

# **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

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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 (PPAR $\alpha$ ), 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 ketoconazole (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  $\mu\text{M}$ . 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. PPAR $\alpha$  was investigated in carp *in vivo* using CFA as a mammalian PPAR $\alpha$ -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 PPAR $\alpha$ -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 PPAR $\alpha$  in fish (carp) to regulation of xenobiotic metabolism. The work presented has provided further characterisation of PXR and PPAR $\alpha$  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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## 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 ( <i>Cercopithecus aethiops</i> ) 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 <sub>50</sub>	Concentration for half the maximal effect
EE2	Ethinylestradiol
E <sub>max</sub>	Maximal effect
ER	Estrogen receptor
EROD	Ethoxyresorufin-O-deethylase
EU	European Union
Fa2N-4	Human ( <i>Homo sapiens</i> ) liver cell line
FHM	Fathead minnow ( <i>Pimephales promelas</i> ) epithelial cell line
FXR	Farnesoid X receptor
GCL	Grass carp ( <i>Ctenopharyngodon idellus</i> ) 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-density lipoprotein
K <sub>ow</sub>	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 ( <i>Poeciliopsis lucida</i> ) 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 ( <i>Oncorhynchus mykiss</i> ) gonad cell line
RTH-140	Rainbow trout ( <i>Oncorhynchus mykiss</i> ) 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- <i>p</i> -dioxin
TZD	Thiazolidinedione
UGT	UDP-glucuronosyltransferase
US	United States
VDR	Vitamin D receptor
VTG	Vitellogenin
WWTW	Waste water treatment works
XME	Xenobiotic metabolising enzyme

