Effects of pharmaceuticals in fish: *In vitro* and *in vivo* studies

Submitted by Jenna Frances Corcoran, to the University of Exeter as a thesis for the degree of Doctor of Philosophy in Biological Sciences, March 2013

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Fish may be exposed to an array of pharmaceuticals that are discharged into the aquatic environment, paralleling advances in medical knowledge, research and technology. Pharmaceuticals by their nature are designed to target specific receptors, transporters, or enzymes. Nuclear receptors (NRs) are often a key component of the therapeutic mechanism at play, and many of these are conserved among vertebrates. Consequently, fish may be affected by environmental pharmaceutical exposure, however there has been relatively little characterisation of NRs in fish compared with in mammals. In this thesis common carp (*C. carpio*) were exposed to selected pharmaceuticals *in vitro* and *in vivo* to investigate effects centred on the pregnane X receptor (PXR) and peroxisome proliferator-activated receptor alpha (PPARα), two key NRs involved in organism responses to pharmaceutical exposure. The PXR acts as a xenosensor, modulating expression of a number of xenobiotic metabolising enzymes (XMEs) in mammals. In a primary carp hepatocyte model it was shown that expression of a number of XMEs was altered on exposure to rifampicin (RIF), as occurs in mammals. This response was repressed by addition of ketoconaozle (KET; PXR-antagonist), indicating possible PXR involvement. The genes analysed showed up-regulation on exposure to ibuprofen (IBU) and clofibric acid (CFA), but not clotrimazole (CTZ) or propranolol (PRP). The lack of response to mammalian PXR-agonist CTZ was unexpected. In contrast, the same XME genes were found to be up-regulated *in vivo* after 10 days of exposure of carp to CTZ, although this response occurred only for a relatively high exposure concentration. CTZ was found to concentrate in the plasma (with levels up to 40 times higher than the water). Development

and application of a reporter gene assay to measure PXR activation in carp (cPXR) and human PXR showed CTZ activation of cPXR, supporting data from the *in vivo* studies. Furthermore, activation was seen at concentrations as low as 0.01 µM. Interestingly RIF did not induce a response in the cPXR reporter gene assay, contrasting with the hepatocyte culture work. Taken together, the data presented here suggests divergence in the PXR pathway between mammals and fish in terms of ligand activation and downstream gene targets. PPARα was investigated in carp *in vivo* using CFA as a mammalian PPARα-agonist. Overall the resulting data suggested a broadly similar role for this NR in lipid homeostasis in fish as for mammals, with a number of PPARα-associated genes and acyl-coA oxidase (ACOX1) activity up-regulated in response to CFA exposure. A number of XMEs were also up-regulated by CFA (*in vivo* and *in vitro*), potentially extending the role of PPARα in fish (carp) to regulation of xenobiotic metabolism. The work presented has provided further characterisation of PXR and PPARα in fish. Elucidation of these pathways is vital to provide meaningful data in terms of establishing toxicity and mechanism-of-action data for pharmaceuticals and other compounds in fish, to allow validation of read-across approaches and ultimately aid in their environmental risk assessment. *In vitro* approaches are attractive ethically, financially and can provide useful mechanistic characterisation of compounds and the primary hepatocyte model and reporter gene assays used here show potential for the screening of pharmaceutical compounds in fish. However, further understanding of the metabolism of drugs and chemicals in fish is required to establish the true value of these methods for informing on possible effects in fish, *in vivo*.

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To beautiful, wonderful Louis - thank you for keeping me going. You make my heart smile and your hugs have helped me through the most dismal of days. This thesis is dedicated to you.

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List of (non-research paper) Figures

Author's Declaration

Statement: I, Jenna Frances Corcoran, was involved in the following manner in the papers presented in this thesis: I planned and wrote the Introduction, Discussion and Review Paper. I planned and carried out the experiments and statistical analysis for Research Papers I, II, III and IV. I was responsible for writing, and co-ordinating the manuscripts for Research Papers I, II, III and IV with valuable input from co-authors.

Dr Shinichi Miyagawa carried out the cloning of the human PXR and construction of the pGL4-PXRE reporter plasmid, as well as providing guidance for the development of the reporter assay for Research Paper III.

Rob Cumming carried out the mass spectrometry analysis of water and plasma samples in Research Papers II and IV.

For all chapters and papers my primary supervisor Prof. Charles Tyler played an advisory and editorial role, advising on the planning, design and implementation of the experiments conducted and editing manuscripts and thesis chapters where necessary. My second supervisor, Dr. Matthew Winter, also played a similar role and in addition provided detailed guidance and help with the *in vivo* exposures for papers II and IV. Dr Anke Lange provided invaluable support and guidance in the planning and implementation of the experiments conducted as well as in editing of the manuscripts and thesis chapters.

List of abbreviations

ABC	ATP-binding cassette
ACOX1	Acyl-coA oxidase
ADME	Absorption, Distribution, Metabolism and Excretion
AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of variance
APO	Apolipoprotein
AR	Androgen receptor
B-AR	Beta adrenergic receptor
BCF	Bioconcentration factor
CAR	Constitutive androstane receptor
cDNA	Complementary DNA
CFA	Clofibric acid
COS-7	African green monkey (*Cercopithecus aethiops*) kidney cell line
COX	Cyclooxygenase
cPXR	Carp PXR
CTZ	Clotrimazole
CYP	Cytochrome P450
DBD	DNA-binding domain
DEX	Dexamethasone
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EC_{50}	Concentration for half the maximal effect
EE2	Ethinylestradiol
E_{max}	Maximal effect
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
EU	European Union
Fa2N-4	Human (*Homo sapiens*) liver cell line
FHM	Fathead minnow (*Pimephales promelas*) epithelial cell line
FXR	Farnesoid X receptor

GCL	Grass carp (*Ctenopharyngodon idellus*) liver cell line
GR	Glucocorticoid receptor
GST	Gluthathione-S-transferase
HAT	Histone acetyltransferase
HDL	High-density lipoprotein
hPXR	Human PXR
HSI	Hepatic (or liver) Somatic Index
IBU	Ibuprofen
KET	Ketoconazole
LDH	Lactate dehydrogenase
LBD	Ligand-binding domain
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDL	Low-denisty lipoprotein
K_{ow}	Octanol-water partitioning coefficient
LPL	Lipoprotein lipase
LXR	Liver X receptor
MDR1	Multidrug resistance 1
MEF1	MDR1 promoter-enhancing factor 1
mRNA	messenger RNA
MRP	Multidrug resistance-associated protein
NBT	Nitro-blue tetrazolium
NF-κB	Nuclear factor kappa beta
NR	Nuclear receptor
NSAID	Non-steroidal anti-inflammatory drug
OATP	Organic anion transporter protein
OSPAR	The Convention for the Protection of the Marine Environment of the North-East Atlantic
PAH	Polycyclic aromatic hydrocarbon
PBS	Phosphate buffered saline
PCB	Polychlorinated biphenyl
PCN	Pregnenalone-16α-carbonitrile
PCR	Polymerase chain reaction
PEC	Predicted environmental concentration
P-gp	P-glycoprotein
PLHC-1	Topminnow (*Poeciliopsis lucida*) hepatoma cell line

PNEC	Predicted no effect concentration
PP	Peroxisome proliferator
PPAR	Peroxisome proliferator-activated receptor
PPRE	PPAR response element
PR	Progesterone receptor
PRP	Propranolol
PXR	Pregnane X receptor
PXRE	PXR response element
RACE	Rapid amplification of cDNA ends
REACH	Registration, evaluation, authorisation and restriction of chemicals
RIF	Rifampicin
RNA	Ribonucleic acid
RO	Reverse osmosis
RTG-2	Rainbow trout (*Oncorhyhnchus mykiss*) gonad cell line
RTH-140	Rainbow trout (*Oncorhyhnchus mykiss*) hepatoma cell line
RT-qPCR	Real time quantitative PCR
RXR	Retinoid X receptor
SEM	Standard error of the mean
SOD1	Cu,Zn-superoxide dismutase
SSRI	Selective serotonin reuptake inhibitor
ST	Sulfotransferase
SXR	Steroid and xenobiotic receptor
TCDD	2,3,7,8-tetrachloro-dibenzo-*p*-dioxin
TZD	Thiazolidinedione
UGT	UDP-glucuronosyltransferase
US	United States
VDR	Vitamin D receptor
VTG	Vitellogenin
WWTW	Waste water treatment works
XME	Xenobiotic metabolising enzyme

Chapter 1
Introduction

1. Introduction

1.1 Pharmaceuticals in the aquatic environment

The aquatic environment is a natural sink for a great variety of chemicals that are released as a consequence of our modern lifestyle. Organisms that live in the aquatic environment are exposed to these compounds via the water, sludge and sediments in which they live, the food that they consume, and even during development as a consequence of maternal transfer into gametes (principally into eggs). Paralleling advances in medical knowledge, scientific research and technology, global consumption of human pharmaceuticals has risen significantly (Khetan and Collins, 2007). An inevitable consequence of this is an increased level of these biologically active compounds in surface and ground waters and an associated potential to induce biological effects, adverse or otherwise, in aquatic wildlife.

Pharmaceuticals are of particular concern as, unlike other environmental contaminants, they are designed specifically to exert particular biological effects in a vertebrate system (i.e. humans), and furthermore many are relatively novel in terms of our understanding of the targets and signalling pathways involved in their therapeutic mode of action (Fent *et al.,* 2006). Many of these pathways are conserved amongst vertebrates and so in these cases the same systems may be targeted by a pharmaceutical compound in environmentally more relevant vertebrate species such as fish. In addition, although pharmaceuticals are generally designed to have low toxicity to humans, there may be unpredicted effects in non-target species, such as fish, owing to differences in comparative physiology. As a consequence, over the past few years there has been increasing interest in the effects of pharmaceutical compounds on fish and other

environmentally-relevant organisms. From this research, there is growing evidence that some pharmaceuticals detected in the aquatic environment have the potential to cause ecological harm (discussed in detail in Chapter 2) with a number of these shown to have clear biological effects in fish and other aquatic biota as a consequence of exposure in the laboratory.

Compared to many chemical contaminants, pharmaceuticals are found at relatively low concentrations in the aquatic environment (Herberer, 2002). However, as they are generally designed to be potent, in many cases biological effect levels will be concomitantly low. In a handful of cases it has been shown that some drugs can produce a toxic effect at environmentally relevant concentrations. For example, ethinylestradiol (EE2), a synthetic oestrogen, can alter the gonadal development and induce plasma vitellogenin in fish at exposure concentrations below 1 ng l^{-1} *(*Pawlowski *et al.,* 2004) well within the range of concentrations which have been detected in wastewater treatment works effluent and surface waters (Shved *et al.,* 2008). Similarly, chronic toxicity studies with diclofenac (an anti-inflammatory agent) have shown that this compound can induce effects in fish at concentrations as low as 1 µg l^{-1} (Mehinto *et al.,* 2010). In addition to their generally high biological potency, the contamination of waterways with pharmaceuticals is characterised by their continuous release (Jones *et al.,* 2002). Exacerbating this, many are designed to be relatively stable to more effectively exert their therapeutic effect within the body, and as a consequence they can be resistance to metabolic degradation in the environment (Khetan and Collins, 2007). As such there is clearly the potential for chronic effects in aquatic organisms and, as pharmaceuticals often occur in the environment in combinations and with their metabolites and other

environmental pollutants, there is the potential for additive or synergistic combinational effects (Cleuvers, 2003).

The issue of pharmaceuticals in the aquatic environment is discussed in more detail in Chapter 2, which reviews some of the main therapeutic classes detected in the aquatic environment, and critically compares the measured environmental concentrations with the effects seen in fish in the laboratory in an attempt to gauge the scale of the potential problem. Overall the evidence suggests that, in some cases, effects in fish from laboratory based studies correlate well with known effects in mammals, although water exposure concentrations are typically much higher than those found in surface waters in the environment. There are, however, exceptions (as mentioned above) and the risks to wild fish populations have not been thoroughly characterised, especially in terms of the likely chronic nature of the exposures, or the potential for mixture effects.

1.2 Nuclear receptors

Nuclear receptors (NRs) are a diverse family of phylogenetically related proteins, present in all animals, which are pivotal in the regulation of many different functions including reproduction, lipid metabolism, drug biotransformation, homeostasis, apoptosis and development. As pharmaceutical drugs are generally designed to target an endogenous pathway, receptor or enzyme, NRs are often a critical part of the therapeutic mechanism involved, controlling many of the functions associated with major diseases (Robinson-Rechavi *et al.,* 2003). As such, NRs have been pursued by the pharmaceutical industry as one of the most promising potential drug target

categories (Metpally *et al.,* 2006). The NR superfamily includes the steroid, retinoid and thyroid hormone receptors, but also many receptors which as yet have no known endogenous ligands, termed orphan receptors (Kliewer *et al.,* 2002).

Although each NR has a distinct (sometimes overlapping) role they generally function via the same mechanism, sharing a common structural organisation (*Figure 1.1*). NRs are ligand-activated transcription factors and contain both a highly conserved DNA-binding domain (DBD) at the N terminus, and a moderately conserved ligand-binding domain (LBD) at the C terminus. Upon ligand binding the NR is able to bind specific response elements in the promoter region of target genes to increase transcription of those genes and to ultimately bring about a biological response. As such, NRs provide a direct link between signalling molecules and transcriptional responses (Robinson-Rechavi *et al.,* 2003). NRs in the NR1 sub-family (located in the nucleus) bind as an obligate heterodimer with retinoid X receptor (RXR) to specific response elements consisting of two copies of the AG(G/T)TCA motif (half sites) which may be arranged as a direct or everted (mirror image) repeat spaced by 3 – 8 nucleotides (Kliewer *et al.,* 2002). Each NR heterodimer binds a different response element and thus confers the specific gene induction profile. For example vitamin D receptor heterodimer (VDR-RXR) binds to the two half sites arranged as a direct repeat, spaced by 3 nucleotides (DR3), whereas the pregnane X receptor (PXR-RXR) commonly binds as an everted repeat spaced by 6 nucleotides (ER-6). There are also various chaperone proteins, transporters, co-activators and co-repressors involved in the intricate activation/supression mechanisms of each individual receptor.

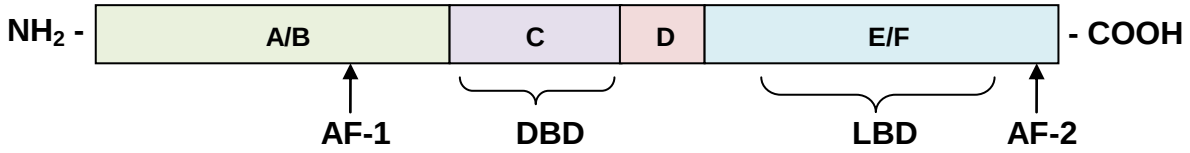

Figure 1.1: Common structure of a nuclear receptor. Region A/B is the N-terminus domain which is highly variable in length and sequence between NRs and contains the AF-1 motif (activation function 1); Region C is the highly conserved DBD (DNA-binding domain); Region D is the flexible hinge region; Region E/F is the relatively conserved LBD (ligand binding domain) which contains AF-2 (activation function 2) at the extreme C-terminus, necessary for ligand-dependant activation.

Typically, NRs act in three steps to activate transcription of a target gene. When NRs are not ligand-bound they are often associated with a co-repressor complex. Upon ligand binding this co-repressor complex dissociates and the NR recruits the first co-activator complex (histone acetyltransferase or HAT) which is involved in chromatin decondensation, thought to be a necessary step for activation of the target gene. The HAT complex then dissociates, the second co-activator complex is assembled and establishes contact with the basal transcription machinery, including RNA polymerase, to stimulate transcription of the target gene(s) (Laudet and Gronemeyer, 2002).

Some NRs are the drug target itself or in other cases the essential mechanism behind the drug's therapeutic action. For example the peroxisome proliferator-activated receptor α (PPARα; NR1C1) is directly targeted by fibrate drugs to regulate genes involved in lipid metabolism and cholesterol homeostasis with the desired therapeutic effect to lower lipid plasma levels in patients with hyperlipidaemia (Staels *et al.,* 1998). Similarly, the PPARγ form (NR1C3) regulates genes involved in fatty acid storage and glucose metabolism and is the target of many insulin sensitizing drugs such as the thiazolidinedions (TZDs) used in the treatment of type II diabetes to lower blood glucose (Spiegelman, 1998); and gluocortcoid drugs target the glucocorticoid receptor (GR; NR3C1) which simultaneously activates and represses various target genes to bring about an anti-inflammatory response (Cato *et al.,* 2004). Alternatively, some NRs are activated as a generalised response to a pharmaceutical rather than being the intended target. For example, the pregnane X receptor (PXR; NR1I2) regulates a series of genes involved in xenobiotic metabolism and excretion and acts as a xenosensor in that it is activated by a diverse range of pharmaceuticals and other compounds, many of which are themselves

substrates for the metabolising pathways encoded by the target genes (Handschin and Meyer, 2003). Critically this can result in potentially dangerous drug-drug interactions where one drug alters the metabolism of a second drug (discussed below).

Fish share a great number of closely conserved genes for NRs with mammals, and in general there seems to be a higher representation of nuclear receptors in fish with, for example, 68 in the genome of the puffer fish *Fugu rubripes* (Maglich *et al.,* 2003), and 71 in *Tetraodon nigoviridis* (Metpally *et al.,* 2006) compared with 48 in humans. At least one ortholog for each human receptor, with the exception of constitutive androstane receptor (CAR; NR1I3) and liver X receptor (LXR; NR1H2), has been identified in the *Fugu* genome (Maglich *et al.,* 2003*)*. As such it is reasonable to assume that some of the NR pathways targeted by pharmaceuticals in mammals may also be affected in fish, either as a direct consequence or an unintended effect of these compounds in a non-target species group. However, with the exception of the estrogen receptor (ER) which is well studied in fish, there is considerably less knowledge on the functions, mechanisms and pathways surrounding the NRs in fish and further research is required to characterise these important systems. Indeed, a better understanding of NR responses and associated pathways of action of pharmaceuticals in fish will help identify biomarkers of exposure and effect for human pharmaceuticals for both *in situ* laboratory studies and the monitoring of wild fish populations.

1.2.1 Pregnane X receptor (PXR)

The pregnane X receptor (PXR; NR1I2), also referred to as the steroid and xenobiotic receptor (SXR), is pivotal in the body's defence against exposure to xenobiotic compounds, acting as a xenosensor and modulating gene expression in response to changes in chemical signals in the cell. In mammals the PXR is known to regulate expression of a number of genes involved in xenobiotic metabolism and transportation (*Figure 1.2*) including members of the phase I oxidising cytochrome P450 (CYP) subfamilies *cyp3a*, *cyp2b* and *cyp2c*, the phase II conjugating enzymes such as UDP-glucuronosyltransferase (e.g. *ugt1a1*), glutathione-S-transferases (e.g. *gsta*), sulfotransferases, and the phase III transporter proteins multidrug resistance protein (*mdr1; abcb1*), MDR-related protein 2 (*mrp2; abcc2*) and organic anion transporter protein 2 (*oatp2*), (Maglich *et al.,* 2002). In this way the activation of the PXR by xenobiotics provides an adaptive feed-forward regulatory mechanism which results ultimately in the metabolism and excretion of these potentially harmful compounds, and so plays a protective role for the organism against this type of toxic threat. There has been much characterisation of CYP3A genes in particular. In humans CYP3A4 is responsible for the oxidation of at least 60% of human pharmaceuticals (Maurel, 1996), and as such it is a critical target of PXR ligand-activation by xenobiotics and is implicated in drug-drug interactions (discussed below).

The PXR is unusual among NRs in that it has a remarkably broad range of ligands; most other NRs respond to a much more specific range of ligands. Consequently, the PXR is termed a 'promiscuous receptor' as it is activated by numerous and diverse xenobiotic compounds. These include many human

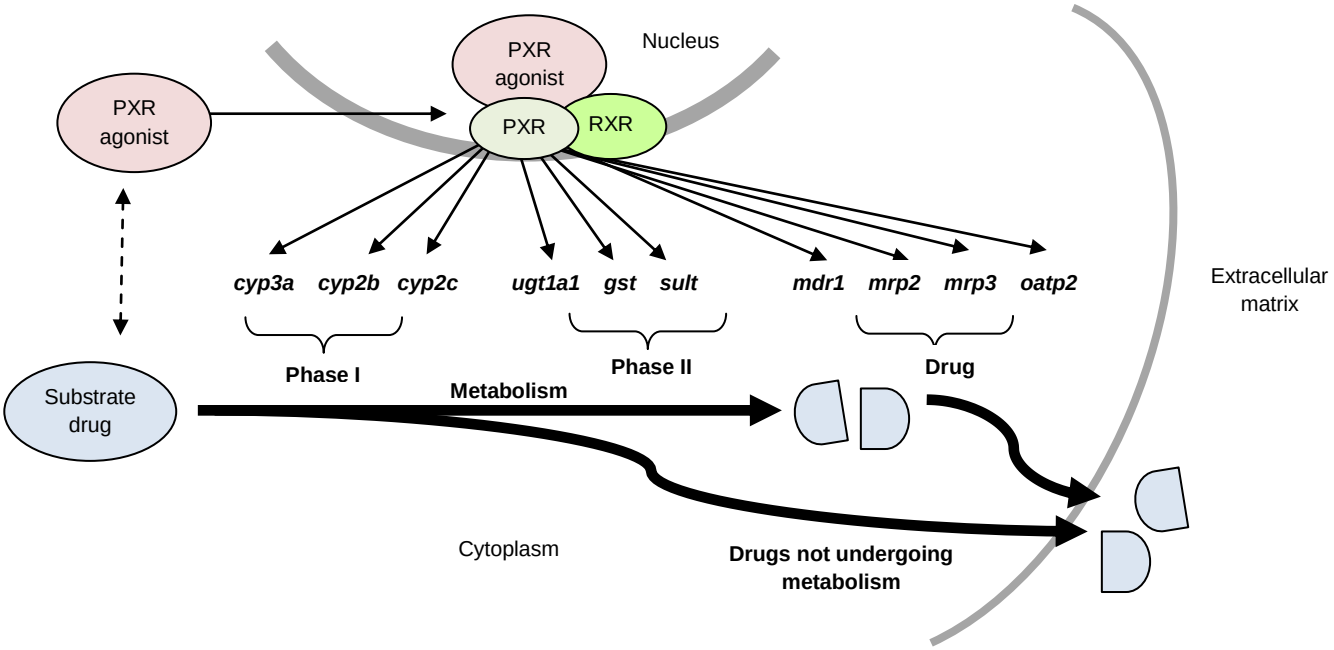

Figure 1.2: Overview of the major gene targets of PXR activation in mammals and their involvement in the xenobiotic metabolism and transport pathway; namely members of cytochrome P450s 3a, 2b and 2c subfamilies(*cyp3a, cyp2b, cyp2c*); UDP-glucuronosyltransferase (*ugt1a1*); Certain isoforms of glutathione-S-stranferase (*gsta*, *gstp*); Sulfotransferase (*sult*); multidrug resistance 1 (*mdr1*); MDR-associated protein 2 and 3 (*mrp2, mrp3*) and organic anion transporter protein 2 (*oatp2*)

drugs currently in use such as clotrimazole (CTZ; an azole antifungal), phenobarbital (an anticonvulsant) and the antibiotic rifampicin (RIF); certain steroids; as well as the industrial chemicals bisphenol A and nonylphenol (reviewed in Lehmann *et al.,* 1998; Kliewer *et al.,* 2002). Some ligands appear to be species specific, for instance RIF is a potent ligand of the human and also rabbit PXR, whereas pregnenalone 16α-carbonitrile (PCN) and dexamethasone (DEX) are strong PXR activators in rodents. The PXR DBD is highly conserved between vertebrates, however the LBD less so, which may account for the species differences in activation profiles. This also correlates closely with CYP3A gene expression and activity induction profiles between species (Kliewer *et al.,* 2002).

Many of these ligands are substrates of the biotransformation genes whose transcription is activated by the PXR, and so essentially they induce their own metabolism. Others act as inducers (or even inhibitors) of these genes without being substrates and *vice versa*, and so although, as mentioned, the role of the PXR is generally a protective one, the vast number of ligands and substrates can be responsible for adverse drug-drug interactions in humans. For instance, a drug which activates the PXR may accelerate the metabolism of a second drug by inducing expression of CYP3A and other drug metabolising genes. Conversely some drugs act as inhibitors of the PXR and/or CYP3A, and so may reduce the clearance of a second drug. In both cases this has the potential to alter the bioavailability and tissue exposure levels of the second drug, and can lead to unintended and sometimes dangerous side effects. For example, hyperforin, a component of popular herbal antidepressant St John's Wort (*Hypericum perforatum*) is an established PXR activator and inducer of CYP3A as well as MDR1. Consequently, when taken in conjunction with oral

contraceptives containing EE2 (a substrate of CYP3A) hyperforin speeds the clearance of EE2 by almost 100%, leading to a dramatically decreased therapeutic effect and reports of 'miracle babies' (Hall *et al.,* 2003). Alternatively, co-administration of the potent anti-fungal CYP3A inhibitor ketoconazole with the opioid analgesic Fentanyl (used in cancer treatment and metabolised by CYP3A4) causes decreased clearance and over exposure to Fentanyl with severe side effects including respiratory depression (Zeisenetz *et al.,* 2011). Understandably, a great deal of medical research has been directed to this field, with the pathways and mechanisms of PXR activation and CYP3A induction in humans an important aspect in both drug discovery as an intended therapeutic target and safer drug development as a result of unintended interactions.

1.2.1.1 PXR and xenobiotic metabolism in fish

In fish information on the PXR and associated gene signalling pathways or their roles in the metabolism and transport of pharmaceuticals and other xenobiotics is relatively scarce. The PXR has been identified in various species of fish, with full PXR (NR1I2) sequences identified in zebrafish (*Danio rerio*), (Moore *et al.,* 2002; Bainy and Stegeman, 2004), rainbow trout (*Oncorhynchus mykiss*), (Wassmur *et al*., 2010) and Pufferfish (*Fugu rubripes*), (Maglich *et al.,* 2003) and a partial sequence in fathead minnow (*pimephales promelas*), (Milnes *et al*., 2008). A cell based reporter assay based on the LBD from zebrafish PXR showed activation by some of the same ligands as in mammals, including PCN, CTZ, nifedipine, phenobarbital, and several steroids such as 5B-pregnane-3,20-dione and androstanol (Moore *et al.,* 2002). In line with this, various compounds have been shown to induce CYP3A gene expression and/or enzyme activity in

fish, including PCN, RIF, DEX, ketoconazole (KET), and alkylphenol (Bresolin *et al*., 2005; Christen *et al*., 2009; 2010; Tseng *et al.,* 2005; Li *et al.,* 2008; Schlenk *et al.*, 2008; Hegelund *et al*., 2004; Hasselberg *et al.,* 2004), which indicates that the PXR may have a similar role in fish as in mammals. However these studies have also highlighted discrepancies, not only between fish and mammalian activation profiles, but also differences between fish species and between PXR activation and corresponding CYP3A induction. For example, CTZ and nifedipine are both potent mammalian PXR activators although neither of these compounds had a significant effect on the expression of *pxr*, *cyp3a* or *mdr1* mRNA in zebrafish (Bresolin *et al.,* 2005) and CTZ showed no induction of a *cyp3a* isoform in sea bass (*Dicentrachus labrax*), (Vaccaro *et al.,* 2007). In contrast, CTZ has been shown to activate zebrafish PXR *in vitro* (Moore *et al*., 2002), so the case is far from certain. Regarding species differences, the prototypical human PXR activator RIF has been shown to be ineffective in activating CYP3A mRNA *in vitro* in rainbow trout cell line RTH-149 (Wassmur *et al.,* 2010), and similarly no induction of CYP3A protein was noted in PLHC-1 fish hepatoma cell line (*Poeciliopsis lucida*), (Celander *et al.,* 1996). In contrast, RIF exposure has been shown to increase CYP3A enzyme activity *in vitro* in PLHC-1 cells (Christen *et al.,* 2010) as well as in the grass carp (*Ctenopharyngodon idella*) liver cell line (GCL) and primary hepatocytes (Li *et al.,* 2008), and to induce CYP3A mRNA expression in zebrafish larvae (Tseng *et al.,* 2005). These examples emphasise the fact that the mammalian PXR activation profile is not necessarily predictive of that in fish and underscores the importance of characterising the associated gene responses in a range of fish species before any conclusions can be drawn.

Crucially, many of the identified CYP3A activators are detectable in the aquatic environment, and are often present as part of a cocktail of xenobiotic chemicals, which potentially places fish at risk in terms of activation of the PXR xenobiotic metabolising pathway. In turn, the modulation of the PXR target genes involved in biotransformation may affect the pharmacokinetics, clearance and homeostatic balance of both endogenous and xenobiotic compounds in the fish (Schlenk *et al.*, 2008), which akin to drug-drug interactions in humans, has the potential to impact on the ability of the fish to cope with exposure to other environmental contaminants. This type of interaction has been demonstrated in rainbow trout co-exposed to the azole antifungal drug KET (a CYP3A antagonist in mammals) and to the synthetic oestrogen EE2 (a substrate of CYP3A in mammals). KET increased the sensitivity of the fish to EE2 exposure by compromising the activity of CYP3A and CYP1A (Hasselberg *et al*., 2008). The expression profiles of a number of mammalian PXR target genes are investigated in common carp *in vitro* in Chapter 3 utilising a primary hepatocyte model exposed to RIF and a number of environmentally-relevant pharmaceutical compounds from different therapeutic classes, namely CTZ, clofibric acid (CFA), ibuprofen (IBU) and propranolol (PRP).

The mRNA responses to CTZ and CFA are further characterised *in vivo* in Chapter 4 and Chapter 6, respectively. In Chapter 5, a carp PXR reporter gene assay was then developed and employed to investigate PXR activation and underpin some of the observed responses while at the same time allowing a direct comparison with human PXR activation.

1.2.2 Peroxisome proliferator activated receptors

Peroxisome proliferator-activated receptors (PPARs) are critical regulators in lipid and carbohydrate metabolism and so play important physiological roles in energy homeostasis. There are three subtypes, PPARα, β (or δ) and γ (NR1C1, 2 and 3), which show different expression patterns and have different functions and gene targets. For example PPARα is fundamental in the clearance of circulating lipids, regulating key genes involved in lipid metabolism, PPARβ is involved in lipid oxidation and cell proliferation, and PPARγ promotes adipocyte differentiation to enhance blood glucose uptake, with a key role in glucose metabolism (Berger and Moller, 2002). PPARs are activated by endogenous ligands including fatty acids, eicosanoids and leukotriens, as well as being induced by high fat diets. In addition, PPARα and γ isoforms are major targets in the treatment of dyslipidemia and diabetes respectively, and PPARβ has shown potential as a target in regulating inflammatory processes (Bishop-Bailey and Bystrom, 2009). The importance of these NRs in physiology and as drug targets has prompted much research in the field (Kota *et al.,* 2005) and as such, the PPARs have now been well characterised in mammalian model systems.

1.2.2.1 PPARα

PPARα coordinates the regulation of a number of genes involved in hepatic lipid metabolism, including those governing fatty acid uptake and activation, intracellular fatty acid trafficking, fatty acid oxidation and ketogenesis and tricylgeride storage (Rakhshandehroo *et al.,* 2010), and as such has a crucial function in lipid homeostasis. Additionally, PPARα has been implicated in lipoprotein metabolism, glucose metabolism, liver inflammation, hepatocyte

proliferation, amino acid metabolism, cholesterol and bile acid metabolism (Mandard *et al.,* 2004). *Figure 1.3* shows an overview of the various pathways mediated by PPARα in mammals. Natural ligands of the PPARα include fatty acids and their derivatives, acyl-coAs, eicosanoids and phytanic acid, whilst the PPARα is targeted therapeutically in humans by fibrate drugs, (e.g. clofibrate, fenofibrate, bezafibrate, gemfibrozil, Wy14643) which are used clinically to lower blood plasma lipid levels by reducing plasma triglycerides and increasing plasma high density-lipoproteins (HDLs) in the treatment of hypolipidemia (Staels *et al.,* 1998). Fibrates are one of a structurally diverse group of compounds termed peroxisome proliferators (PPs, also including phthalate ester plasticizers, certain insecticides and herbicides) due to their ability to cause proliferation of the cellular organelles, peroxisomes, in rodents.

This effect is mediated via PPARα, which upon ligand activation causes induction of a large set of genes encoding enzymes involved in peroxisomal β oxidation of fatty acids (e.g. *acox1, ehhadh and acaa1*) as well as genes involved in peroxisomal biogenesis (*pex* genes). PPs have also been associated with oxidative stress and hepatocarcinogenesis, due to the increased production of hydrogen peroxide (Rao and Reddy, 1991) although this is also species-specific and not reported in humans. PPARα activation also induces numerous genes involved in the mitochondrial β-oxidation and microsomal ω-oxidation of fatty acids, cholesterol homeostasis and bile acid synthesis as well as triglyceride clearance. Disruption of the pathways regulated via PPARα may have detrimental effects; for instance, a side effect of long term fibrate use in humans is the formation of cholesterol gallstones, thought be due to PPARα mediated repression of *cyp7*α and *cyp27*α involved in the bile acid synthesis pathway (Francis *et al.,* 2003). Additionally, disturbances to hepatic

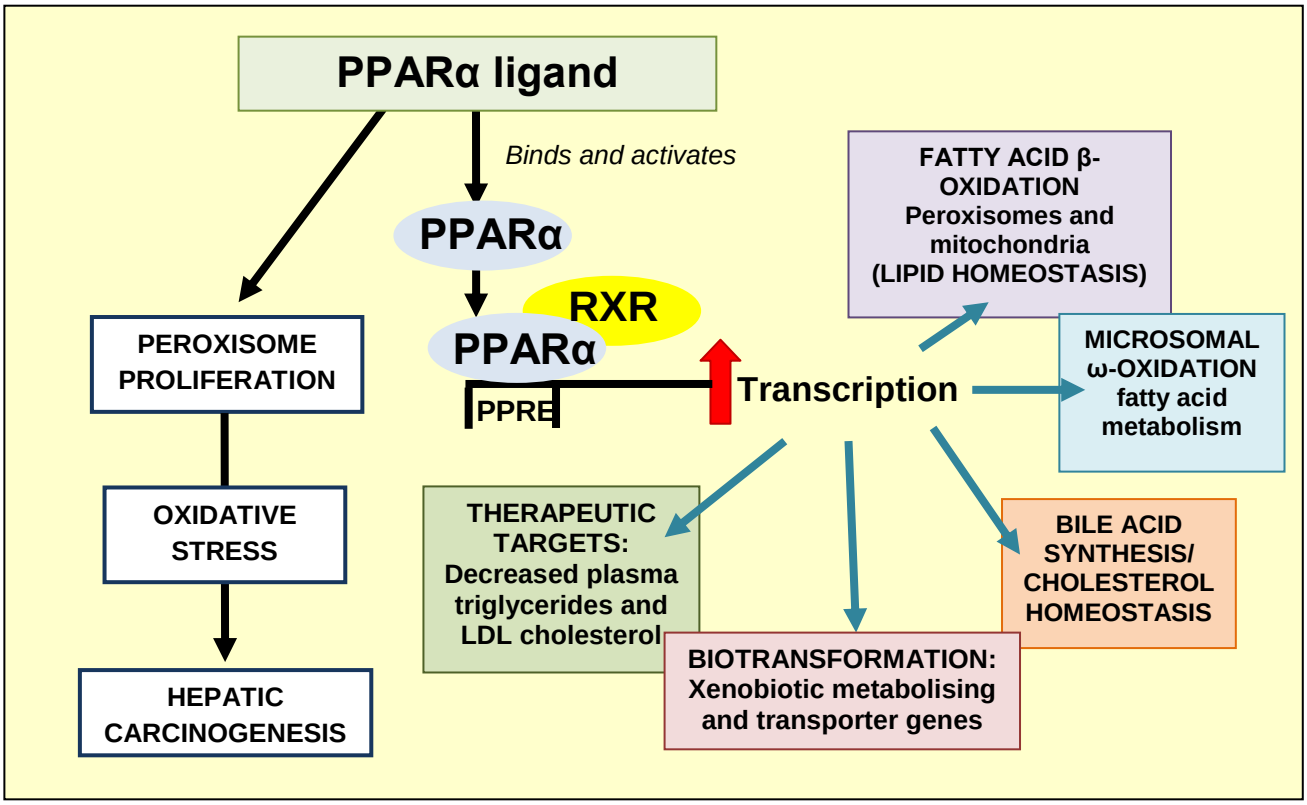

Figure 1.3: Overview of the major pathways regulated via the PPARα

lipid metabolism are the basis for alterations in plasma lipoprotein levels leading to hepatic steatosis (Rakhshandehroo *et al.,* 2010).

1.2.2.2 PPARs in fish

Three PPAR isoforms have been reported in various fish species, which are analogous to PPARα, -β and -γ (Leaver *et al.,* 2005; Boukouvala *et al.,* 2004; Andersen *et al.,* 2000; Maglich *et al.,* 2003*)* and have tissue and cell distribution similar to that of the mammalian PPAR subtypes (Ibabe *et al.,* 2002). There are also indications that some of the same PPAR- regulated pathways involved in lipid metabolism may be induced in fish on exposure to PPs. For example, exposure to fibrates has been shown to increase the activity of acyl-coA oxidase (ACOX1), the rate limiting enzyme of peroxisomal β-oxidation, in fathead minnow (Weston *et al*., 2009) and also in salmon hepatocytes (Ruyter *et al*., 1997), and the phthalate plasticiser DEHP induced expression of *acox1* as well as enoyl-coA, hydratase/3-hydroxyacylcoA dehydrogenase (*ehhadh;* part of the peroxisomal β-oxidation pathway) in zebrafish (Uren-Webster *et al.,* 2010). In contrast, clofibric acid was not found to alter protein levels of CYP4 in *Fundulus heteroclitus* (Emblidge and DeLorenzo, 2006) illustrating possible differences between the fish and mammalian response to clofibric acid exposure.

Many chemicals and substances that are discharged into the aquatic environment are known to be PPs in rodents, for example certain pharmaceuticals, oestrogens, phthalate ester plasticizers, polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), alkylphenols, certain pesticides, bleached kraft pulp and paper mill effluents (Lai, 2004) and

furthermore there is evidence that some may cause peroxisome proliferation fish and other aquatic organisms, which may be associated with oxidatiave stress and hepatocarcinogenesis (Cajaraville *et al.,* 2003). There are surprisingly little data on the effects of these compounds in fish, and limited characterisation of the role of the PPARα, response pathways and associated gene expression in fish, identifying a significant knowledge gap. Chapter 6 investigates the *in vivo* responses of common carp to the commonly-detected mammalian PPAR ligand drug CFA in an attempt to further characterise some of these pathways in fish.

1.3 *In vitro* approaches

In vitro systems are proving of increasing value in studies of predictive drug metabolism, biotransformation and toxicity in humans, offering many advantages over *in vivo* studies. For example *in vitro* systems are generally faster to run with higher throughput, cheaper than *in vivo* testing and sometimes better suited for deducing the mode-of-action of a chemical due to the absence of complicating factors such as ADME (Absorption, Distribution, Metabolism and Excretion) and the ability to maintain (and alter) specific defined experimental conditions.

In vivo testing also uses large numbers of animals, is time consuming, relatively expensive, and doesn't always provide details of the chemicals mode-of-action, despite being the most biologically holistic approach. In contrast, *in vitro* models can never be completely representative of the intact animal and there is often much disagreement as to how accurately *in vitro* data can be extrapolated to the *in vivo* situation. Nevertheless, *in vitro* models, specifically those utilising

cultured cells, are now showing increasing potential in aquatic species and applied ecotoxicology, particularly where meeting legislative requirements would be prohibitively expensive (and ethically difficult to justify) using *in vivo* models alone. One example is the perceived effort to meet the requirements of Regulation (EC) No 1907/2006 which concerns the Registration, Evaluation, Authorisation and restriction of CHemicals (REACH). This guideline is a positive step forward in terms of regulating the release of chemicals into the environment, but at the same time potentially means a large increase in the numbers of animals used to demonstrate each chemical's toxicological properties. With this in mind, the development of reproducible, reliable and representative *in vitro* models for assessing toxicology in aquatic species is of great value. In fact, an important feature identified in the REACH guidelines is the encouragement to move away from whole animal (*in vivo)* testing, supporting the well known concept of the 3Rs in animal testing: replacement, refinement and reduction (Russell and Burch, 1959*)*.

At the same time, utilising *in vitro* models in ecotoxicology studies has great potential in terms of aiding the understanding of toxic mechanisms for these compounds, knowledge of which is lacking in fish in most cases. When considering the nature of the *in vitro* preparation to be developed and used for such work, a key feature is the capacity to make reliable extrapolations to the i*n vivo* situation. As the liver is the main organ targeted for studies of the nuclear receptors, genes and enzymes involved in xenobiotic metabolism, lipid and carbohydrate metabolism, bile acid synthesis and certain reproduction endpoints, cell lines and primary cultures of hepatocytes, therefore, show the most potential in fish in terms of toxicity testing. The relative merits of both are discussed in more detail below.

1.3.1 Primary cell culture in fish

Primary hepatocytes are derived from normal liver tissue freshly isolated from the animal. Once isolated they can either be used in suspension straight away, in which case they are viable for 2 to 4 hours; or alternatively they can be plated onto dishes as a primary culture where the cells are able to attach, in some cases becoming confluent (*Figure 1.4*), and are generally viable for up to a week (Pesonen and Andersson, 1997*).* In terms of drug metabolism studies, human hepatocytes in culture are reportedly the closest *in vitro* model to human liver, retaining many differentiated *in vivo* characteristics and liver-specific functions, including inducible drug metabolising enzymes and transporter proteins and producing a metabolic profile of a compound that is very similar to that found *in vivo* (Gomez-Lechon *et al.,* 2008).

Hepatocytes from various species of fish have been shown to be a very useful model for use studying biotransformation enzymes (Segner and Cravedi, 2001) endocrine-disrupter associated vitellogenin induction (Christinson-Heiska and Isomaa, 2008; Navas and Segner, 2006; Bickley *et al.,* 2009*)*, reporter gene assays (Marlatt *et al.,* 2006*)*, DNA damage (Tollefsen *et al,* 2006*),* cytotoxicity, and lipid peroxidation *(*Gagne *et al.,* 2006*)*, often with dose-response relationships close to values obtained *in vivo.* A major drawback in mammalian primary hepatocyte culture is the decline of many liver-specific functions during culture of more than a few days (Gomez-Lechon *et al.,* 2008*)* however, primary cultures of fish hepatocytes are generally reported to maintain differentiated *in vivo* characteristics, including metabolic pathways and responsiveness to various hormones, with only minor cytological alterations in the first 5 days (Segner, 1998).

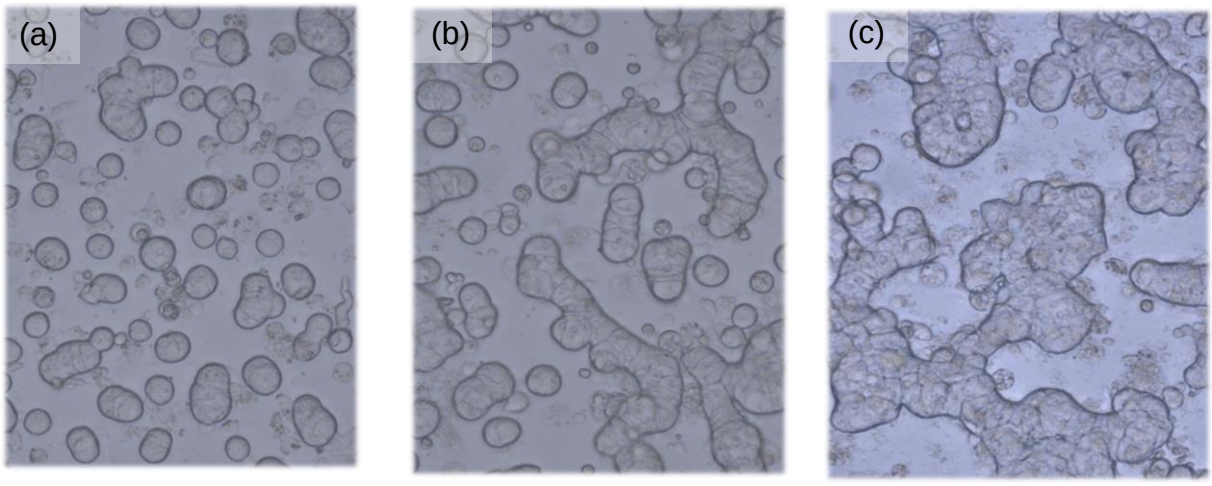

Figure 1.4: Common carp primary hepatocytes used in this thesis, visualised under the light microscope after (a) 24 h (b) 48 h and (c) 72 h in culture.

With regards to toxicity testing, it is obviously a prerequisite that those enzymes involved in aspects of drug metabolism (e.g. Phase I oxidising enzymes such as CYPs, Phase II conjugation enzymes such as UDP) are stable, in order for the *in vitro* results to correlate as closely as possible with those *in vivo.* Whereas in mammals, isolated hepatocytes can quickly lose the capacity for xenobiotic metabolism in terms of levels of enzymes such as CYPs *(*Singh *et al.,* 1996; Guguen-Guillouzo and Guillouzo*,* 1983*)*, in fish, data indicates a good degree of stability of these enzymes (Sturm *et al.,* 2001; Pesonen and Andersson, 1997; Segner *et al.,* 1999; Stegeman *et al.,* 1992; Segner and Cravedi, 2001*)*. In fact levels of CYPs and conjugating enzyme levels have been shown to remain very close to the levels in freshly isolated cells *(*Pesonen and Andersson, 1997). On the other hand primary hepatocytes do not necessarily reflect the intact liver, which contains a variety of different cell types; although hepatocytes account for 80% of the livers cell volume *in vivo*, other cells such as Kupffer cells may be necessary, for example, for cofactor supply (Barndon *et al.,* 2003). In addition, the isolation of cells can be time consuming, laborious and complicated, the primary cells have only short term viability and may show much variation between different cultures, depending for instance on the health, age or sex of the fish and differences in the isolation procedure (Segner, 1998).

1.3.2 Immortalised cell lines in fish

Cell lines are specific cell types artificially maintained in the laboratory for scientific purposes and are originally derived from a primary culture of cells, which has been divided and successfully propagated to establish a new culture. Fish cell lines are generally continuous (Bols *et al.,* 2005*)*, which means they

are 'immortal' in the sense that they have the ability to proliferate indefinitely. Mammalian cell lines are well established for use in biomedical research, and fish cell lines are becoming of growing importance in the field of ecotoxicology (Hightower and Renfro, 1988). There are now over 150 cell lines reportedly in use from 74 species of fish (Fryer and Lannan, 1994; Bols *et al.,* 2005) which have been utilised in measuring a number of endpoints, such as DNA damage (Smolarek *et al.,* 1987;1999; Nehls and Segner, 2001), cell detachment (Babin *et al.,* 2005), neurotoxicity (Evans *et al.,* 2000), endocrine disruption (Fent, 2001; Flouriot *et al.,* 1995), cytochrome P450 induction (Fent, 2001; Huuskonen *et al.,* 1998; Thibaut and Porte, 2008) and β-galactosidase enzyme activity (Babin *et al.,* 2005).

An important requirement for use as an *in vitro* model in toxicity testing is that the cells should retain a good degree of differentiation and so resemble the normal physiology of the originating cells (e.g. hepatocytes) *in vivo,* especially in the case of pharmaceuticals which are generally targeted at a specific target function or cell type. However, one of the most reported problems with cell lines is that they have often lost important structural, functional or metabolic properties of the originating tissue, and so there is some dispute as to how well the *in vivo* situation is really represented. In particular, it has been reported for various cell lines that there is often incomplete or low level enzyme expression of all families of metabolic enzymes (Brandon *et al.,* 2003), including the important phase I and II enzymes involved in xenobiotic metabolism, which are key to understanding biotransformation patterns. This obviously limits the application of cell lines for use in toxicity testing in a quantitative sense. As an example, Caminada *et al.* (2006) used fish cell lines PLHC-1 and RTG-2 to investigate the cytotoxicity of 34 common pharmaceuticals, and found that there

was poor *in vitro/in vivo* correlation. Despite the disadvantages, cell lines are more uniformed, easier to culture and less laborious than primary cultures, and have relatively stable enzyme concentrations, making them potentially more reproducible.

1.3.2.1 Reporter gene assays

As mentioned above, many pharmaceuticals are linked to biological responses through the binding and activation of specific NRs. Cell lines have frequently been utilised in mammals in reporter gene assays which measure direct activation of a NR *in vitro*, providing a sensitive and rapid screening method to detect compounds with a common mechanism of action. In reporter gene assays cells are simultaneously transfected with an expression vector containing the mRNA sequence for the particular NR being investigated, and a reporter vector which is generally the appropriate NR response element coupled to the 'reporter' gene. Ligand activation of the receptor allows binding to the response element sequence in the reporter vector, and subsequent transcription of the 'reporter' gene, e.g. luciferase, which can be measured spectrophotometrically or fluorometrically (*Figure 1.5*).

Reporter assays have been developed in fish to study activation of various NRs including the estrogen receptor (ER; e.g. Katsu *et al.,* 2013; Chakraborty *et al.,* 2011; Flouriot *et al.,* 1995), aryl hydrocarbon receptor (AhR; e.g. Abnet *et al,* 1999), PXR (Moore *et al.,* 2002; Milnes *et al.,* 2008), androgen receptor (AR; Eckman *et al.,* 2012) and glucocorticoid receptor (GR; Bury *et al.,* 2003). Overall, reporter assays in fish have demonstrated great potential as screening tools in environmental risk assessment and equally in elucidating the mode-of-

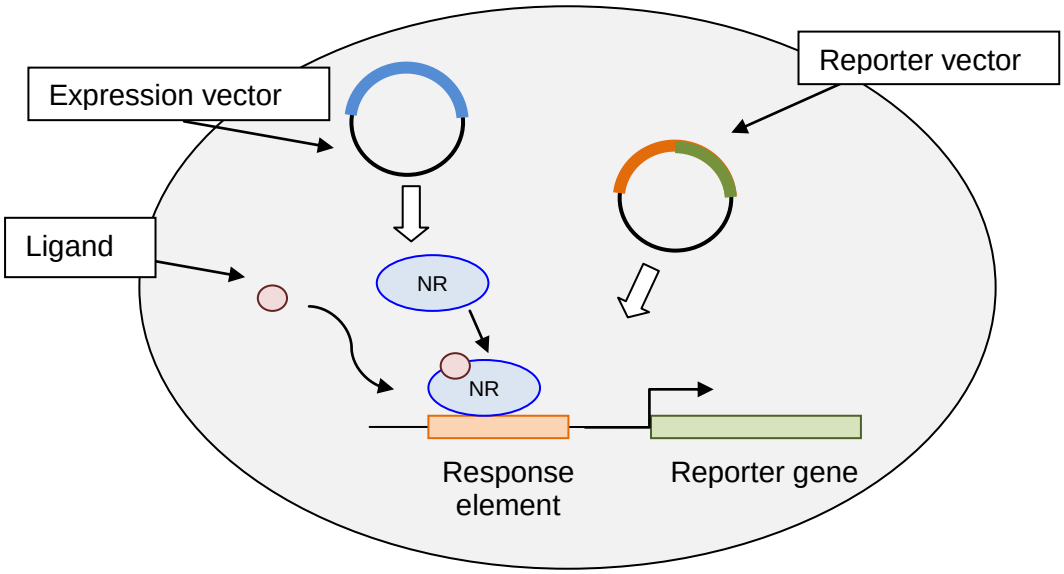

Figure 1.5: Schematic of a generalised reporter gene assay system. The expression vector consists of a plasmid expressing the nuclear receptor (NR). The reporter vector is a plasmid carrying sequences for the NR response element (i.e. the NR binding site) and the reporter gene. A ligand binds and activates the NR which binds to the response element in the promoter of the reporter gene, initiating transcription (and subsequent translation) of the reporter gene.

action of compounds, including pharmaceuticals. Importantly, these assays can be used to compare the potency of single compounds, mixtures or environmental samples, incorporating both agonistic and antagonistic effects on a receptor, thus potentially providing results of high environmental relevance and as such could prove particularly valuable in the field of ecotoxicology.

1.3.3 *In vitro* approach: summary

There is still a certain amount of ambiguity as to how well data obtained from either cell lines or cells in primary culture can be extrapolated to the *in vivo* situation, particularly in any quantitative way. However, primary hepatocytes appear to retain key features essential to the study of toxicity, most notably the maintenance of xenobiotic metabolism enzymes, at levels nearer to the *in vivo* situation (albeit over a relatively short duration), whereas in contrast, the number of fish cell lines that possess specialised functions remains limited. This points to primary hepatocyte cultures as being the method of preference over cell lines for use in fish toxicity testing at present. In Chapter 3, a common carp primary hepatocyte model is employed to investigate drug metabolising genes *in vitro* in response to a number of pharmaceutical compounds. These *in vitro* responses are compared to expression profiles for the same genes *in vivo* for two case study compounds, clotrimazole (Chapter 4) and clofibric acid (Chapter 6) in order to further validate the model and its applicability in this field of research in fish. Nevertheless, the homogeneity of cell lines gives less variation than with hepatocytes and they are therefore more amenable for toxicogenomic studies, for example those utilising reporter gene technologies, microarrays, etc. In Chapter 5, the mammalian cell line COS-7 is utilised in a reporter assay with

human and carp PXRs in order to establish the activation profile of this nuclear receptor.

1.4 Summary

In summary, some aquatic organisms are exposed to a complex cocktail of pharmaceuticals, together with a wide variety of other synthetic chemicals, many of which exert their therapeutic action on targets conserved between vertebrates; not least the super-family of nuclear receptors which govern many major physiological, metabolic and developmental processes. There is relatively little characterisation of these pathways in fish, but it is likely that many of the same receptors, genes and enzymes may be modulated by exposure to these compounds, which may place fish at risk to diverse and in some cases potentially detrimental effects. The elucidation of these pathways in environmentally-relevant species groups would provide meaningful data, not only directly in terms of toxicity of pharmaceutical compounds and mechanisms-of-action in fish, but also in comparing these responses to those mammals, and for risk assessment when determining toxicity regulations. In line with this, *in vitro* testing has many advantages over *in vivo* exposures, including ethically and financially, and has greater potential to provide useful mechanistic characterisation of compounds. Primary cell cultures are routinely used in toxicity studies in mammals, and further development of this model in fish would be beneficial in studies of ecotoxicology.

1.5 Aims of thesis

Broadly, the aim of this thesis is to investigate pathways associated with important nuclear receptors PXR and PPARα in common carp (*Cyprinus carpio*) *in vitro* and *in vivo* with the view to gaining a greater understanding of the potential effects caused by modulators of these pathways in fish, when exposed in the environment. A secondary aim was to gain a greater understanding of the value of an *in vitro* fish hepatocyte preparation as a viable alternative to the use of *in vivo* fish studies for understanding the potential impact of pharmaceuticals in the environment. With these aims in mind, the thesis is arranged into the following chapters:

Chapter 2 provides a critical review of the literature on the presence of select therapeutic classes of human pharmaceuticals reported in the aquatic environment, as well as effect concentrations of these pharmaceutical compounds in fish exposed in the laboratory, essentially providing a comparison between environmental concentration and effect concentration to try and assess potential risk to fish exposed in the wild. Potential mechanisms of action are also discussed and compared to those known in mammals.

Chapter 3 summarises studies measuring selected biotransformation genes in carp *in vitro,* which have been associated with activation of the PXR in mammals, namely cytochrome P450s (*cyp2k, cyp3a*), gluthathione-S-trasnferases (*gsta, gstp*) and multidrug resistance transporters *mdr1* and *mrp2*. A primary carp hepatocyte model was utilised in order to investigate this pathway in fish and to characterise expression of these genes in response to rifampicin exposure as a positive control for PXR activation in mammals, as well as to various environmentally-relevant pharmaceutical compounds.

The Null Hypotheses tested in this chapter are:

The PXR-associated biotransformation genes analysed (*cyp2k, cyp3a, gsta, gstp, mdr1* and *mrp2*) are not induced in response to rifampicin and/or the selected pharmaceuticals *in vitro*.

The hepatocyte cell culture model is not suitable to study gene expression in fish.

Chapter 4 compares the responses of the same key biotranformation genes measured *in vitro* (Chapter 3) on exposure of carp to clotrimazole *in vivo*. Clotrimazole is an azole antifungal drug known to potently activate the PXR in mammals, however evidence in fish is lacking and this chapter aims to better characterise this response. In this chapter bio-concentration of clotrimazole in the plasma is also measured in order to correlate with the observed hepatic gene responses.

The Null Hypotheses tested in this chapter are:

Clotrimzole is not a PXR ligand in carp and does not have an effect on expression of the biotransformation genes (*cyp2k, cyp3a, gsta, gstp, mdr1* and *mrp2*) *in vivo.*

Clotrimazole does not bioconcentrate in carp.

Chapter 5 further investigates those gene responses observed in carp *in vitro* and *in vivo* (Chapters 3 and 4 respectively) which suggest possible implication of the PXR in fish in biotransformation gene induction. In this chapter a reporter gene assay is developed to measure PXR activation in carp and validated by direct comparison with responses in human PXR.

The Null Hypotheses tested in this chapter are:

The carp PXR is not activated by human PXR ligands and is not implicated in induction of biotransformation genes.

The PXR reporter assay developed is not a suitable model for screening compounds for activation of carp and/or human PXR.

Chapter 6 investigates the involvement of PPARα and associated pathways for an exposure to clofibric acid *in vivo* in carp. Additionally, expression of a suite of key biotransformation genes were compared to those responses established *in vitro* (Chapter 3).

The Null Hypotheses tested in this chapter are:

Clofibric acid at the tested exposure concentrations has no effect on PPARα associated gene expression and enzyme activity or on biotransformation genes measured in carp *in vivo*.

Chapter 2
Review Paper

(pages 49 – 66)

Please refer to Appendix A to see ammendments to this chapter

Link to review paper:

http://informahealthcare.com/doi/abs/10.3109/10408440903373590

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Chapter 3
Research Paper I

(pages 67 – 77)

Link to Research Paper I

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Research Paper I

Supporting Information

(pages 81 – 87)

Link to Research Paper I

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Chapter 4

Research Paper II

Bioavailability of the anti-fungal pharmaceutical clotrimazole and its effects on the expression of hepatic biotransformation genes in common carp (*Cyprinus carpio*)

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Abstract

Clotrimazole (CTZ) is an imidazole antifungal agent, and an established potent ligand of the human PXR. CTZ is also frequently detected in the aquatic environment, alongside other imidazoles, is known to be persistent and predicted to bioconcentrate in fish. As such chronic exposure to CTZ may result in activation of the PXR in fish, and could pose a health threat by impacting on the organisms' ability to deal with other ligands of this key detoxification pathway component. We investigated the bioavailability of CTZ in common carp following exposure via the water and measured the hepatic responses of key downstream target genes of the PXR in mammals. The genes investigated were key drug biotransformation genes (e.g. cytochrome P450s *cyp2k* and *cyp3a* and glutathione-S-transferases *gsta* and *gstp*), and drug transporter genes (multidrug resistance 1 (*mdr1; abcb1*) and MDR-related protein 2 (*mrp2; abcc2*)). All the biotransformation genes analysed were up-regulated and the drug transporter genes altered in their expression patterns after exposure to 14.6 µg CTZ l^{-1} (measured water concentration) for 10 days, indicating the possible activation of the carp PXR by waterborne exposure to CTZ. The corresponding measured plasma concentration of CTZ was 1 µM suggesting a similar sensitivity of response in the carp PXR, with that shown in mammals. Alterations in expression of the drug transporter genes *mdr1* and *mrp2* also occurred after exposure to a water concentration of just 1 µg l^{-1} CTZ, which is within the range reported in waste water treatment works (WWTW) effluent. CTZ is one of a number of imidazoles detected in the aquatic environment, and as such, although generally present in the low ng l^{-1} range (<100 ng l^{-1}) there is the potential for PXR activation and alterations to downstream gene expression in environmentally-exposed fish, if only through potential additive effects.

Introduction

Clotrimazole (CTZ) is an imidazole used therapeutically in humans as a topical antifungal agent. The fungicidal action of imidazoles is via the inhibition of ergosterol synthesis, which leads to impairment of the cell membrane and ultimately arrest of fungal growth. Specifically, the imidazoles interfere with the cytochrome P450 (CYP) dependant 14-α-demethylation of lanesterole, the main step in the synthesis of ergosterol. This CYP inhibition is non-specific and imidazoles, including CTZ, have been shown to interact with several key CYP enzymes involved in steroid homeostasis, and drug-metabolism invertebrates.[1] Imidazoles, therefore, have the potential to affect the endocrine system in vertebrates, including fish and in 2002, CTZ was included in the OPSAR list of chemicals for priority action.[2] Furthermore, by compromising the function of drug metabolising CYPs in this way imidazoles may have effects on the metabolic clearance of other chemicals, including pharmaceuticals, pesticides and other contaminants processed by CYPs, as well as on the homeostasis of natural substrates such as steroids and bile acids. In humans imidazoles, including CTZ, are known to inhibit CYP3A4, which can result in adverse drug-drug interactions and unanticipated drug toxicities.[3] Similarly in fish, the imidazole drug ketoconazole has been shown to inhibit CYP3A in rainbow trout (*Oncorhynchus mykiss*)[4], killifish (*Fundulus heteroclitus*)[4] and Atlantic cod (*Gadus morhua*)[5] and the agricultural imidazole prochloraz has been shown to inhibit both CYP3A and CYP1A activities in rainbow trout hepatocytes.[6] Further, exposure to ketoconazole has been shown to increase the sensitivity of rainbow trout to ethinylestradiol (EE_2) exposure,[7] and exposure to CTZ resulted subsequently in increased bioaccumulation of the pro-carcinogen

benzo[a]pyrene in gizzard shad (*Dorosoma cepedianum)*[8] probably as a result of CYP inhibition.

In contrast with findings that imidazoles inhibit CYPs, CTZ has also been demonstrated to induce both *cyp3a4* mRNA expression and CYP3A4 enzyme activity in human hepatocytes.[9] In humans, CTZ is now established as a potent agonist of the pregnane X receptor (PXR; NR1I2), an important transcription factor which regulates expression of *cyp3a* as well as a several other genes involved in xenobiotic metabolism including oxidation, conjugation and transport.[10] Accordingly, many of these genes have been shown to be induced by CTZ exposure in mammals, including *cyp3a* as well as P-glycoprotein (multidrug resistance 1; *mdr1)* and MDR-related protein 2 (*mrp2)*.[11,12,13] CTZ has been shown to activate the ligand binding domain of zebrafish and fathead minnow PXR in transient transfection assays[14,15], which suggests that CTZ is also an agonist of the teleost PXR. However studies on the expression of genes thought to be regulated via the PXR in fish have so far not supported this.[16,17,18]

Around 10 tonnes of CTZ are produced annually in the EU, and the drug is available for purchase over the counter as well as via prescription.[1] Absorption through the skin after topical application is low and the main route of this drug into the aquatic environment is via bodily wash-off, with little, if any metabolism, and via waste water treatment work (WWTW) effluents. Imidazoles are also used in horticulture and agriculture as pesticides, and CTZ and various other imidazole compounds are therefore ubiquitous in surface waters and present in the ng l^{-1} to µg l^{-1} concentrations (reviewed in[19]). Unlike many pharmaceuticals which are designed to be relatively non-lipophilic to prevent their accumulation

in the body, CTZ is lipophilic in nature (log K_{ow} >4) to facilitate its entry through skin cell membranes.[20] CTZ is therefore relatively persistent in the environment, and reasonably resistant to biodegradation, with a predicted half life greater than 60 days.[2] The uptake and pharmacokinetics of CTZ in fish have not been studied, however, CTZ is predicted to have a moderate bio-concentration factor (BCF) of 610[2] and as such chronic exposure may pose a potential health threat in receiving biota.

In this study we investigated the bioavailability of CTZ in common carp (*Cyprinus carpio*) following exposure via the water and measured the responses of key genes involved in the biotransformation and transport of drugs in the liver. Bioavailability, in terms of pharmacology, is defined here as the fraction of the administered dose of (unchanged) drug, in this case CTZ, reaching the systemic circulation. Two exposure concentrations were adopted; the first (2.87 µg l^{-1}; 7.5 nM) was selected to be approaching an environmentally relevant concentration. For example, CTZ has a Predicted Environmental Concentration (or PEC) of 0.2 µg l^{-1}, and a Predicted No Effect Concentration (or PNEC) of 1 µg l^{-1}, and has been measured in the environment at concentrations up to 1.8 µg l^{-1}.[21] The second, higher concentration (34.48 µg l^{-1}; 100 nM) was selected to allow us to make comparisons with induction of the PXR pathway-associated biotransformation genes in mammals. Uptake, and subsequent clearance of CTZ was measured in the blood, and the expression of six genes thought to be regulated via the PXR, namely cytochrome P450 3A, and 2K (*cyp3a, cyp2k*), glutathione-S-transferases (*gst*) a and p, and the ABC drug transporters p-glycoprotein (multridrug resistance; *mdr1* or *abcb1*) and multidrug resistance associated protein (*mrp2* or *abcc2*), were measured by RTq-PCR.

Materials and methods

Animals

Juvenile common carp (*Cyprinus carpio*) with a mean weight of 2.5 ± 0.48 g (mean ± SEM, n=360) and a mean fork length of 49 ± 4.3 mm (mean ± SEM) were held in aerated tanks in a flow-through water system, at 22 °C, maintained under a 16:8 light: dark cycle and fed daily *ad libitum* with pelleted feed (Biomar Incio Plus 08.mm). Fish were supplied by the husbandry unit at AstraZeneca Brixham and were acclimated for 8 weeks prior to being transferred into experimental tanks at the start of the exposure experiment.

Test chemicals

Test compounds were all obtained from Sigma Aldrich, Poole, UK, unless stated otherwise.

In vivo exposure to CTZ

Fish were exposed to CTZ (CAS: 23594-75-1) via a flow through system. On day zero, individual carp were randomly allocated to one of three duplicated treatment groups: 34.48 µg l^{-1} CTZ, 2.87 µg l^{-1} CTZ or dilution water controls, and placed into 9L working volume glass tanks at a density of 10 fish per tank for each of the 3 sampling time points. This gave a total of 6 tanks per treatment. CTZ stock solution was prepared in a 120 L tank in dechlorinated tap water to a concentration of 580 µM (maximum solubility) without a solvent. This stock solution was diluted and delivered to each mixing cell at appropriate flow

rates, together with dilution water, to give the desired in-tank nominal concentrations of 34.48 µg l^{-1} and 2.87 µg l^{-1}. The nominal CTZ solutions were delivered to each individual tank at a rate of 60 ml min^{-1}. For dilution water controls, dechlorinated dilution water only was delivered to the mixing cell, and delivered to tanks as above. The stock solution was made fresh from powdered CTZ, and replenished daily. All exposure solutions were maintained at 22°C ± 0.2°C, with water temperature, dissolved oxygen and pH measured in all tanks at least twice weekly during the exposure period (see table 1).

Sampling

Duplicate tanks of fish were sampled at day 4 and day 10 of the exposure period. The remaining cohort of fish were then depurated for a further 4 days (exposed to dilution water only) and sampled on day 14. On each sampling day, 20 fish (10 from each of two tanks) were sampled from each treatment group. Fish were anaesthetised in MS222 solution (500 mg l^{-1} dechlorinated water, 1 g l^{-1} $NaHCO_3$, aerated, pH 7.28) in dilution water, weighed, fork length measured and bled from the caudal vein using heparinised (5000 U ml^{-1}) capillary tubes. Fish were terminated without recovery by destruction of the brain. The blood was spun immediately (7000g, 4 mins; Haematokrit 210, Hettich) and plasma separated, snap frozen and stored at -80 °C, until required. The liver was rapidly dissected out, weighed, snap frozen and stored at -80 °C until further analysis.

Water and plasma analysis

CTZ was determined in water samples from each individual tank, and in the plasma isolated from the sampled fish, using liquid chromatography tandem mass spectrometry (LC-MS/MS). Water and plasma samples were prepared using acetonitrile (ACN), and an internal standard added prior to analysis. LC-MS/MS analyses were performed using a CTC PAL autosampler (Thermo) with a MS gradient Pump (Thermo) interfaced to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo) equipped with a heated ESI probe. Compound detection was by selected reaction monitoring (SRM) using argon at 1.5 torre as a collision gas. Quantitation was by peak area with reference to standards of known concentration of CTZ using an internal standard method. See the Supporting Information for full details on the methodology. Measured water concentrations are reported in table 1. Measured plasma CTZ concentrations are reported in table 2.

Gene expression analysis

Total RNA was isolated from liver samples and reverse transcribed to cDNA using random hexamers (Eurofins MWG Operon) and MMLV reverse transcriptase (Promega). Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out on cDNA samples for each of the treatments at each of the three sampling points (N = 8 for each treatment) for the target genes using Absolute QPCR SYBR Green Fluorescein mix (ABgene) according to the protocol described previously.[22] Ribosomal protein L8 (*rpl8)* was used as a 'housekeeping' gene, to normalize the target gene expression, using a development of efficiency correlated relative quantification as described previously[22], as it was found not to alter following exposure CTZ ($p>0.05$).[16] See

the Supporting Information for full details of the methodology. Details of primers used for RT-qPCR are shown in table S1 (Supporting Information).

Data analysis

Data are presented as mean ± standard error of the mean. All statistical analyses were carried out using SigmaPlot® software (Systat Software, Inc., Chicago, USA). Data were tested for normality/equal variance and log transformed when necessary. Effects of test compounds on levels of gene expression and morphological endpoints were determined using one-way ANOVA followed by Fisher LSD multi comparison procedure, where appropriate. Where data did not meet assumptions of normality and/or homogeneity of variance, data were analysed using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post hoc analysis. In all cases, $p<0.05$ was considered statistically significant.

Results

CTZ concentrations in the exposure water and fish plasma

The mean measured water concentrations of CTZ were approximately 40% of the nominal concentrations for both high and low exposure concentrations (see table 1). After 4 days depuration the CTZ concentration in the low concentration group was below the limit of quantification (0.13 µg l^{-1}), whereas in the high concentration group there was a very low, but still measureable, concentration of 0.54 µg l^{-1} (approximately 4% of the measured water concentration during the exposure).

Measured concentrations of CTZ in the plasma are summarised in table 2. After 4 days of exposure there were 30-fold higher levels of CTZ measured in the blood plasma of the low exposure concentration group, and 21-fold higher levels in the high exposure concentration group, compared with in the water for the respective treatment. After 10 days of exposure there were approximately 23-fold higher levels of CTZ in the plasma of the high exposure concentration group and 44-fold higher levels in the low exposure concentration group, compared with that measured in the water. After 4 days depuration, the levels in the plasma were below the limit of quantification in all treatment groups.

Morphometric parameters

After 4 days of exposure there was no significant effect of CTZ concentration on any of the morphometric parameters measured (see table 2). After 10 days of exposure there was also no significant effect on any of the morphological parameters measured with the exception of liver weight, where fish exposed to either concentration of CTZ had a significantly reduced liver weight compared with the controls. After 4 days of depuration this difference was still apparent. There was no effect of CTZ exposure on any other of the morphological endpoints measured after depuration (day 14).

Expression of hepatic biotransformation genes

Figure 1 shows expression the biotransformation genes analysed at each sampling time point (days 4, 10 and 14) for each treatment group. After 4 days of exposure, the expression of *cyp3a* was significantly lower in the high exposure concentration group by approximately 2-fold relative to controls, but

there was no difference in expression of *cyp2k, gsta* or *gstp* between any treatment groups. After 10 days of exposure *cyp3a, cyp2k, gsta* and *gstp* were all expressed at higher levels relative to the control with significant differences at the high exposure concentration for all genes (1.6-fold, 2-fold, 2.6-fold and 1.7-fold respectively). After 4 days of depuration there was no significant difference between treatment groups for any of the biotransformation genes, although all showed a non significant trend for lower expression at the higher exposure concentration.

Expression of hepatic drug transporter genes

Figure 2 shows expression of the drug transporter genes *mdr1* and *mrp2* analysed at each sampling point (days 4, 10 and 14) for each treatment group. After 4 days of exposure, both drug transporter genes were significantly up-regulated at the high exposure concentration, by 3-fold and 1.8-fold relative to controls for *mdr1* and *mrp2,* respectively. After 10 days of exposure, *mrp2* was still expressed at a higher level relative to controls at both concentrations, and was 2.5 fold higher at the high concentration compared with controls. *Mdr1* however, was expressed at a significantly lower level (down-regulated) compared with controls at the highest concentration (6-fold lower compared with the controls). After 4 days of depuration (day 14) both drug transporter genes were significantly down-regulated at both exposure concentrations relative to the controls, with *mdr1* showing up to an 8-fold lower expression at the lower test concentration, and *mrp2* showing a 3.4-fold lower expression at the highest test concentration.

Discussion

Plasma bio-concentration of CTZ

There are no previously published data regarding the pharmacokinetics or bioaccumulation of CTZ in fish. CTZ is generally applied topically in humans to treat local dermatological fungal infections and as such systemic exposure and therapeutic levels for humans are considered negligible. However, non-target aquatic species may be exposed to CTZ via the water and associated additional uptake routes that may lead to systemic exposure (e.g. oral, transbranchial, transdermal), and therefore knowledge of the bioavailability of this compound to fish is important in understanding the potential for effects in non-target (wildlife) species. As previously stated, CTZ is amongst the compounds listed by OSPAR as of high priority in terms of environmental monitoring due to its persistence and resistance to degradation, with a published predicted bio-concentration factor in fish (BCF_{fish}) of 610 based on its LogK_{ow}.[2]

Here we calculated the predicted bio-concentration factor for CTZ in fish plasma ($P_{blood:water}$) to be 129 using the 'Fish Plasma Model'.[23] This model has been used in the literature to predict fish plasma concentrations of a number of human drugs found in the aquatic environment with a view to estimating the potential for resultant pharmacological effects at environmentally relevant water concentrations.[24] This model has been shown to be reasonably accurate in predicting plasma concentrations for fish exposed to a number of compounds including various non-steroidal anti-inflammatory drugs (NSAIDs) and gembfibrozil[25], and for 11 out of 14 pharmaceuticals detected in sewage effluent[26]. However the relationship with the Log*K*ow is critically important in calculating these values, and Kow >3 compounds should be compared using a different blood water partition relationship to those of lower lipophilicity.[23]

The concentrations of CTZ measured in the plasma in our study were lower than those suggested by the fish plasma model, with concentrations 21 – 30 times greater than measured water concentrations after 4 days of exposure and 23 – 44 after 10 days. We do not know however, whether a steady-state plasma concentration had been reached after 10 days and based on this, it is possible that further chronic exposure could result in a higher plasma concentrations. That said, in rainbow trout exposed to various triazole fungicides, steady state between water and plasma was reached after just 1 day of exposure, albeit via dietary, rather than water exposure.[27] Interestingly the plasma concentration: water concentration ratio (effectively the BCF_{plasma}) for the lower exposure concentration employed in the current study (1 µg l^{-1} measured water concentration) was approximately twice that for the high exposure concentration (14.6 µg l^{-1} measured water concentration). This may reflect the fact that the highest concentration is approaching a steady-state concentration, where the rates of metabolism and elimination are equal to the rates of uptake and accumulation, and consequently the plasma concentration has begun to plateau.

PXR activation and expression of hepatic biotransformation genes

CTZ is well established as a potent activator of the PXR in humans and other mammals and accordingly shown to induce the mRNA expression of PXR-regulated drug metabolising cytochrome P450 3A4 (*cyp3a4*).[12] In fish, although CTZ has been investigated in terms of endocrine disruption owing to the inhibition of aromatase (CYP19) and other CYPs involved in steroid synthesis[28,29], there has been considerably less characterisation of CTZ as a PXR-ligand and/or inducer of biotransformation genes and enzymes. Here we

found that expression of several hepatic biotransformation genes, namely *cyp3a, cyp2k*, *gsta* and *gstp*, were induced after 10 days of exposure to the highest concentration of CTZ (measured water concentration of 14.6 µg l^{-1}), equating to a measured plasma concentration of approximately 1 µM (336 µg l^{-1}). In contrast, there was no induction of these genes at the lower exposure concentration, or after 4 days of exposure to the higher concentration, in line with lower plasma concentrations (0.09 – 0.92 µM).

EC_{50} values ranging from 0.8–2.5 µM have been reported for the activation of human PXR by CTZ *in vitro*[30-32] which correlate well with the expression profiles for the biotransformation genes found in the present study. This suggests that CTZ activation of the PXR may also occur in teleosts, as supported by studies in other fish species which have demonstrated 6- to 8- fold activation of the PXR in zebrafish (*Danio rerio*)[14, 15] and up to 34-fold PXR activation in fathead minnow (*Pimephales promelas*)[15] at concentrations between 0.5 and 50 µM. The apparent lack of response of the carp biotransformation genes at the lower exposure concentration employed in our study suggests a similar sensitivity of the carp PXR to CTZ as occurs in humans. These data are based on carp plasma CTZ concentrations only, and the hepatic concentrations of CTZ correlating with the induction of gene expression observed may have been much higher. Indeed in general, responses of biotransformation genes and receptor pathways in fish are thought to be less sensitive to xenobiotic exposure than in mammals.[33] That said, both zebrafish and fathead minnow PXR have been shown to be activated by CTZ at concentrations as low as 0.5 µM.[15] In contrast with these studies, a reporter assay using rainbow trout PXR showed no activation on exposure to 10 µM CTZ.[34] This may indicate differences in the responsiveness of this pathway between cyprinid and salmonid species.

However, in this study there was either weak or no response to any of the PXR-ligands tested, which the authors concluded may be due to the human PXRE used in the reporter construct being unable to recognise trout PXR.[34]

Despite the fact that PXR activation has been demonstrated in fish *in vitro*, investigation of *cyp3a* response to CTZ *in vivo* or *in vitro* has so far not supported this, and so the effect of CTZ exposure on biotransformation pathways in fish is not clear. CTZ failed to induce *cyp3a* mRNA *in vivo* in fathead minnow exposed to 1 µM by injection.[35] Similarly *Bresolin et al.*[18] showed no significant induction of expression of *pxr*, *cyp3a* or *mdr1* genes in zebrafish exposed to relatively high levels of CTZ (20 mg kg^{-1} by injection). PLHC-1 cell line showed no induction of *cyp3a* following exposure to 25 µM CTZ[36] although in this study there was no induction observed following exposure to any PXR ligand tested, and basal expression of *cyp3a* was shown to be low. This raises uncertainty as to the usefulness of this cell line for measuring expression of *cyps*. Indeed a lack of response for *cyp3a* to typical PXR ligands has been reported for this cell line previously.[37] Additionally, our previous work using carp primary hepatocytes showed no induction of any of the analysed biotransformation genes by CTZ at either test concentration (0.01 and 1 µM).[16] This lead to the conclusion that CTZ may not induce these genes in fish, highlighting possible differences in the PXR gene induction pathway between mammals and fish. In the present study measured plasma concentrations of CTZ below 1 µM coincided with a lack of induction of the biotransformation genes analysed. Furthermore, the hepatic concentrations of CTZ associated with this response may have been much higher. As such, it may be that the test concentrations of CTZ used *in vitro*[16] were simply not high enough to elicit an inductive response in these genes.

Interestingly *cyp3a* expression was slightly down-regulated in the present study following 4 days of exposure to the higher CTZ concentration, (equating to a plasma concentration of 0.92 µM). Similarly *in vitro* exposure of hepatocytes to 1 µM CTZ for 3 days showed slight but significant down-regulation of *cyp3a* as well as *cyp2k* and *gsta* expression[16] and *Krago and Clapper*[35] observed down-regulated *cyp3a* and *pxr* mRNA in fathead minnow exposed to 1 uM CTZ. This down-regulation is difficult to explain. It has been shown that another azole compound ketoconazole can inhibit PXR at the transcriptional level by disrupting the interaction between hPXR and steroid receptor co-activator 1 (SRC-1) *in vitro*[38] and it is possible that this sort of interaction could account for the down-regulation observed in these studies at lower concentrations of CTZ.

Expression of hepatic drug transporter genes

In contrast to the expression profiles for the biotransformation genes analysed, the drug transporter genes analysed here *mdr1* and *mrp2* were up-regulated at the lower water and corresponding plasma concentrations. In particular, *mrp2* expression was up-regulated in the low concentration exposure group after 10 days (measured water concentration of 1 µg l^{-1}) in line with much lower measured plasma concentrations of 0.13 µM. Expression of both *mdr1* and *mrp2* is known to be regulated by the PXR in mammals.[39] In line with this, it has been demonstrated that PXR ligand pregnenolone-16α-carbonitrile (PCN) up-regulated *mdr1* in zebrafish *in vivo* in line with *pxr* and *cyp3a* expression, indicating a similar association between these targets in fish as in mammals.[18] Interestingly, in the same study there was no response of any of these genes to CTZ.

The responses of *mdr1* and *mrp2* to CTZ exposure observed in the present study may signify a greater sensitivity of these genes to PXR activation compared with the biotransformation genes. However, unlike *cyp3a* expression, which is generally considered a biomarker for PXR activation, the multidrug resistance genes are transcriptionally regulated by a complex array of other pathways in addition to the PXR. These include those involved in inflammation and the general stress response in mammals[40] and a number of transcription factors are implicated, including SP1, NF-Y, YB1, MEF1 (MDR1 promoter-enhancing factor 1), P53, NF-R1 and NF-κB.[41] As such, it is possible that the up-regulation of *mdr1* and *mrp2* seen here after 4 days of exposure to CTZ, and where the other measured genes involved in biotransformation were not up-regulated, could signify a general chemical stress response to CTZ exposure rather than specific PXR activation.

Whereas *mdr1* is predominantly functional in the transport of xenobioitics, *mrp2* is involved in the transport of conjugates, including drug metabolites.[42] Here, *Mrp2* is up-regulated after 10 days exposure, even in the low exposure concentration group and we speculate that this may relate to the onset of increased CTZ metabolism and in turn the generation of greater levels of phase I metabolites.

In contrast *mdr1* expression was reduced after 10 days exposure to CTZ where the other biotransformation genes (and *mrp2*) appear to be simultaneously up-regulated. As mentioned above, a number of receptors are implicated in *mdr1* expression in mammals, and it is possible that this down-regulation may result from crosstalk between the PXR and another receptor. For example it has been demonstrated that PXR activation by various ligands, including CTZ,

antagonises NF-κB, a receptor involved in the inflammatory response, and implicated in *mdr1* transcriptional regulation in humans.[43] It is interesting to hypothesise whether this sort of interaction may account for the simultaneous up-regulation of *cyp3a* (and other biotransformation genes) and down-regulation of *mdr1*, as demonstrated in the present study.

In agreement with the data presented here, we reported down-regulation of *mdr1* expression *in vitro* in our previous work with primary carp hepatocytes at an exposure concentration of 1 µM.[16] *Mrp2* on the other hand showed no response *in vitro* which is in direct contrast to responses shown here *in vivo*. This suggests that the hepatocyte model may not necessarily be predictive of the dynamic *in vivo* responses of the drug transporter genes, at least on exposure to CTZ. Clearly, the networks surrounding regulation of the drug transporters are complex, and as yet only poorly understood in fish, and further work is needed to unravel these responses more precisely at a mechanistic level.

The Potential for environmental risk posed by CTZ

As mentioned, the measured concentration of CTZ in carp is lower than the values predicted for fish based on the LogK_{ow} of CTZ, albeit measured here in plasma only (local hepatic and other tissue concentrations may be higher). That said, the plasma concentrations were higher at day 10 than at day 4 of the exposure and so it is not clear if a steady-state plasma concentration had been reached. This highlights the possible need for longer term exposures to CTZ and greater characterisation of this effect in fish.

The biotransformation genes analysed here (*cyp2k, cyp3a, gsta, gstp*) were only up-regulated in the high exposure concentration group (measured water concentration of 14.6 µg l^{-1}) which is a concentration that is considerably higher than any reported in the aquatic environment. As such, although this correlates well with human PXR activation, based on these data this pathway is unlikely to be activated in fish exposed to CTZ in the wild.

On the other hand, expression of both *mdr1* and *mrp2* was modified in fish exposed to the lower exposure concentration (measured water concentration of 1 µg l^{-1}), and this is within the range of concentrations that has been reported in the aquatic environment.[21] OSPAR[2] predict that1 µg l^{-1} represents the no effect concentration (PNEC) for CTZ exposure in fish, however the data presented here shows that, at least at the level of gene expression, both drug transporters that were analysed are affected at this concentration. Alteration of xenobiotic transportation pathways (as indicated by modulated *mdr1* and *mrp2* expression) could impact on the ability of fish to metabolise and eliminate other endogenous or xenobiotic compounds, although this remains speculative without any direct evidence on altered compound absorption, distribution, metabolism and excretion (ADME).

Perhaps importantly CTZ is only one of a number of structurally related imidazole compounds that has been detected in the aquatic environment including human and veterinary drugs, pesticides, as well as those use for other purposes such as aircraft de-icer.[19,44] Although in general concentrations in the environment do not exceed ng l^{-1}, a recent study reported concentrations of CTZ, ketoconazole, fluconazole, econazole and miconazole of between 1 and 1834 ng l^{-1} in effluent in China.[21] Moreover these compounds were found to be

persistent, ubiquitously detected in the aquatic environment, and not effectively removed on passage through WWTWs.[21] In combination, therefore, these related compounds may be present in the high ng l^{-1} to low µg l^{-1} range. Critically, many of these are structurally similar to CTZ and some have been shown to activate the PXR, for example the antifungal drugs ketoconazole, fluconazole and enilconazole[38, 45] plus a number of other novel azole compounds with PXR-activation EC_{50} values between 1.1 and 4.4 µM.[46] In line with this, *cyp3a* as well as *cyp1a* mRNA expression, in addition to protein levels, were up-regulated in rainbow trout injected with 25–100 mg kg^{-1} ketoconazole.[4] The authors further state that CTZ, miconazole and propiconazole showed similar up-regulation of *cyp* expression.[4] Consequently it could be hypothesized that these compounds, present as a mixture, may have the potential to cause additive effects to exposed fish with respect to the gene responses measured in the present study.

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Supporting Information

Supporting information contains a more detailed description of the methods used as well as table S1 referred to in the text.

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	Nominal water concentration of CTZ [µg l^{-1}]		
	control	2.59	34.48
Measured concn. (mean ± SE, *n*=18)	<LOQ*	1.02 ± 0.03	14.63 ± 0.6
% nominal concn.	-	39	42
Measured concn. during depuration	<LOQ	<LOQ	0.54 ± 0.07
pH (mean ± SE, *n*= 18)	7.64 ± 0.04	7.61 ± 0.04	7.59 ± 0.04
O2 %sat. (mean ± SE, *n*=18)	80.52 ± 0.25	78.82 ± 0.09	77.85 ± 0.93

Table 1: Nominal and measured water concentrations of clotrimazole (CTZ), pH and oxygen saturation during the 10 day exposure period and subsequent 4 day depuration period. Measured CTZ concentrations, oxygen saturation and pH values are given as means and standard errors. LOQ = limit of quantification. LOQ = 13 µg l^{-1}.

	Nominal water concentration CTZ								
	control			2.59			34.48		
	Day 4	Day 10	Day 14	Day 4	Day 10	Day 14	Day 4	Day 10	Day 14
Plasma [CTZ] (µg l^{-1})	<LOQ	<LOQ	<LOQ	30 ± 3.09	44 ± 2.08	<LOQ	318 ± 15.82	336 ± 9.57	<LOQ
Liver weight (g)	0.043 ± 0.01	0.049 ± 0.01	0.052 ± 0.01	0.043 ± 0.01	0.038 ± 0.01*	0.046 ± 0.01*	0.039 ± 0.01	0.044 ± 0.01*	0.040 ± 0.01*
Fish wet weight (g)	2.41 ± 0.11	2.96 ± 0.16	3.19 ± 0.15	2.62 ± 0.16	2.55 ± 0.14	2.97 ± 0.14	2.11 ± 0.14	2.79 ± 0.19	2.79 ± 0.15
HSI	1.83 ± 0.08	1.70 ± 0.06	1.65 ± 0.06	1.66 ± 0.07	1.52 ± 0.07	1.51 ± 0.08	1.91 ± 0.10	1.63 ± 0.08	1.47 ±0.11
Fork length (mm)	49.25 ± 0.86	52.16 ± 1.05	53.63 ± 0.90	50.05 ± 1.11	49.66 ± 0.88	52.43 ± 0.88	47.02 ± 1.09	51.47 ± 1.20	51.01 ± 1.00
Condition factor (K)	2.00 ± 0.06	2.04 ± 0.04	2.03 ± 0.04	2.07 ± 0.04	2.05 ± 0.03	1.96 ± 0.10	1.99 ± 0.01	2.00 ± 0.01	2.67 ± 0.04
Haematocrit % (RBC:total blood)	33.69 ± 1.13	34.34 ± 0.86	33.33 ± 0.44	30.77 ± 1.25	33.53 ± 0.81	33.30 ± 0.89	34.01 ± 1.40	35.95 ± 0.89	32.69 ± 0.75

Table 2: Fish morphometric and physiology data. All data presented as mean ± standard error; HSI = hepatic somatic index, calculated as (liver weight/fish wet weight)x100; Condition factor (K) calculated as fish wet weight/(fork length)^3; Haematocrit calculated after centrifugation of the blood in capillary tube (see methods section) and defined as a ratio of red blood cell (RBC) vol. to total blood volume, and expressed as a percentage of total blood volume. LOQ = limit of quantification (13 µg l^{-1}). *N* = 20 in all cases. * indicates significant difference to control value (p<0.05).

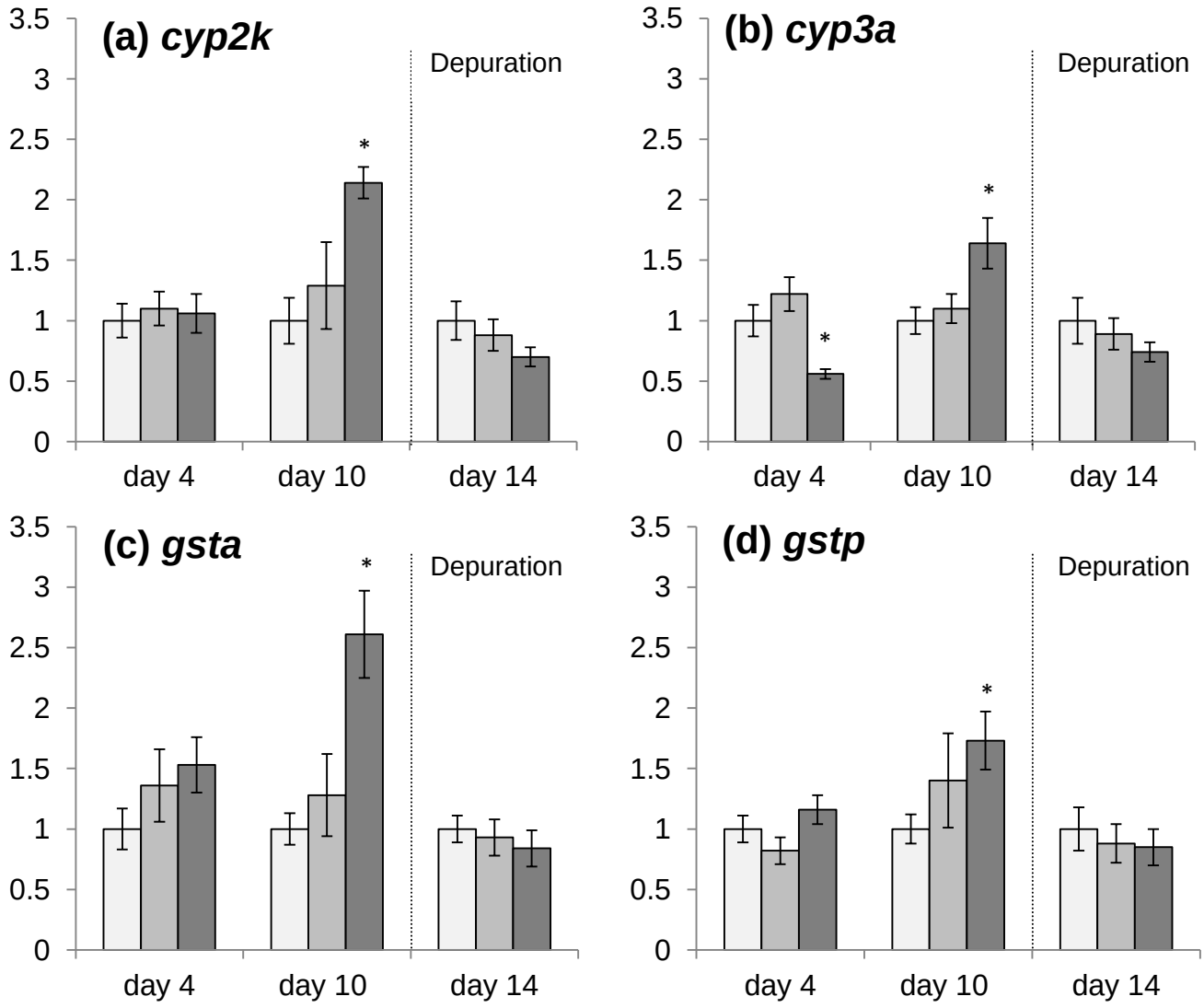

Figure 1: Expression of drug biotransformation genes: (a) *cyp2k,* (b) *cyp3a,* (c) *gsta* and (d) *gstp,* after 4 and 10 days exposure to clotrimazole at concentrations of 2.87 µg l^{-1} (light grey bars), 34.48 µg l^{-1} (dark grey bars) or dilution water controls (white bars); and after 4 days depuration (day 14). Values are mean expression relative to control groups. Error bars represent standard error. An asterisk denotes a significant difference to the control group

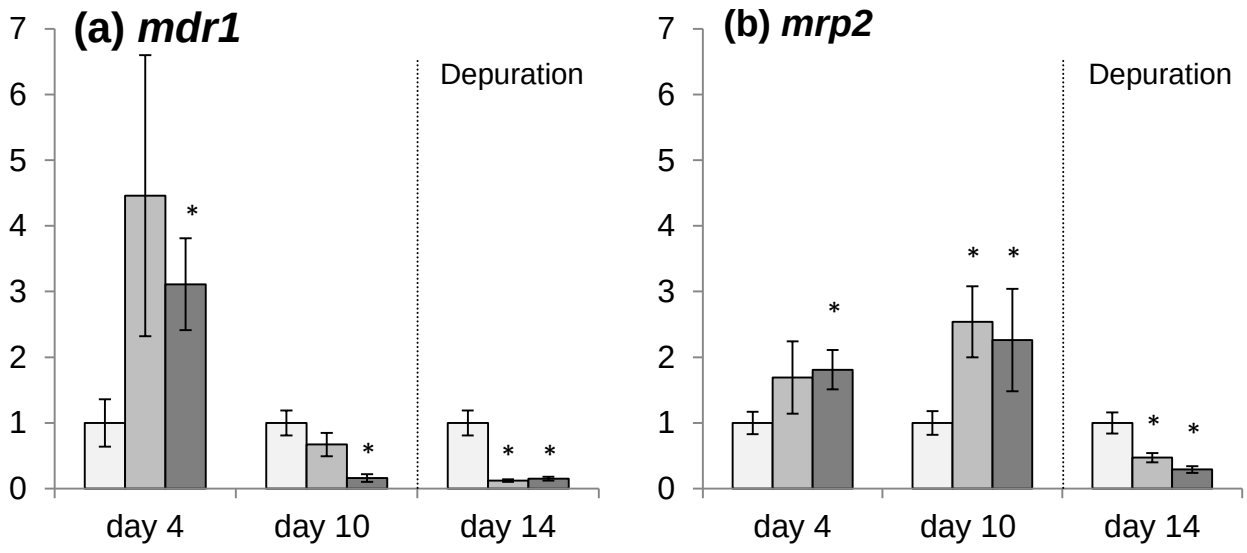

Figure 2: Expression of drug transporter genes: (a) *mdr1* and (b) *mrp2* after 4 and 10 days exposure to clotrimazole at concentrations of 2.87 µg l^{-1} (light grey bars), 34.48 µg l^{-1} (dark grey bars) or dilution water controls (white bars); and after 4 days depuration (day 14). Values are mean expression relative to control groups. Error bars represent standard error. An asterisk denotes a significant difference to the control group (p<0.05). N = 8 in all cases.

Chapter 4

Research Paper II

Supporting Information

Bioavailability of the anti-fungal pharmaceutical clotrimazole and its effects on the expression of hepatic biotransformation genes in common carp (*Cyprinus carpio*)

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This Supporting Information contains:

Supplemental experimental section

Water and plasma analysis

Clotrimazole (CTZ) was measured in water samples from each individual tank and in plasma isolated from the sampled fish, using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Sample preparation: water samples

For samples from all treatment groups, 800 µl of each water sample was transferred to a 96 deep well plate. 200 µl of ACN, containing 50 nM of internal standard was added prior to analysis by LC-MS/MS.

Sample preparation: plasma Samples

10 µl plasma from individual fish in the high dose (34.48 µg l^{-1} CTZ) samples, or 50 µl plasma (pooled from five fish) for dilution water control and low dose (2.87 µg l^{-1} CTZ) samples were added to a 96 deep well plate along with 450 µl ACN and the sample extracted using a Genogrinder (Spex) at 1000 stokes/min for 3 min. The plate was centrifuged at 4000 rpm for 30 min and 200 µl of supernatant removed and evaporated to dryness (Turbovap). The residue was re-suspended in 200 µl 80:20 Water:ACN (equivalent to x10 or x50 dilution for the low dose and dilution water control samples or the high dose samples respectively); containing 10 nM internal standard, ready for analysis by LC-MS/MS.

Instrumental analysis.

LC-MS/MS analyses were performed using a CTC PAL autosampler (Thermo) with a MS gradient Pump (Thermo) interfaced to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo) equipped with a Heated ESI probe. After injection of 20 µl, chromatographic separation was achieved using a

Hypersil Gold C18 column (50 x 2.1 mm, 3 µm (Thermo) running gradient elution at 500 µl min^{-1} as follows:

Water samples

Time (min)	(A) 0.1% formic acid in water	(B) 0.1% formic acid in methanol
0	80%	20%
1.5	0%	100%
3	0%	100%
3.01	80%	20%

The retention times for CTZ and the internal standard were 1.8 min and 1.6 min, respectively.

Plasma samples

Time (min)	(A) 0.1% formic acid in water	(B) 0.1% formic acid in methanol
0	80%	20%
1.5	80%	20%
10	0%	100%
12.5	0%	100%
12.51	80%	20%

The retention times for CTZ and the internal standard were 6.2 min and 5.5 min, respectively.

The mass spectrometer was operated in negative ion, electrospray ionization mode using the following parameters:

Capillary temperature: 270°C

Vaporiser temperature : 350°C

Spray voltage: 3750V

Sheath gas: nitrogen at 50 (arbitrary units)

Auxilary gas: nitrogen at 30 (arbitrary units)

Compound detection was by selected reaction monitoring (SRM) using argon at 1.5 torre as a collision gas and the following transitions monitored; For CTZ the precursor ion was the protonated molecular ion m/z = 277 and the product ion was m/z = 165 at a collision energy of 25V. For the internal standard, the precursor ion was the protonated molecular ion m/z = 408 and the product ion was m/z = 174 at a collision energy of 18V. Quantitation was by peak area with reference to standards of known concentration of CTZ using an internal standard method.

Gene expression analysis

Frozen 10 mg aliquots of liver were homogenised directly in Tri-reagent[1] and total RNA was isolated following manufacturer's instructions. The amount of RNA was quantified using a NanoDrop spectrophotometer and RNA quality was determined both by electrophoresis on an ethidium bromide-stained 1.5% agarose gel and through the measurement of A_{260}/A_{280} ratio. 1 µg RQ1 DNase treated (Promega) total RNA was subsequently reverse transcribed to cDNA using random hexamers (Eurofins MWG Operon) and MMLV reverse transcriptase (Promega), according to the protocol described previously.[2]

*Real-time quantitative polymerase chain reaction (RT-qPCR***)**

RT-qPCR was carried out for each of the target genes on cDNA samples for each of the three sampling time points (*N* = 8 for each sampling point), in triplicate, as described previously.[2] Briefly, primer pairs were optimized for annealing temperature (Ta), specificity confirmed by melt curve analysis, and

the detection range, linearity and amplification efficiency (*E*) established using serial dilutions of carp liver cDNA. RT-qPCR was carried out using Absolute QPCR SYBR Green Fluorescein mix (ABgene), with an initial activation step of 95 °C for 15 minutes followed by 30 – 40 cycles of denaturation (95 °C, 10 seconds) and annealing (appropriate Ta, 45 seconds) and final melt curve analysis. Ribosomal protein L8 (*rpl8)* was used as a 'housekeeping' gene, to normalize the target gene expression, using a development of efficiency correlated relative quantification as described previously[2], as it was found not to alter following exposure CTZ ($p>0.05$).[3] Details of primers used for RT-qPCR are shown in table S1.

Gene		NCBI accession # or reference	Sense primer (5'-3')	Antisense primer (5'-3')	Ta (°C)	PCR efficiency	Product size (bp)
Cytochrome P450 2K	*cyp2k*	GU19996	GCTCTTCCTGTTCTTC	TGTGACTTCTACTACTC	60.0	2.07	103
Cytochrome P450 3A	*cyp3a*	GU19997	CCAAGGACCACAAGAAGAAG	AGCCGCCGAAGATGAAG	60.0	1.921	159
Glutathione-S-transferase α	*gsta*	DQ411310	TACAATACTTTCACGCTTTCCC	GGCTCAACACCTCCTTCAC	61.5	1.979	149
Glutathione-S-transferase π	*gstp*	DQ411313	GTCCTTTGCTCTGCCTCTCTG	TTACTGCTTGCCATTGCCATTG	60.5	2.103	141
P-glycoprotein	*mdr1 (abcb1)*	AY999964	TTGCGGCTGTGGGAAGAG	GTGGATGTTCAGTTGCTTTGTG	58.5	2.104	109
Multidrug resistant protein 2	*mrp2 (abcc2)*	AY679169	TTCGGCTCTAATCTGGATG	CTCACCCGCTGTTTCTG	58.5	2.08	149
Ribosomal protein 8	*rpl8*	See[4]	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	60.0	2.14	N/A

Table S1: Details of primers used with RT-qPCR; Ta is annealing temperature; PCR efficiency represents the 'E' value

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Chapter 5
Research Paper III

Molecular cloning of the pregnane X receptor and development of a transactivation reporter assay in common carp (*Cyprinus carpio*)

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Abstract

The pregnane X receptor (PXR), plays a key role in regulating the expression of a suite of genes involved in drug biotransformation. Although the PXR is well characterised in mammals, information on this response pathway in fish is limited. It has been established that the PXR is present in fish and some of the genes thought to be regulated by this transcription factor have been shown to be up-regulated by ligands of the mammalian PXR. However, direct involvement of the PXR in this response in fish has yet to be established. Here, the full length PXR sequence was isolated from common carp (*Cyprinus carpio*) and used in a luciferase reporter assay in order to better understand the role of the PXR in xenobiotic metabolism in carp. A reporter assay for the human PXR (hPXR) was also established to compare transactivation of this receptor with the carp PXR (cPXR). COS-7 cells transfected with either hPXR or cPXR were exposed to rifampicin (RIF) or clotrimazole (CTZ), both established as potent agonists of the hPXR. Accordingly hPXR was activated by both compounds with EC_{50} values of 2.04 µM and 0.88 µM respectively. There was no activation of cPXR by RIF at concentrations up to 10 µM. However, CTZ did activate cPXR, in line with that reported in other fish species. CTZ was more potent in activating the cPXR compared with the hPXR, with an EC_{50} of 0.024 µM, within the range of concentrations of CTZ measured in the aquatic environment. The results indicate that the cPXR may differ from the hPXR in its responses/sensitivity to induction by different environmental chemicals, with impacts for risk assessment. The model established here could potentially be useful in screening a variety of known mammalian PXR-ligands and other environmentally-relevant pharmaceuticals to assess their potential in regulating gene expression through the teleost PXR.

Introduction

Fish may be exposed to a wide range of xenobiotic compounds present in the aquatic environment, including human pharmaceuticals and personal care products. As a means to combat these exposures, fish and other vertebrates have an inducible pathway of xenobiotic metabolism involving a number of enzymes including: the cytochrome P450 superfamily (notably members of cyp1,2,3 and 4 subfamilies); conjugation enzymes (e.g glutathione S transferases, UGTs, sulfotransferases); as well as transporter proteins such as P-glycoprotein and other multidrug resistance-associated proteins and organic anion transporter proteins. The ultimate aim is de-toxification and subsequent excretion of the xenobiotic compounds from the exposed organism.

This pathway is well conserved in vertebrates, and in mammals is known to be mediated, at least in part, by the pregnane X receptor (PXR; NR1I2). The PXR is an orphan nuclear receptor, well characterised in humans and non-human mammalian models as having a pivotal role in transcriptional regulation of downstream detoxification pathways. In particular CYP3A, an enzyme implicated in the metabolism of over 60% of pharmaceuticals in humans (Goodwin *et al.,* 2002), is well established as being transcriptionally regulated by the PXR.

The PXR is activated by a range of structurally diverse ligands, owing to a large hydrophobic and flexible ligand binding domain (LBD; Kobayashi *et al*., 2004). There are however, species differences in the inducible nature of the PXR even for model compounds in mammals and this is likely to extend to other vertebrate groups. For example, whereas rifampicin (RIF) is a powerful activator of PXR in humans and rabbits, it fails to activate rat or mouse PXR. On the other hand

pregnenolone 16α-carbonitrile (PCN) effectively activates the PXR in rodents, but shows much less activity on humans or rabbit PXR (Blumberg *et al.,* 1998; Jones *et al,.* 2000; Lehmann *et al.,* 1998; Savas *et al.,* 2000). The basis for this divergence is thought to be mainly due to differences in the amino acid sequences in the ligand binding domain (Kliewer *et al.,* 2002). Thus, one cannot assume that test systems that protect for effects of chemicals on metabolic pathways in one species will protect for effects in other vertebrate species.

Although the PXR pathway is well established in mammals, information on the role of this response pathway in fish is limited. It has been established that the PXR is present in some species, including zebrafish (*Danio rerio*), Pufferfish (*Takafugu rubripes*), fathead minnow (*Pimephales promelas*) and medaka (*Oryzias latipes*), and has been shown to be activated by some of the same ligands as in humans (Moore *et al.,* 2002; Maglich *et al*., 2003; Bainy and Stegeman, 2004; Milnes *et al.,* 2008). However, the downstream effects of PXR activation in fish are not fully understood.

We have previously shown that expression of a number of biotransformation genes (c*yp2k, cyp3a, gsta, gstp, mdr1* and *mrp2*) was up-regulated on exposure to RIF in carp primary hepatocytes (Corcoran et al., 2012; Chapter 3). Furthermore these genes were up-regulated on exposure of carp to the mammalian PXR-agonist CTZ *in vivo* (Chapter 4, manuscript in preparation) although interestingly not *in vitro*.

Similarly expression of *pxr, cyp3a* and *mdr1* was found to be induced by PCN in zebrafish *in vivo* (Bresolin *et al.,* 2005). It has also been demonstrated that RIF increased CYP3A enzyme activity in primary hepatocytes from grass carp (*Ctenopharyngodon idellus*) and largemouth bass (*Micropterus salmoides*), (Li

et al., 2008) and in a fathead minnow (FHM) cell line (Christen *et al.,* 2010). However, direct involvement of the PXR in this response pathway in fish has yet to be established.

In order to better understand the role of the PXR in xenobiotic metabolism in carp, cDNA encorporating the full-length PXR coding region in carp was isolated, and the transactivation function of the PXR determined by establishing a carp PXR (cPXR) luciferase reporter assay expressing this receptor in transiently transfected cultured cells. *In vitro* nuclear receptor reporter assays have previously been established for various nuclear receptors, including estrogen, androgen, thyroid and glucocorticoid receptors in fish. (e.g. Bury *et al.,* 2003; Lange *et al.,* 2012; Oka *et al.,* 2012; Todo *et al.,* 1999). These bioassays have been applied to establish the roles of specific nuclear receptors in hormone signaling in fish as well being used as efficient chemical screening systems.

Here we investigated activation of cPXR by the mammalian PXR-model ligands, RIF and dexamethasone (DEX), and subsequently also the effects of CTZ, a potent activator of human PXR. We further set out to establish a PXR reporter assay for the human PXR (hPXR) to validate the test system and to compare transactivation of this receptor with the carp PXR for the ligands tested.

Methods

Obtaining the carp PXR sequence

The carp PXR (cPXR) mRNA sequence was not available and so degenerate primers were initially designed based on conserved regions from aligned PXR sequences in other, closely related species. Total RNA was isolated from frozen

aliquots of carp liver using Tri-reagent (Chomczynski, 1993) following manufacturer's instructions and 1µg RNA reverse transcribed using random hexamers (MWG Eurofins) and MMLV reverse transcriptase (Promega). This cDNA was used as template in PCR using GoTaq DNA polymerase (Promega) with the degenerate primers (table 1) to obtain a partial cPXR sequence of approximately 400 base pairs (bp). The following PCR protocol was employed: 96˚C for 2 minutes, followed by 35 cycles of denaturation at 94˚C for 1 minute, annealing at 55˚C for 1minute and elongation at 72˚C for 3 minutes. The resulting PCR product was purified using NucleoSpin Extract II columns (Macherey Nagel) according to manufacturer's instructions. Sequencing of the product (MWG Eurofins) and characterisation using BLAST (Altschul*, et al.,* 1997) confirmed it was cPXR.

Gene specific primers (GSP1, GSP2, GSP3; table 1) were designed based on the partial cPXR sequence, and rapid amplification of cDNA ends (RACE) PCR employed to extend the initial fragment in both the 5' and 3' direction. The sequence was extended by approximately 800 bp to the extreme terminal 5' end (5' RACE) and approximately 1100 bp to include the 3' end of the protein coding region (3' RACE).

RACE was carried out using the SMARTer RACE cDNA Amplification Kit (Clontech). Briefly, total RNA from carp liver was used in reverse transcription using SMARTScribe Reverse Transcriptase (a specialised MMLV) according to manufacturer's instructions, to generate two separate cDNA populations for 5' RACE and 3' RACE. In both cases, the reverse transcription incorporates the 'SMARTer' oligonucleotide sequence (Clontech) into the resulting cDNA, which is subsequently utilised as a priming sequence in RACE PCR, in which a

universal primer mix provided by the manufacturer can recognize the SMARTer sequence in conjunction with the GSP. PCR was carried out using Advantage 2 Polymerase mix (Clontech), according to the following protocol: 5 cycles of denaturation at 94˚C for 5 seconds, annealing at 70˚C for 10 seconds and elongation at 72˚C for 3 minutes; followed by 25 cycles of denaturation at 94˚C for 5 seconds, annealing at 68˚C for 10 seconds and elongation at 72˚C for 3 minutes. Both 5' and 3' RACE products were purified as described above, sequenced, and characterised using BLAST and Clustal W (Altschul*, et al.,* 1997; Larkin *et al.,* 2007).

cPXR expression plasmid

RACE PCR generated the 3' and 5' sequences as two separate products, and so primers were then designed at the extreme ends of the sequence, incorporating restriction enzymes (*Bam*HI and *Eco*RV) as well as the Kozak sequence (which enhances ribosomal binding at the start codon during trasnscription) in the case of the 5' primer. cDNA reverse transcribed from 1 µg carp liver RNA was used as template in PCR, using the primers described (Cc_PXR_F_K_BamHI, Cc_PXR_R_EcoRV; table 1) using Advantage 2 DNA polymerase mix (Clontech) to generate the full coding sequence of cPXR. The following PCR protocol was used: 95°C for 1min followed by 30 cycles of denaturation at 95°C for 15 seconds and annealing/elongation at 68°C for 3 minutes. The resulting PCR product was purified and sequenced to confirm the full sequence length.

Purified PCR products were initially ligated into pGEM-T Easy vector (Promega) following the manufacturer's recommendations and cloned to obtain a greater

volume of product. Briefly, pGEM-T Easy vector/cPXR ligation products were transformed into DH5α competent cells (Invitrogen) by heating to 42°C for 45 seconds, followed by addition of SOC medium (Sigma-Aldrich) and subsequent incubation on LB/ampicillin/IPTG/X-gal plates at 37°C overnight. Selected colonies were then cultured in 3 ml LB broth at 37°C for a further 8 hours and plasmid DNA isolated using Wizard Plus SV minipreps DNA Purification system (Promega) according to manufacturer's instructions.

The cPXR was subsequently ligated into the pcDNA3.1 (+) vector (Invitrogen) by incubation of vector and insert DNA with the appropriate restriction enzymes (*Bam*HI and *Eco*RV; New England Biolabs) and T4 DNA ligase (Promega) at room temperature for 1 hour. The pcDNA3.1(+) vector containing the cPCR sequence was subsequently used as cPXR expression vector.

hPXR expression plasmid

Human PXR (hPXR) clone cDNA was purchased from Promega and 1 ng used as template DNA for PCR using PrimeStar Max DNA polymerase (Takara) with primers (hPXR_F_BamHI, hPXR_R_XbaI; table 1) containing restriction enzyme sites *(Bam*HI and *XBa*I) in order to amplify the full hPXR sequence. The following PCR protocol was employed: 21 cycles of denaturation at 98˚C for 10 seconds, annealing at 60˚C for 5 seconds and elongation at 72˚C for 15 seconds. The purified product was then sub-cloned into the pcDNA3.1 (+) vector using the appropriate restriction enzymes to create the hPXR expression vector, as described for cPXR (5.2.2).

Construction of pGL4-PXRE-reporter plasmid

A reporter vector was constructed based on several variations of the PXR response element (PXRE; figure 1). The PXRE occurs either as a direct or everted repeat of the consensus motif AGTTCA, spaced by between 3 and 8 nucleotides. The PXRE sequence created here was based partly on that reported in Xie *et al.,* (2000). Initially two complementary long chain oligos (table 1) were annealed to create double stranded DNA, containing four PXRE motifs. To maximise reporter efficiency a further two PXRE motifs were then added. 1 ng of the annealed DNA was used as template for polymerase chain reaction (PCR) using PrimeStar Max DNA polymerase (Takara) and primers (PXRE1, PXRE2; table 1) designed containing restriction enzyme sites (*Kpn*I and *Hind*III respectively). The following PCR protocol was employed: 21 cycles of denaturation at 98˚C for 10 seconds, annealing at 60˚C for 5 seconds and elongation at 72˚C for 30 seconds. The product was then ligated into the pGL4.24[*luc2P*/minP] vector (Promega) which contains the firefly (*Photinus pyralis*) luciferase (*luc2P*) gene, using the appropriate restriction enzymes *Kpn*I and *Hind*III to create the pGL4-PXRE reporter construct.

Culture of COS-7 cells

All reagents used for cell culture were obtained from Invitrogen. COS-7 cells were a gift from Inês Castro, University of Exeter. COS-7 cells were cultured in phenol red-free Dulbeccco's Modified Eagles medium (DMEM) containing 1000 mg l^{-1} glucose, supplemented with 10% foetal bovine serum and 1% L-glutamine. Cells were sub-cultured when they reached approximately an 80% confluence. Briefly, cells were rinsed with calcium and magnesium-free Dulbecco's phosphate-buffered saline (DPBS), dissociated with 0.05% trypsin/EDTA for 3 min at 37 °C, the trypsin subsequently neutralised with fresh

cell culture medium and cells collected by centrifugation for 5 min at 1000 rpm at room temperature. Cells were then resuspended in culture medium, counted and seeded into 75 cm^3 cell culture flasks (at a 1:10 ratio) or into 24 well plates and used in the reporter gene assay (see below).

Transfection for reporter gene assay

Reporter gene assays were performed using COS-7 cells seeded in 24-well plates at $5x10^4$ cells $well^{-1}$ in phenol-red free DMEM (supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone). After 24 h, the cells were transfected with 200 ng of either pcDNA3.1(+)/cPXR or pcDNA3.1(+)/hPXR, 400 ng of reporter construct (pGL4-PXRE) and 100 ng of pRL-TK (containing the strong herpes simplex virus thymidine kinase promoter driving *Renilla reniformis* luciferase gene as an internal control to normalise variations in transfection efficiency; Promega) using Fugene HD transfection reagent (Promega) in serum-free medium according to the manufacturer's protocol. Four hours after transfection, cells were treated with PXR agonists (see below).

Chemical Screening

Initially transcriptional assays were used to measure activation of both cPXR and hPXR by the prototypical human PXR ligands RIF and DEX at concentration of 10^{-6}, comparing two separate reporter constructs in assessing for PXRE function. In addition to the pGL4-PXRE reporter construct, a commercially available MMTV/luc2/pGL4 reporter vector (Promega) was tested as a reporter plasmid (i.e. for PXRE function), as it is known to contain various nuclear receptor response elements in the promoter region.

The pGL4-PXRE construct showed greater induction by both hPXR and cPXR in the initial comparison, pGL4-PXRE and was subsequently used as the reporter construct. Transcriptional assays were used to establish dose-response curves for the receptor agonists RIF and CTZ in the concentration ranges of 10^{-11} M to 10^{-5} M for both cPXR and hPXR.

All compounds (obtained from Sigma-Aldrich) were dissolved and diluted in DMSO and added to the medium. The final solvent concentration was 0.1% DMSO. Control wells were dosed with 0.1% DMSO only.

Dual-Luciferase Assay

After an incubation of 44 h post dosing (i.e. 48 h after transfection), the luciferase activity of the cells was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, wells were rinsed with 1 ml PBS and cells lysed with 100 µl 1x passive lysis buffer. 10 µl of lysed cells were added to 50 µl substrate buffer and firefly luciferase (reporter) activity measured. Subsequently this luminescence was quenched by addition of 50 µl Stop & Glo® reagent and *Renilla reniformis* luciferase activity was measured. Measurements were carried out using a Tecan MS200 plate reader. Promoter activity was normalised by calculating firefly (*P. pyralis*) / sea pansy (*R. reniformis*)-luciferase activity and expressed as fold activation relative to the control group.

Data Analysis

Data are presented throughout as mean ± standard error of the mean (SEM). All transfections were performed in triplicate and repeated three times on cells with different passage numbers. Dose–response data using a four-parametric

curve fitting and EC50 (for agonists) were analysed using GraphPad Prism (Graph Pad Software Inc.). All other statistical analyses were carried out using SigmaPlot® software (Systat Software, Inc.). Data were tested for normality/equality of variances, and log transformed if necessary. Effects of test compounds on activation of PXR were determined using Students T test to compare two groups, or one-way ANOVA to compare multiple groups, followed by Fisher LSD multi comparison procedure, where appropriate. In all cases, $p<0.05$ was considered statistically significant.

Results

cPXR sequence analysis

The cDNA sequence for cPXR was obtained between the extreme 5'-terminus and approximately 1700 bases downstream, incorporating the full coding region, translating into a 430 amino acid sequence. The cPXR has a highly conserved DNA binding domain (DBD) consisting of two C4-type zinc fingers and including a P-box motif (CEGCKG; a sequence essential for DNA-binding specificity) and a conserved ligand-binding domain (LBD) including the AF-2 motif (PLxxEx), essential for co-regulator interaction during ligand binding of transcription factors. BLASTp analysis (Altschul, *et al.,* 1997) confirmed sequence identity and revealed a high homology between the DBD amino acid sequence of cPXR and zebrafish PXR (98%) and 74% sequence similarity with the human PXR DBD. Figure 2 shows the alignment of the cPXR amino acid sequence with other species.

Comparison of reporter plasmids

Initially both cPXR and hPXR reporter assays were set up to trial two different reporter vectors, the pGL4-PXRE construct and the commercial MMTV/luc2/pGL4 plasmid, to test for response to PXR activation (i.e. for a functional PXRE), (Figure 3). The test system transfected with pGL4-PXRE showed significant activation of the hPXR in those cells exposed to 1 µM RIF compared with the control. cPXR activity was also higher in cells exposed to 1 µM RIF than in control cells, but this was not significant (p=0.05). In contrast, the test system transfected with the MMTV/luc2/pGL4 plasmid showed no activation significantly greater than controls of either hPXR or cPXR, by any treatment. 1 µM DEX showed no significant activation of cPXR or hPXR in either test system. Subsequently pGL4-PXRE was used as the reporter plasmid in the transactivation dose response assays.

Dose responses

Figure 4 shows dose response curves for cPXR and hPXR activation on exposure to RIF or CTZ between 10 µM and 10 pM (10^{-5} and 10^{-11} M).

RIF activated hPXR with an E_{max} (maximal effect) of 6.4 fold activation relative to control and EC_{50} (the concentration that gives half the maximal effect) of 2.04 µM. Analysis using one-way ANOVA showed a significant effect of RIF concentration on hPXR activation, with post-hoc testing revealing a significant receptor activation compared to controls at test concentrations of 1µM (10^{-6} M) and 10µM (10^{-5} M) specifically. RIF failed to activate cPXR, showing no significant difference to controls at any test concentration.

CTZ activated hPXR (E_{max} 4.8, EC_{50} 0.88 µM) and analysis with one-way ANOVA showed a significant effect of CTZ on hPXR activation, with test

concentrations of 1µM (10^{-6} M) and 10 µM (10^{-5} M) significantly activating hPXR compared with controls. CTZ also activated cPXR (E_{max} 10.9, EC_{50} 0.024 µM) and one-way ANOVA demonstrated a significant effect on cPXR activation. Post-hoc analysis showed significant activation of cPXR at test concentrations of 0.01 µM (10^{-8} M) CTZ and higher compared with controls. There was no significant difference between concentrations of 0.1, 1 or 10 µM CTZ, which indicates maximum activation of cPXR by CTZ at concentrations of 0.1 µM (10^{-7} M) and higher.

Discussion

Activation of the hPXR

The hPXR was shown here to be activated by RIF and by CTZ, both well established as potent PXR agonists in humans, using the pGL4-PXRE construct as reporter plasmid. Dose responses in the present study showed that hPXR was activated by RIF with an EC_{50} of 2.04 µM, and E_{max} of 6.4 µM, both of which are comparable to the range previously reported in humans (EC_{50} 0.71 – 3µM; E_{max} 3.5 – 23 fold compared with controls; Blumberg *et al.,* 1998; Milnes *et al.,* 2008; Moore *et al.,* 2000; Svecova *et al.,* 2007). Similarly, CTZ activated hPXR with an EC_{50} of 0.88 µM, within the range reported in other studies (0.8 – 2.5 µM) and an E_{max} of 4.8 µM, again very similar to that measured elsewhere (3 – 5.1 µM), (Bertilsson *et al.,* 1998; Lemaire *et al.,* 2004; Moore *et al.,* 2000; Svecova *et al.,* 2007). 1 µM DEX failed to significantly activate the hPXR, although there was a non significant trend for activation, with levels around 1.5 - fold higher compared with controls. Although DEX is a potent agonist of the rodent PXR, it is only a weak agonist of hPXR (Kliewer *et al.,* 2002) and this

response is comparable to a previous study showing a approximately 1.8-fold (although in this case significant) induction of hPXR by 2 µM DEX (Luo *et al.,* 2002). Taken together, the activation profiles for the hPXR transfection assay developed here to RIF, CTZ and DEX, substantiates the use of this model to measure PXR activation, at least in terms of responsiveness of the hPXR.

Activation of cPXR by RIF

cPXR did not respond to RIF in the present study. This is in agreement with a study which similarly found no activation of the zebrafish PXR by RIF (Moore *et al.,* 2002*)* and together this suggests that RIF may not activate the PXR in certain (cyprinid) fish species. However, in contrast, RIF (50 µM) was shown to activate PXR from the pufferfish *Takifugu rubripes* by up to 5.8-fold (Milnes *et al.,* 2008). RIF is also a species-specific PXR ligand in mammals, for example RIF activates PXR in humans and rabbits much more strongly than in rodents (Kliewer *et al.,* 2002), and this is thought to be due to sequence differences in the ligand binding domain. Carp and zebrafish PXR share high sequence similarity (79%) whereas Fugu Sp. PXR shares only 52% and 51% sequence homology with carp and zebrafish PXR respectively. As such, there may be a similar basis for species differences in PXR responses to RIF in fish. Critically, this species-specificity of PXR ligand activation is generally paralleled by the response (or lack of) of *cyp3a* expression to the same ligands (Kliewer *et al.,* 2002; Luo *et al.,* 2002) and accordingly, *cyp3a* is well established to be transcriptionally regulated directly by the PXR in mammals.

Conversely, the lack of cPXR response to RIF seen here is in stark contrast to previous gene expression profiles for *cyp3a* and a number of other PXR-

associated gene targets on exposure to RIF in a carp primary hepatocyte model (Corcoran *et al.,* 2012; Chapter 3). Using the hepatocyte model, a number of genes associated with the PXR in mammals were dose-dependently up-regulated by RIF, in some cases at concentrations as low as 0.1 uM, which pointed to the involvement of the PXR in this response. Furthermore, this induction was inhibited on co-exposure to ketoconazole, which is known to antagonise the interaction of RIF with the PXR which strengthened this assumption. In agreement with the hepatocyte study, RIF (10 µM) has also been shown to up-regulate expression of a novel *cyp3a* isoform, c*yp3a65,* in zebrafish larvae (Tseng *et al.,* 2005). In addition, RIF was demonstrated to induce CYP3A enzyme activity in the FHM cell line (Christen *et al.,* 2010) and also in primary hepatocytes from grass carp and largemouth bass, albeit at relatively high exposure levels (EC_{100} 45.28 and 52.43 µM respectively; Li *et al.,* 2008). As such, the lack of response of the cPXR to RIF in the present study is unexpected, and suggests that in contrast to the previous conclusions, the observed up-regulation of teleost biotransformation genes and enzymes by RIF may involve non-PXR mechanism(s).

In mammals, although the PXR is identified as the key regulator of *cyp3a* and other genes involved in xenobiotic metabolism, there is evidence for a considerable crosstalk between other nuclear receptors and transcription factors, including farnesoid X receptor (FXR), glucocorticoid receptor (GR) and vitamin D receptor (VDR), in the regulation these genes (Pascussi *et al.,* 2008). This sort of interaction may account for the discrepancy between the responses of teleost CYP3A at the gene and enzyme level previously observed and the PXR activation profile presented here. For example in rodents the glucocorticoid receptor (GR) was demonstrated to regulate expression of *cyp3a*, accounting

for the inconsistencies between potent induction of *cyp3a* expression yet only weak activation of the PXR by DEX (a GR ligand), (Quattrochi and Guzelian, 2001).

Interestingly, in a study by Tseng *et al.* (2005), expression of c*yp3a65* was up-regulated by 1 nM 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD), an established ligand of the Aryl hydrocarbon receptor (AhR). This response was abolished by repressing AhR2 translation. This suggests a role for the AhR2 signalling pathway in *cyp3a65* transcriptional regulation, e.g. in response to AhR ligands. The authors hypothesised that the TCDD-activated AhR may target the *cyp3a65* gene directly through an (uncharacterised) upstream regulatory element, rather than via activation of the PXR. (Tseng *et al.,* 2005)

Furthermore, PXR activation, as for other nuclear receptors, often requires various co-factors, co-repressors, transcription factors and complexes that interact with the PXR or the response element (Quattrochi and Guzelian, 2001). It may be that one of these factors, necessary for the activation of cPXR by RIF, wasn't present in the transfection assay in the present study.

Activation of carp PXR by CTZ

As has been mentioned above, CTZ is a potent agonist of the PXR in humans and other mammalian models. In the present study cPXR was activated by CTZ, with an EC_{50} of 0.024 µM and an E_{max} of 10.9. Furthermore, CTZ showed significant activation of the PXR compared with controls at exposure concentrations as low as 0.01 µM, almost 100-fold lower than the concentration of CTZ shown to significantly activate hPXR, suggesting that cPXR is comparatively much more sensitive to activation by CTZ. In good agreement

with this study, CTZ has been shown to activate the zebrafish PXR by up to 8-fold at concentrations between 0.5 and 50 µM using transactivation assays (Milnes *et al.,* 2008; Moore *et al.,* 2002). Similarly FHM PXR was activated by CTZ, with up to 35-fold higher activation than controls on exposure to 50 µM CTZ (Milnes *et al.,* 2008). In contrast, Wassmur *et al.,*(2010) reported no response to 10 µM CTZ using a rainbow trout PXR reporter assay, although it should be noted that this assay showed either weak or no activation by any of the PXR-ligands tested, questioning its robustness.

Fish may be exposed to CTZ in effluent and surface waters; environmental concentrations of CTZ are generally in the low nanogram l^{-1} range, but have been reported in sewage effluent up to 1.8 µg l^{-1} (Peng *et al.,* 2012), which is approaching the lowest effect concentration reported here. Moreover, CTZ has been shown to bio-concentrate in fish (Chapter 4, manuscript in preparation), with a plasma bioconcentration factor (BCF_{plasma}) of between 20 and 45, leading to the possibility of higher internal concentrations of this compound in exposed fish.

Although PXR activation by CTZ has been demonstrated in fish, the expression of PXR-associated genes, in particular *cyp3a*, does not parallel this response, with no induction demonstrated in response to CTZ *in vivo* or *in vitro* (Bresolin *et al.,* 2005; Krago and Clapper, 2011; Wassmur *et al.,* 2013; Corcoran *et al.,* 2012 (Chapter 3)). On the contrary, we have previously demonstrated up-regulated expression of a number of biotransformation genes, including *cyp3a,* in carp exposed to measured water concentrations of 17 µg l^{-1} CTZ for 10 days *in vivo* (correlating with plasma concentrations of 1 µM), (Chapter 4, manuscript in preparation). Nonetheless, inconsistency between PXR activation and gene

response is still apparent, with cPXR activated here by concentrations of CTZ 100-fold lower than those which were shown to induce expression of biotransformation genes *in vivo*. Taking this into account, it is possible that a certain level of PXR activation is required to lead to induction of the downstream gene targets. Alternatively it could be that the PXRE construct in this system is not reflective of the sensitivity of carp PXRE motifs in target genes *in vivo*.

On the other hand, it may be that activation of the teleost PXR does not necessarily lead to induction of biotransformation genes as it does in mammals. Indeed, this lack of direct correlation between PXR activation and gene expression is reflected in the response of carp to RIF as discussed.

Summary

Overall the data presented here suggests that the reporter gene assay developed for activation of cPXR has potential as a useful model with which to characterise the ligand activation profile of the PXR in carp. Furthermore this assay could be applied for screening pharmaceuticals or environmental samples for PXR activation, which holds potential for risk assessment purposes.

At the same time, this study has highlighted possible differences between the PXR associated xenobiotic-metabolising pathway in mammals and fish. Most notably, the well established correlation between PXR activation and *cyp3a* mRNA induction in mammals does not appear to be fully conserved in carp. CTZ was shown here to activate cPXR, but has shown no or little activation of *cyp3a* and other PXR-associated genes in carp *in vivo* or *in vitro.* On the other hand, RIF failed to activate cPXR in the present study, but demonstrated concentration-dependant up-regulation of a number of genes associated with

the PXR in carp hepatocytes. This warrants further investigation, not only in profiling of the ligands of teleost PXRs, but in order to clearly establish the regulatory role of this nuclear receptor in biotransformation pathways in fish.

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Name	Description	Sequence	Direction	Primer Ta (°C)
Cc_PXR_F	Degenerate primers for amplification of cPXR partial sequence	TYTTCAGRMGKGCSATGAAR	Sense	55
Cc_PXR_R		CCHGGVYGRTCTGGDGARAA	Antisense	55
Cc_GSP1	RACE PCR to generate full coding sequence of cPXR	ACTATGAAAGCTGGAGGATGGGGACGAG	Antisense	68
Cc_GSP2		CTCACTGCACATCACAAGACCTTCGACA	Sense	68
Cc_GSP3		CCGCAACCAGGAAATAGTAGCACTCACC	Sense	68
Cc_PXR_F_K_BAMHI	Cloning of cPXR for cPXR expression vector	GC**GGATCC**GCCACCATGTGCTTGCTTCAGCTCAGG	Sense	68
Cc_PXR_R_ECORV		CC**GATATC**GTCCTCGCTGGTTTTGACTG	Antisense	68
hPXR_F_BamHI	Cloning of hPXR for hPXR expression vector	GGATCCGCCATGACAGTCACCAGGACTC	Sense	60
hPXR_R_XbaI		TCTAGATCAGCTACCTGTGATACCGAACAA	Antisense	60
Long chain oligo DNA 1	Construction of pGL4-PXRE reporter plasmid	TGAGAGCTCTGAACTTCATCAAGGTCAGGGACTGAACTTTCCTGACCTTGGCACAGTGCCACCATGAACTTGCCTGACCTGCTGCAGTTCAACAGAGTTCACTCGAGGGT	Sense	N/A
Long chain oligo DNA 2		ACCCTCGAGTGAACTCTGTTGAACTGCAGCAGGTCAGGCAAGTTCATGGTGGCACTGTGCCAAGGTCAGGAAAGTTCAGTCCCTGACCTTGATGAAGTTCAGAGCTCTCA	Antisense	N/A
PXRE1		TGAGGTACCTGAACTTTTGATGGGTCATGAGAGCTCTGAACTTCATCAAGG	Sense	60
PXRE2		CCTAAGCTTTGAACTCGAATGAACTGCACCCTCGAGTGAACTCTGTTG	Antisense	60

Table 1: Details of the primers and oligonucleotides used (referenced in text). *For degenerate primers, letters denote where the sequence may be more than one specific base as follows: D (A, G or T), H (A, C or T), K (G or T), M (A or C), R (A or G), S (G or C), V (A, C or G), Y (C or T). Restriction sites are indicated in bold letters and Kozac sequence is underlined.

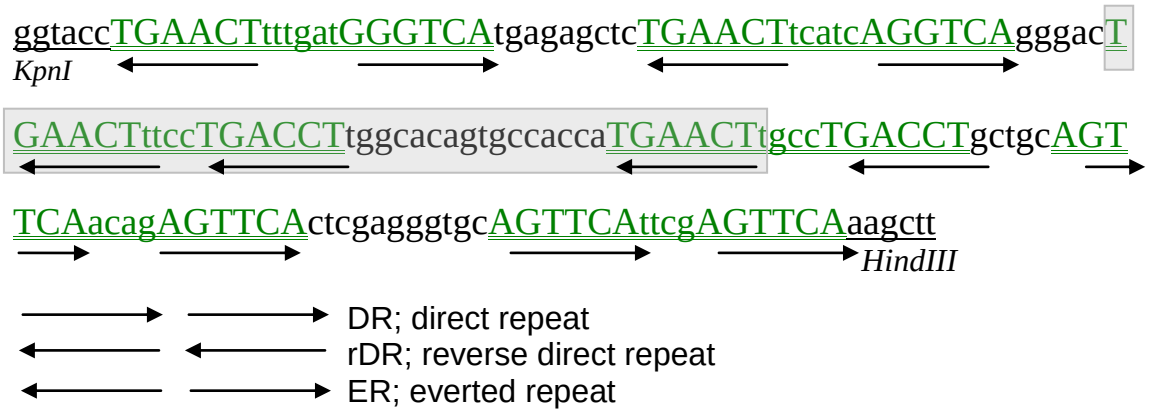

Figure 1: Sequence used for the PXRE reporter construct. Bases shown in green are PXRE sequences, with capital letters showing the conserved motif. Boxed section is a DR4 based on that published by *Xie et al., (2000)*. This sequence was inserted into pGL4.24 vector (Promega) using *Kpn*I and *Hind*III restriction sites to create the PXRE reporter vector. Arrows indicate direction of nuclear response element repeat motif (see figure legend).

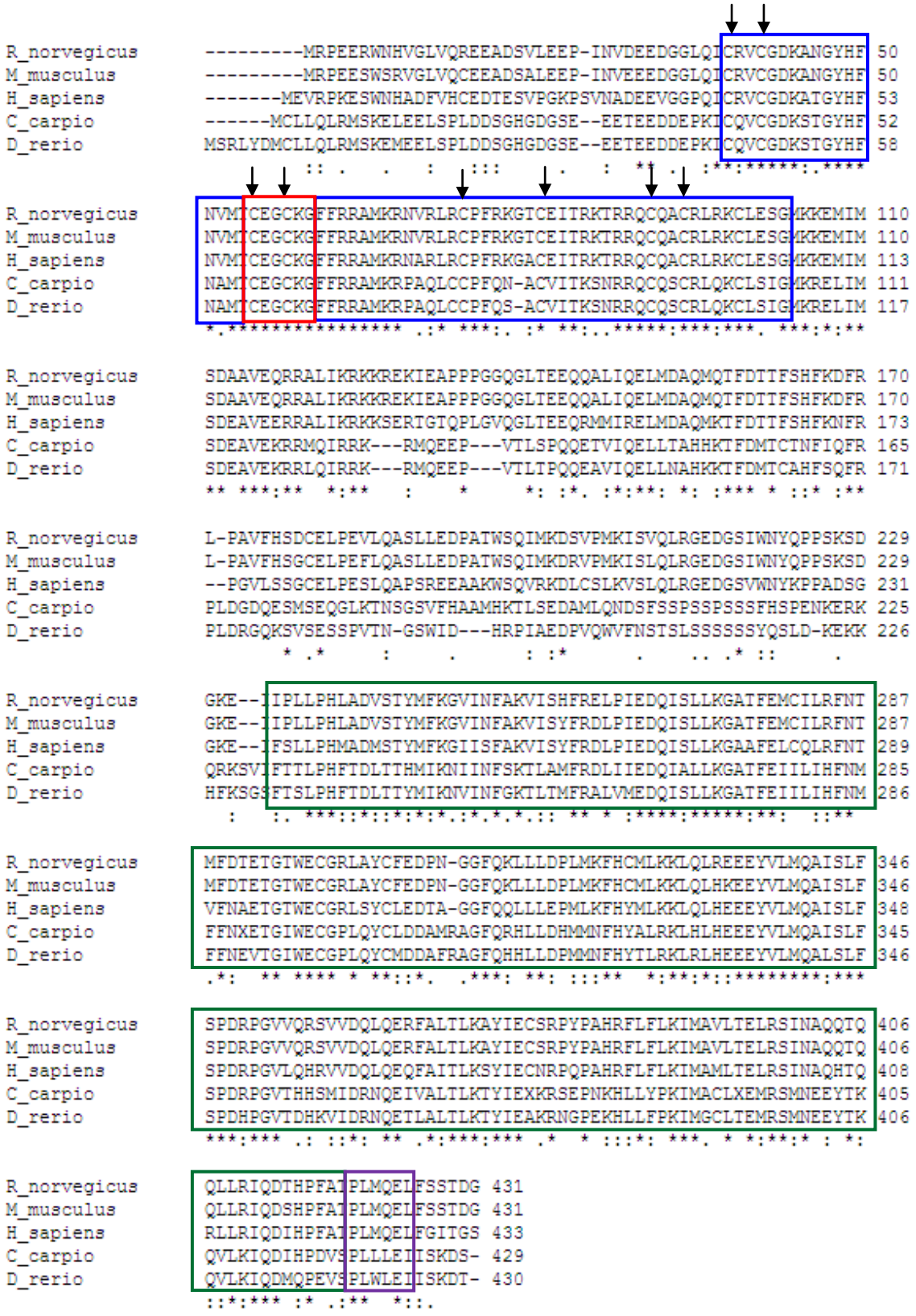

Figure 2: cPXR amino acid sequence aligned with PXR sequences of other species. The highly conserved DBD (blue outline) consists of two C4-type zinc fingers and includes a P-box motif (red outline). The conserved LBD (green outline) includes the AF-2 motif (purple outline). Accession numbers of sequences used for alignment: AAH17304 (*H. sapiens);* NP_443212 (*R. norvegicus*); NP_035066 (*M. musculus*); NP_001092087 (*D. rerio*)

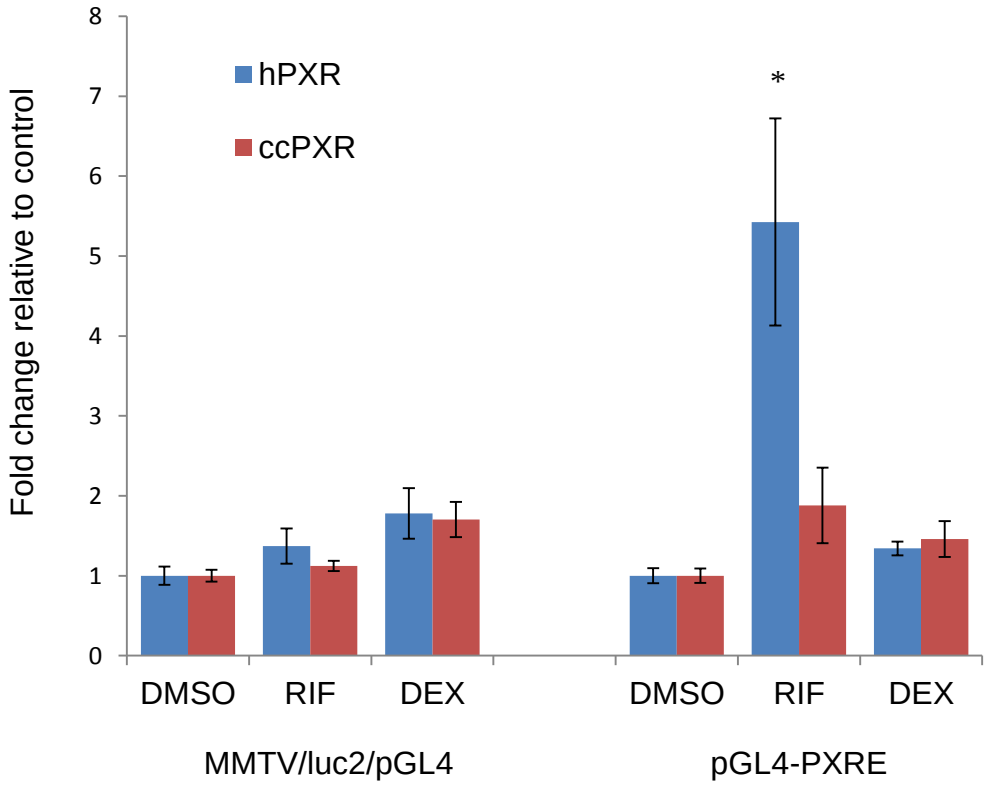

Figure 3: Activation of cPXR and hPXR by rifampicin (RIF) and dexamethasone (DEX) mediated by two different luciferase vectors. An asterisk denotes the treatment is significantly different from the corresponding DMSO control group (p<0.05)

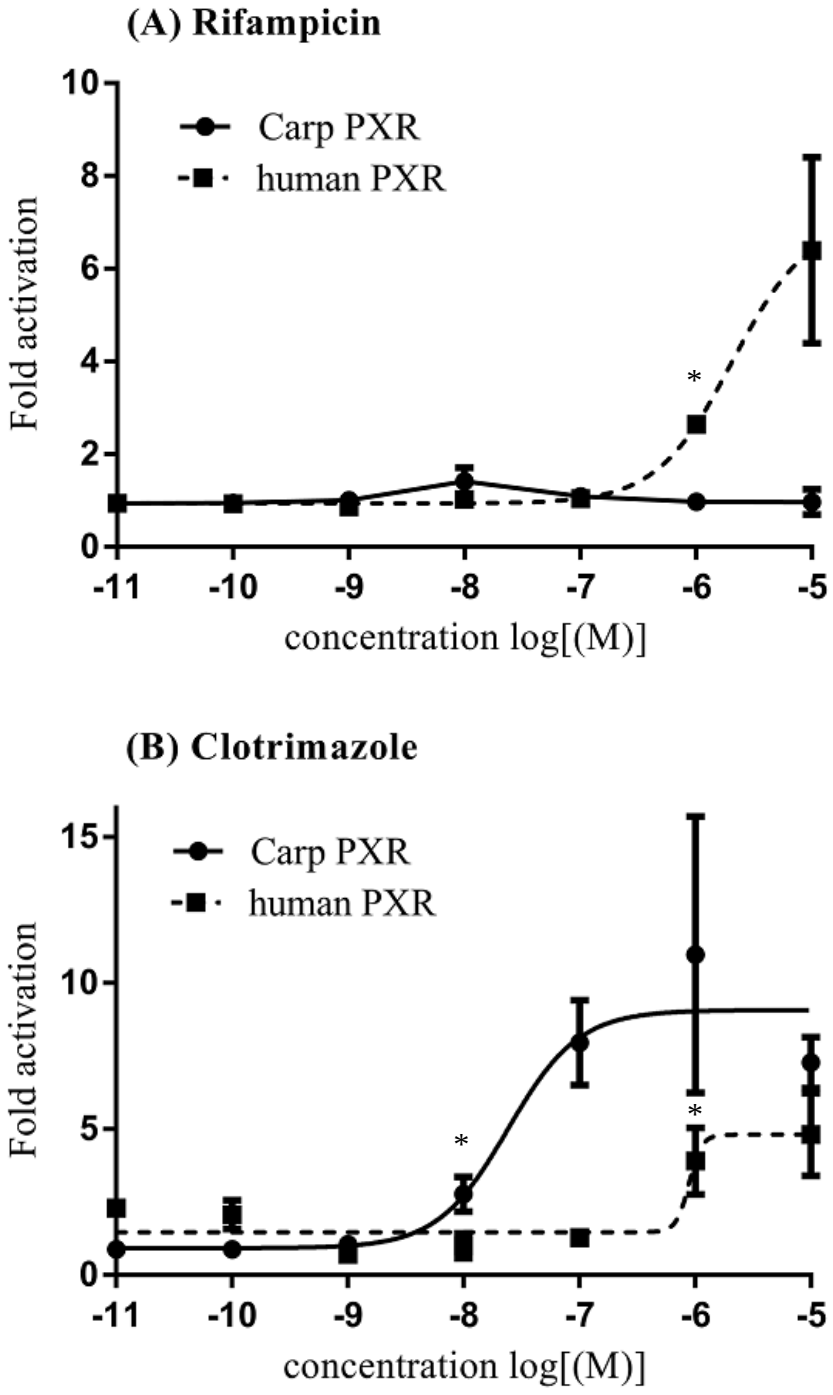

Figure 4: Dose response curves for carp (solid line) and human (dashed line) PXR activation on exposure to (A) rifampicin and (B) clotrimazole for 44 hours at concentration between 10 µM and 10 pM (10^{-5} and 10^{-11}M). Data are presented as x-fold activation relative to DMSO control. An asterisk above a data point denotes the lowest concentration at which the response was significantly different to DMSO control for that treatment (p<0.05).

Chapter 6
Research Paper IV

The teleost PPARα: Mode-of-action effects of the lipid lowering drug clofibric acid in common carp (*Cyprinus carpio*)

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Abstract

In mammals, the peroxisome proliferator-activated receptor α (PPARα) plays a key role in the regulation of a number of genes involved in lipid metabolism, bile acid synthesis and cholesterol homeostasis, and is activated by a diverse group of compounds collectively termed peroxisome proliferators (PPs). Fish can be exposed to certain PPs in surface waters, however, information on the response of downstream pathways is limited. Here we investigated the effects of exposure to the human PPARα agonist clofibric acid (CFA) on the expression of PPARα target genes, activities of the enzymes acyl-coA oxidase (ACOX1) which is involved in peroxisomal fatty acid metabolism, and the antioxidant Cu,Zn-superoxide dismutase (SOD1); as well as a suite of genes involved in xenobiotic metabolism in carp (*Cyprinus carpio*). CFA induced all measured PPARα target genes, and affected ACOX1 activity, indicating a similar role for PPARα in lipid metabolism in fish compared with mammals. SOD1 activity was not affected. In addition, a number of genes involved in xenobiotic metabolism were up-regulated on exposure to CFA paralleling reports in mammals and providing evidence of an extended functional role of PPARα in fish. Certain genes, along with ACOX1 activity, were up-regulated at exposure concentrations comparable with those found in some effluent discharges. Considering the important physiological pathways associated with PPARα, PPs such as CFA may, therefore, have biological consequences in fish exposed in the wild. Overall, *in vivo* hepatic expression profiles for target genes involved in drug biotransformation and transport were in good agreement with those reported for previous *in vitro* carp hepatocyte cultures.

Introduction

Peroxisome proliferator-activated receptors (PPARs) are transcription factors activated endogenously by fatty acids and their derivatives, which have been well characterised (in mammals) due to their potential importance in the development of hypolipidemic and antidiabetic drugs. There are three PPAR subtypes, α, -β and –γ, which have different roles in lipid and energy dynamics, show different expression patterns and have different downstream gene targets. PPARα plays a role in clearance of circulating lipids via regulation of the expression of genes involved in lipid metabolism, PPARβ is involved in lipid oxidation and cell proliferation, and PPARγ promotes adipocyte differentiation to enhance blood glucose uptake.[1]

In mammals, PPARα mediates the action of a structurally diverse group of compounds known as peroxisome proliferators (PPs). PPs bind to the PPARα which in turn binds response elements (PPREs) in the promoter region of target genes that include various enzymes involved in lipid metabolism.[2] Of these, most notable is acyl-coA oxidase (ACOX1), which has an important role in peroxisomal β oxidation, and several enzymes involved in other stages of fatty acid catabolism via both peroxisomal and mitochondrial β-oxidation pathways (including several cytochrome P450 (CYP) 4 isoforms), lipoprotein metabolism, triglyceride clearance (e.g. lipoprotein lipase) and cholesterol catabolism (CYP7A1, CYP27A).

In addition PPREs have been identified in the promoter regions of a number of genes involved in inflammation, gluconeogenesis, biotransformation, ketogenesis, lipogenesis, and amino acid metabolism.[3] As the term suggests, PPs also cause peroxisome proliferation in some species and have been

associated with oxidative stress and hepatocarcinogenesis resulting from an increased production of hydrogen peroxide (H_2O_2).[4] PPARα is targeted therapeutically in humans by fibrate drugs, which are used clinically to lower blood plasma lipid levels, via transcriptional activation of a number of genes regulated by PPARα.[2]

As with many of the orphan nuclear receptors, PPAR has been well conserved during vertebrate evolution and fish have three isoforms analogous to mammalian PPARα, -β and –γ.[5-8] A wide range of chemicals discharged into the environment are known to be PPs in mammals, including pharmaceuticals, phthalate ester plasticizers, PAHs, PCBs, alkylphenols, certain pesticides.[9] Complex effluent discharges from bleached kraft pulp and paper mills have also been shown to induce peroxisome proliferation in fish and other aquatic species.[10] Despite this, there are surprisingly little data on the effects of PPAR-active compounds in fish, and even less is known with regards to the role of the PPARα and its associated response pathways. There is some indication that PPARα activation and /or peroxisome proliferation by PPs does occur in fish[10] but there is as yet relatively little characterisation of the mechanisms involved.

In this study we investigated responses in the PPAR*α* system in carp (*Cyprinus carpio*) exposed, *in vivo*, to clofibric acid (2-(4)-chlorophenoxy-2-methyl propionic acid; CFA) the active metabolite of the lipid lowering fibrate Clofibrate, and compared the responses shown with those described in mammals. First marketed in the US in 1967, Clofibrate was widely prescribed, and although now largely superseded by other lipid regulating drugs such as gemfibrozil and the statins, CFA is still commonly detected in the aquatic environment at concentrations ranging from the high ng l^{-1} to low µg l^{-1} range in both sewage

effluents and surface waters.[11-16] CFA has also been detected in the North Sea in the low ng l^{-1} range[17] and in groundwater and drinking water at levels up to 4 µg l^{-1} and 270 ng l^{-1}, respectively[18] illustrating it is highly mobile in the environment. In addition CFA is regarded as a relatively persistent drug residue with an estimated lifespan in the environment of 21 years.[11, 17, 19, 20]

In the current study, carp were exposed to CFA via the water and various parameters measured including resultant plasma CFA concentrations, and a range of temporal changes in expression of hepatic *ppara* and target genes known to be regulated via the PPAR*α* in mammals. These target genes were: acyl-coA oxidase (*acox1)* thiolase (*acaa1)* and cytochrome P450 4 (*cyp4)* which are involved in lipid metabolism; sterol 27-hydroxylase (*cyp27a)* and apolipoprotein-A1 (*apoa1)* which play a role in cholesterol homeostasis; lipoprotein lipase (*lpl*) which is involved in lipoprotein metabolism; and Cu/Zn superoxide dismutase (*sod1)* a cellular antioxidant. Acyl-coA oxidase (ACOX1) activity, and Cu,Zn superoxide dismutase (SOD1), a key antioxidant enzyme controlled via the PPAR*α* in mammals were also measured. The *in vivo* expression of several hepatic genes involved in drug metabolism (*cyp2k*, *cyp3a*, *gsta*, *gstp*) and transport (*mdr1, mrp2*) were also compared with those previously measured *in vitro* in carp primary hepatocytes in our laboratory.[21] Two exposure concentrations of CFA were employed in the current study. A high nominal concentration (20 mg l^{-1}) was employed to facilitate assessment of bioavailability (uptake from the water into the blood circulation and the subsequent clearance from the plasma) and for comparative effects analyses with established responses in mammals (therapeutic plasma concentrations in humans are between 50 – 250 µg ml^{-1}).[22] A second, much lower nominal

exposure concentration of 4 µg l^{-1} was adopted to provide analyses with environmental relevance in terms of the risk potential for exposed fish.

Materials and methods

Animals

Juvenile common carp (*Cyprinus carpio*) with mean weight of 2.5, ± 0.48 g (mean, ± SEM, n=360) and mean fork length of 49, ± 4.3 mm (mean, ± SEM) were held in aerated tanks in a flow-through water system, at 22 °C, maintained under a 16:8 light: dark cycle and fed daily *ad libitum* with pelleted feed (Biomar Incio Plus 08.mm). Fish were supplied by the husbandry unit at AstraZeneca Brixham and were acclimated for 8 weeks prior to being transferred into experimental tanks at the start of the exposure experiment.

Test chemicals

Test compounds were all obtained from Sigma Aldrich, Poole, UK, unless stated otherwise.

Experimental design

Carp were exposed to CFA (CAS: 882-09-7) via a flow through system. On day zero, carp were randomly allocated to one of three duplicated treatment groups; 20 mg l^{-1} CFA, 4 µg l^{-1} CFA or dilution water controls; in 9 L glass tanks at a density of 10 fish per tank for each of the 3 sampling time points, giving a total of 6 tanks per treatment. Two separate stock solutions were prepared for the two dosing regimes. For the high exposure (20 mg l^{-1}), a 2.5 g CFA l^{-1} stock solution was prepared in reverse osmosis (RO) water using 0.6 g l^{-1} NaOH in

order assist dissolution. For the 4 µg l^{-1} exposure, a 2.5 mg CFA l^{-1} stock was prepared from the first solution. These stock solutions were diluted and delivered to each mixing cell at appropriate flow rates, along with RO water, to give the desired nominal concentrations of 20 mg l^{-1} and 4 µg l^{-1} respectively. The nominal CFA solutions were delivered to the individual tanks at a rate of 60 ml min^{-1}. To balance the concentration of NaOH across all treatments, a stock of RO water containing NaOH [0.6 g l^{-1}] was also delivered to the dilution water control and 4 µg l^{-1} treatments. The stock solutions were replaced every two days. Water was maintained at 22, ± 0.2°C (mean, ± SEM), with water temperature, dissolved oxygen and pH measured in all tanks at least twice weekly during the exposure (table S1, Supporting Information).

Sampling

Duplicate tanks of fish were sampled at day 4 and day 10 of the exposure. The remaining fish were then depurated for a further 4 days (exposed to dilution water only) and sampled on day 14. On each sampling day, 20 fish (from two tanks) were sampled from each treatment group. Fish were anaesthetised in MS222 solution (500 mg l^{-1} in dechlorinated water, adjusted with 1 g l^{-1} $NaHCO_3$ to pH 7.3), weighed, fork length measured and blood collected from the caudal vein using heparinised (5000 U ml^{-1}) capillary tubes. Blood was centrifuged immediately (7000g, 4 mins; Haematokrit 210, Hettich) and plasma separated, snap frozen and stored at -80 °C until further analysis. Fish were then terminated according to UK Home Office regulations, the was liver dissected out, weighed, snap frozen and stored at -80 °C until required.

Water and plasma analysis

CFA was determined in water samples from each individual tank, and in the plasma isolated from the sampled fish, using liquid chromatography tandem mass spectrometry (LC-MS/MS). Water and plasma samples were prepared using ACN, and internal standard added prior to analysis. LC-MS/MS analyses were performed using a CTC PAL autosampler (Thermo) with a MS gradient pump (Thermo) interfaced to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo) equipped with a heated ESI probe. Compound detection was by selected reaction monitoring (SRM) using argon @ 1.5 torre as a collision gas. Quantitation was by peak area with reference to standards of known concentration of CFA using an internal standard method. See the Supporting Information for full details on the methodology.

Gene expression analysis

Total RNA was isolated from liver samples and reverse transcribed to cDNA using random hexamers (Eurofins MWG Operon) and MMLV reverse transcriptase (Promega). Real-time quantitative polymerase chain reaction (RT-qPCR) was carried out on cDNA samples for each of the treatments at each of the three sampling points (N = 8 for each treatment) for the target genes using Absolute QPCR SYBR Green Fluorescein mix (ABgene) according to the protocol described previously.[23] Ribosomal protein 8 (*rpl8)* was used as a 'housekeeping' gene, to normalize the target gene expression, using a development of efficiency correlated relative quantification as described previously[21], as it was found not to alter following exposure CFA ($p>0.05$).[23] See

the Supporting Information for full details of the methodology. Details of primers used for RT-qPCR are shown in table S2 (Supporting Information).

Biochemical analyses

ACOX1 (EC 1.3.99.3) activity was quantified in liver homogenates for each sample (*N*= 8 per treatment per sampling point) based on previously published work[24]. Briefly, the production of H_2O_2 generated specifically by the peroxisomal β-oxidation pathway using lauroyl-CoA as the enzymatic substrate was determined by measurement of the oxidation of 4-hydroxyphenylacetic acid to a fluorescent product in a horseradish peroxidise-coupled reaction. H_2O_2 concentrations were determined relative to a standard curve of known amounts of H_2O_2 incubated without substrate.

SOD1 (EC 1.15.1.1) activity was measured in liver cytosol for each sample (*N*= 8 per treatment per sampling point) according to the protocol described previously[25] based on inhibition of the reduction of nitro-blue tetrazolium (NBT) by superoxide, using xanthine as the substrate. Absorbance at a wavelength of 560 nm was then recorded spectrophotometrically and units of SOD activity calculated by comparison to a standard curve of SOD activity using known concentrations of SOD between 0.001 and 1 U/ml. In both cases, protein concentrations in the cell homogenates were determined for each sample using Bradford reagent and enzyme activities expressed as units of enzyme activity per $minute^{-1}$ mg $protein^{-1}$. Full methodologies are provided in the Supporting Information.

Data analysis

Data are presented throughout as mean ± standard error of the mean (SEM). All statistical analyses were carried out using SigmaPlot® software (Systat Software, Inc., Chicago, USA). Data were tested for normality/equality of variances, and log transformed if necessary. Effects of test compounds on levels of gene expression and morphological endpoints were determined using one-way ANOVA followed by Fisher LSD multi comparison procedure, where appropriate. The two test concentrations were not compared directly as each represented a separate experimental paradigm for assessment against the relevant group in isolation. Where data did not meet assumptions of normality and/or homogeneity of variance, data were analysed using Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's post hoc analysis. In all cases, $p<0.05$ was considered statistically significant.

Results

Clofibric acid concentrations in the exposure water and fish plasma

The mean measured water concentrations of CFA were very close to nominal concentrations (between 93 and 115 %) for both high and low exposure concentrations (Table S1, Supporting Information). After 4 days depuration CFA concentrations in the water from all treatment groups was below the limit of quantification (1.3 µg l^{-1}).

Concentrations of CFA in the plasma of carp are summarised in Table S3 (Supporting Information). Concentrations in the low exposure (4 µg l^{-1}) and dilution water control groups were below the limit of quantification at all time

points measured. In the high concentration exposure group (20 mg l^{-1}), after 4 days exposure, the blood plasma levels of CFA was approximately 28% of the measured water levels and after 10 days exposure there was little, if any change (approximately 30%). After 4 days depuration, the levels in the plasma were below the limit of quantification in all treatment groups. The mean recovery from fortified plasma samples was 103% with a relative standard deviation (RSD) of 3.0.

Morphometric parameters

There was no significant effect of CFA concentration on the size, growth or condition of the fish at any of the time points sampled (Table S3, Supporting Information). There was also no effect of the treatment on liver somatic index (HSI). Haematocrit value was significantly higher with increasing concentration of CFA at day 4 (ANOVA, $p<0.05$) but there were no significant effects at later time points.

Hepatic expression of PPARα-related genes

Figure 1 shows expression of a suite of genes regulated by the PPARα in mammals, analysed for each treatment at each sampling point during the exposure (days 4, 10). Expression was also measured after 4 days depuration; however there was no significant difference in expression for any of the genes measured across the treatments at this time point (i.e. no treatment effects, data not shown).

After 4 days exposure, the expression of *ppara* was significantly elevated in the high exposure concentration group (by approximately 2.5-fold relative to

controls). *Acox1* expression was significantly higher relative to control at both exposure concentrations, up to approximately 4-fold 20 mg l^{-1}. Expression of *cyp4* and *lpl* were significantly up-regulated at 4 µg l^{-1} only (2.5-fold and 3.5-fold, respectively). *Cyp27α* was significantly reduced, by approximately 2-fold relative to controls in the high exposure group. There was no significant change in expression of *acaa1, apoa1* or *sod1.*

After 10 days exposure *acox1, apoa1* and *cyp27α* expression was significantly higher relative to control at both exposure concentrations (up to 3.5-fold, 2-fold and 2-fold, respectively). Expression of *acaa1, cyp4* and *lpl* were increased relative to controls at 4 µg l^{-1} only (2.5, 2.5 and 4- fold respectively) and *sod1* was higher (by 2-fold) at 20 mg l^{-1} only. There was no change in the expression of *ppara* at either exposure level.

Acyl-coA oxidase (ACOX1) activity

After 4 days exposure to CFA, ACOX1 activity was significantly higher (by 2.5-fold) in the high exposure group compared with controls and this was also the case in both exposure concentrations after 10 days (at between approximately 2-and 2.5- fold higher; Figure 2). After a further 4 days depuration ACOX1 activity remained significantly higher (approximately 2-fold) in the high exposure group relative to controls.

Cu,Zn Superoxide dismutase (SOD1) activity

There was no significant effect of CFA treatment on SOD activity at any sampling point during the exposure or depuration (Figure 3). However, interestingly the SOD activity was higher on day 4 than on subsequent days in

all treatment groups, with a mean activity of between 20 and 23 units per minute per mg protein compared with between 11 and 13 units per minute per mg protein on days 10 and day 14 (after 4 days of depuration), respectively.

Expression of hepatic biotransformation genes

After 4 days exposure to CFA all of the analysed drug biotransformation and transport genes (*cyp2k*, *cyp3a*, *gstp*, *mdr1, mrp2*), except *gsta*, showed significantly higher expression levels compared with controls (Figure 4). The patterns of expression differed, however; *cyp2k* and *mrp2* were induced at 20 mg l^{-1} (by 1.9 -fold and 2.7 fold, respectively) whereas *cyp3a, gstp* and *mdr1* were elevated only at 4 µg l^{-1} (1.4 -fold, 5-fold and 5 –fold, respectively).

After 10 days exposure to CFA all of these genes were significantly more highly expressed compared with controls, with the exception of *mdr1* which was down-regulated with increasing CFA concentration (3- fold at the highest exposure concentration). *Cyp2k* and *gstp* showed up-regulated expression at 20 mg l^{-1} (2.2- fold in both cases) whilst in contrast *gsta* and *mrp2* were most highly expressed at the 4 µg l^{-1} (4.3- fold and 4.6- fold, respectively) and *cyp3a* was induced only at 4 µg l^{-1} (1.8- fold relative to control). No significant difference in expression across any of the genes measured was seen after 4 days depuration (data not shown).

Discussion

In mammals, PPARα activation results in modified transcription of various genes associated with the inducible β-oxidation pathway in peroxisomes (and to a lesser extent in mitochondria). These include the key genes acyl-coA oxidase

(*acox1*), enoyl-coenzyme A hydratase/3-hydroxyacyl coenzyme A dehydrogenase (*ehhadh)* and thiolase (*acaa1*), as well as fatty acid transport protein (*fatp1*) and long-chain acyl-coA synthetase (*acs*). This response serves to increase fatty acid uptake, conversion and oxidation in the liver, resulting in a lower availability of fatty acids for triglyceride synthesis.[2]

ACOX1 induction in particular is a key, rate-limiting step in the peroxisomal β-oxidation pathway in mammals, and is also suggested to be a rapid and specific marker of exposure of aquatic organisms to PPs.[10] Here we found *acox1*, and to a lesser extent *acaa1*, were up-regulated on exposure of carp to CFA, including at the lower exposure concentration tested, indicating activation of this PPARα-regulated pathway in fish even potentially at environmentally-relevant levels of the drug. Elevated expression of *acox1* also translated into increased ACOX1 enzyme activity which is in agreement with previous studies on other fish species reporting increased ACOX1 activity in fathead minnow[26] and in salmon hepatocytes[27] after exposure to CFA.

Linked with this pathway, in mammals several members of the cytochrome P450 4 (CYP4*)* family are known to be transcriptionally regulated by the PPARα. These enzymes have an important role in microsomal ω-oxidation of fatty acids which are converted to dicarboxylic acids and subsequently metabolised by the peroxisomal β-oxidation system[28] and not surprisingly there is a close association between induction of CYP4 and the induction of the peroxisomal fatty acid metabolising system.[29] In fish, a link between CYP4 and PPARα has not been established, however in the present study *cyp4* gene expression was up-regulated on CFA exposure. This activation however, only occurred at the lower exposure concentration, which could indicate some sort of

negative feedback response at the higher concentration. This finding is in contrast to a study in *Fundulus heteroclitus* where exposure to 10 µg CFA l^{-1} did not alter CYP4A protein levels during a 17 day exposure.[30]

In line with the induction of hepatic peroxisomal β-oxidation, in rodents it is well documented that PPs including fibrates increase peroxisomal number and volume (peroxisome proliferation) which can in turn result in oxidative stress and hepatocarcinogenesis.[4] The occurrence of peroxisome proliferation and associated oxidative stress appears to be limited to certain species, however, and is not reported in humans. In rainbow trout, it has been demonstrated that CFA induces oxidative stress (in hepatocytes[31]), the fibrate drug, Ciprofibrate increases peroxisomal volume[32] and gemfibrozil increases liver/body weight ratio[33] indicating that fish may be susceptible to peroxisome proliferation.

The resultant oxidative stress associated with peroxisome proliferation is thought to be due, in part, to the increased peroxisomal production of H_2O_2 via increased ACOX1 activity.[34] As such, it would be reasonable to assume that the apparent induction of the peroxisomal β-oxidation pathway we report in this study, notably increased ACOX1 activity, could lead to an increase in reactive oxygen species (ROS) and associated oxidative stress. To investigate this further, we measured *sod1* gene expression as well as SOD1 enzyme activity. As described above, *sod1* encodes Cu,Zn SOD and is an important antioxidant enzyme converting superoxide ions in the cell to H_2O_2 and oxygen, thus playing a crucial role in the homeostasis of these ROS.[35] *Sod1* has been identified as a gene target of PPARα and has been shown to respond to fibrate treatment in rodents[36, 37]. As such PPARα may have a protective role against oxidative damage on exposure to PPs. Here in our studies on the carp, although there

was a modest induction of *sod1* mRNA at day 10 at the highest exposure concentration, this did not appear to be translated into elevated enzyme activity. This could suggest that other antioxidant defence mechanisms played a greater role than SOD1 in response to PP exposure in our fish. Supporting this, exposure of carp to a PP herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) caused oxidative stress, but SOD1 was induced only in the gill, whereas in other tissues there was increased activity of catalase, glutathione peroxidase and glutathione-S-transferase.[38] Further supporting this, in the current study we found that two glutathione-S-transferase isoforms (*gsta* and *gstp*) were up-regulated (discussed below).

Expression of the *ppara* gene is believed to be auto-regulated in mammals.[39] Here the *ppara* gene was more highly expressed after 4 days of exposure to CFA, however there was no *ppara* induction seen at later time points although a number of other downstream targets remained up-regulated. This aligns well with a previous study in fathead minnow where it was found that *ppara* gene expression was not significantly up-regulated after 21 days of exposure to 108.9 mg l^{-1} CFA, despite ACOX1 activity being enhanced.[26] Together these studies suggest a transient activation of the *ppara* gene. It should also be noted that changes in expression of the *ppara* gene do not necessarily parallel ligand activation of the PPARα receptor.

Therapeutically, in addition to reducing triglyceride synthesis, fibrates have been shown to alter lipoprotein metabolism and cholesterol homeostasis in humans, leading to an increase in plasma high-density lipoprotein (HDL) or ‘good’ cholesterol and a decrease in low-density lipoprotein (LDL). This is achieved via a number of PPARα regulated targets, including the

apolipoproteins AI and AII (APOA1, APOA2), lipoprotein lipase (LPL), as well as various enzymes involved in bile acid synthesis (cholesterol catabolism) and transport in the liver. Cholesterol is an essential component of the cell membrane, as well as being necessary for a number of normal cell functions, and the precursor to all steroids, oxysterols and bile acids[40] and so clearly disruption of cholesterol homeostasis could have knock-on effects on various important metabolic pathways.

In humans, fibrate exposure leads to PPARα-mediated up-regulation of LPL[2] as well as increased production of APOA1 and APOA2. These are major components of HDL cholesterol[41] and their up regulation contributes to increased circulating plasma HDL and more efficient efflux of cholesterol to the liver for excretion (reverse cholesterol transport). The *apoa1* gene in rodents, however, is unresponsive to PPARα activation.[42] Here *lpl* and *apoa1* were both up-regulated in carp on exposure to CFA which implies that both genes are activated by the PPARα in fish as they are in humans. This response pathway in fish has not previously been investigated, however teleost apolipoproteins including *apoa1* are known to be regulated by exposure to oestradiol-17β and are thought to play a role in vitellogenesis.[43] As such, regulation of *apoa1* expression by PPs could potentially lead to alterations in oocyte development in females.

Bile acids are derived from cholesterol in the liver, generating bile flow from the liver to the intestines (enterohepatic circulation). Normal bile acid synthesis depends largely on the balance of cholesterol and bile acid secretions, and so impairment of this balance may disrupt bile flow and lead to disrupted cholesterol homeostasis.[40] In mammals, it has been demonstrated that fibrates

disrupt bile acid synthesis via the PPARα mediated repression of enzymes involved in both the classic and alternative bile acid synthesis pathways: cholesterol 7α–hydroxylase and sterol 27-hydroxylase.[44] CYP7A1 catalyses the first and rate-limiting step in the classical pathway, converting cholesterol into 7α hydroxyl cholesterol; CYP27A mediates sterol side chain oxidation in the classic pathway and catalyses the first two steps of the alternative pathway. The simultaneous inhibition of these enzymes after long term fibrate treatment has been linked to imbalanced bile acid and cholesterol secretions leading to a higher risk of gallstone formation in human patients.[45] Here, *cyp27a* was shown to be down-regulated in carp after 4 days of exposure to CFA suggesting a similar role for the PPARα in suppressing bile acid synthesis in fish. However after 10 days exposure, *cyp27a* expression was up-regulated, implying a possible feedback mechanism which may reflect changes in circulating cholesterol and bile acid levels, and indicates possible differences with the response pathway in mammals. In accordance with this, circulating bile acids in the plasma of roach (*Rutilus rutilus*) have been shown to be up-regulated as a result of effluent exposure, which was suggested could be a reflection of modulation of bile acid synthesis and metabolism, or of changes to enterohepatic circulation.[46] In that study it was also noted that bile acids are regulatory molecules of a number of nuclear receptors including the farnesoid X receptor (FXR), pregnane X receptor (PXR) and vitamin D receptor (VDR), and as such changes to the levels of bile acids could lead to alterations in their associated pathways.

Although PPARα has been extensively studied as a modulator of lipid homeostasis and associated effects in terms of activation of the peroxisomal-β-oxidation and bile synthesis pathways, a role as regulator of xenobiotic-

metabolising enzymes (XMEs) has not been well investigated. As mentioned above, fatty acids are natural ligands of PPARα in mammals[47] and as fatty acids are endogenous substrates of phase I and phase II metabolising enzymes, it seems reasonable therefore that PPARα is able to regulate these pathways. Indeed, recent studies in mammals indicate that PPARα participates, with other nuclear receptors (e.g. PXR, CAR), in regulating the genomic expression of a number of XMEs. For example, various cytochrome P450 and phase II conjugating enzymes were altered on treatment of human hepatocytes with PPARα activators[47] and exposure to three fibrate drugs has been shown to induce mRNA levels of *cyp3a4*, *cyp2c8* and *ugt1a1* in human hepatocytes as well as respective enzyme activities.[48] Crucially, no PXR activation was observed, leading to the conclusion that fibrates induced transcriptional activation of these enzymes via non PXR-activation mechanisms.[48] This is in contrast with other reports that suggested PPARα may indirectly modulate XMEs via a functional PPRE in the PXR promoter.[49, 50]

After 4 days of exposure to CFA, the overall *in vivo* hepatic expression profiles in carp for the target genes *cyp2k*, *cyp3a*, *gsta, gstp, mdr1* (*abcb1)* and *mrp2* (*abcc2*) which are involved in drug biotransformation and transport, was in good agreement with that reported for previous *in vitro* carp hepatocyte cultures[21]. All the genes studied were up-regulated with the exception of *gsta*, demonstrating good comparability between the *in vitro* model and the *in vivo* response reported here. After 10 days all genes were up-regulated with the exception of *mdr1* which was down-regulated. *In vitro* there was no significant induction of any of the analysed genes at exposure concentrations below 1 µM (214 µg l^{-1})[21], however a significant induction was seen here across all analysed genes on exposure to 4 µg l^{-1} implying an overall greater sensitivity of these genes *in*

vivo. Overall, the *in vitro* and *in vivo* responses together imply that the PPARα may be involved in regulation of a number of XMEs in fish as is hypothesised in mammals.

The study presented here demonstrates that exposure of common carp to the mammalian PPARα ligand CFA effects the expression of number of genes known to be regulated via the PPARα in mammals, as well as modifying the activity of the key peroxisomal enzyme ACOX1, which has previously been suggested as a biomarker of exposure to PPs. The findings suggest a similar role for the PPARα in lipid metabolism in response to PPs in fish, although a direct link in terms of PPARα activation is yet to be demonstrated. Measured plasma concentrations in the high exposure group were approximately 6 mg l^{-1} (30% of the measured water concentration) which is at least 10-fold lower than the therapeutic plasma concentrations of CFA reported in humans.[22] As such the observed hepatic gene and enzyme responses suggest that the teleost PPARα and associated pathways may be more sensitive to regulation by CFA than mammals. Furthermore, several of these gene responses occur at a water concentration of 4 µg l^{-1}, in line with some of the concentrations reported in the aquatic environment. Importantly, some of the targets investigated here have different physiological roles in fish, compared with mammals, which may lead to deleterious effects not previously considered. One such example is the role of APOA1 in vitellogenin synthesis in fish.

It should also be recognised that CFA is only one of a number of fibrate drugs to have been detected in the environment, for example fenofibrate, bezofibrate and ciprofibrate have all been measured at similar concentrations in surface waters. This raises the possibility of additive effects due to strong similarities in

their mechanisms of action. Moreover, many other compounds classed as PPs and acting via similar mechanisms, enter the aquatic environment including herbicides, plasticisers and wood pulp compounds, adding to the possible mixture effects of PPs on these target endpoints in fish.

As a final note, the study presented here suggests that PPARα may be involved in regulating the expression of a number of important XMEs in carp in common with what is known in mammals, and this potentially extends the role of the PPARα not only as regulator of lipid homeostasis but also as a key modulator of hepatic xenobiotic metabolism.

Acknowledgments and declaration of interest

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Supporting Information

Supporting information contains a more detailed description of some of the methods used as well as supporting figures and tables referred to in the text. This information is available free of charge via the Internet at http://pubs.acs.org.

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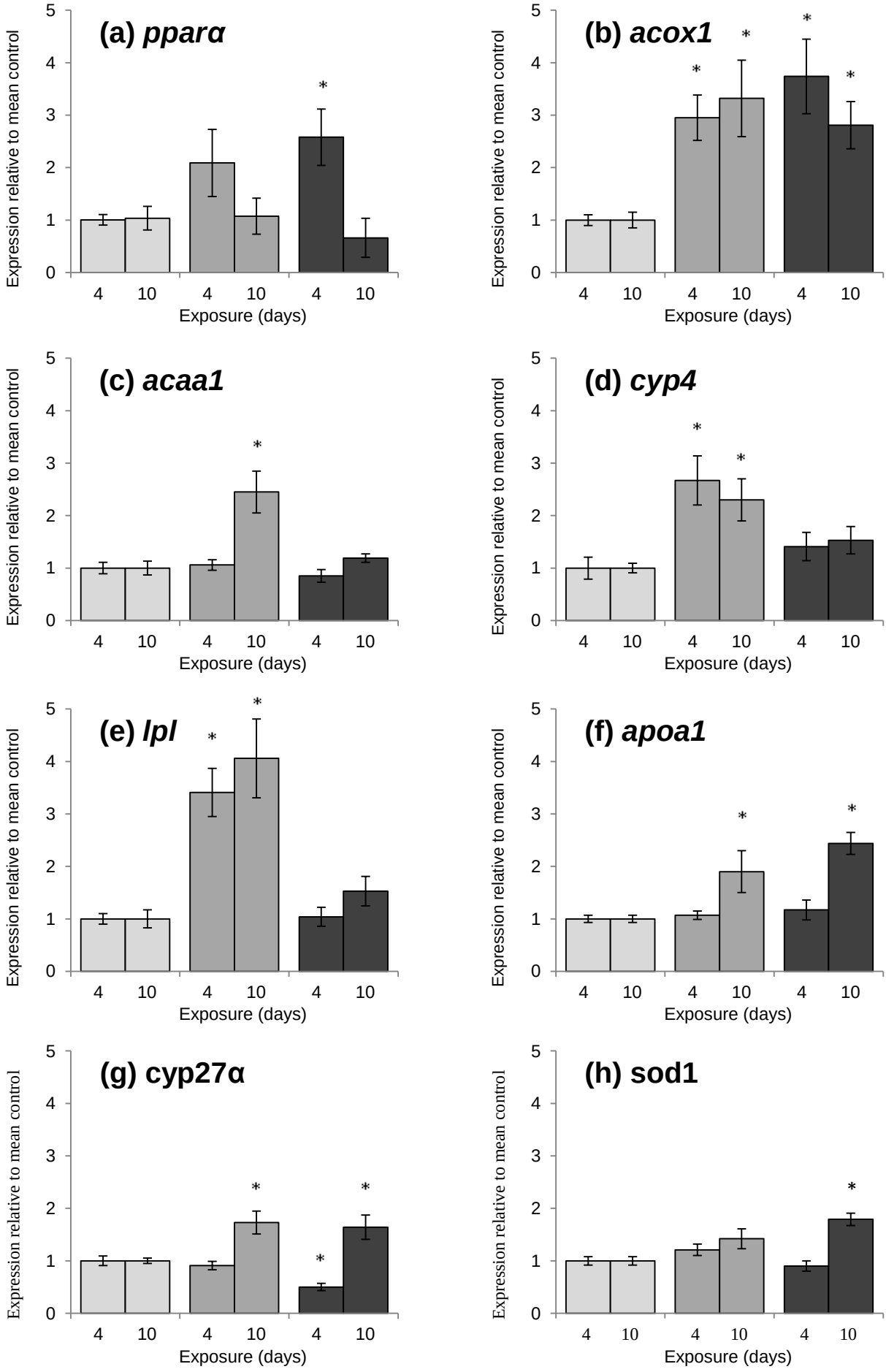

Figure 1: Expression of genes associated with PPARa activation on exposure of carp to clofibric acid at 20 mg l-1 (dark grey bars), 4 ug l-1 (light gray bars) and dilution water controls (white bars). Expression is measured as mean fold difference relative to control. Fish were sampled at day 4 and day 10 of the exposure. *N* = 8 for each treatment. Error bars represent standard error. An asterisk above the bar indicates significant difference to the control group ($p<0.05$).

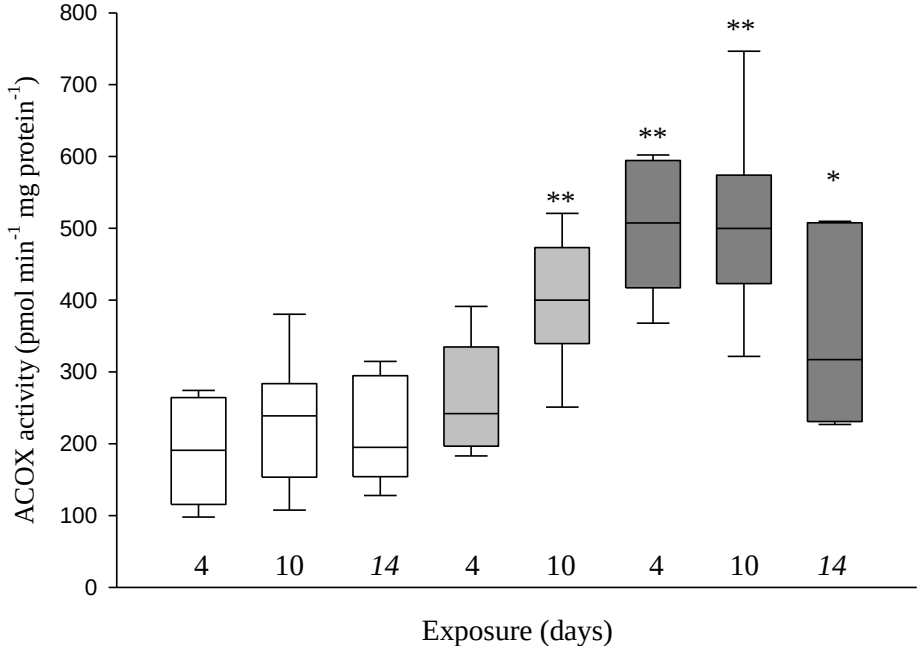

Figure 2: Activity of acyl-coA oxidase in carp exposed to clofibric acid at 20 mg l^{-1} (dark grey boxes), 4 µg l^{-1} (light grey boxes) and dilution water controls (white boxes) after 4 and 10 days exposure and a further 4 days depuration (day *14*). There was no depuration data for 4 µg l^{-1} treatment. Activity is expressed as pmol H_2O_2 per minute per mg protein as described in methods. *N* = 8 in each case. Box represents inter-quartile range, bars represent maximum and minimum values and horizontal line represents median value for each treatment. Asterisks above boxes indicate significant difference to control group (* $p<0.05$, ** $p<0.001$).

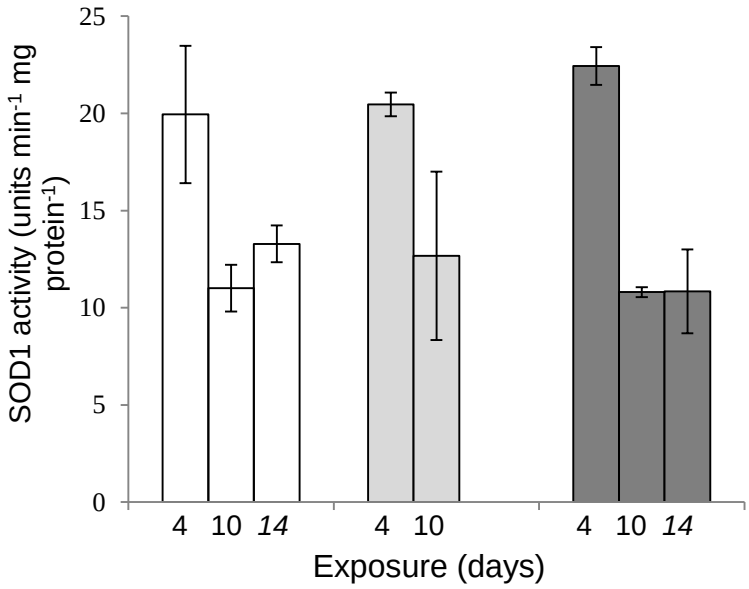

Figure 3: Enzyme activity of Cu,Zn superoxide dismutase (SOD1) in livers of carp exposed to CFA [20 mg l^{-1} (dark grey bars), 4 µg l^{-1} (light gray bars) and dilution water controls (white bars) after 4 and 10 days exposure and after 4 days depuration (day 14). SOD1 was measured via inhibition of the reduction of NBT and expressed as units of SOD1 activity per minute per mg protein. One unit of SOD activity was defined as the amount of enzyme resulting in 50% inhibition of NBT reduction as calculated by use of a standard curve with known amounts of SOD. Error bars represent standard error. *N* = 3 for each treatment. There was no low concentration exposure group for day 14.

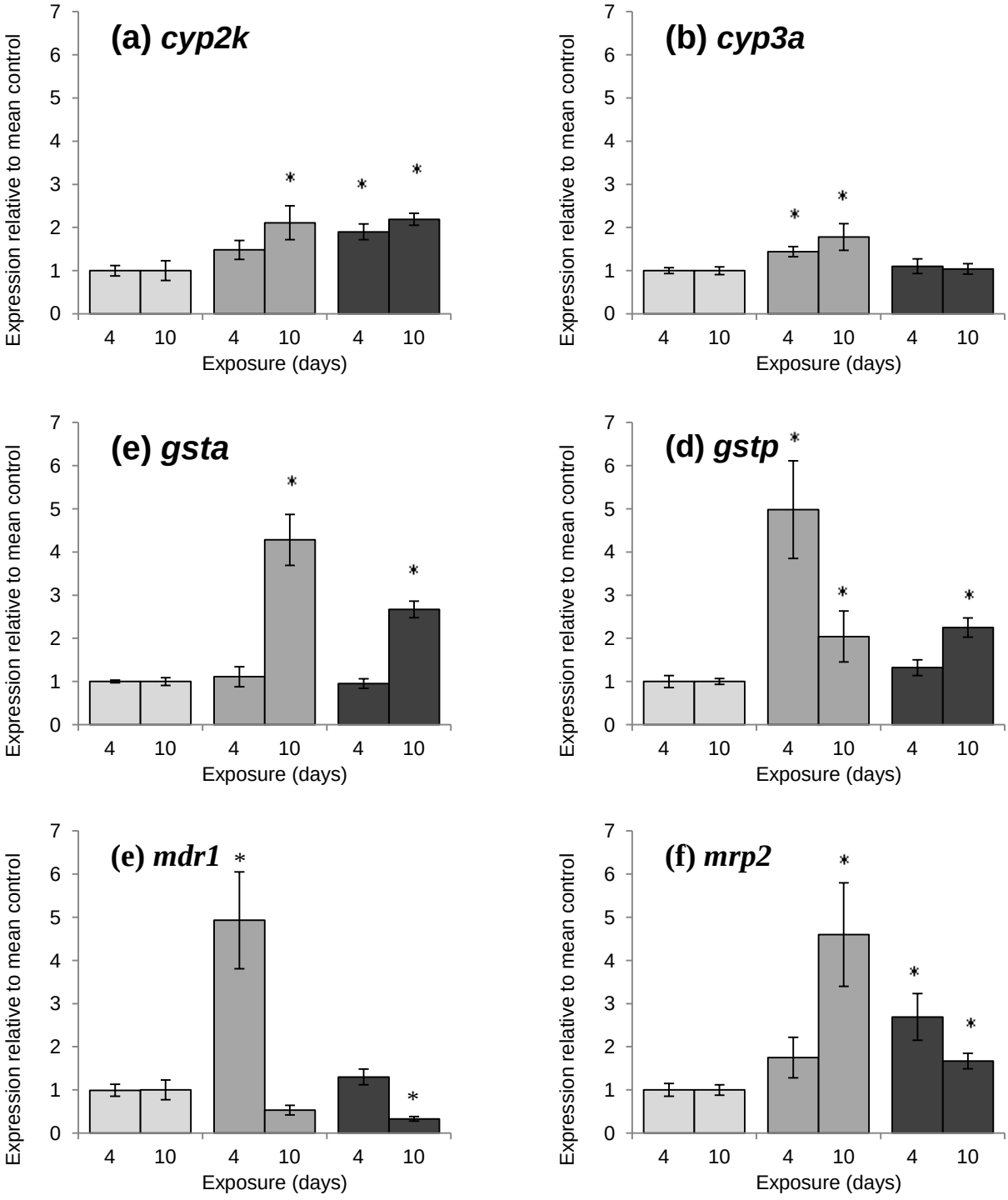

Figure 4: Expression of genes involved in xenobiotic metabolism on exposure of carp to clofibric acid at 20 mg l-1 (dark grey bars), 4 ug l-1 (light grey bars) and dilution water controls (white bars). Expression is measured as mean fold difference relative to control. Fish were sampled at day 4 and day 10 of the exposure. *N* = 8 for each treatment. Error bars represent standard error. An asterisk above the bar indicates significant difference to the control group ($p<0.05$).

Research Paper IV
Supporting Information

The teleost PPARα: Mode-of-action effects of the lipid lowering drug clofibric acid in common carp (*Cyprinus carpio*).

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This Supporting Information contains:

Supplemental experimental section

Water and plasma analysis

Clofibric acid (CFA) was measured in water samples from each individual tank and in plasma isolated from the sampled fish, using liquid chromatography tandem mass spectrometry (LC-MS/MS).

Sample preparation: water samples.

For dilution water control and low dose (4 µg l^{-1} CFA) samples, 800 µl of each water sample was transferred to a 96 deep well plate. 200 µl of ACN, containing 50 nM of internal standard was added prior to analysis by LC-MS/MS. For high dose (20 mg l^{-1} CFA) samples 50 µl of each water sample was transferred to a vial containing 10 ml of 10 nM Internal Standard in 80:20 water:ACN. 1 ml was then transferred to a 96 deep well plate for analysis by LC-MS/MS.

Sample preparation: plasma Samples

10 µl plasma from individual fish in the high dose (20 mg l^{-1} CFA) samples, or 50 µl plasma (pooled from five fish) for dilution water control and low dose (4 µg l^{-1} CFA) samples were added to a 96 deep well plate along with 490 µl ACN and the sample extracted using a Genogrinder (Spex) at 1000 stokes/min for 3 min. The plate was centrifuged at 4000 rpm for 30 min and 200 µl of supernatant removed and evaporated to dryness (Turbovap). The residue was re-suspended in 80:20 Water:ACN; 200 µl for the low dose and dilution water control samples and 400 µl for the high dose samples (equivalent to x10 or x100 dilution respectively); containing 10 nM internal standard, ready for analysis by LC-MS/MS.

Instrumental analysis.

LC-MS/MS analyses were performed using a CTC PAL autosampler (Thermo) with a MS gradient Pump (Thermo) interfaced to a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo) equipped with a Heated ESI probe. After injection of 20 µl, chromatographic separation was achieved using a Hypersil Gold C18 column (50 x 2.1 mm, 3 µm (Thermo) running gradient elution at 500 µl min^{-1} as follows:

Water samples

Time (min)	(A) 0.1% formic acid in water	(B) 0.1% formic acid in methanol
0	80%	20%
1.5	0%	100%
3	0%	100%
3.01	80%	20%

The retention times for CFA and the internal standard were 1.9 min and 1.6 min, respectively.

Plasma samples

Time (min)	(A) 0.1% formic acid in water	(B) 0.1% formic acid in methanol
0	80%	20%
1.5	80%	20%
10	0%	100%

12.5	0%	100%
12.51	80%	20%

The retention times for CFA and the internal standard were 6.4 min and 5.5 min, respectively.

The mass spectrometer was operated in negative ion, electrospray ionization mode using the following parameters:

Capillary temperature: 270°C

Vaporiser temperature : 350°C

Spray voltage: 2850V

Sheath gas: nitrogen at 50 (arbitrary units)

Auxilary gas: nitrogen at 30 (arbitrary units)

Compound detection was by selected reaction monitoring (SRM) using argon at 1.5 torre as a collision gas and the following transitions monitored; For CFA the precursor ion was the deprotonated molecular ion m/z = 213 and the product ion was m/z = 127 at a collision energy of 18V. For the internal standard, the precursor ion was the deprotonated molecular ion m/z = 406 and the product ion was m/z = 172 at a collision energy of 31V. Quantitation was by peak area with reference to standards of known concentration of CFA using an internal standard method.

Gene expression analysis

Frozen 10 mg aliquots of liver were homogenised directly in Tri-reagent[1] and total RNA was isolated following manufacturer's instructions. The amount of RNA was quantified using a NanoDrop spectrophotometer and RNA quality was

determined both by electrophoresis on an ethidium bromide-stained 1.5% agarose gel and through the measurement of A_{260}/A_{280} ratio. 1 µg RQ1 DNase treated (Promega) total RNA was subsequently reverse transcribed to cDNA using random hexamers (Eurofins MWG Operon) and MMLV reverse transcriptase (Promega), according to the protocol described previously.[2]

*Real-time quantitative polymerase chain reaction (RT-qPCR***)**

RT-qPCR was carried out for each of the target genes on cDNA samples for each of the three sampling time points (*N* = 8 for each sampling point), in triplicate, as described previously.[2] Briefly, primer pairs were optimized for annealing temperature (Ta), specificity confirmed by melt curve analysis, and the detection range, linearity and amplification efficiency (*E*) established using serial dilutions of carp liver cDNA. RT-qPCR was carried out using Absolute QPCR SYBR Green Fluorescein mix (ABgene), with an initial activation step of 95 °C for 15 minutes followed by 30 – 40 cycles of denaturation (95 °C, 10 seconds) and annealing (appropriate Ta, 45 seconds) and final melt curve analysis. Ribosomal protein 8 (*rpl8)* was used as a 'housekeeping' gene, to normalize the target gene expression, using a development of efficiency correlated relative quantification as described previously[2], as it was found not to alter following exposure CFA (*p>0.05*). Details of primers used for RT-qPCR are shown in table S2.

Acyl-coA oxidase (ACOX1) activity

The ACOX1 (EC 1.3.99.3) activity assay quantified production of the reactive oxygen species (ROS) hydrogen peroxide (H_2O_2, generated specifically by the peroxisomal β-oxidation pathway), measured fluorometrically using a

modification of the method described by *Poosch et al.*[3] Lauroyl-CoA was used as the enzymatic substrate and production of H_2O_2 via the direct transfer of electrons to oxygen by ACOX1 determined by measurement of the oxidation of 4-hydroxyphenylacetic acid to a fluorescent product (6,6'-dihydroxy-(1,1'-biphenyl)-3,3';-diacetic acid) in a horseradish peroxidise-coupled reaction. Ten mg frozen liver aliquots were homogenised in buffer containing 0.3 M mannitol, 10 mM HEPES, 1 mM EGTA (pH7.2) in a hand-held homogeniser. Homogenates were centrifuged at 3000 g for 5 minutes to remove unbroken tissue and heavy mitochondria. Assays were performed in a 96-well plate with 10 µl of each sample added to wells containing 50 µl of assay cocktail comprised of 60 mM potassium phosphate buffer (pH7.4) containing 4 U/ml horseradish peroxidise, 1 mM 4-hydroxyphenylacetic acid, 100 µM lauoryl-CoA, 20 µM flavin adinine dinucleotide (FAD) and 0.2 mg/ml triton X-100. Samples were added to wells with assay cocktail containing no substrate as controls. Samples were added under indirect light as FAD is light sensitive, incubated in the dark at 37^{o}C for 30 minutes and the reactions terminated by the addition of 1.5 ml of chilled 100 mM sodium carbonate buffer (pH10.5) containing 2 mM potassium cyanide. All samples were analysed in triplicate. Fluorescence was measured at room temperature with excitation at 318 nm and emission at 405 nm (Tecan MS200); the difference in fluorescence with and without lauroyl-coA in assay cocktail was used to indicate ACOX1 activity. H_2O_2 concentrations were determined relative to a standard curve of known amounts of H_2O_2 incubated with substrate free assay cocktail. H_2O_2 standards were made daily by serial dilution of 30% H_2O_2 in deionised water. Protein concentrations in the cell homogenates were determined for each sample using Bradford reagent (an

assay based on[4]) and bovine serum albumin (Fisher) as reference standard protein. ACOX1 activity was expressed as activity per min^{-1} mg $protein^{-1}$.

Cu,Zn superoxide dismutase (SOD1) activity

SOD1 (EC 1.15.1.1) catalyses the dismutation of superoxide into oxygen and hydrogen peroxide. The SOD1 activity assay is based on the ability of SOD1 to inhibit the reduction of nitro-blue tetrazolium (NBT) by superoxide as described in[5]. The activity of SOD is expressed as units of SOD with one unit defined as the amount of enzyme causing half the maximum inhibition of NBT reduction. Frozen samples of liver (~10 mg) were homogenised in 1 ml PBS (pH7.4), centrifuged at 14,000 g for 10 minutes and the supernatant (cytosol) separated to measure SOD1 activity only; SOD1 makes up 90% of cellular SOD and is present in the cytosol whereas SOD2 is present in the mitochondrial fraction. The cytosol fraction was diluted 1:10 in PBS and 25 µl loaded into a 1 ml cuvette with 1.25 µl xanthine oxidase and 965 µl substrate solution containing 0.1 mM xanthine, 0.1 mM EDTA, 0.05 mg BSA ml^{-1} and 0.025 mM NBT. The absorbance at a wavelength of 560 nm was then recorded using a spectrophotometer and units of SOD activity calculated by comparison to a standard curve of SOD activity using known concentrations of SOD between 0.001 and 1 U ml^{-1}. Protein concentrations were determined for each sample using Bradford reagent (as above) and SOD1 activity was expressed as units of enzyme activity per $minute^{-1}$ mg $protein^{-1}$.

	Nominal water concentration of CFA		
	control	4 µg l^{-1}	20 mg l^{-1}
Measured concn. (mean ± SD, *n*=18)	<LOQ*	4.61 ± 0.58	18.58 ± 4.51
% nominal concn.	-	115.25	92.90
Measured concn. during depuration	<LOQ	-	<LOQ
pH (mean ± SD, *n*= 18)	7.61 ± 0.16	7.51 ± 0.04	7.63 ± 0.01
O2 %sat. (mean ± SD, *n*=18)	79.07 ± 0.86	80.54 ± 0.72	79.95 ± 0.75

Table S1: Nominal and measured water concentrations of CFA and pH and oxygen saturation during the 10 day exposure period and subsequent 4 day depuration. Measured concentrations, oxygen saturation and pH values are given as means and standard errors. There was no depuration period for the 4 µg l^{-1} treatment group.*LOQ = limit of quantification. LOQ = 1.3 µg l^{-1}.

Gene		Accession number	Sense primer (5'-3')	Antisense primer (5'-3')	Ta (°C)	PCR efficiency	Product size (bp)
Peroxisome proliferator-activated receptor *α*	*ppara*	FJ849065	GGGAAAGAGCAGCACGAG	GCGTGCTTTGGCTTTGTT	62.0	2.032	105
Acyl-coA oxidase	*acox1*	CF660510	ACAGCACAGCAAGAGCAATG	ACAGAGTGGACAGCCGTATC	59.0	1.971	104
Thiolase	*acaa1*	See[6]	TTGCCTGTGGTGTGGAG	CAACATTCTCTGAGGTTATTCC	59.0	2.200	90
Cytochrome P450 4	*cyp4*	GU046698	TTGACCTCTGCCACTTG	CTGATAACTTCCGCTGTATG	57.0	2.110	138
Lipoprotein lipase	*Lpl*	FJ716101	TTGGGTTACAGTCTTGGTGCTC	AAAGGGCATCATCGGGAGAAAG	62.0	2.110	104
Apolipoprotein A1	*apoa1*	AJ308993	GCCGAAGAAGGTGAAGC	GGTGGCAAGGAAGAAAGG	57.0	2.012	82
Sterol 27*α* hydroxylase	*cyp27a*	CF660988	GAGCCACGAAAGTTCAAACC	CATCTCCAGTTCAGCAATGC	56.0	2.012	88
Cu,Zn superoxide disumutase	*sod1*	CA964628	GGAATACTCGGTCATTGG	ACTGAGTGATGCCTATAAC	54.0	2.036	100
Cytochrome P450 2K	*cyp2k*	GU19996	GCTCTTCCTGTTCTTC	TGTGACTTCTACTACTC	60.0	2.070	103
Cytochrome P450 3A	*cyp3a*	GU19997	CCAAGGACCACAAGAAGAAG	AGCCGCCGAAGATGAAG	60.0	1.921	159
Glutathione-S-transferase α	*gsta*	DQ411310	TACAATACTTTCACGCTTTCCC	GGCTCAACACCTCCTTCAC	61.5	1.979	149
Glutathione-S-transferase π	*gstp*	DQ411313	GTCCTTTGCTCTGCCTCTCTG	TTACTGCTTGCCATTGCCATTG	60.5	2.103	141
P-glycoprotein	*mdr1 (abcb1)*	AY999964	TTGCGGCTGTGGGAAGAG	GTGGATGTTCAGTTGCTTTGTG	58.5	2.104	109
MDR related protein 2	*mrp2 (abcc2)*	AY679169	TTCGGCTCTAATCTGGATG	CTCACCCGCTGTTTCTG	58.5	2.080	149
Ribosomal protein 8	*rpl8*	See[7]	CTCCGTCTTCAAAGCCCATGT	TCCTTCACGATCCCCTTGATG	60.0	2.140	N/A

Table S2: Details of primers used with RT-qPCR; Ta is annealing temperature; PCR efficiency represents the 'E' value

	Nominal water concentration CFA							
	control			4 µg l^{-1}		20 mg l^{-1}		
	Day 4	Day 10	Day 14	Day 4	Day 10	Day 4	Day 10	Day 14
Plasma [CFA] (µg l^{-1})	<LOQ*	<LOQ*	<LOQ*	<LOQ*	<LOQ*	5537	6113	<LOQ**
Liver weight (g)	0.043 ± 0.01	0.052 ± 0.02	0.053 ± 0.01	0.052 ± 0.01	0.065 ± 0.02	0.048 ± 0.01	0.049 ± 0.01	0.061 ± 0.01
Fish wet weight (g)	2.40 ± 0.77	2.83 ± 0.67	3.07 ± 0.57	3.14 ± 0.82	3.78 ± 0.82	2.48 ± 0.47	2.92 ± 0.64	3.46 ± 0.54
HSI	1.87 ± 0.29	1.85 ± 0.35	1.83 ± 0.36	1.71 ± 0.33	1.74 ± 0.54	1.98 ± 0.42	1.70 ± 0.46	1.77 ± 0.33
Fork length (mm)	48.55 ± 5.32	51.44 ± 3.76	52.99 ± 3.61	53.61 ± 4.92	55.82 ± 3.64	49.24 ± 3.81	52.04 ± 3.87	55.19 ± 3.55
Condition factor (K)	2.04 ± 0.13	2.04 ± 0.13	2.05 ± 0.05	2.01 ± 0.16	2.15 ± 0.13	2.07 ± 0.02	2.05 ± 0.16	2.05 ± 0.14
Haematocrit % (RBC:total blood)	33.49 ± 1.48	35.69 ± 0.74	34.74 ± 1.19	34.53 ± 1.01	35.74 ± 0.89	37.69 ± 0.90*	36.62 ± 0.89	33.00 ± 1.04

Table S3: Fish morphometric and physiology data. All data presented as mean ± standard error; HSI = hepatic somatic index, calculated as liver weight/fish wet weight; Condition factor (K) calculated as fish wet weight/fork length^3; Haematocrit calculated after centrifugation of the blood in capillary tubes (see methods section) and defined as a ratio of red blood cell (RBC) vol. to total blood volume, and expressed as a percentage of total blood volume. LOQ = limit of quantification (1.3 µg l^{-1}). * indicates significant difference to control value (p<0.05).

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Chapter 7
Discussion

7. Discussion

7.1. The roles of PXR and PPARα in fish

The major aim of this project was to investigate the nuclear receptors PXR and PPARα in common carp (*Cyprinus carpio*) with the view to gaining a greater understanding of the potential effects of human pharmaceuticals, acting via these pathways, in fish exposed in the aquatic environment. The results presented in this thesis expand the current knowledge on these key nuclear receptors in fish, characterise effects of selected pharmaceutical compounds, providing information on their mechanisms-of-action in fish, and provides a comparison of the responses in fish, with those that are known in mammals.

A number of nuclear receptors mediate the responses of an organism, at the transcriptional level, to diverse physiological stimuli including altered levels of circulating steroids, hormones, lipids, and/or xenobiotics chemicals. As such, these transcription factors are pivotal in the regulation of major biochemical and physiological systems, including energy homeostasis, xenobiotic metabolism and sexual development. The mammalian PXR, in particular, has a major role in regulating the transcription of a variety of important xenobiotic metabolising enzymes (XMEs), most notably CYP3A, in response to exposure to various, structurally-unrelated compounds. As such, the PXR response pathway provides an effective defence mechanism against toxic threat from a diverse range of xenobiotics that may be encountered. In fish relatively little work has been published on the characterisation of the PXR system, with recent literature reporting the identification of a number of teleost PXR ligands and presenting tentative links to altered gene expression (for example Bresolin et al., 2005; Wassmur et al., 2010; Moore *et al.,* 2002; Maglich *et al*., 2003; Bainy and

Stegeman, 2004; Milnes *et al.,* 2008). However, any direct link between PXR activation by xenobiotics, and downstream regulation of drug metabolising gene expression in fish, remains to be conclusively demonstrated.

The data presented in this thesis (Chapters 3, 4 and 5; Research papers I, II and III) have further characterised the PXR response pathway in fish in terms of PXR activation by selected pharmaceuticals, as well as expanding our understanding of the expression of associated XMEs, in common carp. At the same time, this work has highlighted apparent differences between the PXR xenobiotic-metabolising pathway in mammals and fish (specifically carp). Most notably, the well established correlation between PXR activation and *cyp3a* mRNA induction in mammals does not appear to be fully conserved in carp (discussed below).

In Chapter 3, primary hepatocytes were isolated from carp and cultured for use as an *in vitro* model to examine the transcriptional response of *cyp3a* and other XMEs on exposure to rifampicin (RIF), a human PXR ligand. These data demonstrated that RIF induced the expression of *cyp3a* and the other XMEs analysed in a dose- and time-dependant manner and further, that co-exposure with the human PXR antagonist ketoconozole (KET) reduced this response. Overall, this led to the conclusion that the PXR was likely implicated in the observed gene responses.

Nevertheless, although RIF is a potent ligand of human PXR, it shows weak or no activation of the PXR in rodents (Blumberg *et al*., 1998) and so care is needed in interpreting these responses and PXR involvement cannot unambiguously be assured based on these data alone. Indeed, this is underscored by subsequent work described in Chapter 5 using a reporter carp

PXR gene transactivation assay. Here, activation of carp PXR by RIF was investigated in order to support the hypothesis that this pathway was responsible for the gene responses measured *in vitro* in Chapter 3. Here, however, RIF failed to activate carp PXR at concentrations up to 10 µM, which is in contradiction to the conclusions suggested based on the hepatocyte work. As discussed in Chapter 5, this may mean that an essential co-factor is missing in the test system and highlights the care that should be taken in extrapolating data based on *in vitro* test systems to the *in vivo* situation. In the same study, however, carp PXR was shown to be activated on exposure to CTZ, indicating that this model did respond in terms of activation of the carp PXR. The test system was further validated by responses of the human PXR to established ligands which were comparable with data in other studies. Based on this it seems more likely, perhaps, that the PXR does not necessarily play such a pivotal role in the regulation of XMEs in carp, and that the intrinsic association of PXR activation and expression of biotransformation genes established in mammals may not be conserved in fish. This is in agreement with a study showing a lack of response of zebrafish PXR to RIF (Moore *et al.,* 2002).

This is also further highlighted in the work presented here on CTZ where, again, there was a lack of any statistical correlation between PXR activation and associated gene response. CTZ, a potent ligand of the human PXR, was demonstrated here to activate carp PXR (Chapter 5) relatively potently, with significant activation on exposure even at 0.01 µM CTZ. However, this was not reflected in the expression profiles of *cyp3a*, or indeed other PXR-associated biotransformation genes, in carp either *in vitro* or *in vivo.* To illustrate, there was no significant effect of CTZ exposure on expression of these genes *in vitro* at concentrations up to 1 µM (Chapter 3). Up-regulation of these genes was

observed *in vivo,* but only following 10 days exposure to CTZ, corresponding with measured plasma concentrations of approximately 1 µM (i.e. 100-fold higher than the concentration shown to activate carp PXR), with no gene induction seen at lower exposure concentrations (Chapter 4). These results are in line with those reported in other species (discussed in Chapter 5). Again, this lack of correlation suggests that activation of the teleost PXR does not necessarily lead to induction of biotransformation genes, as is the case in mammals.

From the data presented in this thesis on the carp PXR further investigation is warranted, not only in profiling of the ligands of teleost PXRs, but to establish the regulatory role of this nuclear receptor in biotransformation pathways in fish. The nature of xenobiotic exposure in fish is quite different to that in mammals, not only due to the different primary routes and timescales involved (i.e. potentially continuous chronic exposure via the water in fish; predominantly via the diet in mammals), but also the huge range of xenobiotic compounds present in the aquatic environment, often in mixtures. As such, it may be that systems such as the PXR have diverged during evolution, in terms of both ligand activation and resultant gene responses, in order to equip the respective organism with the detoxification system necessary for their specific ecological circumstances. This is evident in the apparent differences in PXR response pathways, not only between mammals and fish, but also between fish species (for example RIF appears to activate PXR in pufferfish *Takifugu rubripes* (Milnes *et al*., 2008) but not in zebrafish (Moore *et al*., 2002).

In contrast, the central pathways governing energy homeostasis appear to be highly conserved between species (Wang *et al*., 2011). This is reflected here by

the similar response of a number of PPARα-associated genes in fish and those in mammals (Chapter 6; Research Paper IV). The *in vivo* exposure of carp to mammalian PPARα ligand clofibric acid (CFA) resulted in the up-regulation of a number of genes associated with this receptor in mammals, as well as ACOX1 enzyme activity often referred to as an enzyme biomarker for activation of PPARα. In fish there is only scarce data on this receptor, especially with regards to downstream transcriptional effects. With the exception of *acox1*, many of the genes analysed here have not previously been investigated in response to pharmaceuticals or other PPARα ligands in fish. Therefore, although a direct link is yet to be demonstrated, the data presented here is novel and supports the notion that the PPARα is likely to play a similar role in lipid homeostasis in fish (carp), as has been described in mammals.

Interestingly CFA demonstrated up-regulated expression of a number of XMEs, including *cyp3a*, both *in vitro* and *in vivo* (Chapter 3 and Chapter 6 respectively). As discussed in Chapter 6, this suggests crosstalk between the PXR and PPARα as has been speculated to occur in mammals. Further, the same biotransformation genes were up-regulated on exposure to ibuprofen (IBU) *in vitro* (Chapter 3), which may have occurred via the same mechanism as this compound is thought to be a PPARα agonist in mammals (Lehmann *et al*., 1997).

7.2. The feasibility of an *in vitro* approach

A second aim of this thesis work was to gain a better understanding of the value of an *in vitro* fish hepatocyte model as a viable alternative to the use of *in vivo*

fish exposures for understanding the potential impact of pharmaceuticals in the environment.

Clearly, an *in vitro* model can never be completely representative of the intact animal and there is disagreement regarding how accurately *in vitro* data can be extrapolated to the *in vivo* situation. Nevertheless, *in vitro* testing has certain advantages over *in vivo* exposures, including ethically and financially, and has greater potential to provide useful mechanistic characterisation of compounds. As such, primary cell cultures are routinely used in toxicity studies in mammals (Gomez-Lechon *et al*., 2003) and are showing increasing potential in fish, including for the study of biotransformation (e.g. Laville *et al*., 2004; Pesonen and Andersson, 1997; Sadar and Andersson, 2001; Segner and Cravedi, 2001; Wassmur et al., 2010). In this sense*, in vitro* models hold great potential in ecotoxicology, in terms of aiding the understanding of toxic mechanisms for pharmaceuticals as well as many other pollutants, knowledge of which is lacking in fish in most cases.

Isolated carp hepatocytes in primary culture were utilised here in order to assess their applicability as a model for studying the PXR pathway in fish, by measuring expression of a number of genes involved in biotransformation (Chapter 3). These genes include those involved in phase I (*cyp2k, cyp3a*) and phase II (*gsta, gstp*) xenobiotic metabolism, as well as multidrug resistance transporter proteins *mdr1* (*abcb1*) and *mrp2* (*abcc2*), all of which are linked to the PXR in mammals. Primary cultures of mammalian hepatocytes have been shown to serve as a sensitive model for analyzing the regulation of CYP modulation by drugs and other chemicals, maintaining constitutive CYP activity at levels close to those in freshly isolated hepatocytes (Brown *et al*., 2003;

Gomez-Lechon *et al.,* 2003; LeCluyse *et al*., 2000). In agreement with this, the carp hepatocytes here showed good cell viability (>90%) over the 96 hour culture duration and moreover, stable constitutive expression of *cyp2k* and *cyp3a* was demonstrated implying that this model is suitable for studies on expression of these genes.

As discussed in Chapter 3, hepatocytes were exposed to RIF as a prototypical PXR ligand (positive control), and all the biotransformation and drug transporter genes analysed were up-regulated, to a degree demonstrating similar sensitivity and responsiveness to the analogous genes in mammals. Based on this, it was concluded that the carp primary hepatocyte model could serve as a useful system for screening responses in these target genes which are involved in drug metabolism. This model was subsequently used to screen a further four environmentally-relevant pharmaceuticals from different therapeutic classes, some of which also induced the biotransformation and drug transporter genes analysed.

A key feature when assessing an *in vitro* model, particularly when proposed as a whole animal surrogate, is the ability to make reliable extrapolations to the *in vivo* situation. For that reason, some of the responses observed using the carp primary hepatocyte model were further explored *in vivo* as part of subsequent studies into two of the pharmaceuticals screened: CTZ (Chapter 4) and CFA (Chapter 6). This served to provide some validation of the *in vitro* model by comparison. In general the carp hepatocyte model showed good general representation of the *in vivo* gene response profiles; however there were some clear differences as well as drawbacks evident from the comparisons, which are discussed below.

As mentioned, exposure of hepatocytes to CTZ *in vitro* showed no induction of any of the biotransformation or drug transporter genes at either of the test concentrations (0.01 and 1 µM). This was surprising given that CTZ is a potent agonist of the PXR in mammals and so the same target genes were measured in liver of carp exposed to waterborne CTZ *in vivo* (Chapter 4). In this study all the biotransformation genes were up-regulated, albeit only after 10 days of exposure to (measured) water concentrations of 15 µg l^{-1} (equating to a plasma concentration of approximately 1 µM). This somewhat contradicts the data presented in the hepatocyte study and indicates that CTZ may activate the PXR-regulated biotransformation pathway in carp after all. This finding was subsequently supported by the reporter gene assay which demonstrated that CTZ significantly activates carp PXR *in vitro* (Chapter 5), as well as by similar reports in zebrafish and fathead minnow (Moore *et al.,* 2002; Milnes *et al.,* 2008). Both drug transporter genes were also up-regulated *in vivo* after 4 days of exposure to water concentrations of 15 µg l^{-1}CTZ (plasma concentration of 0.9 µM), which again does not reflect the responses observed using the hepatocyte model; *in vitro*, *mdr1* was down-regulated whilst *mrp2* showed no response at exposure concentrations up to 1 µM. Taken together, the data presented here indicates that the carp primary hepatocyte model was not suitable for predicting expression of the genes analysed, in response to CTZ exposure. It should, however, be noted that this may be in part due to the limited duration of the cell culture (cell viability declined after 96 h in culture). Indeed, after only 4 days of exposure to CTZ *in vivo* there was no up-regulation of the biotransformation genes, and so in this sense it could be argued that the lack of transcriptional response in the *in vitro* model accurately reflected the *in vivo* situation. Furthermore, *in vivo* the biotransformation genes did not respond

to plasma CTZ exposure at concentrations below 1 µM, which may indicate a threshold for gene responses that was not reached at the concentrations used in the *in vitro* model. Nevertheless, based on the data generated, for the purpose of mechanistic studies of CTZ on the PXR pathway, the *in vitro* hepatocyte model used here did not appear to be suitable.

In contrast with this, the studies presented here investigating exposure of carp to CFA have demonstrated a good overall correlation between gene responses *in vivo* and *in vitro.* Utilising the hepatocyte model, exposure to CFA was demonstrated to significantly up-regulate expression of all the biotransformation and drug transporter genes analysed *in vitro*, with the exception of *gsta*. The same gene targets were measured in liver of carp exposed to waterborne CFA *in vivo* (Chapter 6). As predicted by the *in vitro* responses, all measured genes were up-regulated after 4 days of exposure, with the exception of *gsta*. However, *in vivo* the genes analysed responded to water concentrations as low as 4 µg l^{-1}, *in vitro* there was no significant response for any of the genes at exposure concentrations below 1 µM (214 µg l^{-1}). This indicates that although the expression profiles for these genes showed a similar overall trend, the *in vitro* model appeared to show much lower sensitivity in terms of transcriptional responses to CFA exposure.

It should be noted that the carp primary hepatocyte model presented some practical challenges, in that a certain amount of practice and expertise was required to carry out the isolation procedure, and that the resulting primary cells did not have the robustness, stability or ease of use compared with immortalised cell lines. Despite this, once the model was established it proved to be an elegant, higher throughput and responsive system, at least for the

purpose of this study. The xenobiotic metabolising genes analysed here using the carp primary hepatocyte model were responsive to the PXR agonist RIF, as well as other pharmaceuticals, and as such there is certainly potential for the use of this model in the screening of compounds for induction of biotransformation genes. This sort of screening may help to identify effect pathways in fish and so further development of this model would be beneficial in studies of ecotoxicology. However, as evidenced in the case studies on CFA and CTZ, the responses *in vitro* did not always correlate with those *in vivo*, in some cases showing a completely contradictory trend. For this reason, an *in vitro* approach alone does not fully represent the *in vivo* situation with respect to the studied biotransformation pathways.

This is probably hindered by the fact that the complex pathways surrounding the PXR, and regulation of these genes in fish, are not yet well established, and perhaps for the measurement of endpoints which are rather better characterised in fish, the model would prove even more representative of whole animal studies. For example, carp primary hepatocytes were demonstrated to be useful in investigating responses to environmental estrogens, as investigated in our laboratory previously (Bickley *et al.,* 2009).

The reporter gene assay developed here, utilising a mammalian cell line, showed great potential for measuring the activation of PXR in carp *in vitro* and furthermore the data generated were well supported by the responses of human PXR using the same system (Chapter 5). This model could facilitate the establishment of ligand activation profiles for the carp PXR in response to pharmaceuticals or other compounds, or to environmental samples. Indeed, this sort of model has been successfully employed to study other nuclear receptors

in fish, such as estrogen, androgen, thyroid and glucocorticoid receptors (Bury *et al.,* 2003; Lange *et al.,* 2012; Oka *et al.,* 2012; Todo *et al.,* 1999). As such, with further characterisation, *in vitro* models such as the reporter gene assay and fish hepatocyte cell culture, in combination with measurement of gene responses as used here, do hold potential for use as part of an integrated approach to aquatic risk assessment.

7.3. Pharmaceuticals and fish: A summary

Fish may be exposed to a wide range of pharmaceutical drugs in surface waters, as reviewed in Chapter 2 (Review Paper). Concentrations reported are generally low compared with other xenobiotics, and generally a few magnitudes lower than concentrations showing effects in the lab (although there are some exceptions, for example EE2 and possibly diclofenac; see Chapter 2). However fish are, arguably, particularly vulnerable to pharmaceutical exposure due to the evolutionary conservation of a great many enzymes, receptors, signalling pathways, biochemical and physiological systems between vertebrates. As such, there is potential for this emerging class of environmental contaminants to have effects in fish exposed to these compounds, especially considering the desired properties of most drugs (specific biological activity, high potency, reasonable stability etc.). However, whether or not such effects are adverse, or could lead to adverse effects for the exposed organisms, or indeed for the population of organisms, is less clear.

Current legislation governing environmental toxicity testing does not taking into account the vast array of (very specific) biological targets that pharmaceuticals are designed specifically to target. Consequently, understanding the

mechanisms of action of pharmaceuticals, and other compounds present in the aquatic environment, will help identify more appropriate indicators of exposure and effect in fish (and other organisms) for both *in situ* studies and studies on wild populations. Indeed, in the last few years a great deal of research has been generated utilising this 'read across' type approach to try to deduce effects of pharmaceuticals on fish, taking into account the diverse but specific pathways targeted by these compounds. For example, here, read across may have shown some validity in the case of CFA, a fibrate drug almost ubiquitously detected in surface waters, and which targets the PPARα in mammals. As described above the responses, in general, reflected those established in mammals. This suggests that read across of data from mammals to fish could prove useful, both in deducing the mechanisms by which compounds are most likely to act in fish, but also in guiding the screening of compounds and environmental samples.

On the other hand, a number of biotransformation genes more classically associated with the PXR than with the PPARα were found to be up-regulated by CFA *in vitro* and *in vivo.* These responses would not have been predicted based purely on a read across type approach, and instead were identified by screening a number of pharmaceuticals for gene responses utilising the carp primary hepatocyte model. Furthermore, studies in this thesis focused on the teleost PXR and associated biotransformation genes identified some differences between mammals and fish, as well as between *in vitro* and *in vivo* test systems. This not only highlights the caution that should be exercised in extrapolating between species and indeed between test systems, but also the complex nature of the nuclear receptor pathways which may be involved in an organisms response to xenobiotic exposure .

In order to gain the best possible understanding of the effects of a particular pharmaceutical in fish it, therefore, seems logical to screen compounds for general toxicological endpoints such as for oxidative stress or common nuclear receptor pathways which may be induced, e.g. the biotransformation pathway focused on here, but in combination with this, to use the compound's mode-of-action to add endpoints which are specifically associated with the effects of the compound in other species (including man in the case of human drugs). In line with this, and as discussed above, further work is required in order to develop effective, reproducible and validated *in vitro* systems as alternatives to whole animals for ecotoxicity testing. This in turn could help to initiate a more compound-based approach to risk assessment of pharmaceuticals in the aquatic environment.

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Appendix A: Corrections to Chapter II (Review Paper)

As this manuscript is already published it is not possible to include the recommended corrections in the text, however these are listed below:

- P288: “Recent reports” includes a 1988 study – this reference should not be included as it does not constitute a ‘recent report’
- P290: “Cytomchrome P450” should be amended to the correct spelling of “cytochrome P450”
- P291: 4.2 and 0.6 ng^{-1} missing a “litre”; should read 4.2 and 0.6 ng L^{-1}
- P291: “to” should not be superscript
- P294: The word “lead” should be amended to “led”; “carcases” should be corrected to “carcasses”
- P294: The “head kidney” (also called the pronephros) is the first kidney to form during development in vertebrates. In fish and amphibians it remains the primary blood filtration and osmoregulatory organ in free-swimming larvae.
- P295: “steroidogensis” should be amended to the correct spelling of “steroidogenesis”
- P296: The bracket is in the wrong place in the final sentence; should read: “Similarly, it has been shown that exposure to gemfibrozil (1.5 mg L-1 for 96 h) reduces testosterone by more than 50% in goldfish testes (Mimeault et al., 2005).”
- P296: In the final sentence, the comma after the word “and” should be removed
- P297: The words “per se” and P298: the words “in vitro” should be italicised

- P298: In the reference to “Johnston et al. (2007)” the author’s name is spelt incorrectly, and should be “Johnson et al. (2007)”, as per the listed reference