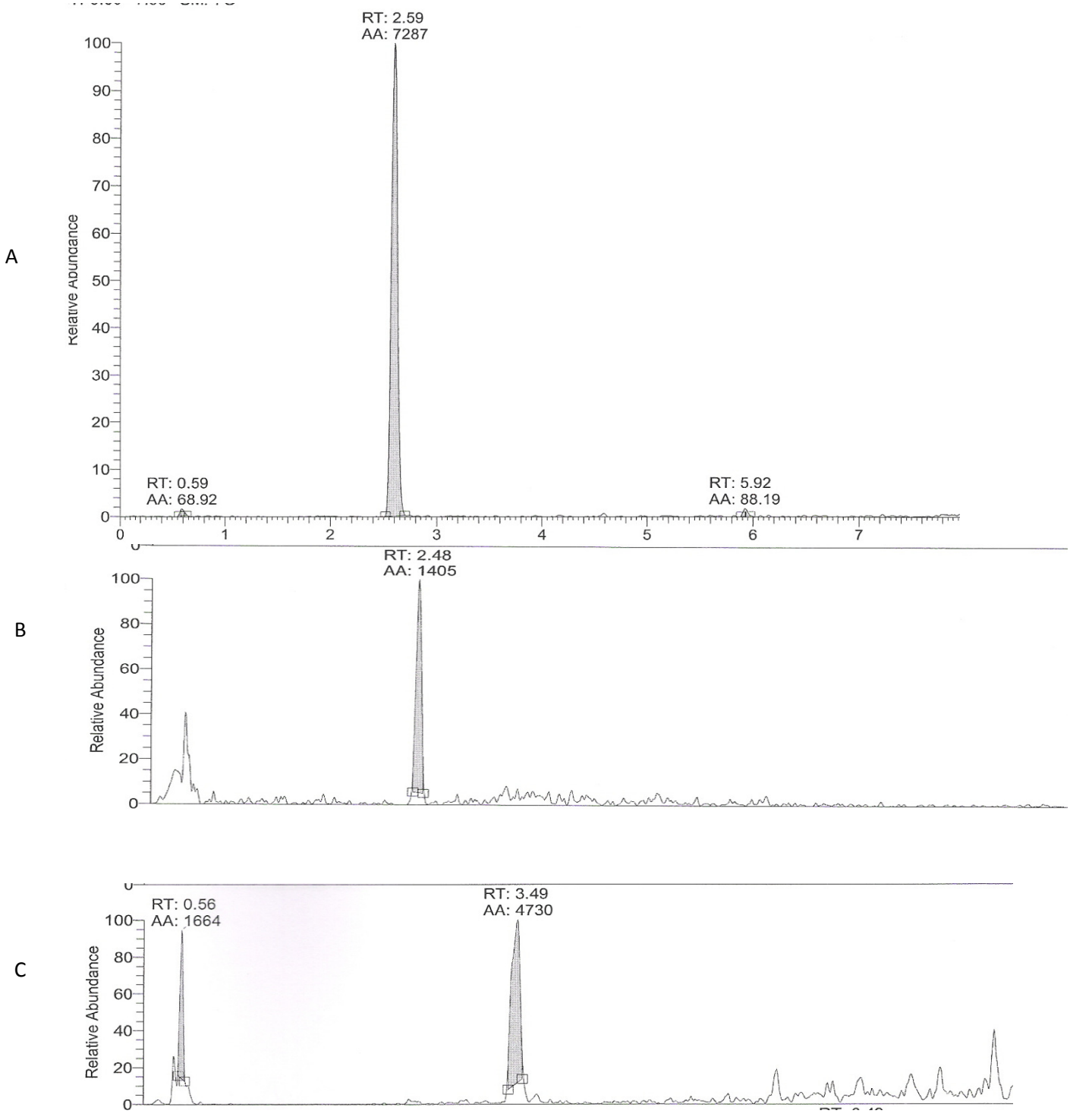


Figure 4.3: The metabolism of acetaminophen by zebrafish larvae assessed by LC-MS. An analytical standard for acetaminophen sulfate (A) was detected at *m/z* 230. An ion of corresponding *m/z* and retention time was identified in larval extracts (B). An ion of *m/z* 455 was also detected (C) and identified as acetaminophen glutathione.

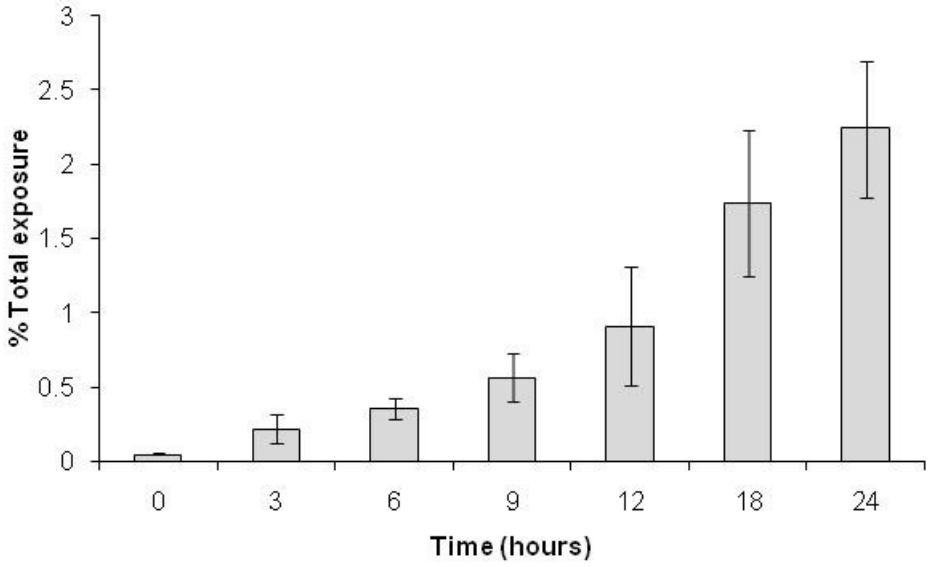


Figure 4.4: Mean ± standard error of the mean % of the total dose of ^{14}C-ibuprofen incorporated into zebrafish larvae exposed to 100 µg ^{14}C ibuprofen/l, over a 24 hour period. (n = 3 replicates, each containing 10 larvae).

Table 4.1: The *m/z* ratio measured by LC-MS(MS) for acetaminophen, ibuprofen and the metabolites of these compounds

A)

Putative identity	Theoretical *m/z*	Measured *m/z*
Acetaminophen	152.06	152
Acetaminophen sulfate	230.02	230
Acetaminophen Glutathione	455.13	455

B)

Putative identity	Theoretical *m/z*	Measured *m/z*
Ibuprofen	205.12340	205.12427 (larval extracts) 205.12447 (water samples)
Ibuprofen	161.13357	161.13429 (larval extracts) 161.13455 (water samples)
Hydroxy-ibuprofen	221.11832	221.11906 (larval extracts) 221.11932 (water samples)
Hydroxy-ibuprofen	177.12948	177.12946 (larval extracts) 177.12956 (water samples)
^{14}C-Ibuprofen	207.12	207
^{14}C-Ibuprofen	161.13	161
^{14}C-Hydroxy-ibuprofen	223.12	223
^{14}C-Hydroxy-ibuprofen	177.13	177

The m/z ratios assessed for (A) acetaminophen and (B) ibuprofen, including the m/z ratio for the metabolites of each parent compound identified in this study. Where appropriate, the accurate m/z ratio is given. Otherwise the m/z ratio used to detect the ion is given.

4.3.3 Assessment of larval extracts for ibuprofen metabolites using LC-MS

Using an analytical standard for ibuprofen sodium salt the protocol described in the methods section (Chapter 2, section 2.12) was optimised. Detection of a MSMS transition *m/z* 205 → 161 corresponded to a loss of *m/z* 44, which was attributed to the loss of carbon dioxide. The use of accurate ion mass determination was used to confirm the empirical formulae of both parent (*m/z* 205) and fragment (*m/z* 161) ions. Following exposure to 100 µg ibuprofen/l for 6 hours no metabolites of ibuprofen were detected. Ibuprofen was detected in larval extracts after six hours exposure (100 µg/l) by LC-MS(MS) and accurate ion mass determination (retention time 5.3 minutes). After 24 hours exposure to 100 µg ibuprofen/l, hydroxy-ibuprofen was identified in larval extracts (retention time 4.0 minutes, Figure 4.5). This identification was based upon the detection of parent (*m/z* 221) and fragment (*m/z* 177) ions, corresponding to a loss of *m/z* 44 (as observed for ibuprofen, attributed to the loss of carbon dioxide). The accurate ion mass determination for both parent and fragment ions of the identified hydroxy-ibuprofen confirmed the empirical formulae of both these ions. No other metabolites were identified in the larval extracts.

The use of ^{14}C-ibuprofen as a standard resulted in identification of ^{14}C-ibuprofen based on a MSMS transition of *m/z* 207 → 161, corresponding to a loss of 46, which was attributed to a loss of $^{14}CO_2$. Hydroxy-ibuprofen was identified after 24 hours exposure to 100 µg ^{14}C-ibuprofen/l (retention time 4.2 minutes), with this identification based upon the detection of a MSMS transition *m/z* 223 → 177 (Figure 4.6), in agreement with the detection of a radio-trace signal (retention time = 4.2 minutes). No other metabolites of ibuprofen were observed in these samples, although ibuprofen was identified in these samples based upon an MSMS transition *m/z* 207 → 161 (loss of *m/z* 46, retention time 5.7 minutes). Both parent compound and the hydroxy-ibuprofen metabolite were

quantified using scintillation counting of fractions separated by the LC method used in the LC-MS analysis (Table 4.1).

4.3.4 Assessment of water samples for ibuprofen metabolites using LC-MS

Ibuprofen was identified in water samples after both 6 and 24 hours exposure to 100 µg ibuprofen/l. Hydroxy-ibuprofen was also detected only after 24 hours exposure (retention time = 4.0 minutes), with this identification based upon the detection of parent and fragment ions (*m/z* 221 → 177) and confirmation of the empirical formulae of both these ions by accurate ion mass determination. No other metabolites were identified in the water samples (Figure 4.7).

After exposure to 100 µg ^{14}C-ibuprofen/l (24 hours) hydroxy-ibuprofen was identified based on retention time (4.2 minutes) and MSMS fragmentation (*m/z* 223 → 177). A second potential hydroxy-ibuprofen metabolite was also observed based upon this fragmentation transition that was not present in the solvent control or larval extracts (retention time = 5.6 minutes, Figure 4.8). This second metabolite co-eluted with ibuprofen, thus it was not possible to use radio-trace or scintillation counting to assess if this metabolite contained ^{14}C. The amount of hydroxy-ibuprofen present in water samples was quantified by scintillation counting of fractions separated by LC (Table 4.1).

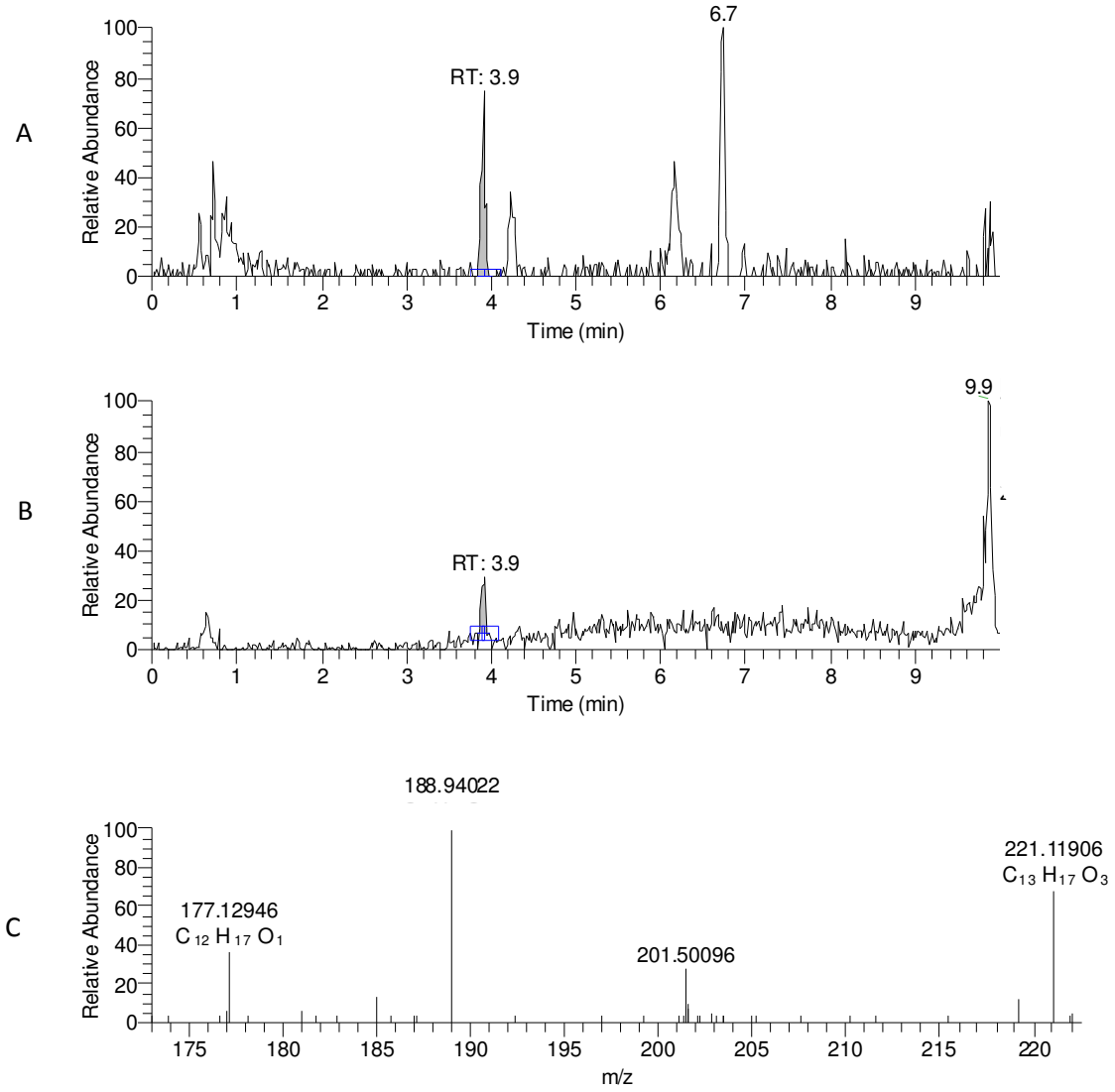


Figure 4.5: Identification of hydroxy-ibuprofen in larval extracts, from fish exposed to 100 µg ibuprofen/l for 24 hours. Identification of parent ion (*m/z* 221, A), fragment ion (*m/z* 177, B) and accurate ion mass determinations for both ions (C) used to identify hydroxy-ibuprofen.

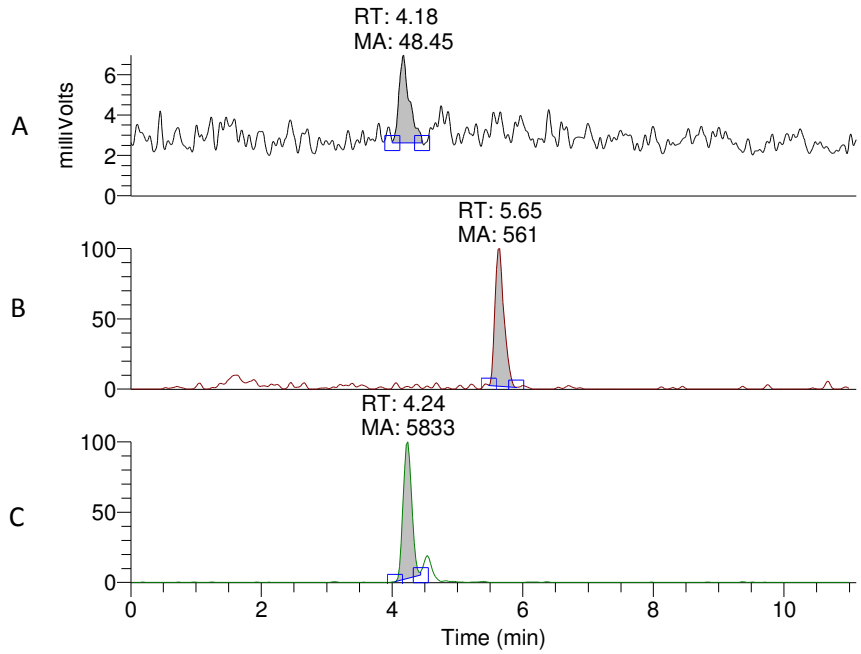


Figure 4.6: The detection of ^{14}C-hydroxy-ibuprofen using LC-MS(MS) in larval extracts from 24 hours exposure to 100 µg ^{14}C-ibuprofen /l. The hydroxy-ibuprofen metabolite was identified based upon radio-trace (A) and detection of MSMS transition 223 → 177 (C). Panel B shows the detection of ^{14}C-ibuprofen in larval extracts using MSMS transition 207 → 161. Panels B and C measured as relative abundance.

Table 4.2: Quantification of ibuprofen and hydroxy-ibuprofen in water samples and larval extracts.

Sample	1	2	3	Mean	Standard error of the mean
Water samples					
Ibuprofen (nmoles)	29.1	39.3	39.9	36.1	3.50
(% of total)	75.9	99.9	98.6	91.7	
Hydroxy-ibuprofen (nmoles)	9.2	0	0.5	3.2	2.99
(% of total)	23.9	0	1.3	8.2	
Ratio of Hydroxy-ibuprofen: ibuprofen	0.32	0.00	0.01	0.09	0.10
%	31.62	0.00	1.25	8.86	10.34
Larval extracts					
Ibuprofen (pmoles)	9.5	9.5	10.1	9.7	0.20
(% of total)	0.03	0.02	0.03	0.03	
Hydroxy-ibuprofen (pmoles)	44.4	27.9	26.6	32.9	5.73
(% of total)	0.12	0.07	0.07	0.08	
Unknown Metabolite (pmoles)	6.3	3.8	3.2	4.4	0.95
(% of total)	0.02	0.01	0.01	0.01	0.00
Ratio of Hydroxy-ibuprofen: ibuprofen	4.67	2.94	2.63	3.39	0.64
%	467.37	293.68	263.37	339.18	63.55
Ratio of unknown metabolite: ibuprofen	0.66	0.40	0.32	0.45	0.10
%	66.32	40.00	31.68	45.36	10.44

Quantification of ibuprofen derivatives identified in zebrafish larval extracts and water samples by LC-MS and scintillation counting. One hundred pooled larvae were used per replicate, with 100 µg/l of ^{14}C-ibuprofen added to the water. Larvae were extracted using dichloromethane and water samples retained after 24 hours exposure to the radiolabelled ibuprofen.

4.3.5 Assessment of ibuprofen metabolites in larval extracts and water samples using scintillation counting of LC fractions

The larval extracts and water samples from the ^{14}C-ibuprofen exposures were separated into fractions using the LC method from the LC-MS(MS) procedure, with each fraction analysed using scintillation counting for the presence of ^{14}C. There was no detection of radioactivity in either larval extracts or water samples from the solvent controls. For larval extracts there were significant peaks of ^{14}C detected in fractions 2 to 3 minutes, 3 to 4 minutes, 4 to 5 minutes and 5 to 6 minutes ($p < 0.05$ Mann-Whitney U test, Figure 4.9A). The 3 to 4 and 4 to 5 minutes fractions were attributed to hydroxy-ibuprofen (as this peak elutes at approximately 4 minutes and can therefore result in signals in both 3 to 4 and 4 to 5 minute fractions) and the peak in the 5 to 6 minute fraction has been attributed to the parent compound ibuprofen based upon the retention time from LC-MS(MS) experiments (Retention Time = 5.6 minutes). The peak identified at 2 to 3 minutes could not be identified based upon LC-MS(MS) experiments. The water sample fractions had detectable peaks in fractions 3 to 4 minutes and 5 to 6 minutes respectively (Figure 4.9b). These peaks were assigned to hydroxy-ibuprofen (3 to 4 minutes) and ibuprofen (5 to 6 minutes) based upon retention times. It should be noted that the peak in 3 to 4 minutes fraction is not statistically significantly different from the solvent control ($p > 0.05$, Mann-Whitney U test), whereas the peak corresponding to ibuprofen (fraction 5 to 6 minutes) is statistically significant ($p < 0.05$, Mann-Whitney U test).

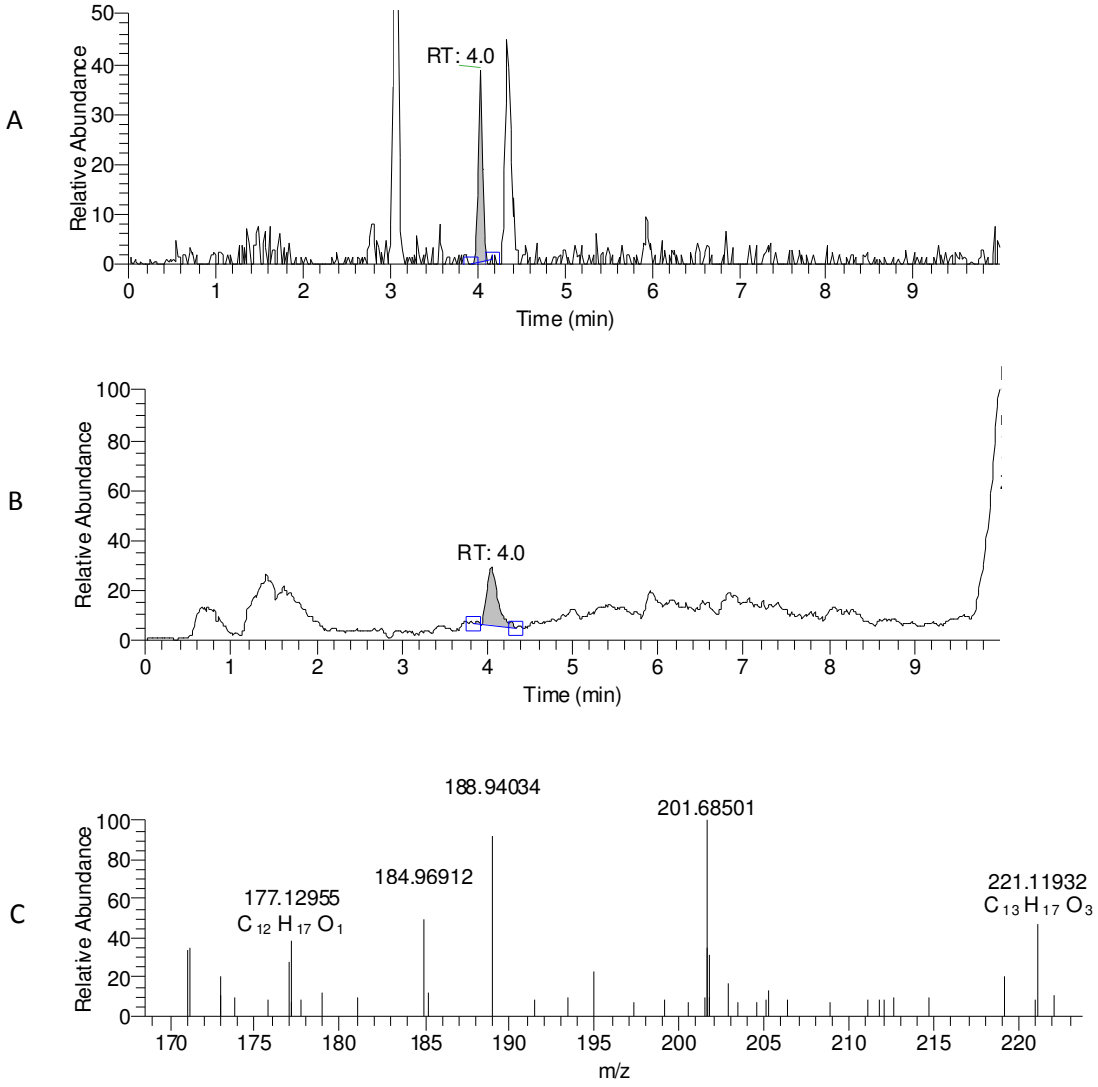


Figure 4.7: Identification of hydroxy-ibuprofen in water samples, from exposures to 100 µg ibuprofen/l for 24 hours. Identification of parent ion (*m/z* 221, A), fragment ion (*m/z* 177, B) and accurate ion mass determinations for both ions (C) used to identify hydroxy-ibuprofen.

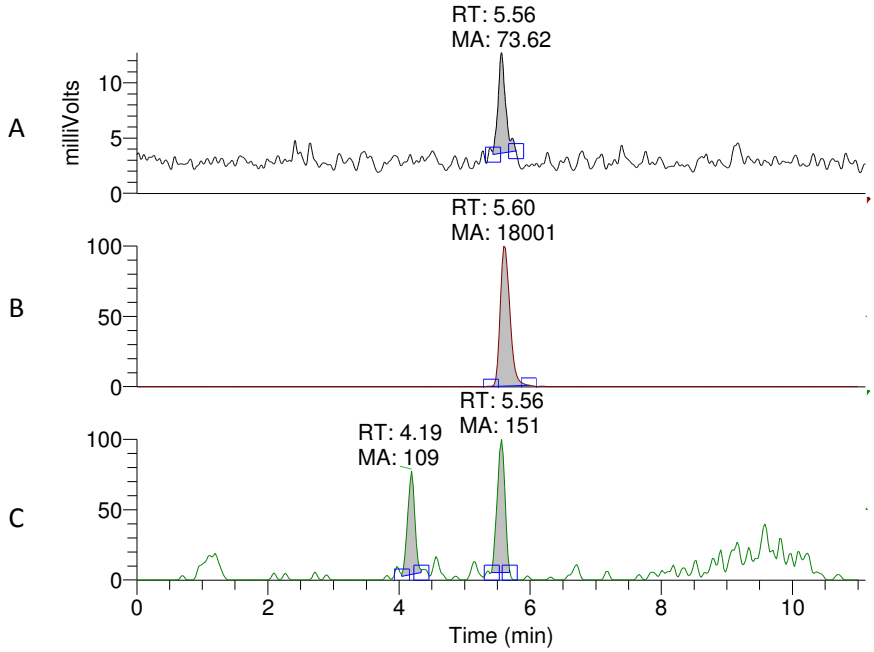
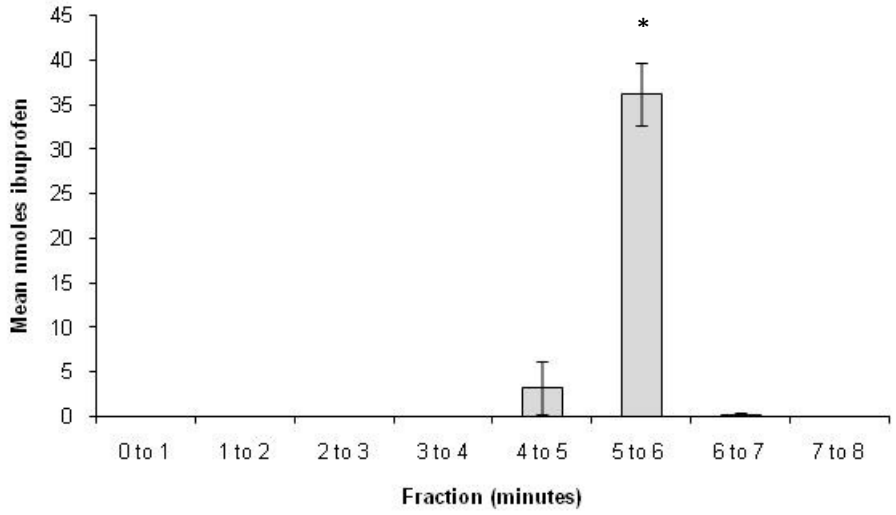


Figure 4.8: The detection of two hydroxy-ibuprofen metabolites in water samples following exposure of zebrafish to 100 µg ^{14}C-ibuprofen/l for 24 hours). Panels A and B show the ^{14}C radio-trace and the detection of ibuprofen. Panel C shows two peaks (retention time = 4.19 and 5.56 respectively) with a MSMS transition of 223 → 177. The peak at retention time = 4.19 minutes corresponds to the hydroxy-ibuprofen metabolite identified in larval extracts. Panels B and C measured as relative abundance.

A



B

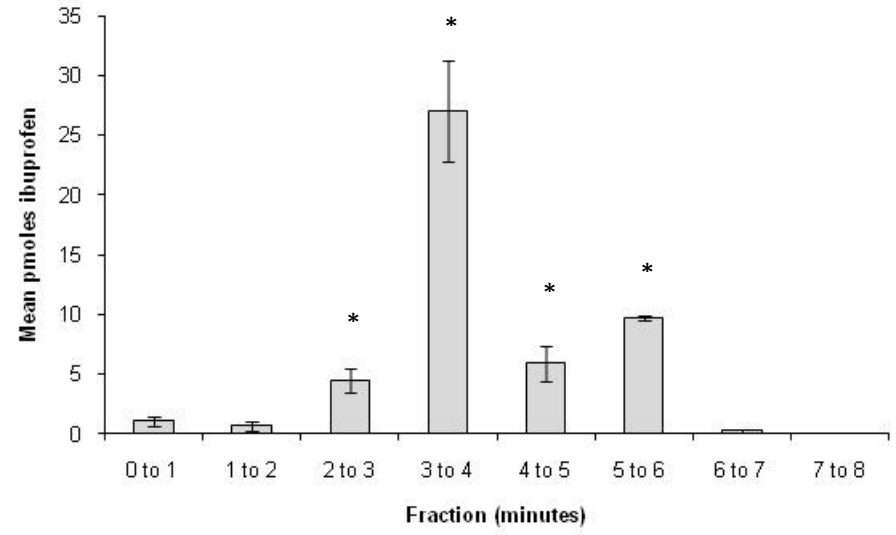
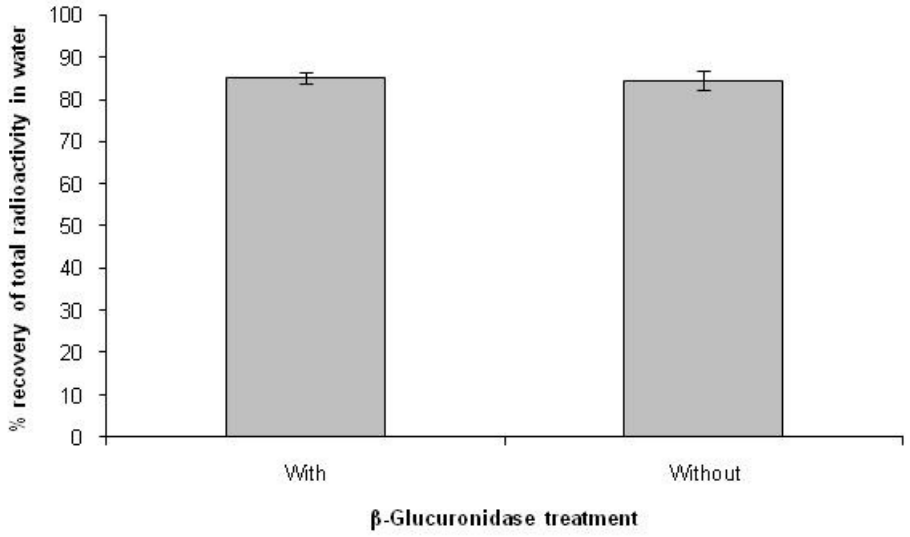


Figure 4.9: Scintillation counting of larval extracts and water sample fractions from 24 hours exposure (100 µg/l). Water samples (A) and larval extracts (B) were fractionated using the LC settings from the LC-MS(MS) method**.** * indicates $p<0.05$.

4.3.6 Assessment of conjugation by scintillation counting

It was determined that the recovery of radioactivity in dichloromethane extracts (non-polar fractions) of larval homogenates and water samples incubated with sodium acetate buffer only or buffer with β-glucuronidase/arylsulphatase added was approximately 86% of the total detected radioactivity in both polar and non-polar fractions of these samples. There was no significant difference between the amount of radioactivity detected in either larval extracts with or without β-glucuronidase/arylsulphatase incubation or water samples with β-glucuronidase/arylsulphatase ($p>0.05$, Figure 4.10 A and B).

A



B

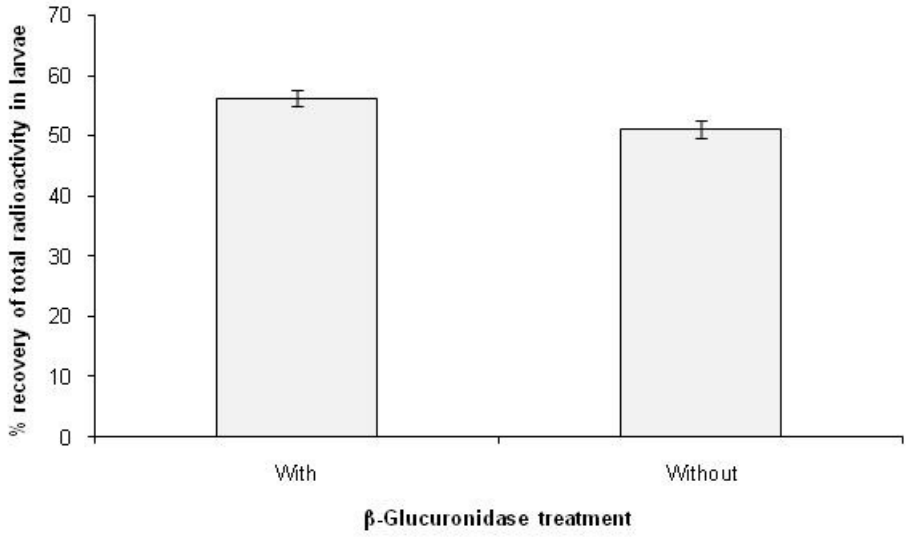


Figure 4.10. The scintillation counting of water samples (A) and larval homogenates (B) extracted with dichloromethane after incubation with β-glucuronidase or buffer. Recovery of radioactivity was assessed by scintillation counting. Mean values (n=3) ± SEM are shown. Relative % of total radioactivity was 92% in the water fractions and 8% in the larval homogenates.

4.4 Discussion

To further assess the potential for xenobiotic metabolism in zebrafish larvae, we have exposed larvae to two model pharmaceuticals, acetaminophen and ibuprofen, and searched for derivatives of these compounds known from mammalian systems using LC-MS(MS). The aim of this work was to identify metabolites that demonstrate a wide variety of metabolic processes. Evidence is provided here that suggests that zebrafish larvae have the ability to metabolise the NSAID acetaminophen and ibuprofen via oxidative and conjugative pathways. To our knowledge this is the first study to assess the metabolism of pharmaceutical compounds in zebrafish larvae.

In mammalian systems, acetaminophen can undergo direct conjugation to both glucuronic acid and sulfate moieties, in addition to cytochrome P450 mediated metabolism followed by glutathione conjugation and degradation to a mecapturic acid derivative (Black 1984). A metabolite identified as acetaminophen sulfate (based upon *m/z* ratio and comparison of retention time with that of an analytical standard) was detected in larval extracts after 24 hours exposure (500 µg/l). It was not possible to assess the concentration of acetaminophen glucuronide conjugates in this study due to a lack of sensitivity for this metabolite. Direct infusion of an analytical standard of acetaminophen glucuronide demonstrated that this particular derivative does not ionise well, and is therefore not amenable to MS detection. A metabolite identified as acetaminophen glutathione (based upon *m/z* ratio of both positive and negative ions) was also observed after 24 hours exposure to acetaminophen (60 µg/l and 500 µg/l). The identification of the acetaminophen glutathione conjugate is supported by the detection of both positive and negative ion forms of this metabolite, and that these ions were not identified in the solvent control samples and therefore related to the exposure to acetaminophen. The tentative identification of a glutathione conjugate of acetaminophen is particularly interesting, as the

formation of this metabolite in mammalian systems is not only indicative of CYP-mediated metabolism, but is also the mechanism responsible for the detoxification of the active metabolite of acetaminophen (Black 1984). Thus the identification of this metabolite implies that zebrafish larvae can activate acetaminophen to a toxic species, known to be the causative agent of the associated hepatic toxicity of acetaminophen (Black 1984). The metabolism of the broad-specificity glutathione-*S*-transferase substrate 1-chloro-2,4-dinitrobenzene has been detected throughout zebrafish development, with significant increases in activity post-hatching, demonstrating that zebrafish larvae have the potential for glutathione conjugation (Weigand et al 2000). Indeed, a glutathione conjugate of the herbicide atrazine has been identified in enzyme extracts from zebrafish embryos (25 hpf), based upon *m/z* ratio of the conjugate only (Weigand et al 2001). Overall, the assessment of acetaminophen metabolism by zebrafish larvae suggests the presence of both oxidative and conjugative metabolism, including evidence for reactive intermediate formation.

The metabolism of ibuprofen by zebrafish larvae is considerably more characterised than that of acetaminophen in this study. The application of radiolabelled ibuprofen has allowed assessment of compound uptake and quantification of its derivatives by scintillation counting, combined with compound identification using LC-MS combined with the fragmentation of ions and accurate ion mass determination where possible. In mammals, ibuprofen can be oxidised to hydroxy-ibuprofen or carboxy-ibuprofen in a CYP2C8/9-dependent manner (Hamman et al 1997). The parent compound, as well as the metabolites, can also undergo glucuronidation (Kepp et al 1997). Ibuprofen was demonstrated to accumulate steadily over 24 hours exposure in zebrafish larvae by scintillation counting, although the majority of the radioactivity was detected in the water samples. Analysis of the water samples and larval extracts identified both the ibuprofen

(*m/z* 205 → 161, retention time 5.3 minutes) and hydroxy-ibuprofen (*m/z* 221 → 177, retention time 4.0 minutes) in both fractions after 24 hours exposure to ^{12}C-ibuprofen. Accurate ion mass determination was used to confirm the empirical formulae of the parent and fragment ions for both metabolites.

Analysis of water samples and larval extracts exposed to ^{14}C-ibuprofen also identified ibuprofen *m/z* 207 → 161, retention time 5.7 minutes) and the hydroxy-derivative (*m/z* 223 → 177, retention time 4.2 minutes) in both water samples and larval extracts, and an additional hydroxy-ibuprofen derivative in the water samples (*m/z* 221 → 177, retention time 5.7 minutes). The detection of hydroxy-ibuprofen in the larval extracts was confirmed by the detection of a radio-trace signal of comparable retention time to that of the parent ion and fragment ion peaks (4.2 minutes). The second identified hydroxy-ibuprofen derivative detected in the water samples could not be confirmed using radio-detection as the retention time for this ion was the same as that for ibuprofen (5.7 minutes). It is possible that this second hydroxy-ibuprofen could be a different isomer to the other detected metabolite, as it has been reported that such isomers can be separated by LC-MS (Quintana et al 2005). All identified metabolites were quantified by scintillation counting of fractions (1 minute per fraction) collected from the LC of water samples and larval extracts. The analysis of these fractions also detected an unidentified metabolite in larval extracts that had not been observed using LC-MS(MS) (retention time 2 – 3 minutes). The quantification of these samples showed that in the water samples the majority of the ibuprofen is in the parent compound form, with a small percentage (8.2%) detected as hydroxy-ibuprofen, with considerable variation observed in the amount of hydroxy-ibuprofen detected between individual replicates. It is not known what may have caused this variability between the water samples. However, in larval extracts the majority of the detected ibuprofen is found as metabolites, predominantly hydroxy-

ibuprofen (3.39 times the amount of ibuprofen detected in larval extracts), with the unknown metabolite being a minor derivative of the parent compound (0.45 times the amount of ibuprofen detected in larval extracts). Although the overall amount of radioactivity in the larval extracts is substantially lower than that detected in the water samples, there is less variability between the replicates for all the derivatives quantified in these samples.

Taken together, these data show that zebrafish larvae can accumulate ibuprofen and metabolise it to a variety of derivatives, which can then be excreted. The identification of hydroxy-ibuprofen in both larval extracts and water samples suggests that zebrafish possess an analogous metabolic system to the mammalian CYP2C8/9 isoforms. Although there is currently no orthologous gene or enzyme with similar substrate specificities to mammalian CYP2C8/9 identified in zebrafish, there has been a large number of CYP2-family genes identified, and the demonstration of an enzyme activity towards ibuprofen supports the hypothesis that zebrafish larvae possess similar drug metabolism system to that observed in mammals. Ibuprofen has been reported to inhibit laurate hydroxylase activities in carp (*Cyprinus carpio*) liver microsomes, corresponding to CYP2K and CYP2M activities, suggesting that these families of enzymes may be involved in the oxidation of ibuprofen (Thibaut et al 2006).

Using the LC-MS(MS) approach to metabolite identification did not provide any evidence to suggest that ibuprofen and its derivatives undergo conjugation in zebrafish larvae. However, this could be due to the use of dichloromethane as an extraction solvent for larval preparations, as it has been observed that dichloromethane is not suitable for the extraction of polar conjugates of steroids (Marwah et al 2001). The comparison of both larval homogenates and water samples incubated with or without a β-glucuronidase/arylsulphatase mix did not alter the detection of ^{14}C signals in dichloromethane extracts of these preparations, suggesting that there is no significant

conjugation of ibuprofen by zebrafish larvae. Although the evidence suggests that no appreciable conjugation occurs using ibuprofen as a substrate, we cannot exclude the possibility that the deconjugation of excreted metabolites can occur. It should be noted that the evidence provided by this study does not mean that these conjugation systems are absent or not functional in larvae, as zebrafish larvae have been demonstrated in Chapter 3 to significantly conjugate xenobiotics such as resorufin and 7-hydroxy-coumarin *in vivo*, and adult zebrafish homogenates have been shown to both glucuronidate and sulfate bisphenol A (Linholst et al 2003). Ibuprofen has also been reported to interfere with the glucuronidation of α-naphthol in carp (*Cyprinus carpio*) liver subcellular fractions, suggesting that fish species may be capable of conjugating ibuprofen (Thibaut et al 2006). In addition to this, the identification of a acetaminophen sulfate metabolite in this study provides further evidence of active conjugative metabolism in zebrafish larvae. The glucuronide conjugates of ibuprofen, hydroxy-ibuprofen and carboxy-ibuprofen are major metabolites of ibuprofen excreted by mammals (Kepp et al 1997). The identification of hydroxy-ibuprofen in both larval extracts and water samples, in addition to the lack of significant glucuronidation observed, suggests that hydroxy-ibuprofen is water-soluble enough to be excreted without prior conjugation by zebrafish larvae, highlighting a species difference between mammals and zebrafish.

The hydroxylation of ibuprofen results in the inactivation of the pharmacological activity of ibuprofen in mammals (Al-Nasser 2000), and thus suggests that zebrafish larvae have the potential to alter the pharmacology of ibuprofen via oxidative metabolism. The observed sulfation of acetaminophen also provides evidence that zebrafish larvae can modulate the pharmacology of xenobiotics, as this process is known to inactivate acetaminophen (Fura et al 2004). Ibuprofen has been detected in surface waters worldwide at a wide range of concentrations, ranging from low ng/l to low µg/l concentrations (Chen et al 2008). In waste water treatment plant effluents in the Hudson basin (New York, USA), ibuprofen has been detected at concentrations ranging from <100 ng/l to >10 µg/l (Palmer et al

2008). Acetaminophen is also detected in surface waters at concentrations ≤10 µg/l in the United States of America (Heberer 2002). The concentrations of ibuprofen and acetaminophen used in this study are 10 times (ibuprofen), 6 times and 50 times the highest detected environmental concentrations, in order to increase the chance of detecting any metabolites of these compounds. As it has been demonstrated that zebrafish larvae are capable of metabolising both pharmaceuticals to pharmacologically inactive species, as well as potentially activating acetaminophen to a toxic species (followed by glutathione conjugation) it is suggested that the pharmacological impacts of ibuprofen and acetaminophen can be limited in fish by metabolism, and that there is also the potential for toxic activation of these pharmaceuticals.

Overall, this study demonstrates that zebrafish larvae can metabolise the NSAIDs acetaminophen and ibuprofen via oxidative and conjugative mechanisms. The hydroxylation of ibuprofen demonstrates the presence of an othologous activity to the mammalian CYP2C8/9 isoform, and the sulfation of acetaminophen has also been demonstrated. It is also suggested that zebrafish larvae are able to conjugate acetaminophen to glutathione, a process requiring CYP-dependent oxidation before conjugation, supporting the observation that zebrafish embryos can conjugate atrazine to glutathione (Weigand et al 2001). This provides compelling evidence that these organisms are well suited for use in toxicity testing and front loaded drug screens. In addition to this, it suggests that there is the potential for the metabolism of NSAID by larval fish in an environmental setting, and therefore modulation of the pharmacological impact of such compounds in fish species.

Chapter 5

The modulation of CYP1A and EROD activity by the organic solvents dimethyl sulfoxide and methanol

5.1 Introduction

Organic solvents are used regularly to solubilise lipophilic compounds, such as pharmaceuticals for use in drug metabolism studies and toxicity testing. It was observed in Chapter 3 that methanol (a commonly used organic solvent, 500 µl/l) significantly repressed CYP and UGT1A1 gene expression and EROD activity after 24 hours exposure compared to unexposed larvae. The OECD guidelines for the testing of chemicals, the fish screening assay for endocrine active substances (2008), recommends the use of minimal solvent concentrations where possible, with 100 µl/L (0.01% v/v) of solvent suggested as a maximum concentration, along with the use of an appropriate solvent control. A major reason for minimising organic solvent concentrations in these types of toxicity tests is that solvents can often have toxic effects upon the test organisms, and thus influence the interpretation of such tests (Hallare et al 2006, Hutchinson et al 2006). However, the implications of solvent effects upon potential xenobiotic metabolism has not been previously explored in fish larvae.

The application of zebrafish larvae to toxicity screening has resulted in the routine use of solvent concentrations up to 1% v/v, required to allow the solubilisation of test compounds into dilution water and thus to allow the exposure of zebrafish larvae to the test compounds (Berghmans et al 2008, Richards et al 2008, Winter et al 2008). Although the comparison of test data with a solvent control is appropriate when considering potential toxic effects of organic carrier solvents, the potential for organic solvents such as methanol to modulate drug metabolism in zebrafish larvae could have an impact upon the predictivity of toxicity of test compounds, as some may require metabolic activation to produce a toxic effect. Additionally, solvent controls cannot take into account the effects of solvent-agent interactions.

This chapter investigates further the previously observed solvent effect of methanol upon drug metabolism systems in zebrafish larvae (see chapter 3). As ethoxy-resorufin-*O*-

deethylase (EROD) activity is well correlated with CYP1A activity in fish, the expression of CYP1A and EROD activity have been used as markers for the effects of methanol exposure upon CYP gene expression and activity (Whyte et al 2000). The modulation of the aryl hydrocarbon receptors (AhRs), known to regulate the CYP1A gene in zebrafish, by methanol exposure has also been assessed in this study. In order to determine if the observed solvent effects of methanol were solvent-specific, the commonly utilised organic solvent dimethyl sulfoxide (DMSO) was also assessed for any potential to reduce CYP1A and AhR expression, as well as for any inhibitory effects upon EROD activity. DMSO was selected as it has also been used in our work to dissolve substrates such as ethoxy-resorufin, ethoxy-coumarin and octyloxymethylresorufin, and CYP inhibitors such as α-naphthoflavone and SKF525A.

5.2 Methods

Zebrafish larvae (72 hpf) were exposed to Aroclor 1254 (100 μg/l, dissolved in methanol with a final concentration of 0.05% v/v), methanol only (0.05%, 0.01% or 0.001% v/v) or DMSO only (0.1% or 0.01% v/v) for 24 hours in dilution water. The exposed fish were then used for RNA extraction using a Qiagen RNeasy kit (as directed by the manufacturer and detailed in chapter 2, section 2.3) for the assessment of gene expression by qPCR, or assessed using the EROD assay. Extracted total RNA was reverse transcribed using superscript II (Invitrogen) and the 250 ng/μl of the synthesised cDNA was loaded per reaction with a PCR mastermix containing SYBR green dye (Quantace) for the assessment of CYP1A, CYP2J26, zgc:153269, UGT1A1, EF1α and RPl13α expression. PCR products were amplified using a program of step one: 95°C (3 minutes), step two: 95°C (30 seconds), 60°C (30 seconds), 72°C (1 minute) and step three: 72°C (5 minutes) using a dissociation protocol. Data was analysed using the ΔΔCt method (Livak and Schmittgen 2001). The EROD activity of zebrafish larvae was determined by incubating 30 larvae per replicate per time-point with 8 μM ethoxy-resorufin and 10 μM dicumarol for up to 10 hours at 28°C. To 750 μl of assay medium, 250 μl diluted β-glucuronidase/arysulfatase was added (666- fold in sodium acetate buffer – 100 mM, pH 4.5), and this mixture was incubated for 2 hours at 37°C. Ethanol (100%, 1 ml) was added to the mixture in a fluorescence curvette, and fluorescence was measured at λ_{ex} 530 nm, λ_{em} 590 nm.

5.3 Results

5.3.1 The effect of methanol upon CYP and UGT gene expression in zebrafish larvae

Methanol (0.05% v/v, 24 hours exposure) significantly reduced the expression of CYP1A (to 6% of untreated levels, $p<0.05$, Mann-Whitney U test), the CYP-gene zgc:153269 (CYP3A, 34% of untreated levels, $p<0.05$, Mann-Whitney U test), and UGT1A1 (21% of untreated levels, $p<0.05$, Mann Whitney U test), but not the expression of CYP2J26, compared to the level of gene expression in untreated larvae (96 hpf, Figure 5.1). In order to determine if the down-regulation of the assessed genes was due to non-specific repression of gene expression, the effect of methanol pre-treatment (0.05% v/v, 24 hours) upon the expression of the validated "housekeeping" genes elongation factor 1α (EF1α) and ribosomal protein l13α (RPl13α) was also assessed. Methanol pre-treatment significantly induced EF1α expression (5.28-fold increase compared to untreated larvae, $p<0.05$, Independent samples t-test) and did not alter the expression of RPl13α (Figure 5.2). Taken together, with the lack of modulation of CYP2J26, this suggests that the reduction in gene expression is a targeted effect of methanol treatment. It was also observed that induction by co-treatment with Aroclor 1254 (100 µg/l) only partially compensated for the loss of expression by methanol of CYP1A ($p<0.05$, Mann-Whitney U test), the CYP-gene zgc:153269 (CYP3A, $p<0.05$, Mann-Whitney U test) and UGT1A1 ($P<0.05$, Mann-Whitney U test, Figure 5.1).

5.3.2 The effect of methanol upon CYP1A expression and EROD activity

The expression of CYP1A in zebrafish larvae was observed to be significantly reduced by treatment, with 0.05% v/v methanol being highly statistically significant after 24 hours exposure ($p<0.05$, Mann-Whitney U test, Figure 5.3). At 0.01% and 0.001% v/v, the reduction in expression was not significant, or significant respectively. Methanol exposure at all doses significantly altered EROD activity in the live larval zebrafish assay (0.05% v/v

–$p<0.05$ at 2, 4, 6 and 8 hours; 0.01% v/v – $p<0.05$ at 2, 4, 8 and 10 hours; 0.001% v/v – $p<0.05$ at 8 hours, Figure 5.4). Co-treatment of zebrafish larvae with Aroclor 1254 (100 µg/l with 0.05% methanol, 24 hours) was found to recover EROD activity to near-basal levels (Figure 5.5).

5.3.3 Assessment of the effects of DMSO on the CYP1A expression and EROD activity

Exposure of zebrafish larvae to 0.1% v/v DMSO (24 hours) resulted in a significant reduction in the expression of CYP1A ($p< 0.01$, Independent samples t-test), whereas 0.01% v/v DMSO did not significantly alter CYP1A expression (Figure 5.6). There was only a minor inhibitory effect of DMSO observed on EROD activity at a concentration of 0.1% v/v ($p<0.05$ at 6 and 8 hours), with no significant inhibition of EROD activity observed at a concentration of 0.01% v/v DMSO (Figure 5.7). It should be noted that there the inhibitory effect of DMSO was not observed after 2 hours in the EROD assay, but was evident at later time-points (e.g. 6, 8 and 10 hours). The cause of this lack of inhibition is unclear, but is unlikely to be due to low sensitivity of the EROD assay, as inhibition was observed by methanol treatment as early as the 2 hour time-point. As DMSO has been used to solubilise substrates and compounds such as ethoxy-resorufin, α-naphthoflavone and SKF-525A throughout this work (see Chapter 3) at a concentration of 0.1% v/v, the effect of DMSO co-exposure upon EROD activity was assessed, with a slight, but not statistically significant, inhibition of EROD activity observed after 10 hours co-exposure to DMSO (Figure 5.8).

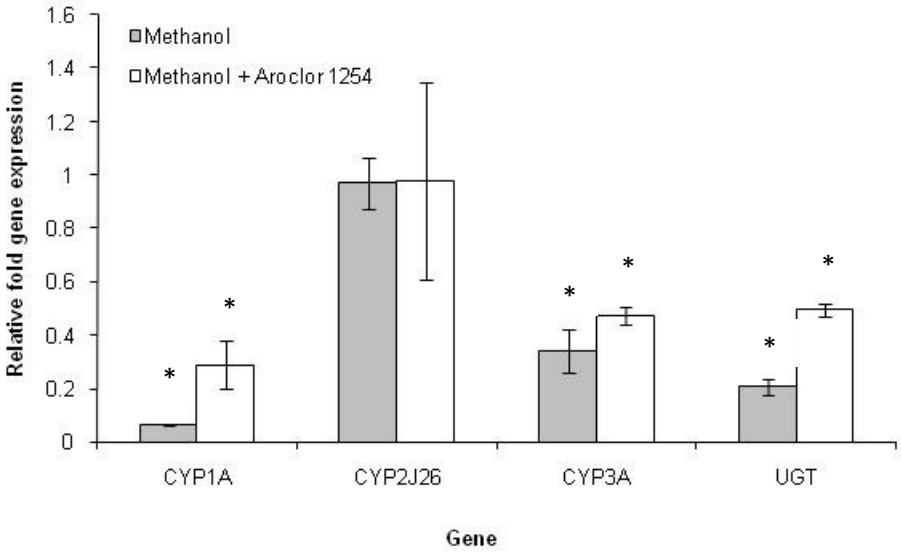


Figure 5.1: The relative fold change in the expression of CYP and UGT1A1 genes in zebrafish larvae after 24 hours exposure to 0.05% v/v of methanol compared to untreated larvae (fold gene expression = 1). Bars represent the mean expression of 100 pooled larvae (n = 3) ± SEM. Gene expression was normalised to that of CYP2J26, as the expression of this gene did not change after exposure to methanol. Statistical significance indicated by * ($p<0.05$) compared to untreated samples.

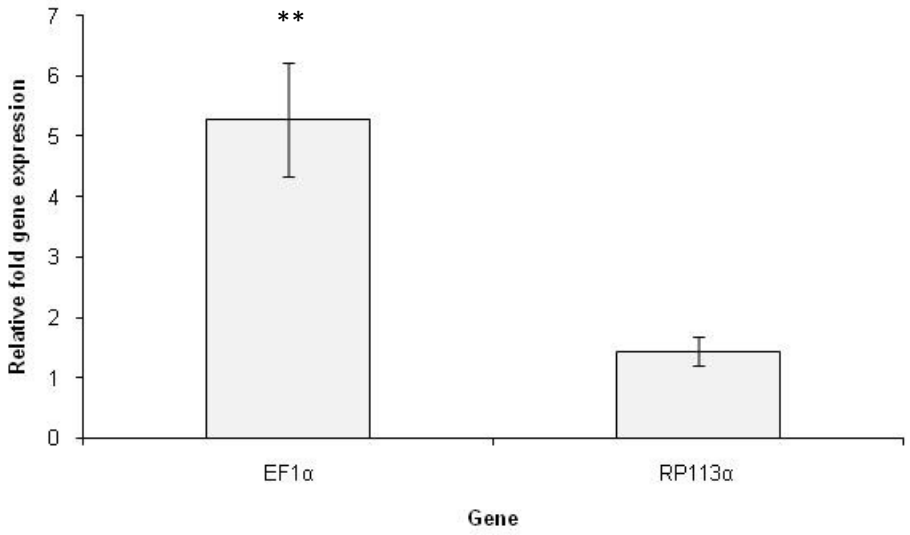


Figure 5.2: The effect of methanol (0.05% v/v) exposure (24 hours) on the expression of the validated reference genes elongation factor 1 alpha (EF1α) and ribosomal protein l13 alpha (RPL13α) compared to untreated larvae (relative fold expression = 1). Bars represent the mean expression of 100 pooled larvae (n = 3) ± SEM. Gene expression was normalised to that of CYP2J26, as the expression of this gene did not change after exposure to methanol. Statistical significance indicated by ** ($p<0.01$) compared to untreated samples.

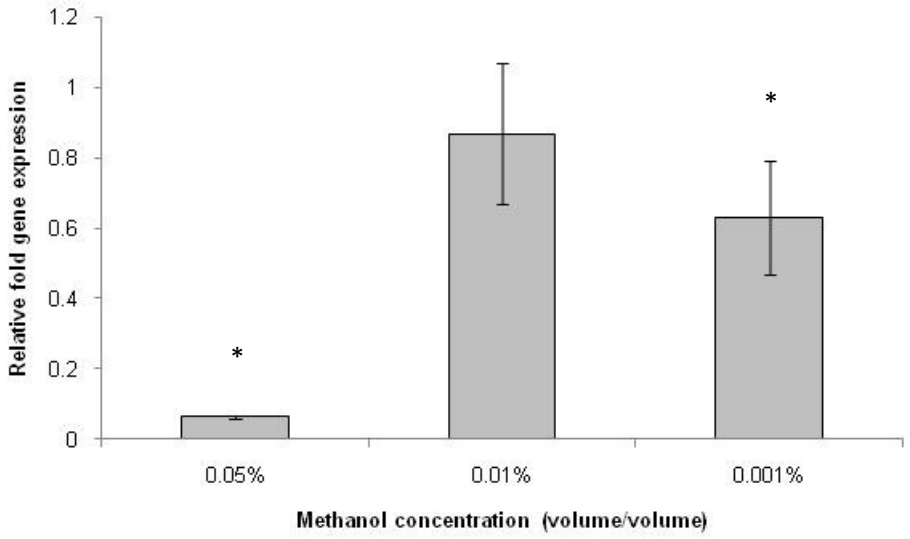


Figure 5.3: The modulation of CYP1A expression by different concentrations of methanol after 24 hours exposure, compared to untreated zebrafish larvae (relative fold gene expression = 1). Bars represent the mean expression of 100 pooled larvae (n = 3 - 5) ± SEM. Gene expression was normalised to that of CYP2J26, as the expression of this gene did not change after exposure to methanol. Statistical significance indicated by * ($p<0.05$) compared to untreated samples.

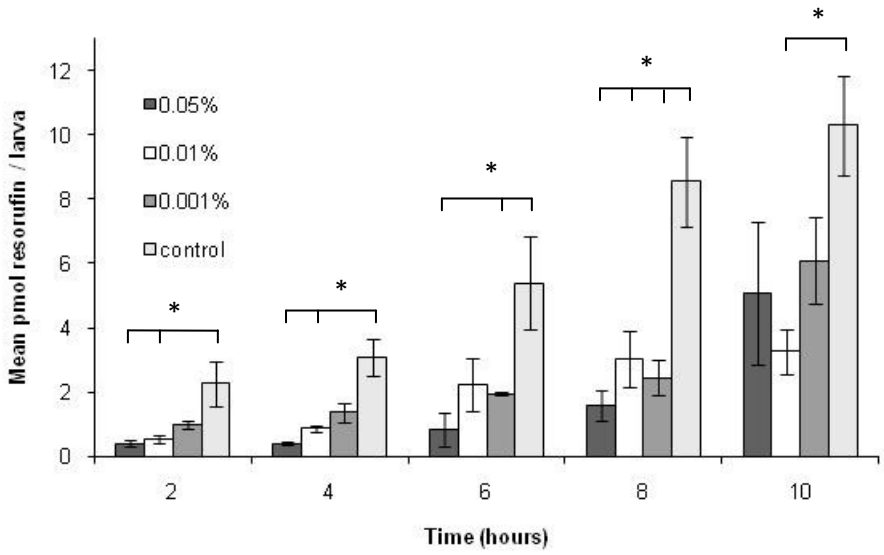


Figure 5.4: The effect of methanol exposure (24 hours) upon EROD activity in zebrafish larvae (96 hpf). Zebrafish larvae (30 per group) were incubated for up to 10 hours with ethoxy-resorufin after exposure to methanol (n = 3). Bars represent the mean pmoles of resorufin detected in the assay water per larva ± SEM. Statistical significance indicated by * ($p<0.05$).

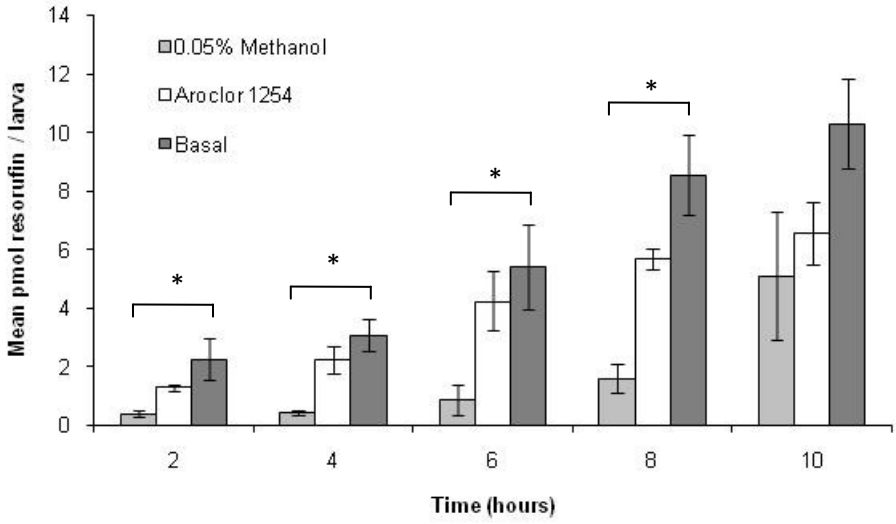


Figure 5.5: The partial recovery of EROD activity in zebrafish larvae (96 hpf) by co-exposure (24 hours) of Aroclor 1254 (100 µg/l) and methanol (0.05% v/v). larvae (30 per group) were incubated for up to 10 hours with ethoxy-resorufin after exposure to methanol and Aroclor 1254 (n = 3). Bars represent the mean pmoles of resorufin detected in the assay water per larva ± SEM. Statistical significance indicated by * ($p<0.05$).

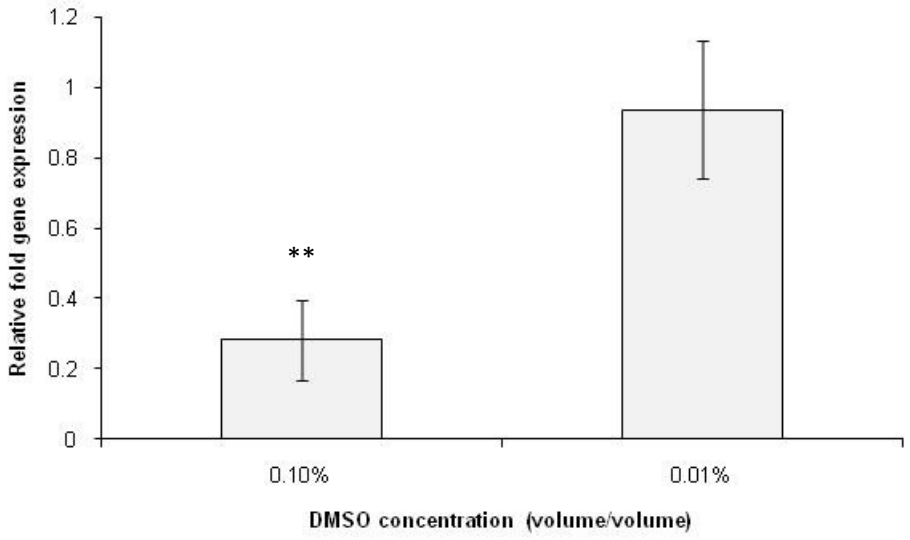


Figure 5.6: The effect of DMSO exposure (24 hours) on CYP1A gene expression compared with untreated larvae (relative fold gene expression = 1). Bars represent the mean expression of 100 pooled larvae (n = 4) ± SEM. Gene expression was normalised to that of CYP2J26, as the expression of this gene did not change after exposure to methanol. Statistical significance indicated by ** ($p<0.01$) compared to untreated samples.

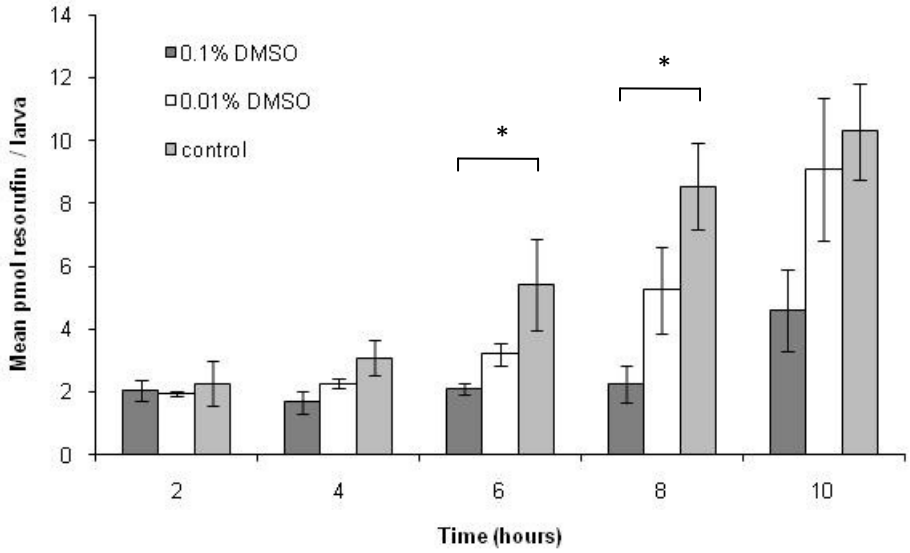


Figure 5.7: The effect of DMSO exposure (24 hours) upon EROD activity in zebrafish larvae (96 hpf). Zebrafish larvae (30 per group) were incubated for up to 10 hours with ethoxy-resorufin after exposure to DMSO (n = 3). Bars represent the mean pmoles of resorufin detected in the assay water per larva ± SEM. Statistical significance indicated by * ($p<0.05$).

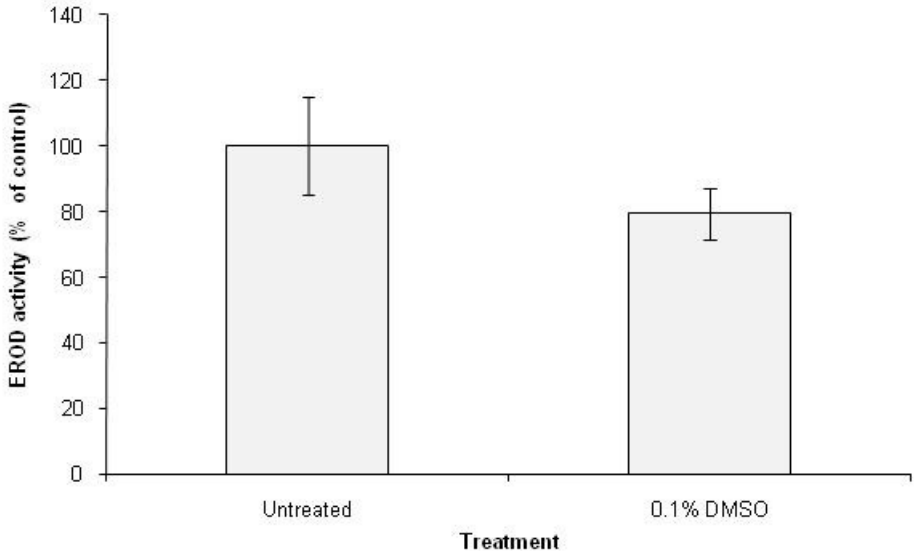


Figure 5.8: The effect of DMSO co-exposure upon the EROD activity of zebrafish larvae (96 hpf). Zebrafish larvae (30 per replicate, n = 3) were incubated with ethoxyresorufin ± an additional 0.1% v/v DMSO. Bars represent the mean EROD activity ± SEM. Activity is expressed as the percentage of the mean EROD activity of untreated fish.

5.3.4 Assessment of the effects of DMSO and methanol on AhR1 and AhR2 expression

Exposure of zebrafish larvae to 0.05% v/v methanol or 0.1% v/v DMSO had been observed to alter CYP1A gene expression and EROD activities. We determined if such an effect might be modulated by the Aryl-hydrocarbon receptors by assessing the expression of these regulators of CYP1A. The exposure of zebrafish larvae to both methanol (0.05% v/v) and DMSO (0.1%) resulted in significantly reduced expression of both AhR1 and AhR2 ($p<0.05$ for all treatments and genes, Mann-Whitney U test, Figure 5.9) assessed by quantitative PCR.

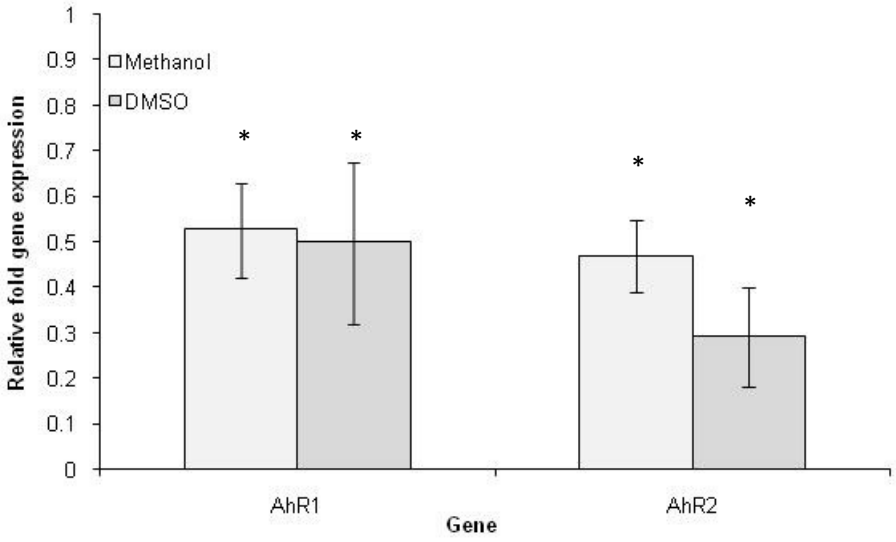


Figure 5.9: The effect of methanol (0.05% v/v) and DMSO (0.1% v/v) exposure (24 hours) on the expression of aryl hydrocarbon receptors 1 and 2 in zebrafish larvae compared to untreated larvae (relative fold gene expression = 1). Bars represent the mean expression of 100 pooled larvae (n = 4) ± SEM. Gene expression was normalised to that of CYP2J26, as the expression of this gene did not change after exposure to methanol. Statistical significance indicated by * ($p<0.05$) compared to untreated samples.

5.4 Discussion

It was observed in Chapter 3 that the common organic solvent methanol had an inhibitory effect upon the expression of CYP and UGT genes, and also EROD activity in live zebrafish larvae (96 hpf). This chapter has further investigated these inhibitory effects, focusing upon the CYP1A gene and EROD activity, due to the association between this gene and enzyme activity. The expression of CYP1A has been assessed after exposure to different concentrations of methanol, as has the expression of the aryl hydrocarbon receptors, known regulators of CYP1A in zebrafish. Additionally, a partial compensatory effect upon gene expression and EROD activity has been demonstrated using the CYP1A inducing agent Aroclor 1254 after exposure to methanol. Finally, the commonly-used organic solvent, DMSO, has been shown to reduce the expression of CYP1A and AhR genes, as well as having a mild inhibitory effect upon EROD activity. Overall, it has been demonstrated that organic solvents have the potential to modulate drug metabolism *in vivo* in zebrafish larvae.

Organic solvents are widely applied throughout the life-sciences to facilitate the solubilisation of a variety of compounds. It has been observed *in vitro*, using human liver microsomes, that organic solvents such as acetone, DMSO, methanol, dimethyl-formamide (DMF) and acetonitrile can modulate CYP activities (Chauret et al 1998, Hickman et al 1998). Indeed, Hickman et al (1998) reported that methanol and DMSO had significant inhibitory effects upon CYP1A2, CYP2C9, CYP2C19 (DMSO only) and CYP2E1 activities at a final solvent concentration of 1% v/v, and Chauret et al (1998) have observed inhibition of CYP2C8/9, CYP2C19 and CYP2E1 activity at a DMSO concentration of ≤0.5% v/v and CYP2C9 and CYP2E1 activities by methanol (0.5% and 1% v/v respectively). There are some discrepancies between these two studies, with no effect of DMSO or methanol exposure observed on CYP1A2 activity by Chauret et al (1998). This may be attributed to the use of different marker activities for this enzyme, with Hickman et al (1998) using caffeine-*N3*-demethylation and Chauret et al (1998) using

phenacetin-*O*-deethylase activity as a marker for CYP1A2. However, where the same marker activities were assessed, there was good reproducibility between the studies (Chauret et al 1998, Hickman et al 1998). It should be noted that the use of microsomes as a model system means that induction and inhibition at the gene expression level cannot be assessed, and also that use of different marker substrates for a particular CYP isoform (for example, diclofenac-4-hydoxylase, tolbutamide-methyl-hydroxylase, phenytoin hydroxylase and celecoxib methyl-hydroxylase activities are all marker activities of CYP2C9) can provide confounding results as to the effects of solvents upon CYP activity (Tang et al 2000). For example, Tang et al (2000) have reported that CYP2C9 activity is inhibited (celecoxib methyl-hydroxylase activity), induced (diclofenac-4-hydoxylase and tolbutamide-methyl-hydroxylase) and not affected by exposure to acetonitrile, depending which marker activity is assessed. Treatment of human hepatocytes with DMSO and methanol resulted in the inhibition of CYP2C9, CYP2C19, CYP2E1 and CYP3A4 (DMSO) and CYP2C9 and CYP2E1 (methanol) activities using solvent concentrations at 1% v/v or greater (Easterbrook et al 2000). There were no observed effects of solvent exposure upon conjugation activities corresponding to UGT or SULT isoforms observed using DMSO, methanol or acetonitrile in these human hepatocytes (Easterbrook et al 2000). The effect of organic solvents upon activities of cDNA–expressed CYP isoforms re-iterates the observations from microsomal incubations, that substantial inhibition of CYP activities can occur at solvent concentrations ≤1% v/v, and that the observed inhibitions are dependent upon the substrate utilised as a marker for CYP isoform activity (Busby et al 1999). It is interesting to note that the majority of these studies recommend methanol as a suitable solvent for use in metabolism studies, and that DMSO is generally found to have the more substantial inhibitory profile however, this conclusion is limited as none of these studies assess the effect of solvents upon the gene transcription and expression.

The use of microsomal fractions to assess the effects of solvents upon CYP activities removes the potential for regulation of gene expression by these compounds. The effects of DMSO exposure upon the gene expression of CYP and UGT isoforms in human primary hepatocytes has also been assessed after 24 hours exposure (Nishimura et al 2003). It was observed that DMSO concentration less than 0.5% v/v did not significantly alter the expression of CYP and UGT genes however, a concentration of 2.5% v/v DMSO did result in mild modulation (Nishimura et al 2003). This modulation was found to be somewhat variable between different donors of hepatocytes, and the genes CYP1B1, CYP2E1 and CYP3A4 were induced by treatment (2 – 3 fold above control) whereas CYP3A7 and UGT1A9 were down-regulated to 0.2 – 0.5 fold of the untreated level (Nishimura et al 2003). Corresponding data on activity was not reported to complement the gene expression data in this study. However, comparisons with other hepatocyte studies (such as that by Easterbrook et al 2000) suggests that, as inhibition of activity is only seen at solvent concentrations of 1% v/v or greater, the regulation of gene expression may be part of the mechanism for organic solvent-mediated inhibition. The direct inhibitory effects of solvent exposure cannot be discounted, due to the effects observed using microsomal preparations.

Using zebrafish larvae, the modulation of CYP and UGT gene expression by the organic solvents DMSO and methanol has been demonstrated *in vivo*, at substantially lower concentrations than those applied to *in vitro* systems, such as cultured hepatocytes or microsomal fractions (0.05% v/v methanol and 0.1% DMSO compared with ≥0.5% v/v for both solvents respectively). Similarly, the inhibition of EROD activity by methanol (0.05%, 0.01% and 0.001% v/v) and DMSO (0.1% v/v) has been demonstrated using a live larval assay, which agrees with the reported inhibition of CYP1A1 activity in cDNA-expressing B lymphoblastoid cells by these solvents (measuring phenacetin-*O*-deethylase activity and using substantially higher solvent concentrations however, cDNA-expressed enzymes are not under the control of the AhR, and are constitutively expressed and thus do not reflect

any regulation of gene expression). It should be noted that for methanol-exposed larvae, inhibition of EROD activity is observed at concentrations where there is no significant change in gene expression comparing to untreated samples (0.01% v/v). This suggests that regulation of gene expression is not the complete mechanism by which solvent-mediated inhibition of EROD activity occurs. However, the use of the CYP gene expression inducing agent Aroclor 1254 does partially compensate for the reduction in gene expression and EROD activity upon co-exposure with methanol. It is possible that the co-treatment with Aroclor 1254 is not preventing or reducing the inhibitory effects of methanol, but simply causes an induction in gene expression and EROD activity, thus masking the inhibition caused by the solvent. The down-regulation of the CYP1A regulators, aryl hydrocarbon receptors 1 and 2 (AhR1 and AhR2) by DMSO and methanol exposures does also imply that genetic regulation of CYP genes by organic solvents is involved in the mechanism for producing the observed effects. The regulation and network of interactions of AhR is highly extensive, and the mechanism of the observed solvent-mediated down-regulation is unclear (Figure 5.10). However, DMSO is known to have some anti-oxidant properties and as methanol is an alcohol it can potentially have anti-oxidant properties (via conversion to a carboxylic acid, it is not known if this is a biologically-relevant mechanism), and it has been suggested, using TAKA-1 cells that AhR expression is down-regulated in the presence of antioxidants (Zhang et al 1998, Santos et al 2003). There is also substantial interaction between the AhR and NF-E2p45-related protein (NRF2), which is a known regulator of anti-oxidant genes and has also been reported to regulate AhR expression in mouse cells (Shin et al 2007). It is thus possible that solvents mediate the observed down-regulation of AhR and its downstream effectors through interactions with NRF2. Taken together with evidence from *in vitro* systems (such as human microsomal fractions and hepatocyte cultures), it appears that zebrafish larvae are sensitive to organic solvent-mediated modulation of CYP activities, partially regulated via alterations in gene expression (mediated via the expression of

nuclear receptors such as the aryl hydrocarbon receptors) and potentially via direct interaction with the enzymes themselves.

Zebrafish larvae are currently being applied in a multi-tier approach as part of the drug discovery and toxicity testing paradigm. Waterborne exposures are a common method for exposing zebrafish (adult and larvae) to pharmaceutical compounds, due to the rapid uptake of small molecules through the skin and gills by embryonic and larval zebrafish (McGrath and Li 2008). It is also common for pharmaceutical compounds to be dissolved in organic solvents, in order to overcome solubility issues, and allow waterborne exposure as a route of administration in toxicity testing assays (Barros et al 2008). Zebrafish larvae are tolerant to DMSO at high concentrations (up to 4%), and concentrations of 1% are routinely used in drug toxicity assays (Winter et al 2008). In view of the observed inhibitory effects of DMSO and methanol observed in this study, and of the apparent sensitivity of zebrafish larvae to solvent-mediated modulation of drug metabolism genes (≤0.1% v/v of solvents elicit inhibitory effects), the use of minimal solvent concentrations, with 0.001% v/v recommended as a maximum concentration applied to assays. Overall, the data presented in this chapter, and in Chapter 3 (induction of gene expression by Aroclor 1254) also highlights the importance of an appropriate solvent control for use in drug metabolism screening *in vivo* using zebrafish larvae, as there is the potential for solvents to modulate the xenobiotic metabolism capabilities of the fish. The implications of the use of solvents in zebrafish toxicity testing assays are the potential for the overestimation (of the toxicity of compounds that are inactivated by metabolism) or underestimation of toxicity (for compounds that require activation by metabolism). There is also potential for the alteration of the qualitative profile of compound metabolism, mediated through the modulation of specific drug metabolism genes.

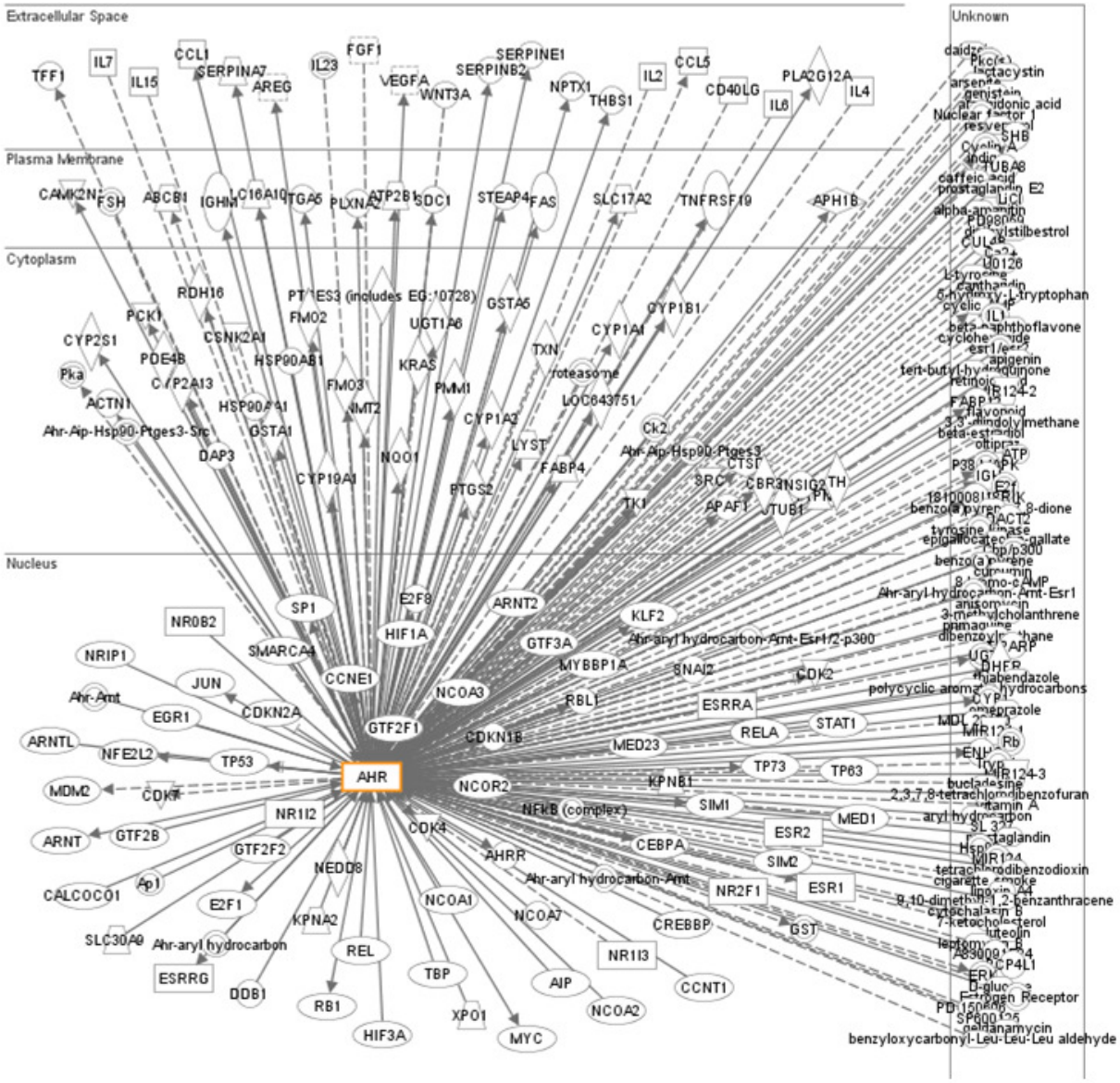


Figure 5.10: The known interactions of the Aryl Hydrocarbon receptor (AhR) with endogenous and exogenous factors. Figure was produced using the Ingenuity Pathway Analysis software (version 8.5, accessed December 2009).

Chapter 6

General Discussion

6.1 General discussion

The application of zebrafish larvae to drug safety assessment along with the identification of pharmaceutical residues in the aquatic environment has resulted in the requirement for the assessment of xenobiotic metabolism in fish species to be assessed. As zebrafish, both adult and larvae, are utilised in both toxicity testing and ecotoxicology (as discussed in Chapter 1), there is now a focus upon characterising the complement of CYP, UGT, GST and SULT enzymes present throughout embryonic development and adult life stages. This project has focused upon the expression of CYP and UGT genes and during embryonic development and larval stages, and assessed xenobiotic metabolism during development and post-hatching.

Zebrafish embryos and larvae have been reported to express several CYP genes including CYP1A, CYP1B1, CYP1C1, CYP1D1, CYP2K6, CYP2J1, CYP3A65 and CYP3C1 (Tseng et al 2005, Wang-Buhler et al 2005, Corley-Smith et al 2006, Jönsson et al 2007, Wang et al 2007, Goldstone et al 2009). These genes have been reported to display distinct patterns of expression throughout embryonic and larval development, in addition to tissue-specific expression patterns. For example, CYP1A expression increases steadily throughout development, whereas CYP1D1 expression is initially relatively high and decreases in the first 24 hours of development followed by an increase in expression by 3 dpf, with a slight decrease to a constant level of expression in adult tissues (Goldstone et al 2009). The CYP2K6 gene has been detected in zebrafish larvae from 5 dpf onwards and in adult tissues, whereas CYP3A65 has been observed to steadily increase in expression throughout development (Tseng et al 2005, Wang-Buhler et al 2005). The developmental expression profiles of SULT genes have been extensively characterised, with isoform-specific patterns of gene expression observed (Ohkimoto et al 2003, Sugahara et al 2003, Liu et al 2005, Yasuda et al 2005a, Yasuda et al 2005b, Yasuda et al 2006, Liu et al 2008, Yasuda et al 2008). Chapter 3 demonstrated that

CYP1A, CYP2J26, CYP-gene zgc:153269 (CYP3A) and UGT1A1 genes are expressed in distinct temporal patterns throughout development, with a general trend for increased expression post-hatching. These observed patterns of expression either confirmed previously-reported expression (CYP1A), or are the first reported study into the expression of these genes throughout development (CYP2J26, the CYP-gene zgc:153269 and UGT1A1). It should be noted that the expression of these genes was assessed in a qualitative manner, although the expression of CYP1A does agree with previously reported temporal patterns. The identification of temporal patterns of expression during development not only suggests a role for these genes during normal development (as in human embryonic development xenobiotic metabolism enzymes have endogenous as well as exogenous substrates such as the metabolism of steroidal hormones (oestrogen and testosterone) and the conjugation of billirubin), but also raises an additional consideration in that certain stages of embryonic development may be better suited, in terms of xenobiotic metabolism, to toxicity testing than others (Miller et al 1996). The presence or lack of drug metabolism systems at different developmental stages suggests that there is variability in the susceptibility of developing organisms to chemical toxicity during development. It is not currently clear as to why there is such variation in the expression of certain CYP activities throughout development however it may be linked to the roles of these systems in organogenesis. It should also be noted that similar regulation of EROD activity has been observed in mice as seen for zebrafish, with the detection of CYP1A1 mRNA without EROD activity, suggesting that the delay in onset of certain drug metabolism activities is a conserved process in normal embryonic development (Mattingly and Toscano 2001). Thus the developmental stages with the widest range of CYP, UGT, GST and SULT isoform expression would be expected to more accurately reflect the extent of toxic activation or inactivation of test compounds, compared to adult mammalian systems.

The highest levels of mRNA for the CYP isoforms identified in this study were detected post-hatching (at 96 hpf). This observation, in addition to the observation of Mattingly and Toscano (2001), that CYP activity towards the substrate ethoxy-resorufin is only detected post-hatching in zebrafish larvae, resulted in the assessment of xenobiotic metabolism activities occurring after the larvae had hatched from the chorion. In addition to this, exposures of zebrafish larvae to inducing agents, inhibitors and substrates of CYP enzymes only utilised hatched zebrafish larvae, in order to reduce any potential reduction of compound uptake or organism exposure by the chorion or perivitteline fluid. It is unknown to what extent the chorion acts as a barrier to xenobiotics, and the detection of the toxic effects upon unhatched zebrafish larvae using chemicals such as TCDD suggests that it is at best a semi-permeable barrier to chemicals (Karchner et al 2005). However, large molecules such as fluorescein dextrans are excluded by the chorion, as are lipopolysaccharides (Scholz et al 2008). It has been shown by monitoring the disposition of cadmium in unhatched zebrafish embryos that only a small percentage of the detected cadmium is associated with the actual embryo, with the majority of this compound associated with the chorion and the perivitteline fluid of the egg (Burnison et al 2006). Other considerations with regards to the absorption of xenobiotics into the embryo or larvae include the presence or absence of functioning gills and the extent (if any) of dietary exposure to xenobiotics. Although zebrafish embryos and larvae develop rapidly, it is not until post-hatching that functional gills develop, and the mouth opens, allowing exposure via the digestive tract (Kimmel et al 1995). Thus, the uptake of compounds preceding the formation of functional gills and the opening of the mouth is probably facilitated by processes such as diffusion, or is dependent upon the lipophilicity of the compound. Overall, the processes involved in the absorption of xenobiotics are poorly characterised and thus substantial investigation into the processes of xenobiotic uptake by zebrafish embryos and larvae are required.

This study has used a live *in vivo* fluorescence-based assay system to assess the metabolism of ethoxy-resorufin, 7-ethoxy-coumarin and octyloxymethylresorufin to demonstrate activities similar to mammalian CYP1, CYP2 and CYP3 respectively. As these assays require the uptake of the substrate and the excretion of the product in order to produce a quantifiable signal, each of these processes has been demonstrated for these compounds (Chapter 3). However, it should be noted that although the substrates used in this study are metabolised by a variety of mammalian CYP isoforms, only a few examples of these oxidative and conjugative processes are presented in this work. Further characterisation using other broad-specificity CYP substrates (such as benzyloxyresorufin, methoxyresorufin and pentoxyresorufin) or by monitoring isoform-specific reactions (for example, the 6β-hydroxylation of testosterone) is required to determine the extent of oxidative and conjugative systems in zebrafish embryos and larvae. We have also demonstrated the uptake of acetaminophen and ibuprofen, as well as the excretion of metabolites of these pharmaceuticals (Chapter 4). There are few examples of the metabolism of pharmaceuticals by fish species, especially at larval stages. For example, the depuration of fluoxetine has been assessed in adult Japanese medaka (*Oryzias latipes*, Paterson and Metcalfe 2008). Although the absorption, metabolism and excretion of characteristic substrates such as ethoxy-resorufin provides evidence demonstrating the presence of processes such as drug uptake and oxidative and conjugative metabolism, the fate of pharmaceuticals in zebrafish is of particular interest due to the application of zebrafish to drug discovery toxicity testing and ecotoxicology. Ibuprofen was demonstrated to accumulate in zebrafish larvae over a 24-hour period using scintillation counting. The identification of acetaminophen derivatives in larval extracts also demonstrates compound uptake, and the identification of ibuprofen metabolites in water fractions from zebrafish exposures is evidence of excretion. There are several potential routes of excretion for xenobiotic compounds and their derivatives. In mammals, the common routes of excretion is in urine or faeces (via the excretion of

compounds into the bile), with minor excretion via sweat or exhalation (Timbrell 2000). The drug transporter proteins such as the ATP-binding cassette (ABC) proteins, organic anion transporter proteins (OATP) and multi-drug resistance proteins (MDR) have a critical role in the excretion of xenobiotics (as well as endogenous substrates) into bile, and thus in excretion (Faber et al 2003). There have been 52 identified ABC transporter proteins identified in the zebrafish genome, in addition to a multi-drug resistance protein (MDR), suggesting that these potential routes of uptake and excretion of xenobiotics are present in the developing zebrafish (Bresolin et al 2005, Annilo et al 2006). It has been observed that the use of the ABC protein inhibitor cyclosporin A results in the accumulation of the fluorescent dye rhodamine red in zebrafish embryos (24 hpf), demonstrating the role of these proteins in the excretion of this compound (Scholz et al 2006).

Chapters 3 and 5 have demonstrated and assessed the induction and inhibition of CYP and UGT gene expression and activities in zebrafish larvae. The modulation of CYP and UGT genes by xenobiotics has been well described in mammals, and these processes are mediated by a variety of nuclear receptors, including the aryl hydrocarbon receptor (AhR), pregnane X receptor (PXR), peroxisome proliferator-activated receptors (PPARs) and constitutively activated receptor (CAR, Xu et al 2005). Unliganded AhR is predominantly located in the cytoplasm of cells, bound to the molecular chaperone heatshock protein 90 (hsp90), and upon ligand binding translocates to the nucleus, exchanging hsp90 for aryl-hydrocarbon receptor nuclear translocator (ARNT) in the process, and binds specific DNA sequences in the promoter regions of target genes (termed response elements, Xu et al 2005). The orphan receptors such as PXR and CAR, form heterodimers with the retinoid X receptor (RXR) within the nucleus upon ligand binding, resulting in the modulation of gene expression (Tompkins and Wallace 2007).

These receptors affect the expression of a variety of stress and xenobiotic metabolism genes, including some (but not all) CYP, UGT, GST and SULT isoforms and drug transporter proteins (Waxman 1999, Xu et al 2005). It should be noted that these nuclear receptors also have endogenous functions, in addition to the modulation of the metabolism of xenobiotics, and are involved in the maintenance of homeostasis and development (Bainy 2007). These receptors also have some constitutive activities in the absence of ligand binding, with CAR having the highest activity in the absence of ligand binding (Bainy 2007). The potential for the activation of these receptors in the early life-stages of zebrafish means that the induction of drug metabolism systems (as well as other downstream targets of these nuclear receptors) by exposure to environmental contaminants, such as polychlorinated biphenyls and PAH, as well as exposure to pharmaceuticals that are ligands of these receptors can be observed and assessed in zebrafish larvae.

The AhR pathway has been extensively characterised in zebrafish larvae, due to the involvement of this receptor in TCDD toxicity (Carney et al 2006). There are three AhR-family members known in zebrafish, named AhR1a, AhR1b and AhR2 respectively (Wang et al 1998, Tanguay et al 1999, Andreasen et al 2002). Unlike mammals, which only have a single AhR gene, it is common for fish to have at least two AhR forms (AhR1 and AhR2), and these fish AhRs differ from mammalian forms by having longer amino acid sequences and a lack of a glutamine-rich region at the C-terminus of the protein (Andreasen et al 2002).

The two identified AhR1 family members, designated AhR1a and AhR1b respectively, have been demonstrated to be expressed throughout zebrafish development with AhR1a and AhR1b expression increasing throughout development from 24 hpf onwards (Andreasen et al 2002, Karchner et al 2005). The modulation of gene expression for both AhR1 forms by TCDD (2 nM) has also been assessed during development, with mild

induction AhR1a but no modulation of AhR1b. Furthermore, the AhR1a form has been demonstrated to be expressed in the liver, heart, swim-bladder and kidney of adult zebrafish (Andreasen et al 2002). The AhR1a isoform has been reported not to be functionally active using a transfected COS-7 cells (these cells are used as a model system due to having minimal AhR regulation) with transfected AhR and its co-activator ARNT2b for activation of a luciferase construct containing an AhR response element (Andreasen et al 2002). Exposure to a variety of model and non-model AhR ligands, such as TCDD, β-naphthoflavone, benzo-[a]-pyrene and 7,12-dimethylbenz-[a]-anthracene resulted in the production of minimal luciferase activity (Andreasen et al 2002). The AhR1b form has been shown to be active upon TCDD treatment using a similar system (Karchner et al 2005). The AhR2 form has also been assessed for gene expression across development, with detectable expression from 24 hpf increasing through to maturity (Tanguay et al 1999). There has also been detection of AhR2 expression in most adult tissues including heart, liver, gill, brain, kidney, swim-bladder, skin, eye and fin (Andreasen et al 2002). The induction of AhR2 expression by TCDD has been demonstrated in both developing embryos (12 – 96 hpf) and a zebrafish liver cell line by Northern blotting (Tanguay et al 1999). It has also been shown using the COS-7 cell transfection system that AhR2 is functionally active upon exposure to a variety of AhR ligands including; TCDD, β-naphthoflavone (β-NF), benzo-[a]-pyrene and 7,12-dimethylbenz-[a]-anthracene. Due to the availability of an AhR2-specific zebrafish antibody, there has also been an assessment of the protein levels and degradation of AhR2 in zebrafish liver cells (Wentworth et al 2004). The degradation of AhR2 has been demonstrated to be mediated by ligand binding (using β-naphthoflavone as a model ligand) and 26S proteosome dependent, as the observed decrease in AhR2 protein levels after β-NF exposure is prevented by co-incubation with the proteosome inhibitor MG-132 (Wentworth et al 2004). The application of this antibody to immunohistochemistry has demonstrated that in untreated cells AhR2 localised to the nucleus and cytoplasm, with

increased nuclear staining upon β-NF exposure (Wentworth et al 2004). Incubation of these cells with a nuclear export inhibitor reduced staining in the cytoplasm, suggesting that AhR2 "shuttles" between both compartments of the cell (Wentworth et al 2004). As well as the different AhRs described above there have been several related factors including AhR interacting protein (AIP), AhR repressors (AhRr) and co-activators such as ARNT have all been identified in zebrafish. The use of morpholino knockdown of the various different isoforms of these factors has been used to dissect the molecular mechanism of TCDD toxicity (Evans et al 2005, Prasch et al 2006).

The zebrafish PXR has also been identified and demonstrated to be activated by a subset of the known ligands of the human PXR (Reschly et al 2007, Ekins et al 2008). This limited set of ligands has been proposed to be due to a smaller ligand binding domain than that of the human nuclear receptor (Ekins et al 2008). Bresolin et al (2005) have reported that PXR is expressed in adult zebrafish liver however, the expression and extent of function of PXR in embryonic and larval tissues, and throughout development has yet to be fully determined. It should be noted that there is currently no identified zebrafish CAR, although other nuclear receptors such as peroxisome proliferator-activated receptors (PPARs) have been identified (Bainy 2007).

This study has primarily used ligands of the AhR such as α-naphthoflavone and Aroclor 1254 to induce CYP and UGT gene expression and activities (Chapter 3). These nuclear receptors have also been implicated as part of the mechanism for solvent-mediated down-regulation of CYP1A gene expression and EROD activities in zebrafish larvae by methanol and DMSO (Chapter 5). The routine use of high concentrations (≥1% v/v) of solvents such as DMSO and methanol in zebrafish safety assessment and toxicity assays (e.g. those of Berghmans et al 2008, Richards et al 2008 and Winter et al 2008) may result in reduced correlations of predictivity (comparing to mammalian species) of these assays, due to altered biotransformation of the test compounds. Thus caution should be

exercised when analysing such data, taking into account if the metabolism of the test compound either promotes toxicity, pharmacological effect, or renders the compound pharmacologically inactive. The induction of CYP and UGT expression, and EROD activity, by Aroclor 1254 is the first demonstration of such effects of this mixture of polychlorinated biphenyls in zebrafish, with Troxel et al (1997) reporting that Aroclor 1254 (using dietary exposure or intra-peritoneal injection) failed to induce CYP expression in adult zebrafish. Chapter 3 surprisingly demonstrated that the CYP inhibitors α-naphthoflavone and SKF525A induced EROD and OOMR activities, and did not inhibit ECOD activity in zebrafish larvae. The induction of EROD activity by α-naphthoflavone has been attributed to the potential for this compound to act as a mild agonist of AhR, and it is suggested that SKF525A may also act as a ligand for another nuclear receptor in order to mediate the induction of OOMR activity. These effects demonstrate that not only do zebrafish larvae express genes and activities for the metabolism of xenobiotics similar to those observed in mammals, but that these activities can also be modulated (induction and inhibition) in a manner similar to those observed using mammalian systems. Although there are only a few examples of such modulation in this thesis, and further examples are required to confirm such findings, these observations not only support the use of zebrafish larvae as a model system for safety assessment with regards to drug metabolism influencing toxicity, but as a result of the inducibility (and inhibition) of such systems, there is the potential to assess and identify any potential drug-drug interactions that a test compound may have.

Another important consideration is the relevance of the work presented in this thesis to the field of ecotoxicology. The identification of pharmaceuticals from a range of therapeutic classes in the aquatic environment has demonstrated that aquatic species are exposed to low concentrations of xenobiotics (Jones et al 2002, Cunningham et al 2008,

Farrè et al 2008). Thus the disposition and fate of such compounds within species such as fish is of relevance to the consideration of the environmental safety of pharmaceuticals. The demonstration of the metabolism of acetaminophen and ibuprofen by larval zebrafish (Chapter 4) suggests that there is potential for the conversion of pharmaceuticals from toxic species to non-toxic species, and *vice versa*, as well as the potential modulation of the pharmacology of compounds. Thus the application of zebrafish larvae (96 hpf) to ecotoxicology toxicity testing is likely to reflect the biotransformation of test compounds along with any toxic effects, although further examples of, and confirmation of, the bioactivation of xenobiotics to toxic derivatives are required to strengthen this conclusion.

The application of model organisms such small fish species including the zebrafish (and in particular embryonic and larval zebrafish) to toxicity testing results from a desire to instigate *in vivo* assessments of compound toxicity, and to increase the attrition of candidate drugs before testing in rodent and non-rodent species (i.e. if a compound is going to fail due to toxicity then it is best to fail in the early stages of toxicity testing). In addition, the use of small fish species complies with the 3R's directive (the reduction, replacement, and refinement of animal model systems) for the use of animals in scientific research. In order for such model organisms to adequately fufil these roles it is important that similar pharmacology, metabolism, distribution and excretion of test compounds as to that observed in humans is reflected by the organisms. In this thesis, it has been reported that zebrafish have similar oxidative (CYP enzymes) and conjugative (such as UGT and SULT enzymes) systems present as those found in mammalian species for the metabolism of xenobiotics, although continued characterisation of the metabolism of xenobiotics using a wider range of characteristic and pharmaceutical substrates is required, and thus suggests that they will be appropriate for use in drug screening and toxicity testing strategies. Finally, this thesis has highlighted an apparent sensitivity of EROD activity and CYP gene expression to DMSO and methanol exposure. This finding

may have implications upon the interpretation of toxicity testing assays using zebrafish larvae.

6.2 Future work

Although, as discussed above, this work strengthens the justification for the application of zebrafish larvae to toxicity testing, further characterisation of the metabolism of xenobiotics in this organism is required. Further examples of the metabolism of pharmaceuticals, in addition to acetaminophen and ibuprofen, from a variety of different therapeutic classes are required in order to strongly compare zebrafish larvae and mammalian species in terms of the metabolism of xenobiotics. The analysis of the metabolism of compounds such as caffeine, coumarin, diazepam, fluoxetine, simvastatin and tamoxifen, using techniques such as LC-MS(MS) and/or nuclear magnetic resonance spectroscopy (NMR) would allow the identification of metabolites produced by specific enzyme isofoms (e.g. human CYP3A4 catalyses the 8-hydroxylation of caffeine, Brown et al 2008). The monitoring of such reactions would not only provide examples of xenobiotics that are metabolised by zebrafish larvae, but would also identify enzyme activities similar to those found in mammals, and thus help characterise to what extent the similarities in substrate specificity between zebrafish and mammals exist. An important consideration, with regards to toxicity, is often the balance between the “phase I” oxidative and reductive activities and the “phase II” conjugative activities of drug metabolism systems, and even between multiple different CYP isoforms for a given substrate. As a result the predictivity of model organisms for human toxicity is never going to be 100% reliable, especially when human inter-individual variability (polymorphic variant for drug metabolism enzymes) is also considered. It should be noted that this is not just an issue for the use of fish as a model organism, as rodent models also suffer from this limitation. The identification of any toxic species and pharmacologically-inactive species would also

provide additional evidence that zebrafish larvae can modulate the pharmacology of foreign compounds. Determining the distribution of xenobiotic compounds within the larvae itself would also provide useful information, and could be achieved using either fluorescent substrates (for example, rhodamine red accumulation has been demonstrated by Scholz et al, 2006), or by autoradiography using radio-labelled compounds. The identification of tissue expression patterns of CYP, UGT, GST and SULT genes in zebrafish embryos and larvae throughout development would also provide useful information, particularly with respect to tissue-specific toxicity. The application of *in situ* hybridisation using DIG-labelled RNA probes specific for a wide range of xenobiotic metabolism genes would allow such an assessment. In addition to the characterisation of the zebrafish xenobiotic metabolism systems there is also potential for the insertion of human drug metabolism genes (e.g. CYP3A4) into the zebrafish. The resulting "humanised" zebrafish may more accurately predict potential compound toxicity than "non-humanised" fish, due to the presence of human enzyme activity and substrate specificities, as well as the zebrafish ones.

The demonstration that the *in vitro* inhibitors α-naphthoflavone and SKF525A induced EROD and OOMR activities in zebrafish highlighted the potential for the assessment of drug-drug interactions *in vivo* using zebrafish larvae. This approach would be of great benefit for drug discovery, as by monitoring specific reactions (e.g. EROD activity) it is possible to assess the effect of compounds upon the metabolism of others. Processes such as induction and inhibition are reflected using an *in vivo* system such as the zebrafish larvae, as demonstrated in Chapters 3 and 5.

Finally, the observed effects of the organic solvents DMSO and methanol highlight an area requiring further investigations. It would be important to determine which solvent, if any, has the least or no modulatory effects upon zebrafish drug metabolism genes and activities. Also, determining whether the observed effect is specific for EROD activity or

CYP activities in general would be of great benefit, as there is evidence provided in this study that the expression of several genes is affected upon solvent exposure. Assessing the effect of solvents upon the GST and SULT isoforms would also be of great benefit. The identification of no-effect levels for solvents would be of great use for providing suitable guidelines for vehicle usage in toxicity testing using zebrafish larvae.

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Appendix

Appendix

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tr|Q6TH23|Q6TH23_DANRE          MALTILPILGPISVSESLVAIITICLVYLLMRLNRTKIPDGLQKLPGPKP 50
gi|4503199|ref|NP_000490.1|     ---MLFPIS--MSATEFLLASVIFCLVFWVIRASRPQVPKGLKNPPGPWG 45
                                    ::**   :*.:* *:* : :***: ::* .*.::*.**:: ***

tr|Q6TH23|Q6TH23_DANRE          LPIIGNVLEIGNNPHLSLTAMSKCYGPVFQIQIGMRPVVVLSGNDVIRQA 100
gi|4503199|ref|NP_000490.1|     WPLIGHMLTLGKNPHLALSRMSQQYGDVLQIRIGSTPVVVLSGLDTIRQA 95
                                 *:**::* :*:****:*: **: ** *:**:**  ******* *.****

tr|Q6TH23|Q6TH23_DANRE          LLKQGEEFSGRPELYSTKFISDGKSLAFSTDQVGVWRARRKLALNALRTF 150
gi|4503199|ref|NP_000490.1|     LVRQGDDFKGRPDLYTFTLISNGQSMSFSPDSGPVWAARRRLAQNGLKSF 145
                                *::**::*.***:**: .:**:*:*::**.*.  ** ***:** *.*::*

tr|Q6TH23|Q6TH23_DANRE          STVQGKSPKYSCALEEHISNEGLYLVQRLHSVMKADGSFDPFRHIVVSVA 200
gi|4503199|ref|NP_000490.1|     SIASDPASSTSCYLEEHVSKEAEVLISTLQELMAGPGHFNPYRYVVVSVT 195
                                * ... :.. ** ****:*:*.  *:. *:.:* . * *:*:*::****:

tr|Q6TH23|Q6TH23_DANRE          NVICGICFGRRHSHDDDELVRLVNMSDEFGKIVGSGNPADFIPFLRILPS 250
gi|4503199|ref|NP_000490.1|     NVICAICFGRRYDHNHQELLSLVNLNNNFGEVVGSGNPADFIPILRYLPN 245
                                ****.******:.*:.:**: ***:.::**::***********:** **.

tr|Q6TH23|Q6TH23_DANRE          TTMKKFLDINERFSKFMKRLVMEHYDTFDKDNIRDITDSLINHCEDRKLD 300
gi|4503199|ref|NP_000490.1|     PSLNAFKDLNEKFYSFMQKMVKEHYKTFEKGHIRDITDSLIEHCQEKQLD 295
                                .::: * *:**:* .**:::* ***.**:*.:*********:**::::**

tr|Q6TH23|Q6TH23_DANRE          ENSNLQVSDEKIVGIVNDLFGAGFDTISTALSWAVVYLVHYPEVQERLQR 350
gi|4503199|ref|NP_000490.1|     ENANVQLSDEKIINIVLDLFGAGFDTVTTAISWSLMYLVMNPRVQRKIQE 345
                                **:*:*:*****:.** *********::**:**:::***  *.**.::*.

tr|Q6TH23|Q6TH23_DANRE          ELDEKIGKDRTPLLSDRANLPLLESFILEIFRHSSFLPFTIPHCTSKDTS 400
gi|4503199|ref|NP_000490.1|     ELDTVIGRSRRPRLSDRSHLPYMEAFILETFRHSSFVPFTIPHSTTRDTS 395
                                ***  **:.* * ****::** :*:**** ******:******.*::***

tr|Q6TH23|Q6TH23_DANRE          LNGYFIPKDTCVFVNQWQVNHDPELWKDPSSFIPDRFLTADGTELNKLEG 450
gi|4503199|ref|NP_000490.1|     LKGFYIPKGRCVFVNQWQINHDQKLWVNPSEFLPERFLTPDG-AIDKVLS 444
                                *:*::***. ********:*** :** :**.*:*:****.**  ::*: .

tr|Q6TH23|Q6TH23_DANRE          EKVLVFGLGKRRCIGESIGRAEVFLFLAILLQRLKFTGMPGEMLDMTPEY 500
gi|4503199|ref|NP_000490.1|     EKVIIFGMGKRKCIGETIARWEVFLFLAILLQRVEFSVPLGVKVDMTPIY 494
                                ***::**:***:****:*.* ************::*:   *  :**** *

tr|Q6TH23|Q6TH23_DANRE          GLTMKHKRCLLRVTPQPVF 519
gi|4503199|ref|NP_000490.1|     GLTMKHACCEHFQMQLRS- 512
                                ******  *
```

Figure A1: The allignment of the predicted amino acid sequences for CYP1A (*Danio rerio*, Q6TH23_DANRE) and CYP1A1 (*Homo sapiens*, NP_000490.1) using the ClustalW software availible at http://www.ebi.ac.uk/Tools/clustalw2/index.html (accessed December 2009). Identical residues indicated by *, conserved substitutions (based upon the chemical properties of the amino acids – e.g. acidic, basic or hydrophobic) indicated by :, and semi-conserved substitutions indicated by .

```
tr|Q9IBG8|Q9IBG8_DANRE          MALENILLHLNSKVWTDAGTILLLFILFLLVSVKLRNRNKPTKTFLLGPT 50
gi|7949031|ref|NP_000758.1|     MELS-VLLFLA----------LLTGLLLLLVQRHPNTHDR----LPPGPR 35
                                * *. :**.*           **  :*:***. : ..:::    :  **

tr|Q9IBG8|Q9IBG8_DANRE          PLPFIGNVFNLDTSQPHICLTKMSDHYGNIFSLRLGSLNTVVVNTYSMVK 100
gi|7949031|ref|NP_000758.1|     PLPLLGNLLQMDRRGLLKSFLRFREKYGDVFTVHLGPRPVVMLCGVEAIR 85
                                ***::**::::*      .: :: ::**::*:::**.  .*::   . ::

tr|Q9IBG8|Q9IBG8_DANRE          KVLNDQGNSFMYRPSNDITERILSKCQGLTFNNGYSWKQHRRFTLSTLKF 150
gi|7949031|ref|NP_000758.1|     EALVDKAEAFSGRGKIAMVDPFF-RGYGVIFANGNRWKVLRRFSVTTMRD 134
                                :.* *:.::*  * .  :.: :: :  *: * **  **  ***:::*::

tr|Q9IBG8|Q9IBG8_DANRE          FGVGKRSLEFIIMEEYKFLHQSILDTNGLPFNPHYIINNGVSNIICSMVF 200
gi|7949031|ref|NP_000758.1|     FGMGKRSVEERIQEEAQCLIEELRKSKGALMDPTFLFQSITANIICSIVF 184
                                **:****:*  * ** : * :.: .::*  ::* ::::. .:*****:**

tr|Q9IBG8|Q9IBG8_DANRE          GRRFEYTDQRFLNMLSLISKALKLQTSVFIQLYAAFPRLMDLLPGPHKEL 250
gi|7949031|ref|NP_000758.1|     GKRFHYQDQEFLKMLNLFYQTFSLISSVFGQLFELFSGFLKYFPGAHRQV 234
                                *:**.* **.**:**.*: :::.* :*** **:  *. ::. :**.*:::

tr|Q9IBG8|Q9IBG8_DANRE          FSCFHQVRAFIKEEVDKHRADWDPSSPRDFIDCYLTEIEKMKDDLEAGFH 300
gi|7949031|ref|NP_000758.1|     YKNLQEINAYIGHSVEKHRETLDPSAPKDLIDTYLLHMEKEKSNAHSEFS 284
                                :. ::::.*:* ..*:***   ***:*:*:** ** .:** *.: .: *

tr|Q9IBG8|Q9IBG8_DANRE          DEGLQYAVLDLFVAGTETTSTTLLWAFVYMMKY-QKSKKVQAEIDKVVGR 349
gi|7949031|ref|NP_000758.1|     HQNLNLNTLSLFFAGTETTSTTLRYGFLLMLKYPHVAERVYREIEQVIGP 334
                                .:.*:  .*.**.********** :.*: *:** : :::*  **::*:*

tr|Q9IBG8|Q9IBG8_DANRE          YRRPSMDDRPCMPYTDAVIHEIQRMGNVVPLSVPRMTNEDTLLEGLFHSL 399
gi|7949031|ref|NP_000758.1|     HRPPELHDRAKMPYTEAVIYEIQRFSDLLPMGVPHIVTQHTSFRG-YIIP 383
                                :* *.:.**. ****:***:****:.:::*:.**::..:.* :.* :

tr|Q9IBG8|Q9IBG8_DANRE          RVLQIIPNLTSVLFDQTKWKTQHSFDPQNFLNAQGKLKSLKLLSPFSLGK 449
gi|7949031|ref|NP_000758.1|     KDTEVFLILSTALHDPHYFEKPDAFNPDHFLDANGALKKTEAFIPFSLGK 433
                                :  :::  *::.*.*   ::. .:*:*::**:*:* **. : : ******

tr|Q9IBG8|Q9IBG8_DANRE          RSCPGESLARMELFLFFTSFLQSFSLSAP--DETQTSLDFKCGMTLSPKP 497
gi|7949031|ref|NP_000758.1|     RICLGEGIARAELFLFFTTILQNFSMASPVAPEDIDLTPQECGVGKIPPT 483
                                * * **.:** *******::**.**:::*   *       :**:   * .

tr|Q9IBG8|Q9IBG8_DANRE          FKICFTPR 505
gi|7949031|ref|NP_000758.1|     YQIRFLPR 491
                                ::* * **
```

Figure A2: The allignment of the predicted amino acid sequences for CYP2J26 (*Danio rerio*, Q9IBG8_DANRE) and CYP2B6 (*Homo sapiens*, NP_000758.1) using the ClustalW software availible at http://www.ebi.ac.uk/Tools/clustalw2/index.html (accessed December 2009). Identical residues indicated by *, conserved substitutions (based upon the chemical properties of the amino acids – e.g. acidic, basic or hydrophobic) indicated by :, and semi-conserved substitutions indicated by .

		Rat		Mouse		Human		Zebrafish		Rainbow trout		
Substrate	**Human Genes**	**Rate**	**References**	**Rate**	**References**	**Rate**	**References**	**Rate**	**References**	**Rate**	**References**	**Notes**
7-Ethoxyresorufin	CYP1A, CYP1B1,	28.2	Chan et al 2009	100 ± 23	Sun et al 2006	100 ± 50	Ueng et al 2002	YES	Billard et al 2006	43.8	Sadar and Andersson 2001	zebrafish larvae assessed using fluorescence microscopy
(O-deethylation)	CYP1D1	99 ± 10	Sun et al 2006	66.96 ± 1.55	Krajka-Kuźniak and Baer-Dubowska 2003	13	Maurice et al 1991	< 20 (adult liver)	Troxel et al 1997	168.6 ± 58.8	Moutou et al 1998	
				280 ± 80	Ueng et al 2002			664 ± 74 (pmol/min/nmol P450)	Chung et al 2004	3130 ± 270	Page and Davies 2002	Zebrafish CYP1A over-expressed in yeast
				74 ± 9	Madra et al 1996			< 1 at (3 and 4 dpf)	Mattingly and Tuscano 2001			Zebrafish larval homogenates (< 5 dpf)
								0.0008 - 0.0016 pmol/min/larvae	Noury et al 2006			Live zebrafish larvae
7-Ethoxycoumarin	CYP2-family	704	Chan et al 2009	11	Greenlee and Poland 1978	520	Maurice et al 1991			19.9	Sadar and Andersson 2001	
(O-deethylation)				24	Greenlee and Poland 1978					42 ± 2.3	González et al 2009	
Methoxyresorufin	CYP1A2	21.5	Chan et al 2009	130 ± 13	Sun et al 2006	70 ± 20	Ueng et al 2002	120 (juvenile)	Arukwe et al 2008	1190 ± 250	Page and Davies 2002	
(O-demethylation)		41 ± 14	Sun et al 2006	145.57 ± 3.87	Krajka-Kuźniak and Baer-Dubowska 2003							
				1420 ± 170	Ueng et al 2002							
Pentoxyresorufin	CYP2B1	20.3	Chan et al 2009	13.6 ± 2.2	Sun et al 2006			50 (juvenile)	Arukwe et al 2008	1720 ± 250	Page and Davies 2002	
(O-deethylation)		21 ± 10	Sun et al 2006	36.38 ± 1.99	Krajka-Kuźniak and Baer-Dubowska 2003							
				7 ± 1	Madra et al 1996							
Benzyloxyresorufin	CYP1A1, CYP2B1,	37.9 ± 6.8	Sun et al 2006	80 ± 17	Sun et al 2006			25 (juvenile)	Arukwe et al 2008	7.3 ± 2.2	Moutou et al 1998	
(O-deethylation)	CYP3A1			30 ± 3	Madra et al 1996					1940 ± 50	Page and Davies 2002	
												Gender differences observed for rat and mouse (Miura et al 1989)
Testosterone	CYP3A4	YES	Miura et al 1989	YES	Miura et al 1989	YES	Miura et al 1989	YES	Reschly et al 2008			Zebrafish liver cell line
(6β-hydroxylation)						21.2 ± 5.6	Fayer et al 2002					Primary human hepatocytes (Fayer et al 2002)
Aflatoxin B1	CYP1A2, CYP3A					20	Buening et al 1981	71 ± 5 (pmol/min/nmol P450)	Wang-Buhler et al 2005	185 ± 2 (pmol/min/nmol P450)	Wang-Buhler et al 2005	Zebrafish CYP2K6 and rainbow trout CYP2K1 over-expressed in insect cells
									Troxel et al 1997	66.6 ± 7.1	Troxel et al 1997	Aflatoxicol and glucuronide metabolites excreted by adult zebrafish (HPLC) and human microsomes
								11.7 ± 1.4 (adult)	Troxel et al 1997			Liver homogenates measuring aflatoxin B1-8,9-epoxide in zebrafish and rainbow trout
Benzo-[a]-pyrene	CYP1A, CYP2C,			580 ± 140	Krajka-Kuźniak and Baer-Dubowska 2003	120 ± 40	Ueng et al 2002	1160 ± 113 (pmol/min/nmol P450)	Chung et al 2004			Zebrafish CYP1A over-expressed in yeast
(hydroxylation)	CYP3A4											

A multi-species comparison of the metabolism of model CYP indicator reactions and xenobiotic compounds assessed in zebrafish adult and larvae, compared with rat, mouse, human and rainbow trout. Activities are expressed as pmol product/minute/mg of tissue unless otherwise stated.

Publications

Research Papers

Jones H, Panter G, Hutchinson T, Chipman JK (2010). *Oxidative and conjugative metabolism in zebrafish larvae in vivo.* Zebrafish, in press.

Conference attendance – poster presentations

British Toxicology Society annual conference (April 2008, Surrey, UK) – Cytochrome P450s and glucuronosyl transferase in zebrafish larvae.

British Toxicology Society annual conference (March 2009, Warwick, UK) – Assessment of non-steroidal anti-iflammatory drug (NSAID) metabolism by zebrafish larvae using liquid chromatography-mass spectrometry (LC-MS).

Pollution Responses In Marine Organisms (May 2009, Bordeaux, France) - Assessment of non-steroidal anti-iflammatory drug (NSAID) metabolism by zebrafish larvae using liquid chromatography-mass spectrometry (LC-MS).

Conference attendance – platform presentations

Society of Environmental Toxicology and Chemistry UK meeting (September 2008, Reading, UK) – Expression and activities of xenobiotic metabolising enzymes in zebrafish larvae: paracetamol metabolism as an example of an environmentally relevant pharmaceutical.

Northern Zebrafish Network Meeting (May 2009, Manchester, UK) – Assessment of ibuprofen metabolism by zebrafish larvae using liquid chromatography-mass spectrometry

Invited presentations

International Society for the Study of Xenobiotics (ISSX) European meeting (May 2009, Lisbon, Portugal), Short course 4: zebrafish for metabolic and toxicity studies – Xenobiotic metabolism and excretion in zebrafish.