Nuclear receptor targets for environmental pollutants

Modulation of transcriptional activity of pregnane x receptor and peroxisome proliferator-activated receptor gamma in polar bears and zebrafish

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Abstract

Liganded nuclear receptors can be viewed as transcriptional dimmers whose activity is adjusted by molecular signals. Since the chemical footprint of humans continue to increase, the numbers of chemicals in the environment that can tune the activity of nuclear receptors inside organisms also keep growing. As several nuclear receptor are central regulators in biological and toxicological pathways knowledge about how xenobiotics can modulate the transcriptional activity of nuclear receptors is important. Studies of nuclear receptor increase insight into toxic mechanisms and also contribute to more precise risk assessment of chemicals. The focus of this study is on how xenobiotics can modulate the activity two xenobiotics activated receptors, the pregnane X receptor (a. k. a. steroid and xenobiotic receptor, PXR/SXR/NR1I2) and the peroxisome proliferator-activated receptor gamma (a. k. a. PPARG/NR1C3).

Paper I is a comparative study of the ability of the PXR orthologs from humans and polar bears to be activated by a 51 compound test panel consisting of pharmaceuticals, pesticides, polychlorinated biphenyls (PCBs), brominated flame retardants (BFRs) and industrial compounds. It was shown that polar bear PXR is activated by xenobiotics and that PXR is a promiscuous receptor that likely functions as a xenosensor in polar bears. Four environmental pollutants, HBCDD, toxaphene, 4-nonylphenol and TBBPA, and the cholesterol lowering drug SR12813, activated polar bear PXR more strongly than human PXR. Our findings show that polar bear PXR is activated by structurally different xenobiotics. While polar bear PXR is promiscuous, it is somewhat less promiscuous than human PXR.

Paper II focuses on how environmental pollutants, present in liver and adipose tissue of polar bears, can modulate the transcriptional activity of PPARG and adipogenesis. Extracts of persistent organic pollutants from polar bear liver and adipose tissue induced lipid accumulation in 3T3-L1 cells. Synthetic mixtures composed to reflect the POP composition of the extract from polar bear adipose tissue did not induce adipogenesis in murine preadipocytes (3T3-L1) nor in adipocyte-derived stem cells from polar bears (ASCs). In contrast, the synthetic

mixtures and some single compounds, such as PCB153, bisphenol A, HBCDD, DDE, oxychlordane and endosulfan, inhibited lipid accumulation. These results suggest that the total burden of persistent organic pollutants in polar bear can modulate adipogenesis in murine preadipocytes.

Paper III focuses on genomic variation in the Pxr gene from four strains of zebrafish, the AB Tübingen (AB/Tü), Singapore wild type (SWT), Tupfel long fin (TL) and a strain of unknown origin (UNK). Due to several missense mutations and indels in the Pxr *gene*s from these strains, functionally different Pxr variants are encoded. PxrAB/Tü was activated more strongly by the antifungal drug clotrimazole, and also formed a stronger interaction with this compound. PxrUNK formed the weakest interaction to butyl 4-aminobenzoate, and was activated the least by this compound. Zebrafish is commonly used as a model species in toxicology. The occurrence of functionally different variants of zebrafish Pxrs could have implications for risk assessment. Based on our results, the choice of strain for use in toxicity testing may therefore be of high importance.

List of publications

Paper I:

Lille-Langøy R, Goldstone JV, Rusten M, Milnes MR, Male R, Stegeman JJ, Blumberg B, Goksøyr A (2015). **Environmental contaminants activate human and polar bear *(Ursus maritimus)* pregnane X receptors (PXR, NR1I2) differently.** Toxicology and Applied Pharmacology, 284(1):54-64. DOI:10.1016/j.taap.2015.02.001.

Paper II:

Routti H, Lille-Langøy R, Berg MK, Fink T, Harju M, Kristiansen K, Rostkowski P, Rusten M, Sylte I, Øygarden L and Goksøyr A. **Environmental chemicals modulates polar bear (*Ursus maritimus*) peroxisome proliferator-activated receptor gamma (PPARG) and adipogenesis *in vitro*.** Environmental Science and Technology. DOI: 10.1021/acs.est.6b03020

Paper III:

Lille-Langøy R, Karlsen OA, Myklebust LM, Goldstone JV, Mork-Jansson A, Male R, Stegeman JJ, Blumberg B and Goksøyr A. **Sequence variations in *pxr* from zebrafish *(Danio rerio)* affect nuclear receptor function.** *In preparation*.

Abbreviations

4BAB	Butyl 4-aminobenzoate (a. k. a. butamben)
Å	Ångstrøm
AB/Tü	AB Tübingen (hybrid strain of zebrafish)
ABC	ATP-binding cassette transporter
ADME	Absorption, metabolism, distribution and elimination
AF	Activation function
AP2	Transcription factor AP-2-alpha
ASC	Adipose-derived stem cell
B[a]P	Benzo[a]pyrene
BADGE	Bisphenol A diglycidyl ether
BAT	Brown adipose tissue
BFR	Brominated flame retardant
BPA	Bisphenol A
CAR	Constitutive androstane receptor
CD36	Fatty acid translocase
CEBP	CCAAT/enhancer-binding protein
CLO	Clotrimazole
COX	Cyclooxygenases
CYP3A4	Cytochromes P450 3A4
CYPs	Cytochromes P450
D-box	Distal zinc finger
DBD	DNA binding domain
DDE	Dichlorodiphenyldichloroethylene
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di-(2-ethylhexyl)phthalate
DHR38	Probable nuclear hormone receptor HR38
DR	Direct repeat
EC_{50}	Effective concentration 50
EE2	17α-ethynylestradiol
EF	Embryonic fibroblast
ER	Everted repeat
ERA	Estrogen receptor α (a. k. a. NR3A1)
ERB	Estrogen receptor β (a. k. a. NR3A2)
FABP	Fatty acid binding protein
FMO	Flavin-containing monooxygenases
FXR	Bile acid receptor (a. k. a. NR1H4)
GAL4	Regulatory protein GAL4
GST	Glutathione S-transferases
H112	Helix 12 of a NR
HBCDD	Hexabromocyclododecane
HNF	Hepatocyte nuclear factor
hPXR	Human PXR
IBMX	3-isobutyl-1-methylxanthine (a. k. a. MIX)
IR	Inverted repeat
KO	Knock out (gene)
LBD	Ligand binding domain
LBP	Ligand binding pocket
LCA	Lithocholic acid
LXR	Oxysterols receptor (a. k. a. NR1H3)
MDI	3-isobutyl-1-methylxanthine, dexamethasone, and insulin
MDR	Multidrug resistance protein

MEHP	Mono-(2-ethylhexyl) phthalate
$MeSO_2$	Methyl sulfone
MRP	Multidrug resistance-associated protein
MSC	Mesenchymal stem cell
MYA	Million years ago
NAPQI	N-acetyl-p-benzoquinone
NGFIB	Nerve growth factor IB (a. k. a. NR4A1)
NR	Nuclear receptor
Nrf2	Nuclear factor erythroid 2-related factor 2
NURR1	NUR-related factor 1 (a. k. a. NR4A2)
OAT	Organic anion transporters
OCT	Organic cation transporters
OH	Hydroxyl
OHC	Organohalogen compounds
P-box	Proximal zinc finger
PBDE	Polybrominated diphenylethers
pbPXR	Polar bear PXR
PCB	Polychlorinated biphenyls
PCN	5-pregnen-3β-ol-20-one-16α-carbonitrile
PEPT	Peptide transporters
PGC1A	PPARG gamma co-activator 1a
POP	Persistent organic pollutant
PPAR	Peroxisome proliferator-activated receptor
PPARA	Peroxisome proliferator-activated receptor α (a. k. a. NR1C1)
PPARD	Peroxisome proliferator-activated receptor δ (a. k. a. NR1C2)
PPARG	Peroxisome proliferator-activated receptor γ (a. k. a. NR1C3)
PPRE	PPAR response element
PXR	Pregnane X receptor (a. k. a. SXR/NR1I2)
R_{max}	Maximum response (luciferase reporter gene assay)
RPL11	60S ribosomal protein L11
RXR	Retinoid X receptor
SLC	Solute carrier
SNP	Single nucleotide polymorphism
SRC1	Steroid receptor co-activator 1
Srebp1	Sterol regulatory element-binding protein 1
SULT	Sulfotransferase
SWT	Singapore wild type (strain of zebrafish)
TBBPA	Tetrabromobisphenol A
TD	Toxicodynamics
TG	Triglyceride
TK	Toxicokinetics
TL	Tupfel long fin (strain of zebrafish)
TNC	Trans-nonachlor
UAS	Upstream activation sequence
UCP1	Uncoupling protein-1
UGT	Uridine diphosphate glucuronyl transferases
UNK	Unknown (here: strain of zebrafish of unknown origin)
UPS	Upstream stimulatory factor-1
VDR	Vitamin D receptor (NR1I1)
VP	Viral protein
WAT	White adipose tissue
zfPxr	Zebrafish Pxr

Contents

1. General introduction

1.1 Nuclear receptors are molecular dimmers

Nuclear receptors (NRs) make up a superfamily of DNA-binding proteins whose primary function is to maintain basal gene expression levels and to modulate expression to meet the temporal needs of a cell, a tissue or an organism. Because of these functions NRs are essential in various biological processes, such as embryonic development, maintenance of cellular phenotypes, immunology, metabolism, homeostasis and cell death (Gronemeyer, Gustafsson, and Laudet 2004). NRs may be referred to as molecular dimmers since they rather than functioning as on-off switches have different modes of action depending on which of four different types of ligands that are bound to activate the receptor (Delfosse et al. 2014; Germain et al. 2006), these types of ligands are described in more detail later.

1.1.1 The origin of nuclear receptors

Since NRs are found in early metazoans, such as Porifera and Placozoa (Trichoplax), but not in fungi, plants and cyanoflagellates, the first nuclear receptor genes are believed to have arisen shortly after the establishment of the metazoan branch approximately 635 million years ago (mya) (Sladek 2011).

In the simplest of animal organisms, such as sponges and trichoplax, there are only a few (1-4) NR genes (Srivastava et al. 2008; Bridgham et al. 2010). In organisms with slightly more complex morphology the number of NRs is higher and in corals and sea anemones there are between 10-17 NR genes (Grasso et al. 2001; Yagi et al. 2003). In bilaterians there are ca. 20 NRs, while 48-50 are found in mammals and approximately 70 in fish (***Figure 1***) (Robinson-Rechavi et al. 2001; Zhang et al. 2004; Bertrand et al. 2007; King-Jones and Thummel 2005).

This difference in the number of NR genes is mainly because of two whole-genome duplications that have taken place during the evolution. The first duplication occured prior to the deuterostome-protostome split approximately 550 mya and lead to the acquisition of multiple families of nuclear receptors. The second duplication occurred within the vertebrate branch, after the arthropod/vertebrate split, and produced paralogs within these subfamilies (Laudet 1997). The resulting superfamily of the NRs are divided into seven families (NR1-6 + NR0) based on sequence homology (Laudet 1997) (***Figure 1***).

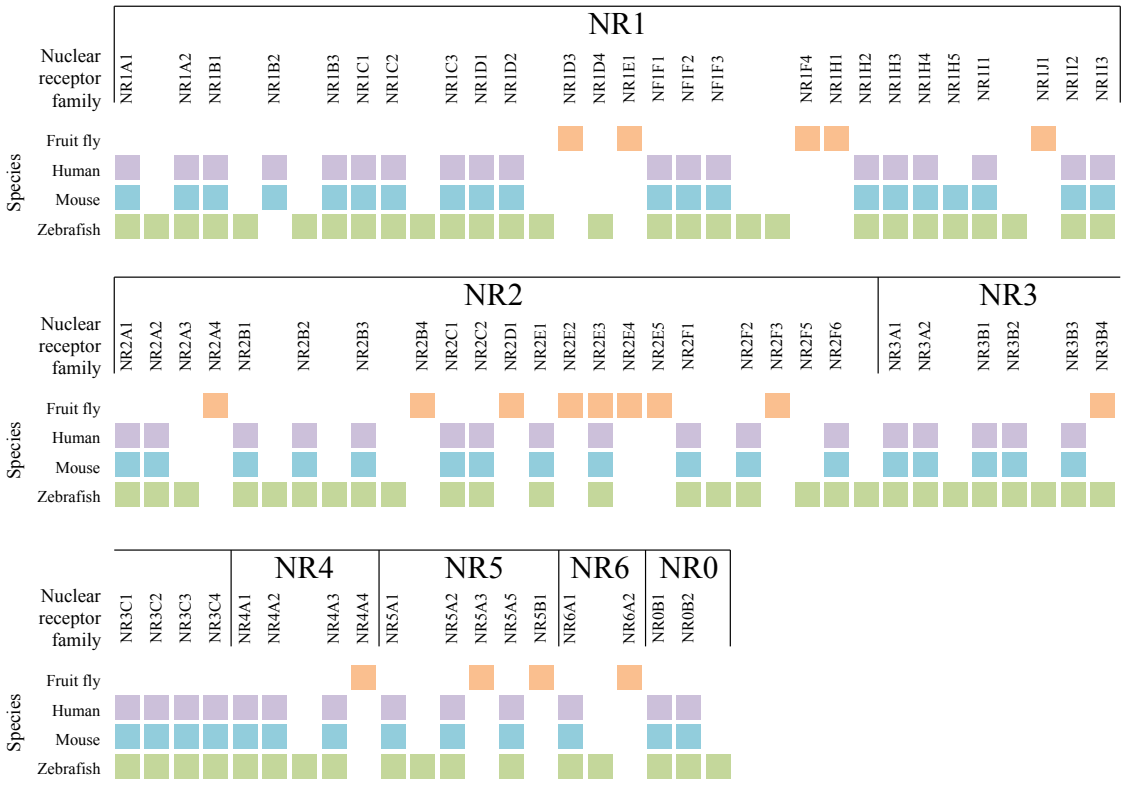

Figure 1 – The six families of nuclear receptors in insects, mammals and fish. Insects have 18-21 NR genes, while mammals have 48-50 and have zebrafish 70 such genes (King-Jones and Thummel 2005; Germain et al. 2006; Zhang et al. 2004; Bertrand et al. 2007).

Of these, NR1 is the largest subfamily and is subdivided into eight groups. Among the NRs of the NR1 subfamily we find three peroxisome proliferator-activated receptors in the NR1C group (PPARs, PPAR alfa, delta and –gamma/NR1C1, -2 and -3) and the pregnane X receptor (a. k. a. steroid and xenobiotic receptor, PXR/SXR/NR1I2) in the NR1I group. In the second

largest subfamily, NR2, we find three retinoid X receptors (RXRA-C/NR2B1-3) and these are important heterodimerizing partners for many NR1s, including PPARs and PXR. Steroid hormone receptors, such as the estrogen receptors (ERA and –B/NR3A1 and -2) compose subfamily NR3. The remaining NR subfamilies, NR 4-6, have one or a few members. Nuclear receptors that contain only one of the two conserved NR domains, the DNA binding or the ligand binding domain, have been placed in a separate subfamily (NR0) irrespective of homology (Nuclear Receptors Nomenclature 1999). It has been suggested that the first ancestral NR probably originated from either the NR2 or NR4 subfamilies because only NRs belonging to these two subfamilies are found in all metazoans (Escriva et al. 1998).

1.1.2 Nuclear receptors share a common structure

Most NRs have a modular structure that consists of five to six regions, denoted A-F (***Figure 2***). The amino terminal A/B domain is highly variable both in length (23-602 AAs) and in sequence, and contains a ligand-independent transactivation function (AF-1) (Tomura et al. 1995). The A/B domain interacts with multiple coregulatory proteins (such as SRC-1, -2, p300 and CBP) to enable a functional synergism between AF-1 and the ligand-dependent activation function, AF-2, located the C-terminal of the NR (Bugge et al. 2009; Gianni et al. 2003). Interestingly, differences in A/B regions cause different affinity to response elements and the transcription initiation complex (Briancon and Weiss 2006; Hollenberg et al. 1996).

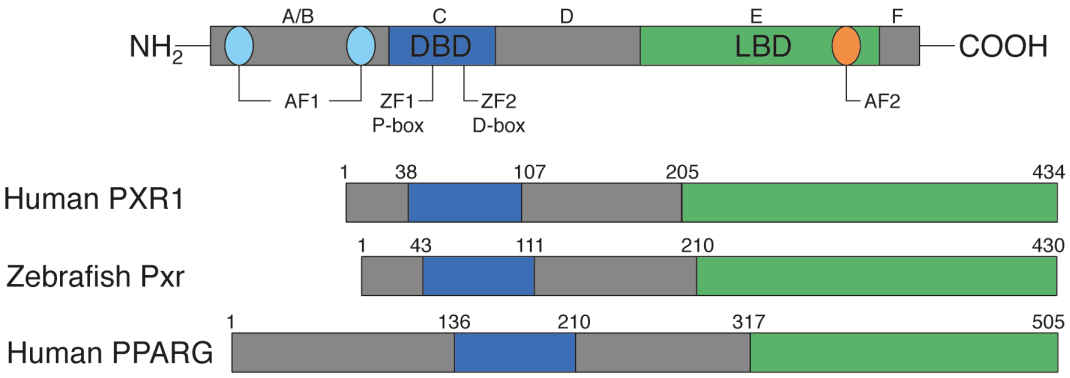

Figure 2 – General structural organization of nuclear receptors. NRs typically consist of five to six regions (A-F). The variable A/B region contains a ligand-independent transactivation function (AF-1). The C-region with its two zinc fingers (ZF1 and -2) interacts with DNA in the major groove in a sequence specific manner. Liganded NR can harbour ligands in the structurally conserved ligand binding domain (LBD)/E-region that contains a ligand-dependent transactivation function (AF-2). In PXR s and PPARs the A/B regions differ in lengths and the F-region is absent.

The C domain is the most conserved region of the NRs, both with regards to amino acid sequence and to structure. The C domain, a. k. a. DNA-binding domain (DBD), binds to DNA in a sequence-specific manner. It has also been proposed that the DBD contains a nuclear export signal (Black et al. 2001). In the junction between the C and D regions is a basic nuclear localisation signal that regulates the subcellular localization of NRs.

The hinge, or region D, that connects the DBD and the LBD, is variable both in sequence and structure. Functionally, the hinge offers flexibility to the NR and interacts with co-regulators (Gray et al. 2006; Puigserver et al. 1998). Interestingly, the hinge of some NRs interact with regulator elements in genes, such as the PPARA-hinge that interacts with PPRE (Chandra et al. 2008). In the estrogen receptors (ERs/NR3As) the hinge has been shown to mediate a synergy between AF-1 and AF-2 that modulates the transcriptional activity of the receptor (Zwart et al. 2010). These functions of the hinge region appear not to be universal for all NRs, possibly due to sequence variation. For instance, the co-repressor RPL11 inhibits transcriptional activity via an interaction surface in the hinge in PPARA but not in PPARB and PPARG (Gray et al. 2006).

The E region, also called the ligand-binding domain (LBD), is well conserved structurally but only moderately conserved in sequence. The LBD contains a ligand-binding pocket (LBP), AF-2, as well as binding sites for co-regulators and dimerization interfaces (Pawlak, Lefebvre, and Staels 2012). The conserved structure of LBDs typically consists of 11-13 α-helices and a β-turn that surround a hydrophobic pocket (Wurtz et al. 1996). Three long α-helices (helices 3, 7 an 10) form the two outer layers. The middle layer is composed of helices 4, 5, 8 and 9 and is missing in the lower parts of the domain, and this creates a cavity that enables ligand binding. In some nuclear receptors, such as the nuclear receptor related 1 protein (NR4A2/NURR1) and the probable nuclear hormone receptor HR38 (NR4A4/dHR38), the framework of the LBD is very tight leaving only a small or no cavity with bulky hydrophobic side chains occupying the LBP (Baker et al. 2003; Wang, Benoit, et al. 2003), suggesting that these are not regulated by ligands. The F-region is poorly understood and not always present in nuclear receptors, such as PXR and PPARs.

1.1.3 DNA recognition by nuclear receptors

NRs interact with DNA via the DBD. A core of 66 amino acids composes two C4-type zinc fingers that each consists of two perpendicular α-helices and a short β-sheet (Freedman et al. 1988). Each zinc finger is stabilized by the coordination of a zinc atom by four cysteins (Helsen et al. 2012). Without the structural stability added by the coordination of zinc, the NR would be unable to form a stable hydrophobic core (Luscombe et al. 2000). Three P-box amino acids in the N-terminal α-helix interact with DNA in the major groove in a sequence specific manner, while the D-box of the C-terminal α-helix forms the dimerization surface (Baumann et al. 1993; Smit-McBride and Privalsky 1994; Luisi et al. 1991; Remerowski et al. 1991).

The specific DNA sequences recognized by NRs typically consist of a group of six bases and commonly used hexads include 5′-AGAACA-3′, 5′-

AGGTCA-3′ or 5′-TGG(T/C)CA-3′. A pair of hexads separated by a nucleotide spacer of various length forms a response element that is found in the relative vicinity of genes, more accurately in promoter regions. The members of the NR protein family can be divided in three groups based on their interaction with DNA (***Figure 3***). The first group consists of NRs that form homodimers that binds response elements with direct, inverted or everted repeats of hexads. Examples are the steroid hormone receptors that typically bind symmetric repeats of 5′-AGAACA-3′ divided by three nucleotide spacer (Zilliacus et al. 1995). The NRs in the second group form heterodimers with RXRs and bind direct repeats of 5′-AGGTCA-3′ separated with spacers of various length (Mangelsdorf and Evans 1995). The third of group NRs binds DNA as monomers, such as the nerve growth factor IB (NGFIB/NR4A1) (Meinke and Sigler 1999). The preference of NR monomers, homodimers and heterodimers for specific hexads sequences, combination and orientation of hexads, allows the transcriptional regulation by NRs to be gene specific.

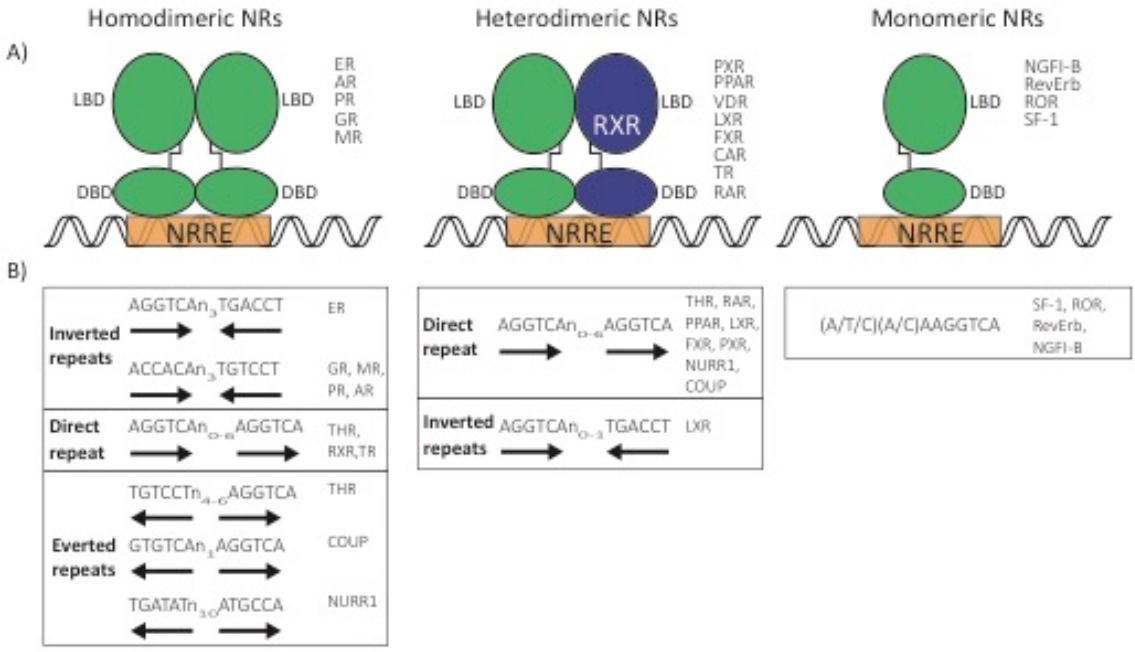

Figure 3 – Modes of DNA binding by nuclear receptors. NRs bind resposne elements in the DNA as monomers, homodimers or heterodimers. Nuclear receptor response elements are composed of direct repeats (DR), inverted repeats (IR) or everted repeats (ER) of sequence hexads separated by a spacer of various length (Helsen et al. 2012).

1.1.4 Ligand binding by nuclear receptors and mechanism of action

Unliganded NRs may be missing the ligand-binding pocket or have pockets that are occupied by lipids, phospholipids or heme (Wisely et al. 2002; Li et al. 2005; Yin et al. 2007), and consequently, unliganded NRs often act as constitutive activators or repressors of transcription (Evans and Mangelsdorf 2014). About half of the 48 NRs in humans are ligand-dependent and require binding of a ligand to exert their function (Germain et al. 2006). The LBD of liganded NRs forms a structurally conserved scaffold that surrounds the ligand-binding pocket (LBP). Within the LBP non-polar amino acid side chains mostly make up the interior surface. The size and shape of the cavity, as well as a limited set of stereo-specific polar contacts in the cavity, contribute to specificity in the ligand binding (Nagy and Schwabe 2004). Although NR LBDs are structurally conserved the volume of the LBP varies greatly between different nuclear receptors. For instance, the LBP is 600 $Å^3$ in the thyroid hormone receptor (THR), 1100 $Å^3$ in PXR and 1300 $Å^3$ in PPARG (Wagner et al. 1995; Nolte et al. 1998; Watkins et al. 2001). Also the shape of LBPs varies. While the LBP of PXR is elliptic, it forms a branched Y-shape in PPARs. It is this Y-shape that allows PPARs to bind branched molecules, such as phospholipids, as well as to bind singly branched ligands, such as fatty acids, in multiple conformations (Xu et al. 1999). Multiple binding conformations are also possible in the LBP of human PXR and the synthetic ligand SR12813 can be oriented with three different conformations within the elliptical cavity of the human PXR (Watkins et al. 2001).

The molecular basis of ligand-modulated agonism and antagonism is described in the helix 12/AF-2 conformational model. According to this model, transcriptional activation relies on recruitment of coactivator proteins to the hydrophobic AF-2 interface composed of helix 3, 4, 5 and 12 (Kojetin and Burris 2013). Binding of an agonist stabilizes the LBD that takes a more compact, rigid and less dynamic structure. Stabilization is obtained either by

direct interaction between agonist and the H12 helix, or by stabilization of the lower parts of the LBD by the agonist (Nagy and Schwabe 2004). In the stabilized active conformation of the NR-agonist complex, recruitment and binding of coactivators is promoted and result in the gathering of the general transcriptional machinery and finally transcription. In contrast, the binding of an antagonist will prevent optimal positioning of H12 with respect to H3, -4 and -5, so that stable coactivators binding site can be formed. When bound by a partial agonist or a partial antagonist the conformational dynamics of H12 remains high and the relative abundance of cofactors determines the level of transcriptional activation obtained (Nahoum et al. 2007). A fourth type of NR ligands, the neutral antagonist, stabilizes structures that promote receptor-corepressor interaction with no successive recruitment of the transcriptional machinery (Germain et al. 2009).

1.2 Pregnane X receptor (steroid and xenobiotic receptor, PXR/NR1I2)

PXR was first described in mice and humans in 1998 and has since been classified a member of the nuclear receptor subfamily 1 group I based on phylogeny (Blumberg et al. 1998; Kliewer, Moore, Wade, Staudinger, Watson, Jones, McKee, Oliver, Willson, Zetterstrom, et al. 1998; Bertilsson et al. 1998; Germain et al. 2006). The human *PXR* (also called *SXR*) is located on chromosome 3q11-13 (Zhang et al. 2001) and at least nine isoforms of human *PXR* have been identified from liver and other tissues (Fukuen et al. 2002; He et al. 2006; Zhang et al. 2001). The most strongly transcribed isoform is *PXR.1* that encodes a 434 AA protein (Lamba et al. 2004). Although detected at very different levels, *PXR* transcripts could be detected in all 21 human tissues studied by Nishimura et. al (Nishimura, Naito, and Yokoi 2004). However, *PXR* transcripts are by far most abundant in the liver, followed by colon (~20% of liver level), small intestine (~12%), stomach and skeletal muscle (~1%). *PXR* is strongly transcribed in fetal liver, indicating that the

ability of ligand-induced modulation of biotransformation may occur even before birth (Lamba et al. 2004; Nishimura, Naito, and Yokoi 2004). *Pxr* transcripts were detected in ~25% of the tissues in mice, and restricted to two tissue systems, the gastroenteric (including tongue, stomach, duodenum, jejunum, ileum, colon, and gall bladder) and metabolic tissue systems (including liver, kidney, muscle, brown and white adipose tissue (Bookout et al. 2006). The highest levels of mouse *Pxr* transcripts are found in duodenum, jejunum, ileum, colon, gall bladder and liver, while lower levels are found kidney, ovary, testis and stomach (Bookout et al. 2006). *Pxr* is not expressed in brown or white adipose tissue in mice (Bookout et al. 2006; Yang et al. 2006).

Several structural features distinguish PXR from other nuclear receptors. PXR has a variable four-residue turn between helices 1 and 3, a large flexible loop in place of helix 6 and two additional β-strands in the structure (Watkins, Davis-Searles, et al. 2003; Watkins, Maglich, et al. 2003; Watkins, Noble, and Redinbo 2002; Watkins et al. 2001). As a result the PXR-LBD is large, elliptic and flexible, and can accommodate an unusually broad range of ligands. PXR ligands include endogenous compounds such as steroids and steroid metabolites (including estradiol, 5-β-pregnane-3,20-dione, progesterones, pregnenolones, corticosterones, testosterone) and bile acids (including litocholic acid), retinoids, thyroid hormone, sterols, dietary compounds (vitamin K, vitamin E), natural compounds including herbal medicine components (hyperforin in St John´s herb), prescription drugs and various environmental pollutants (polychlorinated biphenyls, brominated flame retardants, pesticides, plasticizers, and many more) (Moore, Parks, et al. 2000; Kliewer, Moore, Wade, Staudinger, Watson, Jones, McKee, Oliver, Willson, Zetterstrom, et al. 1998; Xue et al. 2007; Goodwin et al. 2003; Krasowski et al. 2005; Moore, Goodwin, et al. 2000; Milnes et al. 2008; Kojima et al. 2011; Sinz et al. 2006). Interestingly, synergistic activation has been observed when the synthetic oestrogen 17α-ethynylestradiol (EE2) and the pesticide trans-

nonachlor (TNC) are bound simultaneously by human PXR (Delfosse et al. 2015).

The difference in sequence and function between PXR orthologs is far greater than between orthologs of any other NRs, with the possible exception of constitutive androstane receptor (CAR/NR1I3). While a purifying evolutionary pressure has conserved the sequence and function of most NRs, a 7% sub-population of codons encoding amino acids in the LBD of mammalian PXRs have been subjected to positive selection ($\omega=K_a/K_s>1.0$) (Krasowski et al. 2005). This indicates that PXR-LBDs mutations have been advantageous, and suggests that fine-tuning of the ligand-specificity of PXR orthologs in the direction of binding of different compounds in different species. Species-dependent tuning of ligand-specificity of PXR can have resulted in the observed species-dependent ligand activation of PXR reported from several studies (Milnes et al. 2008; Shukla et al. 2011).

PXR binds to DNA as a heterodimer with RXR (Kliewer, Moore, Wade, Staudinger, Watson, Jones, McKee, Oliver, Willson, Zetterstrom, et al. 1998). The PXR-RXR heterodimer can bind a variety of DNA response elements consisting of direct or indirect repeats of AG(G/T)TCA hexads spaced by 3, 4, 5 6 and 8 nucleotides (DR-3, DR-4, DR-5, ER-6 and ER-8) (Orans, Teotico, and Redinbo 2005). Ligand binding does not seem to be absolutely required for interaction between DNA and the PXR-RXR, but increases the frequency of this interaction. PXR has been shown to bind to a low number of specific regions in the promoters of its target genes also in non-exposed mice. However, exposure to pregnenolone-16α-carbonitrile (PCN) strongly enriches binding of PXR to these specific regions (Cui et al. 2010).

Upon ligand and DNA-binding PXR can recruit different co-activators, including steroid receptor co-activator 1 (SRC-1), TIF/GRIP (SRC-2), and peroxisome proliferator activated receptor gamma co-activator 1a (PGC-1a) (Ihunnah, Jiang, and Xie 2011).

Chromatin immunoprecipitation sequencing identified more than 3000 binding sites for PXR in mice targeting approximately 600 genes in mice exposed to PCN (Cui et al. 2010). Furthermore, 220 genes were differentially expressed in a microarray on rat livers exposed to PCN (Guzelian et al. 2006). In human primary hepatocytes exposed to rifampicin 157 differentially regulated genes were identified, of which 110 were induced (Smith et al. 2014). Collectively these studies demonstrate that PXR is involved in the regulation of a large set of genes involved in many biological pathways, including hepatic synthesis of glucose (Kodama et al. 2007), lipid homeostasis by several mechanisms including positive regulation of lipogenesis via regulation of fatty acid translocase (CD36) (Zhou, Zhai, et al. 2006), metabolism and excretion of cholesterol (Li, Chen, and Chiang 2007), metabolism of hormones including gluco- and mineralocorticoids (Zhai et al. 2007), bile acid homeostasis (Xie et al. 2001) and bilirubin clearance (Sugatani et al. 2005; Staudinger et al. 2001). PXR is also involved in vitamin E catabolism and excretion (Kiyose et al. 2001; Swanson et al. 1999), bone homeostasis (Igarashi et al. 2007), retinoic acid metabolism and excretion (Wang et al. 2008), and inflammation (Zhou, Tabb, et al. 2006). Activation of PXR has also been linked to cell migration and mitosis (Kodama and Negishi 2011; Smutny et al. 2014). Importantly, PXR is central in the protection against chemical insults and in this context PXR acts as a sensor for endogenous and exogenous compounds, and regulate transcription of genes involved in biotransformation. PXR target genes include genes of monooxygenases, including CYP3A4, CYP2Bs, CYP2Cs, CYP2A6, carboxylesterases and reductases (Ferguson et al. 2005; Xu, Wang, and Staudinger 2009; Liu, Takahashi, et al. 2009; Wang, Faucette, et al. 2003; Goodwin, Hodgson, and Liddle 1999), several key conjugating enzymes, including UGTs, SULTs and GSTs, as well as transporters, such as multidrug resistance proteins (MDRs) (Naspinski et al. 2008; Geick, Eichelbaum, and Burk 2001).

Genetic variation in human PXR has been reported in the form of alternative splicing and nucleotide polymorphisms. At least 9 different splicing and transcript variants of *PXR* exist (Dotzlaw et al. 1999; Fukuen et al. 2002; Gardner-Stephen et al. 2004; Hustert et al. 2001; Lin et al. 2009; Liu, Ji, et al. 2009; Brewer and Chen 2016; Mensah-Osman et al. 2007; Kurose et al. 2005). The PXR variants differ both in expression profiles, transcriptional activation and protein-protein interactions (Lamba et al. 2004; Lin et al. 2009; Liu, Ji, et al. 2009; Elias, Wu, and Chen 2013; Hustert et al. 2001; Zhang et al. 2001). In addition to transcript and splicing variants, ~2500 single nucleotide polymorphisms and ~240 indels have been described mammalian PXRs (Information 2015). Polymorphisms, in coding or non-coding regions, affect *PXR* transcript levels, the transcriptional activity of PXR/transcript levels of PXR target genes such as *CYP3A4*, as well as rates of drug clearance (Lamba et al. 2008; Swart et al. 2012; Zhang et al. 2001; Siccardi et al. 2008; Koyano et al. 2002). Genetic variation in *PXR* can contribute to the severity of disease conditions, such as non-alcoholic fatty liver disease and intrahepatic cholestasis (Sookoian et al. 2010) and has been associated to increase susceptibility of colorectal cancer (Ni et al. 2015). Recently, we described allelic variation in zebrafish *pxr* (Bainy et al. 2013).

Maintaining homeostasis can mean life or death for cells, tissues and organisms. Since PXR regulates many genes involved in the maintenance of homeostasis this makes PXR a potential target for factors that disrupt homeostasis. The promiscuity in its ligand binding adds to the relevance of PXR as a target for exogenous chemicals capable of disrupting homeostasis.

1.3 Peroxisome proliferator-activated receptor gamma (PPARG)

The PPARs were first described in the early 1990s, and three PPAR paralogs, PPAR alfa (PPARA/NR1C1), PPAR delta (PPARD/NR1C2) and PPAR gamma (PPARG/NR1C3) have now been described (Dreyer et al. 1992;

Issemann and Green 1990; Kliewer et al. 1994). The human PPAR paralogs vary from 441 to 505 AAs in length and considerable divergence in ligand-binding regions has occurred (64-70% identical) (Tachibana et al. 2008). In humans, PPARA is expressed in liver, kidney, small intestine, heart and muscles and regulate fatty acid catabolism and lipoprotein assembly (Mandard, Muller, and Kersten 2004), while PPARD regulate fatty acid oxidation, skin development and wound healing, and is ubiquitously expressed (Tan et al. 2001; Tanaka et al. 2003). All three PPAR paralogs are ligand-activated nuclear receptors that heterodimerize with RXR.

Kubota *et al.* demonstrated that embryonic fibroblasts (EFs) that are lacking PPARG (*PPARG*$^{-/-}$) fail to undergo adipogenesis altogether, while EFs with 50% PPARG (*PPARG*$^{-/+}$) have retained 50% of the ability of wild type EFs to differentiate (Kubota et al. 1999), demonstrating that PPARG is essential in adipogenesis. Heterozygote *PPARG*$^{-/+}$ mice held under standard diet show, in contrast to wild type mice (*PPARG*$^{+/+}$), normal weight and insulin sensitivity but are protected from obesity and insulin resistance when fed high fat diets (Kubota et al. 1999), demonstrating that both PPARG alleles is required for adipocyte hypertrophy and that PPARG is essential for building adipose tissues. Both the *in vitro* and the *in vivo* study are in agreement with the current consensus about PPARG being a major, and mandatory, regulator of adipogenesis and also regulate genes involved in energy balance, inflammation and lipid biosynthesis (Feige et al. 2006).

Four different transcript variants of PPARG have been described in humans and these are translated into two different isoforms, PPARG1 (477 AA) and PPARG2 (505 AA) (Aprile et al. 2014; Lefterova et al. 2010). PPARG2 is exclusively expressed in white adipose tissue (WAT) and brown adipose tissue (BAT), while PPARG1 is expressed in many different tissues, such as macrophages, colon, stomach spleen, heart, brain, liver, muscle, kidneys and pancreas, but the highest levels of PPARG1 are found in in colon, WAT and BAT (Bookout et al. 2006; Mukherjee et al. 1997). Interestingly,

only the expression of PPARG2 is regulated in response to intake of nutrients and obesity (Medina-Gomez et al. 2007). Adipose tissue expansion is ablated in PPARG2 KO mice (Medina-Gomez et al. 2007), and it has been demonstrated that the adipogenic potential of PPARG2 exceeds that of PPARG1 (Feige et al. 2006; Ren et al. 2002; Mueller et al. 2002). Moreover, PPARG2 has a 5-fold stronger ligand-independent activation (AF-1) than PPARG1, which suggests that PPAR2G has the stronger basal transcriptional activity (Werman et al. 1997).

Since the transcriptional activity of PPARG can be modulated by ligands, compounds that can modulate the function of PPARG potentially can influence the building of fat stores. Several endogenous compounds have been demonstrated to modulate the transcriptional activity of PPARG, such as lipid components of oxidised LDL (such as 9- and 13-HODE), unsaturated fatty acids, prostaglandins, linoleic acids, serotonin, lysophosphatidic acid, phospholipid cyclic phosphatidic acid (antagonist) and indole acetates (Bell-Parikh et al. 2003; Waku et al. 2010; Schopfer et al. 2005; McIntyre et al. 2003; Tsukahara et al. 2010; Forman et al. 1995; Nagy et al. 1998; Davies et al. 2001) (***Figure 4***). However, most of these are not present in cellular concentrations necessary to activate this nuclear receptor (Schupp and Lazar 2010).

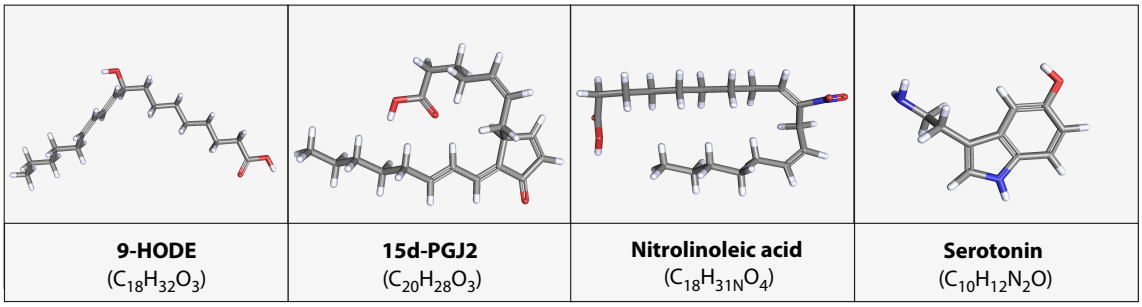

Figure 4 – Structure of selected endogenous compounds that activates PPARG. Examples of endogenous PPARG agonists are 9-hydroxy-10E,12Z-octadecadienoic acid, 15-deoxy-delta-12,14-prostaglandin J2, 10-nitrolinoleic acid and serotonin. Chemical structures were obtained from PubChem (National Center for Biotechnology Information).

Recently several compounds, including pharmaceuticals, pesticides, brominated flame-retardants and industrial chemicals, have been demonstrated to have the ability of modulating PPARG activity and to induce adipogenesis (Taxvig et al. 2012; Kamstra, Hruba, et al. 2014; Bastos Sales et al. 2013; Chamorro-Garcia et al. 2012; Routti et al. 2016). Interestingly, many xenobiotic PPARG ligands consist of aromatic ring structures and differ from the aliphatic endogenous agonists (***Figure 5***).

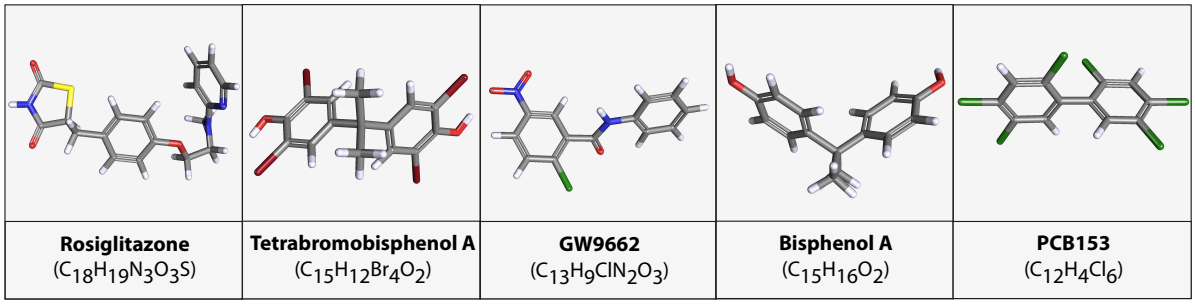

Figure 5 - Structure of selected exogenous compounds with agonistic and antagonistic effect on PPARG. Chemical structures were obtained from PubChem (National Center for Biotechnology Information).

1.3.1 Adipose tissues and adipogenesis

Adipose tissues are not merely passive storage sites of energy but active organs with crucial roles in buffering energy intake, regulating insulin sensitivity and to shield non-adipose tissue from toxic accumulation of fat (Rosen and Spiegelman 2006). Moreover, adipose tissues are endocrine organs that secrete peptide hormones, cytokines and growth factors, such as leptin, adiponectin and others, to communicate with other metabolic tissues (Peirce, Carobbio, and Vidal-Puig 2014). Several different cell types make up adipose tissues, including mature adipocytes, stromal-vascular cells such as fibroblasts, muscle cells, immune cells, pericytes, and preadipocytes (Frayn et al. 2003). There are three main types of adipose tissues, white adipose tissues (WAT), brown adipose tissues (BAT) and beige adipose tissue. Brown adipocytes are packed with mitochondria containing the uncoupling protein-1 (UCP1) that stimulates the respiratory chain to carry out the energy demanding process of thermogenesis in which heat is produced at the expense

of ATP (Harms and Seale 2013). The major function of brown tissue is to maintain the core temperature, but BAT also plays a role in normalizing hyperglycaemia and hyperlipidaemia due to its high capacity of glucose and lipid uptake (Cannon and Nedergaard 2004). Unlike BAT, the WAT is widely distributed in the organism. WAT is the major energy deposit and also an important endocrine organ that produces and stores steroid hormones (Fain et al. 2004). The number of xenobiotic-metabolising enzymes expressed in adipose tissue also indicates a role in elimination of xenobiotics, especially via cytochrome P450s of family 1 (Li, Papadopoulos, and Vihma 2015; Ellero et al. 2010).

Both BAT and WAT may expand in response to either chronic stress or calorie overconsumption, but WAT is by far more plastic and may constitute between 5-60% of total body weight in humans (Ortega et al. 2010; Fleck 1983).

Adipocytes are derived from pluripotent mesenchymal stem cells. Brown adipocytes originate from the same type of stem cells as skeletal muscle ($Pax7^{+}/Myf5^{+}$), while white and beige adipocytes originate from another type of stem cells ($Pax7^{-}/Myf5^{-}$) (Rosen and Spiegelman 2014). The process of transforming undifferentiated precursor cells with adipogenic potential into a mature adipocyte is called adipogenesis, and involves two steps, commitment and differentiation (Ali et al. 2013). This transformation involves six developmental stages including mesenchymal precursors, committed pre-adipocytes, growth-arrested preadipocytes, mitotic clonal expansion, terminal differentiation and mature adipocytes (Pellegrinelli, Carobbio, and Vidal-Puig 2016). Commitment involves that the developmental possibilities of undifferentiated precursor cells, such as a mesenchymal stem cell (MSC), are restricted and limited to the adipogenic lineage (Bowers and Lane 2007). Commitment is a multistep process with activating and inhibiting factors (Tang, Otto, and Lane 2004; Spinella-Jaegle et al. 2001) and will not be discussed in detail in this thesis.

The process of transforming the committed precursor cell into a mature adipocyte is called differentiation and requires an appropriate adipogenic signal (Cristancho and Lazar 2011). Terminal differentiation involves a transcriptional cascade, including CEBPB and CEBPD (***Figure 6***), that eventually leads to the expression of PPARG and CEBPA (Farmer 2006). PPARG can induce adipogenesis in mice embryonic fibroblasts deficient in CEBPA, but CEBPA cannot drive adipogenesis in absence of PPARG (Rosen et al. 2002), which suggests that PPARG is the dominant factor in a single pathway of adipose development including both CEBPA and PPARG. During terminal differentiation CEBPA plays a critical part in feedback loop to maintain expression of PPARG in order to complete adipogenesis (Wu et al. 1999).

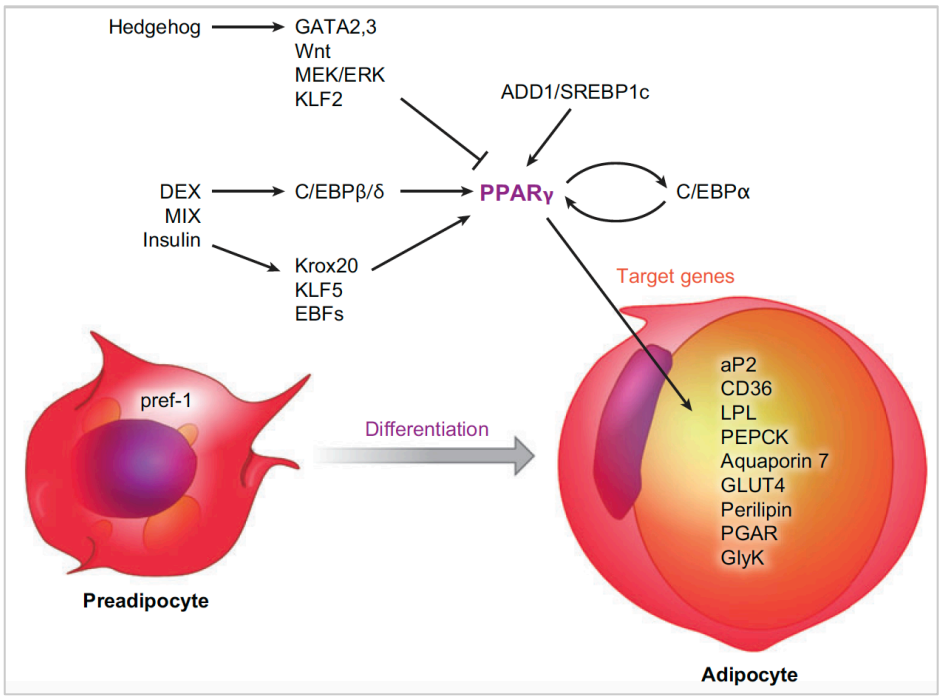

Figure 6 – PPARG is a master regulator of adipogenesis. A committed preadipocyte develops into a mature adipocyte. Transcription factors CEBPA, CEBPB and CEBPD contribute to the expression of PPARG, the master regulator of adipogenic differentiation. Figure from Tontonoz *et al.* (Tontonoz and Spiegelman 2008).

1.4 The defensome and toxic effects

Genes and proteins involved in the maintenance of homeostasis against challenges of physical, chemical or biological nature are collectively termed the defensome (Goldstone et al. 2006). Homeostasis is maintained by a limited number of highly expressed enzymes with broad substrate specificity and low catalytic rates. Recently, the complete chemical defensome of a sea anemone, a sea urchin and a fish species have been described (Goldstone et al. 2006; Goldstone 2008; Goldstone et al. 2010).

1.4.1 Evolution of the chemical defensome

Systems for the metabolism of harmful endogenous and exogenous compounds likely started to evolve in bacteria more than 3.5 billions years ago(Coleman 2010). Initially, the drivers for this evolution probably were increased survival or the gain of additional sources of nutrients. Later, the protection against food toxins probably became more important in the more complex organisms that had more complex diets (Coleman 2010). It has been suggested that development of a defence against harmful lipophilic compounds accelerated with the transition from life in water to life on land with a plant based diet (Coleman 2010).

1.4.2 Biotransformation – metabolism and excretion of chemicals

Without systems for the removal of lipophilic xenobiotics their rates of elimination would be lower than the rates of absorption, and they would accumulate in the organism until unacceptably high levels were to be reached. Reabsorption is a major obstacle in elimination from cells, tissues and organisms. To reduce reabsorption, and to increase rates of elimination, lipophilic compounds are modified in a set of processes often referred to as biotransformation. This is a three-phase process carried out by a limited number of highly expressed enzymes and proteins with broad substrate specificity and low catalytic rates. In general, phase I and II enzymes

transform harmful lipophilic compounds so that they can be excreted from the cell by phase III transporters (Williams et al. 2004; Konig, Muller, and Fromm 2013). Enzymes and transporters involved in the metabolism of xenobiotics are widely distributed in different tissues, but not uniformly. While most tissues have a certain capacity of metabolism, certain specialized tissues and organs have an enhanced metabolic capacity. The main organs for xenobiotic metabolism are the liver, the gastrointestinal system, the kidneys and the lungs. Additionally, the skin holds some metabolic capacity.

Phase I reactions

The overall effect of phase I reactions is a slight reduction in the lipophilicity of the substrates and due to this modest reduction of lipophilicity phase I reactions contribute only marginally to increased elimination directly. However, phase I modifications introduce reactive groups that allows for subsequent reactions that more effectively reduce the lipophilicity of the substrate. Type I reactions take place in endoplasmatic reticulum, cytoplasm, lysosomes and mitochondria, and include different chemical reactions including hydrolysis, reductions and oxidations.

These reactions add, or reveal, functional groups, including –OH, -NH_2, -SH or –COOH, in the substrate. In humans, approximately 65% of all prescription drugs are substrates in phase I reactions. Of these, approximately 75% (appr. 55% of all) are metabolised by cytochrome P450 monooxygenases (CYPs) (Williams et al. 2004). Fifteen human CYPs are involved in xenobiotic metabolism, and the majority of these (13 of 15) belong to three CYP subfamilies, namely CYP1-3 (Guengerich 2008),. Five of these xenobiotic metabolising CYPs, CYP3A4, CYP2C9, CYP2C19, CYP2D6 and CYP1A2, contribute to the vast majority of the oxidations (>95%) of drugs (Williams et al. 2004).

Phase II reactions

Phase II reactions involve the conjugation of reactive moieties in substrates to endogenous metabolites, such as sugars, amino acids, glutathione, sulphate and more. These metabolites greatly add to the water-solubility of the substrates and greatly reduce reabsorption and increase elimination. Glucuronidation is the major mechanism for conjugative clearance. Other important phase II reactions are sulfation, methylation, acetylation and glutathionylation. Just over 10% of all prescription drugs are directly conjugated with glucuronide by uridine diphosphate glucuronyl transferases (UGTs). UGT1A1 and UGT1A3 are the major contributors and perform 50% of all glucuronidations (Williams et al. 2004). Other important phase II enzymes are sulfotransferases (SULTs) and glutathione S-transferases (GSTs).

Phase III efflux transporters

Due to the nature of biological membranes, transport of hydrophilic compounds across lipid membranes is a challenge and requires active transport. Systems for uptake and efflux of hydrophilic compounds exist. Uptake transporters of the solute carrier (SLC) family include the organic anion transport proteins (OATPs), organic anion transporters (OATs), organic cation transporters (OCTs) and peptide transporters (PEPTs). These are expressed in metabolically active tissues, mainly in hepatocytes and in epithelial cells in the intestines, kidneys and brain (Konig, Muller, and Fromm 2013).

To be eliminated from the organism, hydrophilic phase II metabolites must first be actively transported against a concentration gradient across cell membranes to the intracellular space. This task is performed by ATP-binding cassette (ABC) transporters. Humans have at least 48 ABC transporters that can be divided into seven subfamilies. Among the most important are MDR1 (ABCB1/P-glycoprotein) and multidrug resistance-associated protein 1

(MRP1/ABCC1) (Konig, Muller, and Fromm 2013; Beringer and Slaughter 2005).

1.4.3 Xenobiotic activated receptors that regulate biotransforming enzymes

The enzymes of the biotransformation system have high basal expression levels and enzymatic activities that are maintained by transcription factors. These transcription factors include CCAAT/enhancer-binding proteins (such as CEBPA and CEBPB/TCF5), hepatocyte nuclear factors (such as HNF1A/TCF1, HNF3G/TCF3G, HNF4G/NR2A2), transcription factor AP-1 (AP1) and upstream stimulatory factor-1 (USF1) (Jover, Moya, and Gomez-Lechon 2009; Rodriguez-Antona et al. 2003; Martinez-Jimenez et al. 2005; Matsumura et al. 2004; Biggs et al. 2007). This high basal capacity can additionally be induced to accommodate transient changes by the action of xenobiotic-activated or xenobiotic-modulated receptors (a. k. a. xenobiotic receptors) (Ma 2008). Xenobiotic activated receptors regulate the expression of phase I and phase II metabolizing enzymes, as well as uptake and efflux pumps, in response to presence of toxic xenobiotics and endobiotics and their by-products (Tolson and Wang 2010). Examples of xenobiotic receptors are PXR, CAR, PPARA-G, hepatocyte nuclear factor 4 alfa (HNF4A/NR2A1), vitamin D receptor (VDR/NR1I1), bile acid receptor (FXR/NR1H4) and oxysterols receptor LXR alfa (LXRA/NR1H3) (Duniec-Dmuchowski et al. 2007; Gnerre et al. 2004; Matsubara et al. 2008; Rakhshandehroo et al. 2009; Tolson and Wang 2010; Blumberg et al. 1998). Transcription factors other than the nuclear receptors also function as xenobiotic receptors, such as the cytoplasmic transcription factor aryl hydrocarbon receptor (AhR) and the nuclear factor erythroid 2-related factor 2 (NRF2) (Malhotra et al. 2010; Beischlag et al. 2008; Sartor et al. 2009). AhR is central in the regulation of the expression of CYP1-like enzymes, such as CYP1A1, but also contribute to the regulation of phase II enzymes and transporters (Beischlag et al. 2008; Sartor et al. 2009).

Two major xenobiotic receptors are the PXR and CAR. Both are promiscuous with respect to ligand-specificity, although the more spacious LBP of PXR allows it to bind a more diverse array of ligands (Moore et al. 2006). The target genes of PXR and CAR are partially overlapping (Tolson and Wang 2010). The PXR gene is found in some but not all fish species (Eide et. al, Unpublished), while *CAR* is restricted to mammals (Reschly and Krasowski 2006).

1.5 Factors influencing toxicological effects

All living organisms are continuously in contact with a variety of different chemicals. Some of these chemicals have chemical properties that can cause undesired or harmful effects also called adverse effects, to these organisms. More than chemical properties alone determine if adverse effects occur or not.

1.5.1 Toxicokinetics and toxicodynamics

The dose of a chemical that an organism is exposed to affects the toxicity. Aside from the dose, the potential of a compound to exert adverse effects in an organism depends on its toxicokinetics (TK) and toxicodynamics (TD) (***Figure 7***). TK involves the movement of a compound within organisms, including absorption, metabolism, distribution and elimination (ADME), and thus, determine the concentration of a chemical in the target organ. TD, on the other hand, involves the interaction between compound, or its metabolites, and target molecules in the organism, as wells as the biochemical and physiological consequences of these interactions. TK/TD relationships depend on the compounds inherent chemical properties but also of properties of the organism that the chemical enters. Such biological factors, including the type of species, gender, strain, biochemical status and others, therefore can affect the outcome of the exposure of an organism by a chemical.

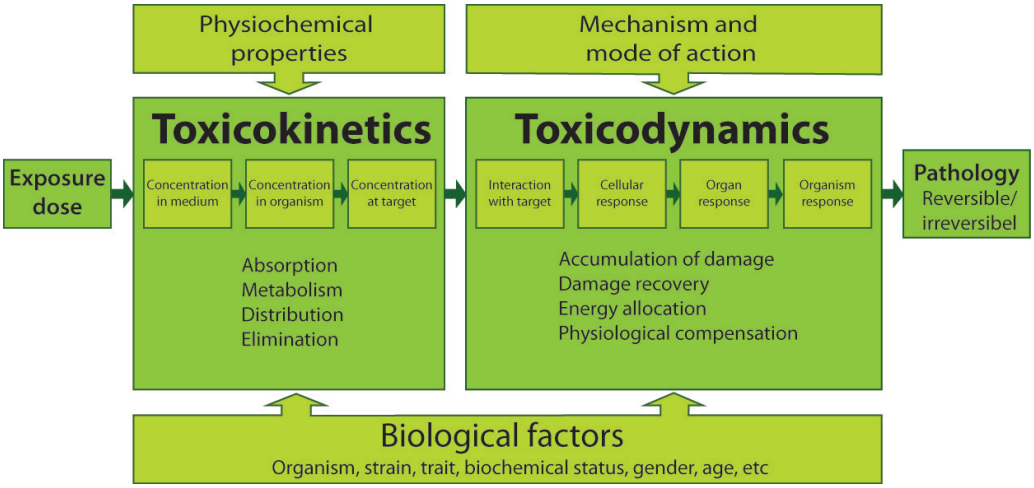

Figure 7 – Schematic overview of adverse effect pathways. Figure was adapted from Ecotoxmodels.org (Ecotoxmodels.org).

Both endogenous and exogenous compounds have the potential of producing toxicological effects. For an xenobiotic compound to enter an organism it must cross external and/or internal barriers. In vertebrates this involves absorption through the skin (or eye), the respiration system (lungs) or the digestion system. Lipophilic compounds are most effectively absorbed due to the nature of biological membranes. When absorbed, the circulation system can distribute the chemicals to their organs of action, or to bio-unavailable storage in plasma (for instance bound to albumin) or in bone, kidney, liver or fat tissues. Circulating lipophilic compounds are reabsorbed in the kidneys or intestines, and could accumulate in the organisms. For this reason only about 25% of prescription drugs are excreted directly via bile and kidneys (Williams et al. 2004). For the remaining, an increase of water-solubility is needed for them to be excreted from the body.

1.5.2 Bioactivation, direct and indirect-acting toxicity

The majority of organic toxicants are indirect-acting and they undergo some kind biotransformation prior to their actions in the target tissue (***Figure 8***) (Park, Lee, and Cho 2014). This biotransformation is most often caused by CYPs, and to a lesser extent of flavin-containing monooxygenases (FMOs) and cyclooxygenases (COX) (Park, Lee, and Cho 2014). Since activation of

PXR increases the transcription of members of the CYP and FMO families, and down-regulates members of the COX family (Rae et al. 2001; Zhou, Tabb, et al. 2006; Tolson and Wang 2010), it is obvious that activation of PXR, in addition to detoxification, can contribute to bioactivation of indirect-acting toxicants.

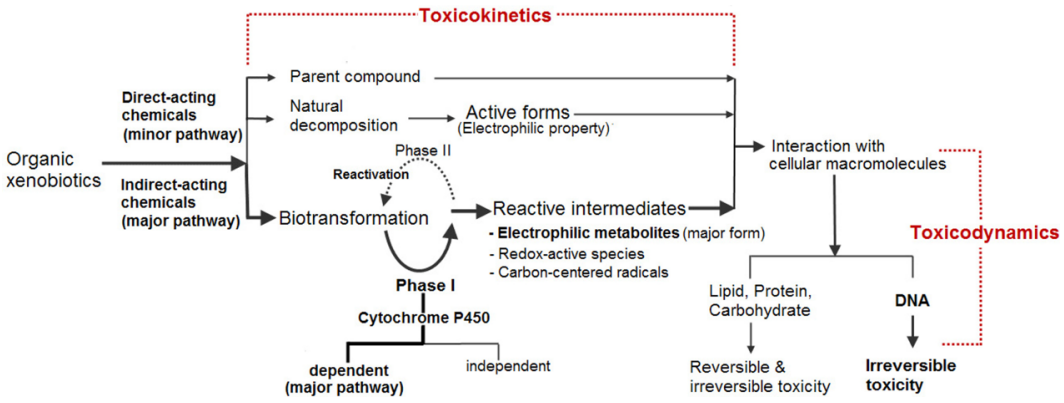

Figure 8 – Toxicokinetics and toxicodynamics in a flowchart of toxicity. Figure from Park (Park, Lee, and Cho 2014).

The polycyclic aromatic hydrocarbon benzo[a]pyrene (B[a]P) is an example of an indirect-acting toxicant. B[a]P toxicity is caused by epoxidized metabolites that are highly carcinogenic (Rose 2004). Toxicants that do not require metabolism to exert their action are direct acting and often act on receptor molecules. Di-(2-ethyl-hexyl)phthalate (DEHP) is a direct-acting toxicant that induce tumorgenesis by targeting PPARA (Takashima et al. 2008; Ito and Nakajima 2008). From this it is clear that whether or not a potential harmful compound can be metabolized is an important determinant of toxicity.

1.6 Zebrafish (*Danio rerio*) as a model system

Zebrafish are teleosts in the order of Cypriniformes that native to the Ganges River in Bangladesh and India, but are also found from Myanmar in the East and to Nepal in the North (Engeszer et al. 2007; Hsu et al. 2007). Zebrafish was established as a laboratory animal model in developmental biology in the 1970 and 1980s and is now a widely used model organism in

various fields of biological research, including genetics, developmental biology, medicine, pharmacology, toxicology, and effects of pollutants (reviewed in (Kamstra, Alestrom, et al. 2014; Carvan et al. 2007; Lieschke and Currie 2007; Quaife, Watson, and Chico 2012; Dai et al. 2014; Walogorsky et al. 2012)). Zebrafish has proven a promising model for predicting toxicity in human cardiac toxicity (Milan et al. 2003) and have been used to study hepatotoxicity (McGrath and Li 2008), endocrine disruption (Bugel, White, and Cooper 2013), reproductive toxicity (King Heiden et al. 2009), neurotoxicity (Wager and Russell 2013), immunotoxicity (Henry et al. 2013) and nanotoxicity (Harper et al. 2011).

The genome of zebrafish was recently sequenced and at least 70% of all human genes have orthologs in zebrafish (Howe et al. 2013). That adverse effects mechanisms have been described in zebrafish on a molecular level and that its chemical defensome has been described, is important for the use of zebrafish in toxicology (Stegeman, Goldstone, and Hahn 2010). While there are broad similarities in the CYPomes of zebrafish and humans, notable dissimilarities exist. Zebrafish have a higher number of CYPs (96 vs. 57) and a greater degree of divergence of biotransforming CYPs (CYP1-4) compared to humans (Goldstone et al. 2010). Despite these differences, activation of Pxr in zebrafish results in a coordinated induction of defensome genes, including *cyp3a* and *mdr1* and *pxr*, in zebrafish as in humans (Bresolin, de Freitas Rebelo, and Celso Dias Bainy 2005), indicating similarities in xenobiotic response pathways in zebrafish and mammals (Stegeman, Goldstone, and Hahn 2010).

The genetic background of zebrafish is more diverse than in mice, possibly because of differences in the methods used to establish lines from the two organisms (Guryev et al. 2006). Wild zebrafish generally have more genetic variation than commonly used laboratory strains of zebrafish (Coe et al. 2009). However, the extent of the genetic diversity differ between different strains of laboratory zebrafish (Coe et al. 2009). Recently, two allelic variants

of the zebrafish Pxr gene, denoted *pxr*1* and *pxr*2*, were described in the Tupfel long fin (TL) strain (Bainy et al. 2013).

The genetic variation in zebrafish may constitute a source of uncertainty with regards to the use of zebrafish as a model species in toxicology, pharmacology and also for regulatory purposes. Fish is currently the most frequently used vertebrate in regulatory toxicology (Knobel et al. 2012) and several OECD guidelines for testing of chemical effects on biotic systems has been developed with zebrafish as a model organism (such as OECD Test Guideline No. 203, 210, 212, 229, 230, 234 and 236) (OECD). However, inadequate description of strains used in toxicology has recently presented as a major obstacle to broad use of fish models in hazard identification and mechanistic evaluations (Planchart et al. 2016). Further knowledge about intra-species variation in biotransforming enzymes, and their regulators, in zebrafish is therefore needed.

1.7 Polar bears (*Ursus maritimus*)

Polar bears are marine mammals and predators on the fifth trophic level in the Arctic marine food webs (Hobson and Welch 1992). During the spring polar bears feed superfluously and accumulate energy reserves that allows them to survive periods with limited access to food. Polar bears in areas with receding sea ice may be fasting up to 6 months (Derocher, Wiig, and Andersen 2002; Mauritzen, Derocher, and Wiig 2001), while gestating and lactating female polar bears may even be fasting for up to 11 months (Thiemann, Iverson, and Stirling 2006). Growth measures, such as body condition and mass, have been linked to survival of cubs (Derocher and Stirling 1996; Rode, Amstrup, and Regehr 2010) and could ultimately affect reproductive success and population recruitment (Durner et al. 2009).

In most polar bear populations ringed seal constitute more than 70% of the diet (Thiemann, Iverson, and Stirling 2008). The high lipid level in the diet

results in that polar bears accumulate environmental pollutants. Polar bears carry some of the highest levels of persistent organic pollutants (POPs) seen in any mammal, much higher than in humans (Verreault et al. 2006; Sonne et al. 2012; Norén, Weistrand, and Karpe 1999; Kim, Marchand, Henegar, Antignac, Alili, Poitou, Bouillot, Basdevant, Le Bizec, Barouki, and Clément 2011). The most prevalent POPs in the blood of polar bears are polychlorinated biphenyls (PCBs) (Kim, Marchand, Henegar, Antignac, Alili, Poitou, Bouillot, Basdevant, Le Bizec, Barouki, and Clément 2011; Salihovic et al. 2012; Bytingsvik et al. 2012; Skaare et al. 2000). Brominated flame-retardants (BFRs) and pesticides, except chlordanes, are less prevalent (Salihovic et al. 2012; Verreault et al. 2008; Bentzen et al. 2008; Goncharov et al. 2011; Lind et al. 2012). Correlations between the concentration of POPs in polar bear plasma and adverse effects, including immune functions (Lie et al. 2005; Lie et al. 2004; Bernhoft et al. 2000), endocrine disruption (Braathen et al. 2004; Haave et al. 2003; Verreault et al. 2009), and tissue pathology (Sonne 2010), have been reported. The concentrations of chlorobenzenes, chlordanes and PCBs in polar bear fat generally increase during fasting (Polischu, Norstrom, and Ramsay 2002).

Polar bears appear to have a certain capacity of metabolising organohalogenated compounds (OHCs), as indicated by low bioaccumulation factors from seal to bears, differences in chlorination pattern of PCB in bears and prey, relatively high liver CYP activities and the depletion of HBCDD from polar bear hepatic microsomes (Muir, Norstrom, and Simon 1988; Letcher et al. 2009; Kannan, Yun, and Evans 2005). In contrast, polar bears appear to be limited in their capacity to metabolize polybrominated diphenylethers (PBDEs) (McKinney et al. 2011; Letcher et al. 2009). However, as the total burden of chlorobenzenes, hexachlorocyclohexanes, chlordanes and PCBs generally remains the same during fasting, xenobiotic metabolism appears to be low in polar bears during fasting (Polischu, Norstrom, and Ramsay 2002). Although polar bears are able to metabolize some of the environmental pollutants they are exposed to, little is known about

the xenobiotic activated receptors, such as PXR and PPARs, and their function in polar bears.

2. Aims of study

Nuclear receptors are central in the interaction of cell, tissue and organs with their environments and in the regulation of cellular processes. Consequently, nuclear receptors are common constituents of toxicity pathways. The study of the contribution of nuclear receptors in toxicological pathways in different species can increase the understanding of processes leading to adverse toxicological effects as well as contribute to risk assessment. This study focuses on the role of PXR and PPARG in toxicity pathways in zebrafish and polar bears, and the following working hypothesis were formulated:

Hypothesis I: *Environmental pollutants have the potential of modulating the transcriptional activity of the polar bear PXR* (**Paper I**). To test this hypothesis we aimed to clone the polar bear *PXR,* to establish a GAL4-UAS luciferase reporter gene assay for polar bear PXR and to test the ability of polar bear PXR to be activated by a selection of environmental pollutants in direct comparison to human PXR.

Hypothesis II: *Environmental pollutants have the potential of modulating the transcriptional activity of PPARG and lipid metabolism in polar bears* (**Paper II**). To test this hypothesis we aimed to clone polar bear *PPARG*, to establish a GAL4-UAS luciferase reporter gene assay for polar bear PPARG and to test the ability of POPs present in polar bear tissues to modulate the transcription activity of polar bear PPARG. Furthermore, we aimed to test the ability of POP extracts from polar bear liver and adipose tissue to induce adipogenesis in 3T3-L1 cells and polar bear adipocyte-derived stem cells.

Hypothesis III: *Genetic variation in the Pxr gene in zebrafish affects the function of the zebrafish Pxr* (**Paper III**). To test this hypothesis we aimed to clone *pxr* from zebrafish of the SWT and AB/Tü strains, to establish GAL4-UAS luciferase reporter gene assays for zebrafish PXR from the TL, AB/Tü,

SWT and an unknown strain and to compare their transcriptional activity when exposed to known agonists of zebrafish Pxr. Additionally, we aimed to express the zebrafish Pxr variants recombinantly and study the receptor-ligand interactions by surface plasmon resonance.

3. Summary of results

3.1 Activation of polar bear PXR by environmental pollutants – a comparative study (Paper I)

To assess how ligand activation of human PXR correlates to activation of the polar bear ortholog, we performed a comparative study of the ligand activation of the orthologs from these two species.

In this paper, we demonstrated that the sequence divergence of polar bear PXR (pbPXR), with regards to human PXR (hPXR), is comparable to that of another caniform such as the dog PXR. As expected, the DBD of hPXR and pbPXR have been well conserved (97% amino acid identity), while the fraction of positionally conserved amino acids in the LDBs of pbPXR and hPXR was 89%. When challenged with single-compound exposures from a 51-compound environmental pollutant test panel, we found that a hPXR-LBD-driven luciferase reporter system was activated by 86% of the test compounds while only 68% activated pbPXR-LBD. The majority (76%) of the compounds that were agonist both for PXR orthologs induced a stronger response via hPXR than via pbPXR. Seven compounds induced responses of similar magnitude via both PXR orthologs and five compounds induced stronger responses via pbPXR than via hPXR. Comparison of a homology model of the pbPXR-LBD and a hPXR-LBD structure indicated that the amino acids substitutions do not cause radical changes in the structure of the pbPXR-LBD ortholog, nor do they occur in regions that are important to nuclear receptor function, such as the interaction surfaces for coactivators and RXR. A comparison of steric and electronic features of the predicted optimal supramolecular interactions for the five most potent agonists for the human and for polar bear PXRs revealed that the polar bear PXR pharmacophore had the shape of a tripod, while the human PXR pharmacophore had pyramidal shape. This indicated that there might be some subtle differences to the ligand-binding site in the two PXR orthologs.

3.2 Modulation of the transcriptional activity of polar bear PPARG and adipocyte metabolism by envrionmental pollutants (Paper II)

PPAR is required for differentiation of preadipocytes and development of adipose tissue (Barak et al. 1999; Rosen et al. 1999). In this study we investigated how environmental pollutants present in polar bears modulate the activity of PPARG and differentiation of preadipocytes as single compounds, extracts and in mixtures.

We found that the hinge and ligand-binding domains of polar bear PPARG are identical to the PPARG ortholog from humans and dogs. Using a GAL4-DBD-UAS-based luciferase reporter gene assay, we demonstrated that certain PBDEs, PBDE-28, -47, -99, -100 and -153, were weak agonists of PPARG that increased the transcriptional activity of PPARG by 20-47%. Tetrabromobisphenol A (TBBPA) induced the transcriptional activity of PPARG by approximately 150%, and could be classified as a moderately strong PPARG agonist, Of the four polychlorinated biphenyls (PCBs) tested for agonistic effects on PPARG only PCB170 showed an effect (16% increased transcriptional activity). Bisphenol A (BPA), HBCDD, PCB153, DDE, oxychlordane and endosulfan all reduced the transcriptional activity of PPARG induced by a strong PPARG agonist, rosiglitazone (0.5 μM), demonstrating that these compounds are antagonists of PPARG. Two different POP-mixtures, with a composition corresponding to neutral and $MeSO_2$ POPs extracted from polar bear fat, also had an antagonistic effect on PPARG.

In absence of adipogenic cocktail, POP extracts (neutral and $MeSO_2$ POP fractions) from both liver and adipose tissues of polar bears induced differentiation of murine preadipocytes (3T3-L1), indicating that the extracts can induce the first wave of transcription factors during adipocyte differentiation. The strongest induction was observed for extracts from polar bear liver and this extract increased 3T3-L1 triglyceride (TG) levels approximately 5-fold, compared to cells exposed to DMSO, and to about half

the TG-level of cells that were exposed to an adipogenic cocktail (MDI) and rosiglitazone. Synthetic mixtures, mimicking extracts from polar bear fat tissues, did not induce formation and storage of TGs in 3T3-L1 cells and thus, did not induce first wave of adipogenesis. In presence of adipogenic cocktail (MDI-mix), synthetic mixtures representing the neutral and $MESO_2$ POP fractions of polar bear fat did not increase TG level. On the contrary, TG levels were reduced in 3T3-L1 cells co-exposed to cocktail and the synthetic mixture of neutral fat tissue POPs, indicating that these mixtures inhibit second wave differentiation.

Neutral POP extracts from polar bear fat increased the lipid content in polar bear adipogenic stem cells from polar bear slightly (~10%), compared to in stem cells exposed to solvent. Neutral POP extracts also reduced the transcript levels of *PPARG* and fatty acid binding protein 4 (*FABP4*). However, as an even more pronounced reduction of *PPARG* transcripts were observed in cells receiving adipogenic mixture and rosiglitazone, these results should be interpreted cautiously.

3.3 Effects of genetic variation on the function of zebrafish Pxr (Paper III)

Lastly, we aimed to investigate if sequence variation in the Pxr gene result in nuclear receptor phenotypes with different properties with regards to ligand-induced transcriptional activity. *Pxr*s were cloned from different strains of zebrafish (zfPxr), a hybrid of the Tübingen and AB strains (AB/Tü), the Singapore wild type (SWT), the Tupfel long fin (TL), as well as a strain of unknown origin (UNK). A comparison of zebrafish Pxr amino acid sequences revealed that DNA-binding domains (region C) were completely conserved, while the hinge and ligand-binding domains were only 94-98% positionally conserved. The ligand-induced transcriptional activity of the zfPxr variants was studied using a luciferase reporter gene assay, and revealed compound-dependent differences in range of response (E_{max}) and in susceptibility for

activation (EC_{50}). As agonists for zfPxr variants, the anti fungal drug clotrimazole (CLO) had higher efficacy (3-9-fold) and potency (~100-fold) than the local anaesthetic 4-butyl-amino benzoate (4BAB). The maximum transcriptional activity induced by CLO via Pxr from the AB/Tü hybrid strain, PxrAB/Tü, was greater than for the maximum response induced via zfPxrs from zebrafish of the Tupfel long fin (PxrTL) and the unknown strain (PxrUNK). CLO had the lower agonistic potency with PxrUNK than with the other zfPxr variants. Surface plasmon resonance was used to quantify the strength of the ligand-receptor interactions, and demonstrated that CLO interacts with much greater affinity to PxrAB/Tü than to the other zfPxr variants.

4. General discussion

4.1 Environmental contaminants activate human and polar bear PXR differently

4.1.1 The polar bear PXR is a promiscuos xenosensor

More than 80% of the available literature on PXRs (PubMed September 2016) focuses on human PXR. Based on its tissue expression, its promiscuity with regards to ligand specificity and its repertoire of target genes the human PXR (hPXR) has been classified a xenobiotic sensor (Bertilsson et al. 1998; Kliewer, Moore, Wade, Staudinger, Watson, Jones, McKee, Oliver, Willson, Zetterström, et al. 1998; Blumberg et al. 1998; Rosenfeld et al. 2003). The susceptibility of hPXR and pbPXR to be activated by environmental pollutants was measured in a luciferase reporter assay. Polar bear PXR proved to be promiscuous with regards to its ligand specificity and together with the expression of polar bear *PXR* in the liver, suggest that PXR functions as a xenosensors also in polar bears.

4.1.2 Differences in the activation of human and polar bear PXR

Fifty-one compounds, including pesticides, PCBs, BFRs, siloxanes and industrial compounds, were tested for their ability to activate the human and polar bear PXR orthologs. Of the tested chemicals, 68% increased the transcriptional activity of pbPXR, while 86% increased the transcriptional activity of hPXR (Paper I), indicating that pbPXR is somewhat less promiscuous than hPXR. Similarly, the dog and macaque PXRs have been shown to be less promiscuous than the hPXR (Milnes et al. 2008). The portion of hPXR agonist in our test panel was comparative to that of a previous study using a test panel of organochlorine pesticides, phthalates and industrial compounds (85%, (Milnes et al. 2008)), and somewhat higher than in studies focusing on pesticides (55%, (Kojima et al. 2011)), and on pharmaceuticals

(54%, (Sinz et al. 2006)). Taken together, it appears that the human PXR is exceptionally promiscuous, and slightly more so than pbPXR.

Out of the agonists in our test panel, ~73% produced greater responses in the luciferase assay for hPXR than in the assay for pbPXR, 16% of the agonist produced similar responses in both assays, and 11% produced greater responses in the assay for pbPXR (Paper I, ***Figure 9***), indicating that binding of an agonist in general induces a stronger response in hPXR than in pbPXR. Similarly, only 22% of pesticide PXR agonist induced a stronger transcriptional activity in mPXR than in hPXR (Kojima et al. 2011).

Figure 9 – Agonists of human and polar bear PXR arranged in a Venn diagram. Test panel compounds that produced a stronger response in the luciferase reporter assay for hPXR are to the left in the Venn diagram, compounds that induced similar responses in both assays are in the middle and compounds that induced a stronger response in the luciferase reporter assay for pbPXR are to the right. The compounds with names in italic activated hPXR but not pbPXR. Figure adapted from Paper I/(Lille-Langoy et al. 2015).

Only five environmental pollutants, including HBCDD, toxaphene, 4-nonylphenol and TBBPA, and the cholesterol lowering drug pharmaceutical SR12813, produced stronger transcriptional activity in pbPXR than in hPXR.

The species-specific ligand activation profiles of PXR orthologs have been suggested to arise from large sequence variation in the LBD of PXR

orthologs (Krasowski et al. 2011; Ekins et al. 2008; Kliewer, Goodwin, and Willson 2002). Interestingly, differences in the LBP amino acids cannot explain the different activation of hPXR and pbPXR by our test panel since the amino acids lining the LBP of hPXR and pbPXR are identical (Paper I). Consequently, the observed differences in activation profiles for pbPXR and hPXR must be caused by substitutions of amino acids elsewhere in the PXR structures. However, we were unable to identify substitutions that could explain the differences in activation of hPXR and pbPXR by our test panel.

4.1.3 Toxicological implications of PXR activation

From a toxicological point of view, maybe the most important consequence of activation of PXR may be the adaptive induction of systems for the detoxification and elimination of endogenous and exogenous compounds. The importance of PXR in detoxification of endogenous compounds can be illustrated by the example of the toxic bile acid lithocholic acid (LCA). Activation of PXR reduces the production of LCA from cholesterol, as well as increasing the metabolism and excretion of LCA (Russell and Setchell 1992; Staudinger et al. 2001; Xie et al. 2001). Mice that are lacking both PXR and CAR ($PXR^{-/-}CAR^{-/-}$ KO mice) show increased sensitivity to LCA toxicity (Uppal et al. 2005). The metabolism of other endogenous compounds, such as steroid hormones is also affected by the activation status of PXR. For instance, exposure of rats and mice to DDE, a PXR agonist, increases the transcription of the genes of CYP3A and CYP2B, enzymes that hydroxylates testosterone (You et al. 1999). Moreover, genes of enzymes involved in the synthesis of corticoids, such as CYP11A1, 3β-hydroxysteroid dehydrogenase, CYP11B1 and CYP11B2, are up-regulated in transgenic ALB-VP-PXR mice that express continuously activated PXR. Consequently, blood and urine levels of aldosterone is elevate in ALB-VP-PXR mice (Zhai et al. 2007). Thus, it is clear that xenobiotic activation PXR can alter both the metabolism and production of endogenous compounds and is involved in homeostatic balancing of bile acids, hormones, vitamin D,

calcium, glucose, cholesterol and vitamin E (Zhai et al. 2007; Niwa et al. 1998; Pascussi et al. 2005; Li, Chen, and Chiang 2007; Landes et al. 2003).

PXR also has an important role in the metabolism of exogenous compounds. A major initial step of detoxification of PCBs is the insertion of –OH group by phase I enzymes, either by monooxygenases such as CYP1A, CYP2B CYP2C and CYP3A or epoxide hydrolase, all regulated by PXR (Xu, Wang, and Staudinger 2009; Tolson and Wang 2010). Hydroxylation appears to be especially important for detoxification of lower chlorinated PCBs that are much more efficiently excreted than their parent compounds, while some higher chlorinated PCBs retain the hydrophobicity and may bioaccumulate (Letcher 2000). Some OH-metabolites are more toxic than their parent compound and are examples of bioactivation. However, there may be species differences in the ability of PXRs to be activated by PCBs. For instance, while PCB184 induced transcription of CYPA1 in rat cells, it did not induce transcription of CYP3A4 in human cells (Tabb et al. 2004). PCB153 is a weak agonist for pbPXR (3.0-fold), indicating that it may have some potential of autoinducing its own metabolism. However, as the biological half-life of PCB-153 is very high, 338 days in humans (Bühler, Schmid, and Schlatter 1988), a timely question is if PCB153 can activate pbPXR also *in vivo*?

To sum up, activation of PXR may modulate the metabolism of the inducer or other compounds, as well as altering the synthesis of endogenous compounds. Our results show that 86% of our test compounds increased the transcriptional activity of hPXR *in vitro*, while 68% increased the activity of pbPXR. This implies that they also could activate PXRs *in vivo*. However, there are some precautions to be taken when extrapolating the *in vitro* PXR transactivation to *in vivo* effects. First, oftentimes the concentration of the test compounds necessary to activate human or polar bear PXR *in vitro* are high and typically in the micromolar range. In a study of the transactivation of hPXR by 170 pharmaceuticals 54% of the compounds demonstrated transactivation at the highest test concentration (Sinz et al. 2006). However,

when *Sinz et al.* employed the hPXR response at the concentration corresponding to the efficacious steady state concentration after a standard dose (C_{max}) of the compounds instead, the percentage of pharmaceuticals that showed transactivation was reduced to 8%. This demonstrates that it is important to take into consideration the concentration of POPs in relevant tissues when trying to predict gene transcription *in vivo*.

It can be challenging to decide what level of *in vitro* transactivation that is to be considered significant. In one study this limit was set to 2.5-fold increase in reporter gene transcription, which corresponds to 43% of the response of 10 μM rifampicin in this study (Moore et al. 2002). The number of false negatives will be large if this limit is set too high. An example of an agonist that would be missed when using this level of significance is the drug tadalifil. Tadalifil exhibits low transactivation at C_{max} (10% of rifamipin) but induces a ~2-fold increase in CYP3A4 levels and activity in primary hepatocytes (Ring et al. 2005). It has been recommended to use 15% of the maximal activation of PXR by rifampicin as cut-off to predict CYP3A4 activity (Sinz et al. 2006). However, if applied it would give a false negative result for 25% of clinical CYP3A4 inducers (Fahmi et al. 2012). These examples demonstrate that even weak *in vitro* PXR transactivation can increase expression and induce the activity of CYP3A4 in hepatocytes. Some compounds can also be inducers of PXR and inhibitors of CYP3A4 at the same time. This may be the case for nifedipine, nitrendipine, roxithromycin, leflunomide, omeprazole and rosiglitazone that all elicit PXR activation (up to 9-fold), but not increased CYP3A4 activity (Fahmi et al. 2012). Thus, to use PXR transactivation to predict CYP3A4 activity for these compounds would give false positive predictions. Clearly, one should be careful in using only NR transactivation to predict effects in cells and *in vivo*.

Micromolar concentrations of POPs are generally not seen in humans (Donaldson et al. 2015). However, up to ~20 μM PCB153 (24316 μg/kg lipid weight), ~24 μM chlordane (15103 μg/kg lipid weight) and ~6 μM DDE (2911

μg/kg lipid weight) have been found in adipose tissue of polar bears (Norstrom et al. 1998). The concentrations in serum is generally lower, up to ~1.5 μM PCB153 (5710 μg/kg lipid weight) has been measured in polar bear plasma (Bytingsvik et al. 2012). The concentration of PCBs in the liver appear to be approximately 16% of that in adipose tissue (Norheim, Skaare, and Wiig 1992). The EC50s for the *in vitro* activation of pbPXR by PCB153 and chlordane were, 25 and 15 μM, respectively (Paper I). Thus, both PCB153 and chlordane has been found in adipose tissue of polar bears at concentrations that activates pbPXR *in vitro*. PXR, however, is not expressed equally in all tissues. The highest levels of PXR expression are seen in the intestines and in the liver, while PXR is not expressed in WAT and BAT (Nishimura, Naito, and Yokoi 2004; Bookout et al. 2006; Yang et al. 2006). Adipose tissue appears to be relative insensitive to activation of PXR. For instance, treatment with rifampicin did not increase the transcript levels of *CYP3A4* in cultured human WAT (Ellero et al. 2010). From toxicological perspective, this could imply that adipose tissue could be a relatively safe storage space for POPs that can modulate the activity of PXR. Additionally, it could imply that that adipose tissue POPs may not be very accessible for metabolism and this may be a possible explanation for why POPs that appear to be able of activating PXR, such as PCB153, have very high half-life in organism.

Fasting/weight loss increases the concentration of POPs in adipose tissue and in plasma of human, polar bears and elephant seals (Debier et al. 2006; Kim, Marchand, Henegar, Antignac, Alili, Poitou, Bouillot, Basdevant, Le Bizec, Barouki, and Clement 2011; Polischu, Norstrom, and Ramsay 2002), indicating that an equilibrium of POPs adipose tissue POPs and plasma is set, likely based on the hydrophilic/hydrophobic properties of the compound. Thus, POPs may be more accessible for metabolism in metabolically active tissues, such as the liver, after fasting.

When considering if a compound may have a biological effect it is important to recognize that chemicals to not occur as single compounds in

nature but in complex mixtures, as evident from the chemical analysis of POP extracts from polar bear liver and adipose tissue (Paper II). Interestingly, it was recently demonstrated that hPXR can bind the synthetic oestrogen 17α-ethynylestradiol (EE2) and the pesticide trans-nonachlor (TNC) simultaneously and that binding of this «supramolecular ligand" augments the effects of the single compounds (Delfosse et al. 2015). Human PXR is much more susceptible to activation by the combination of EE2 and TNC, than by the single compounds, and the EC_{50} for the combined response approximately 1000-times lower than for the single compounds (μM to nM). This synergetic effect was also seen in primary human hepatocytes where combined exposure augmented the expression of *CYP3A4* 15-20 times. These results demonstrates that exposures to combinations of chemicals can give effects at concentrations that are 1000-times lower than the corresponding single compounds does. If combined effects also can occur in polar bears, the concentrations of POPs measured in polar bear tissues are within relevant concentration ranges.

Based on the observed qualitative and quantitative differences in the activation of hPXR and pbPXR, it can be predicted that slightly fewer xenobiotics would be expected to increase the transcriptional activity of pbPXR than of hPXR. Additionally, it can be predicted that xenobiotics would be expected to induce a stronger transcriptional activity in hPXR than in pbPXR, with a few exceptions. The exceptions to this generalization, most notably prominent pollutants such as HBCDD, toxaphene, and TBBPA, may however be of specific concern in risk assessment in arctic ecosystems with polar bears. However, because of large differences in POP burdens of humans and polar bears, the concentration needed to activate PXR *in vitro* is more likely to be reached in polar bears.

4.2 Environmental pollutants modulate the transcriptional activity of polar bear PPARG and adipogenesis *in vitro*

4.2.1 Polar bear *PPARG* has been conserved

The recent sequencing of the polar and brown bear genomes revealed that polar bears since their divergence from brown bears have undergone unusually rapid evolution (Liu et al. 2014). Lui *et al.* showed that genes involved in lipid metabolism have been subjected to the strongest positive evolutionary pressure, and suggest alterations in lipid metabolism genes have enabled polar bears to live in the Arctic. The ligand binding domain of polar bear PPARG is identical to that of human, dog and panda PPARG. Moreover, polar bears have the same PPAR isoforms as humans and mice (Mukherjee et al. 1997; Tontonoz et al. 1994). These findings suggest that a change of PPARG function has not been necessary for this adaption to living in the Arctic. Rather, they indicate that PPARG in humans, polar bears and panda are activated by the same endogenous ligands and that processes regulated by PPARG are important. That the function of PPARG is important is evident from the fact that *PPARG*$^{-/-}$ mice show impaired embryonic development and die 10-12 days *post coitum* (Kubota et al. 1999). However, this critical function may not be related to regulation of lipid metabolism and adipose tissue development, as *PPARG*, according to Kubota *et al.*, is not expressed at the time when the *PPARG*$^{-/-}$ embryos die (Kubota et al. 1999), nor is any adipose tissues developed before later in the pregnancy (third trimester) (Symonds et al. 2003). In contrast, *PPARG* was expressed in the mutant placentas that had poorly developed blood vessels (Kubota et al. 1999). The exact function of PPARG in placental development is still not known, but the critical contribution of PPARG during embryonic development may not be related to angiogenesis based on that activation of PPARG has been shown to inhibit angiogenesis in the cornea of rats and in an *in vitro* angiogenesis assay (Xin et al. 1999).

That the functions of PPARG may have been conserved between mammalian species does not necessarily mean that lipid metabolism is the same in polar bear as in other mammals. Which of polar bear genes that were subjected to positive selection were not specified by Lui and coworkers (Liu et al. 2014), and could include genes involved both in lipid catabolism and anabolism. However, looking into these aspects have not been within the scope of this thesis.

4.2.2 POP extracts from polar bear affect adipogenesis

Extracts of POPs from liver and adipose tissue induced adipogenesis in 3T3-L1 cells at concentrations similar to concentrations in polar bear tissues (Paper II). This demonstrates that mixtures of POPs that are similar to actual POP loads of free-ranging polar bears can induce adipogenesis. Both the total POP extracts from liver and adipose tissue induced more accumulation of lipids in the 3T3-L1 adipogenesis assay than the combined effects by the neutral, $MeSO_2$ and OH-metabolite extracts separately (3.9 vs 1.8 fold), which indicates that compounds from different POP extraction fractions may stimulate each other. A synergistic inhibitory effect on adipogenesis was demonstrated for resveratrol and quercetin (Yang et al. 2008). When testing selected POPs for their ability to modulate pbPPARG *in vitro*, none of the neutral fraction POPs showed agonistic activity. Thus, based on these experiments we could not explain the adipogenic components of the extracts.

Targeted chemical analysis of the extracts showed that the neutral POP fraction contained pesticides, PCBs and PBDEs, while the $MeSO_2$-fraction contained $MeSO_2$-metabolites of PCBs and DDE, and the OH-fraction contained OH-metabolites of PCBs and PDBEs as well as TBBPA and pentachlorophenol. Due to limited amount of polar bear tissues, we were only able to obtain a limited amount of POP extracts. In an attempt to compensate for this, synthetic mixtures were composed based on a targeted chemical analysis of the extract from polar bear adipose tissue. However, the synthetic

mixtures did not induce adipogenesis neither in murine preadipocytes nor in polar bear adipose tissue derived stem cells (Paper II). This indicated that the adipogenic effects of the extracts were not PPARG mediated, or that the adipogenic components of the extract were missing in the synthetic mixtures. That none of the single neutral POPs tested had any agonistic effects on pbPPARG *in vitro* was in support of the latter explanation. Another clue that the extracts contained adipogenic compounds not detected in the targeted analysis came from comparing the POP content and effect of the extracts from the two tissues. Because, while the extracts from the adipose and liver tissue had similar composition, the concentration of identified POPs in the adipose tissue extract was higher than in the liver tissue extract, 7.2 vs 4.1 μM. Yet, the adipogenic potential was higher for the liver tissue extract and that indicated that the adipogenic components of the extracts had not been identified in the targeted chemical analysis. Non-target analysis revealed that in particular the liver extracts were more complex than the initial targeted analysis revealed (Paper II).

A non-targeted chemical analysis of the extracts detected chemicals that had not been detected in the targeted analysis. Several phthalates in the adipose tissue. Phthalates, such as di-2-ethylhexyl-, mono-2-ethylhexyl and benzyl butyl phthalate, are potent PPARG agonists and inducers of adipogenesis (Hao et al. 2012; Schmidt et al. 2012; Yin et al. 2016). And when testing, we found that 10 and 50 μM mono-2-ethylhexyl phthalate (MEHP) induced the lipid accumulation of 3T3-L1 cells and increased levels of the *PPARG* transcript, but did not induce transcription of *FABP4*, a PPARG target gene (Paper II). The lack of induction of *FABP4* transcription may indicate that MEHP induced adipogenesis via a PPARG-independent mechanism. However, it appears that high concentrations id necessary to increase PPARG transactivation *in vitro*. MEHP has been shown to induce the transcription of another PPARG regulated gene, *aP2,* in 3T3-L1 cells, but in this study 100 μM MEHP was needed to induce transcription of *aP2* (Hao et al. 2012).

The non-targeted analysis revealed that the composition of polar bear liver extract were much more complex than the extract from the adipose tissue, possibly reflecting the role of the liver in detoxification and higher metabolic activity of the liver compared to adipose tissue. The liver extract contained several compounds of interest with regards to adipogenesis, including phthalates and 4-nonylphenol, that have been shown to induce the expression of adipogenic genes (*Pparg*, *Leptin* and *Srebp1*) and adipogenesis in rats (Zhang et al. 2014). Consequently, both phthalates and 4-nonylphenol may be the components of the POP extracts from adipose tissue with adipogenic activity.

4.2.3 Xenobiotics and adipogenesis in polar bears

A primary role of PPARG is to regulate adipogenesis and lipid metabolism (Feige et al. 2006), functions that should be particularly important for arctic mammals. In the Arctic the availability of food varies with location and season. For example, pelagic polar bears in the high Arctic may hunt seal all year around, while polar bears in areas with receding sea ice may be fasting up to 6 months (Derocher, Wiig, and Andersen 2002; Mauritzen, Derocher, and Wiig 2001). Gestating and lactating female polar bears may even fast for up to 11 months (Thiemann, Iverson, and Stirling 2006). Thus, polar bear may have a short period of time to hunt and store enough energy to endure longer periods of negative energy balance. The nutritional state of polar bears can have effects both on the individual level and on the population level because the rates of reproduction, the probability of cub survival and the probability of survival through the fasting season depend heavily on the size of energy stores and the body mass of polar bears (Atkinson and Ramsay 1995; Derocher and Stirling 1996).

The expansion of adipose tissue by adipogenesis is a complex process in which PPARG is a central and mandatory regulator (Barak et al. 1999; Rosen et al. 1999; Medina-Gomez et al. 2007). Chemicals that can perturb

lipid metabolism and adipogenesis are termed obesogens or antiobesogens (Grun and Blumberg 2006), and include both pharmaceuticals (such as thiazolidinedione) and industrial chemicals (such as BPA, tributyltin, nonylphenol and phthalates) x. One way for obesogens and antiobesogens to exert their action is to modulate the activity of PPARG. The conservation of PPARG indicates that humans and polar bears have similarities in the lipid metabolism. However, there also appear to be differences. For instance, unlike humans, polar bears have very high levels of circulating lipids and cholesterol, apparently without suffering adverse health effects. Polar bears from Svalbard have up 8.6 mM cholesterol in plasma (Ormbostad 2012), a level that is lethal in dogs and rabbits (Kaduce, Spector, and Folk 1981) and associated with a high risk of coronary heart disease in humans (>6.3 mM). Thus, large fat depots appear to be less of a problem for polar bears than for humans. In fact, the link between body mass and polar bears survival suggests that factors that facilitate growth of fat deposits will be advantageous for polar bears.

There is, however, at least one situation where inappropriate activation of the PPARG pathway could be unfortunate. As the transcription of PPARG target genes results in increased glucose and lipid uptake in adipocytes, there is at least a theoretical possibility that PPARG activation during fasting could counteract the release of energy from fat stores. During fasting polar bear may loose as much as 1kg body mass per day (Atkinson, Nelson, and Ramsay 1996) and lipid content of adipose tissue may be reduced by 35-55% (McKinney et al. 2014). Thus, if activation of PPARG counteracts the consumption of fat stores during fasting, in theory, polar bears may not be able to release enough energy to cover their allostatic loads.

We were not able to test the ability of all the components of the POP extracts to modulate the transcriptional activity of pbPPARG. Thus, we cannot rule out that compounds other that those we tested can activate pbPPARG. However, our impression was that only few xenobiotics are pbPPARG agonists. This is in consistence with a previous study that did not find any

mouse PPARG agonists in a test panel of 200 pesticides (Takeuchi et al. 2006). Nevertheless, the POP extracts from liver and fat of polar bears induced lipid accumulation in murine preadipocytes, proving, at least in principle, that the polar bears of Svalbard holds exogenous compounds that can induce adipogenesis. Presently, it cannot be concluded that the extracts induced adipogenesis via a PPARG-dependent mechanism.

Antiobesogens perturb adipogenesis and the expansion of adipose tissues, and consequently may constitute a serious threat to polar bears. We have demonstrated that single compounds, including BPA, HBCDD, PCB153, DDE, endosulfan and oxychlordane, as well as synthetic mixtures (10 and 44 POP mixtures) from polar bears, reduce the basal transcriptional activity pbPPARG, and the rosiglitazone induced transcriptional activity of pbPPARG *in vitro*. These effects were observed at concentrations that are biologically relevant, thus it is possible that perturbations of PPARG signalling may occur *in vivo* and cause antiobesogenic effects. However, it is important to keep in mind that even if some single compounds and the synthetic mixtures reduced the transcriptional activity of pbPPARG, the POP extracts, that reflects the true content of POPs in polar bear adipose and liver tissue, induced lipid accumulation in 3T3-L1 cells. Unfortunately, we were unable to test the effect of POP extracts on pbPPARG activation due to limited supply. That the extracts were adipogenic, despite containing PPARG inverse agonist, partial agonists and/or neutral antagonists, may indicate that the agonistic potential of the extract outweighs the antagonistic, and masks any inhibitory effects from single components of the extracts. To sum up, it appears that the combined POP load of polar bear liver and adipose tissue may have a weak adipogenic effect, despite containing single compounds that reduce the transcriptional activity of pbPPARG *in vitro.*

4.3 Genetic variations affects the transactivation activity of zebrafish Pxr variants

4.3.1 Genetic variation in PXR genes

Most nuclear receptors are under strong purifying evolutionary pressure and show strong sequence conservation (Zhang et al. 2004). However, a sub-population of codons in the PXR LBD has been subjected to positive selection (Krasowski et al. 2005; Zhang et al. 2004). Consequently, the amino acid sequence conservation of PXR orthologs are generally 10-15% lower than for other NRs (Krasowski et al. 2005). In addition to inter-species variation in *PXR* sequences, significant intra-species variation in *PXR* has also been reported. For instance, ~2700 SNP and indels have been described in human *PXR*, while ~1500 has been described from mouse *Pxr* (Information 2015). Recently we described genetic variation in *pxr* of individuals from a single strain of zebrafish, the Tupfel long fin strain (TL) (Bainy et al. 2013). Fourteen coding sequences polymorphisms were described in the TL fish, six were synonymous, seven were non-synonymous and one introduced an insertion of an amino acid (Bainy et al. 2013). As laboratory strains of zebrafish are less genetically varied than wild fish (Coe et al. 2009), the extent of the genetic variation in the Tupfel long fin strain may be a bit surprising. However, as a laboratory model, zebrafish are less inbreed than their rodent counterparts, and commonly used lines of zebrafish are more genetically diverse compared to other rodent laboratory strains (Guryev et al. 2006). Hence, the existence of polymorphisms in *pxr* of zebrafish may not be very surprising.

4.3.2 Functionally different zebrafish Pxrs

Following our discovery of sequence variations in *pxr* from different strains of zebrafish, we decided to investigate if the variation could be the origin of functional differences. We used an *in vitro* ligand activation assay to study the transcriptional activation of Pxr variants from four strains, and

surface plasmon resonance to study their receptor-ligand interactions. We demonstrated differences in the transcriptional activity of zfPxr variants in the form of altered potency (EC_{50}) and efficacy (measured as E_{max}) of selected zfPxr agonists (Paper III). However, the functional effects of the variation appeared to be compound specific. For instance, the strength of the interaction between PxrAB/Tü and clotrimazole (CLO) is greater than for the same compound and PxrTL, PxrSWT and PxrUNK. PxrAB/Tü was also far more susceptible to activation by CLO than other zfPxr variants, as measured by EC_{50}. Moreover, CLO induced the strongest response via PxrAB/Tü (R_{max}). As for inductions by 4BAB, the three variants from the AB/Tü, SWT and TL strains were equally susceptible to activation (EC_{50}) and were also activated to similar maximum response. PxrUNK was less susceptible to activation by 4BAB and was activated to a lower transcriptional activity.

Polymorphisms in PXR genes from several species, including humans, cattle, mice and pigs, have been described in the SNP database of the NCBI (dbSNP) (www.ncbi.nlm.nih.gov/projects/SNP/). SNPs are much less common in coding regions of human PXR than in non-coding regions (~13%). Missense or nonsense SNPs account for ~9% of the total number of coding-sequence SNPs. In the human PXR, several substitutions, such as R122Q, Q158K and D163G, have been associated with reduced transcriptional activity and lower expression of *CYP3A4* in HepG2 cells. In contrast, the substitutions V140M and A370T result in increased expression of *CYP3A4* and CYP3A4 levels (Hustert et al. 2001; Lim et al. 2005; Zhang et al. 2001). Thus, missense substitutions in coding regions of *PXR* are known to result in differences in the transcriptional activity of hPXR.

4.3.3 Substitutions that alter zfPxr function

As mentioned, PxrAB/Tü was the zfPxr that was most susceptible for activation by CLO. PxrAB/Tü had four unique amino acid positions compared to the other three variants, suggesting that these could be responsible for the

enhanced susceptibility of PxrAB/Tü for being activated by CLO. The four substituted positions were 186 (L>V), 235 (S>T), 417 (L>P) and 421(L>P), where the first letter indicates the amino acid in PxrAB/Tü. As the substitutions in position 186 and 235 involved structurally and chemically similar amino acids, functional effects of these substitutions man not be likely. On the other hand, the substitution of proline by leucine in 417 and 420 may give PxrAB/Tü more flexibility in a part of the molecule (helix 12) that are important for the function of PXR (Nagy and Schwabe 2004). Crystal structures of PPARG bound to a full or a partial agonist, MRL-20 and MRL-24 respectively, have revealed that even subtle differences in chemical structures have grave effect on the agonistic potential of a ligand (Bruning et al. 2007). Bruning *et al.* demonstrated that this difference arose from an alteration in the compound's ability to stabilize helix 12 (H12) via hydrogen bonding to H12-residues. Thus, one may speculate that increased regional flexibility as a result of L>P substitutions could ease the binding of large ligands, such as CLO, and provide a more optimal interaction surface for coactivators or create a more stable interaction with helix-12.

Butyl 4-aminobenzoate was a more potent agonist for PxrAB/Tü, PxrTL and PxrSWT than for PxrUNK. PxrUNK has four unique amino acid positions, 184 (I>S), 218 (C>Y/F), 305 (K>M), 385 (N>H) and one unique insertion (Ile428).Two tools for predicting functional and structural effects of substitutions classified the H385N substitution as deleterious, suggesting H385 is important for transactivation of PxrUNK by 4BAB. Further studies, such as site-directed mutagenesis and studies of regional protein flexibility (for instance by NMR) could give more information about which of the substitutions that contribute to augmented or reduced ligand activation of zfPxr variants.

4.3.4 Ligand dependence of functional differences of zfPxrs

While no significant difference in activation of the zfPxr by nifedipine was seen, CLO activated PxrAB/Tü strongest while 4BAB activated PxrUNK the poorest. Although our test panel was limited, it did represent compounds of diverse size, polarity and structure (***Figure 10***, ***Table 1***). With a reservation concerning the limited test panel, our findings indicated that augmentation or reduction of function was ligand dependent. From various crystal structures it is know that the spacious LBP of the PXRs allows for different orientation of ligands, such as SR12813, hyperforin, rifampicin, colupulone and estradiol (Chrencik et al. 2005; Teotico et al. 2008; Watkins et al. 2001; Watkins, Maglich, et al. 2003; Xue et al. 2007).

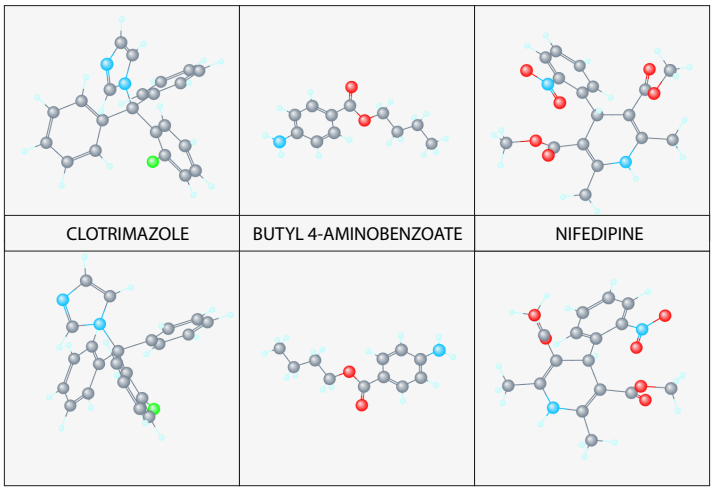

Figure 10 – Chemical structures of test panel compounds used to test zfPxr variant function. Chemical structure from PubChem (National Center for Biotechnology Information).

Bruning *et al.* demonstrated that the orientation of the agonist determine the transcriptional activity of the ligand bound receptor by forcing ERA to orientate WAT169916 in specific ways (Bruning et al. 2010). Since our three test compounds were different structurally different, it may be plausible that CLO, 4BAB and nifedipine are oriented differently in the zfPxr LBP, and that substitutions that are advantageous for the orientation of CLO may not have the same advantage in the interaction with 4BAB or nifedipine.

Table 1 - Chemical properties of test compounds in Paper I. Chemical properties were obtained from PubChem (National Center for Biotechnology Information).

Compound Name (CID)	**CAS No**	**Molecular formula**	**Molecular weight** (g/mole)	**Partition coefficient** (calculated XLOGP3)	**Topological polar surface area** ($Å^2$)	**Molecular volume** (cm^3/mole)
Clotrimazole (CID 2812)	23593-75-1	$C_{22}H_{17}ClN_2$	344.8	5.0	17.8	305.1
Butyl 4-aminobenzoate (CID 2482)	94-25-7	$C_{11}H_{15}NO_2$	193.2	2.9	53.2	179.3
Nifedipine (CID 4485)	21829-25-4	$C_{17}H_{18}N_2O_6$	346.3	2.2	110	272.5

4.4 Quantification of transcriptional activity by reporter gene assays – methodical conciderations

Measuring the ligand activation of PXR and PPARG has been a central part of this work. For this an *in vitro* method that made it possible to screen a relatively large number of compounds, and at the same time was flexible with regards to the type of receptor to analyse, was used. Several *in vitro* methods for assessing NR activation exist. Among these are cell-free ligand binding assays and cell-based assays, such as mammalian two-hybrid system and NR transactivation assays (Pinne and Raucy 2014). Compared to using an *in vivo* method, the most prominent advantages of using an *in vitro* method are that it allows high throughput and reduces the number of laboratory animals that are necessary to use. Disadvantages include variability between assays associated to transfection efficiency and long assay time (Pinne and Raucy 2014).

The most common type of cell-based systems to measure NR transactivation uses transient transfection transactivation (Raucy and Lasker 2013). Most commonly these systems use full-length receptors and native promoter/enhancer in the reporter plasmid. In this work we used a GAL4-UAS

reporter assay as described by Forman *et al.* (Forman et al. 1995). GAL4-UAS reporter assays use a hybrid receptor of the DBD (AA1-147) of GAL4 from yeast and the LBD of the receptor of interest; in this work these were zfPxrs, hPXR, pbPXR and pbPPARG. The GAL4-UAS reporter plasmid has a thymidine kinase promoter with four copies of the GAL4 upstream activation sequence (UAS_G) (Forman et al. 1995). That the same reporter plasmid can be used to study the activation of many different receptors, and that it has enhanced sensitivity (Bainy et al. 2013), are major advantages of using a GAL4-UAS system. Additionally, cross-reactivity from other receptors is low due to the absence of a native promoter in the reporter plasmid (Paguio et al. 2010). However, it is important to keep in mind that both the reporter plasmid and the hybrid receptor used present some limitations to this system. Because of the non-native promoter/enhancer of the reporter plasmid the system will be insensitive to natural regulatory mechanisms in the promoter/enhancer regions. The use of a hybrid receptor likely causes other cellular factors that regulate PXR activity, such as cyclin dependent kinase and HNF4A (Lin et al. 2008; Tirona et al. 2004), to be non-functional. Additionally, synergism between the AF-1 in the DBD and AF-2 of the LBD, as observed in ERA (Zwart et al. 2010), likely not will occur in the hybrid receptor. The GAL4-UAS system is also insensitive to permissiveness. The PXR/RXR heterodimer is permissive and can be activated by RXR agonists (Jones et al. 2000). In contrast, the GAL4-DBD-PXR-LBD hybrid likely binds DNA as a monomer, as GAL4 does in yeast (Campbell et al. 2008). Thus, although the GAL4-UAS system has its advantages it also has some limitations that must be taken into consideration when evaluation the results.

Other factors, more general for all cell-bases NR transactivation assay, can also affect the outcome, and include the choice of cell as well as properties of the test compounds. For instance, the agonistic potential of indirect acting compounds may be underrated if measured in cells with low metabolic capacity, such as simian kidney cells (CV1 and COS7), but also human heptoblastoma cells (such as HepG2) have low CYP3A4 expression

(Rodriguez-Antona et al. 2003; Aninat et al. 2006). Low solubility of the test compound, chemical instability, and high cytotoxicity may all contribute to false negative results in cell-based NR transactivation assays (Sinz et al. 2006).

Several studies have compared PXR activation to CYP3A4 transcription and CYP3A4 activity in primary human hepatocytes and DPX-2/HepG2 cells. The reported correlations are somewhat conflicting. Shukla *et al* found strong correlation (r=0.87) between PXR activation and CYP3A4 activity measured in DPX-2 cells using P450-GLO (Promega) (Shukla et al. 2011). In contrast, the correlation between PXR activation and testosterone 6β- hydroxylation activity in primary hepatocytes was relatively poor (r=0.53), while the correlation between PXR activation and CYP3A4 transcription was moderate (r=0.65) (Luo et al. 2002). These results may indicate that care should be taken when interpreting biological responses from PXR activation data. However, *in vitro* activation of NR can be a very useful tool to explore relative binding potencies of chemicals to specific NRs and to deduct mechanisms of toxicity.

5. Conclusions

PXR was cloned from polar bear liver RNA and it encodes a 434 amino acid protein. Polar bear can be activated by environmental pollutants but is somewhat less promiscuous than human PXR with regards to ligand specificity. The expression of *PXR* in the liver of polar bears and the promiscuity of polar bear PXR, suggest that PXR may function as a xenosensors in polar bears as in humans. Most of the chemicals in our test panel induced stronger transcriptional activities in human PXR than in polar bear PXR. However, four environmental pollutants, HBCDD, toxaphene, 4-nonylphenol and TBBPA, and the cholesterol-lowering drug SR12813, activated a stronger transcriptional activity in polar bear PXR than in human PXR (Paper I).

PPARG was cloned from liver and adipose tissue of polar bears. Two PPARG isoforms were identified. Several of the POPs found in polar bear tissues reduced the transcriptional activity of PPARG in single compound exposures. Synthetic mixtures also reduced the transcriptional activity of polar bear PPARG. Extracts of POPs from polar bear liver and adipose tissue induced lipid accumulation in murine preadipocytes (Paper II).

Genetic variation occurs in the *pxr* of four different strains of zebrafish, the AB Tübingen (AB/Tü), Singapore wild type (SWT), Tupfel long fin (TL) and a strain of unknown origin (UNK). The resulting zebrafish Pxrs are functionally different and differ in their ability of being ligand-activated by clotrimazole and butyl 4-aminobenzoate, as well as in their interaction with these agonists (Paper III).

Together, these findings have given new insights into the role of the NRs PXR and PPARG as targets of endocrine and metabolic disruption in a laboratory model species (zebrafish) and in a wildlife target of POPs (polar bear) through food chain biomagnification.

6. Future perspectives

6.1 Further establish the role of polar bear PXR as a xenosensor

We have demonstrated that polar bear PXR is a promiscuous nuclear receptor that can be activated by xenobiotics and that it is expressed in polar bear liver, strongly indicating a role as a xenosensor. To strengthen the argument that polar bear PXR is a xenosensor, the tissue expression profile of *PXR* in polar bear should be established, for instance by quantitative PCR, microarray or by RNA sequencing. The same methods could be used to prove that activation of polar bear PXR results in increased transcription of genes involved in biotransformation in polar bear hepatocytes, either *in vitro* but ideally *in vivo*. Induction of transcription of biotransformation enzymes could be studied by establishing primary or immortalized cultures of polar bear hepatocytes and expose these for potent polar bear PXR agonists, such as SR12813 or lindane. Endpoint measurements could be transcript level of pbPXR and pbPXR target genes/biotransformation genes (qPCR or RNA sequencing), protein level of pbPXR (WB) and key metabolic enzymes as well as enzyme activity of these (luminescence/fluorescent substrates). Complementary information about the target gene repertoire of polar bear PXR could be obtained by screening for PXR binding sites in the polar bear genome for instance *in silico* methods (response element analysis), or preferably by mapping the PXR cistrome in polar bear liver cells (ChIP-sequencing). These experiments could give knowledge about whether activation of pbPXR results in a similar biological response in polar bears as in humans.

6.2 Effects of combined exposures on modulation of polar bear PXR and PPARG transcriptional activity

Study of activation of nuclear receptors by single compounds is very useful to deduct mechanisms of action, such as toxic pathways. However, exposure to mixtures of POPs, for instance mixtures that are similar to the actual content of POPs in adipose and liver tissue of free-ranging polar bears, may give a more accurate impression of how the sum of POPs modulate the activity of NRs *in vivo*. Endpoint measurement could be transcriptional activity in a reporter gene assay. It was recently shown that human PXR is activated by a supramolecular ligand composed of 17α-ethinylestradiol (EE2) and trans-nonachlor (Delfosse et al. 2015). An alternative strategy to using total mixtures could be to use binary mixtures of compounds, for compounds detected in polar bear tissues. The latter strategy could reveal if binary mixtures of POPs that are present in polar bears can modulate the effect of NRs in a synergetic manner.

6.3 Can POP extracts induce adipogenesis in polar bear cells?

The adipogenic effect of POP extracts from polar bear tissues should be proven in polar bear cells. In paper II this the extracts sufficed only to test the adipogenic effects in murine preadipocytes (3T3-L1 cells). Hence, the adipogenic potential of the extracts have strictly only been proven for mouse cells. New extracts should be prepared and their adipogenic potentials should be tested in polar bear cells, for instance in adipocyte-derived stem cells (ASCs) from polar bears, established through this study. Endpoint measurements could be lipid or triglyceride accumulation and measurements of the transcript levels of adipogenesis related genes, for instance *PPARG*, *FABP4*, lipoprotein lipase (*LPL*) and *CEBP*s.

6.4 What is the adipogenic mechanism of the POP extracts?

The limited supply of extracts forced us to use synthetic mixtures composed to reflect the composition of the extract from polar bear adipose tissue to study the effects on the activity of PPARG. And as mentioned, while the extracts induced adipogenesis in 3T3-L1 cells, the synthetic mixtures did not, nor did the mixture increase the transcriptional activity in the luciferase reporter assay for pbPPARG. Rather, the synthetic mixtures reduced the transcriptional activity of pbPPARG, possible because adipogenic components of the extracts had been left out of the mixtures, such as phthalates. Thus, the mixtures were probably not ideal representatives for the original extracts. New extracts should be prepared and tested for their ability to modulate the activity of polar bear PPARG to provide critical information needed to conclude whether or not a PPARG-dependent mechanism was active when the POP extracts induced lipid accumulation in 3T3-L1 cells.

6.5 Characterization of SNPs in the AB/Tü and SWT strains and establish minor allele frequences

We have previously identified a relatively high number of SNPs in the coding sequence of *pxr* from fish of the TL strain (Bainy et al. 2013), suggesting that genetic variation in *pxr* of zebrafish is quite common. The sequences reported in Paper III likely represent a major *pxr* allele in these strains. It would be interesting to characterize other *pxr* SNPs in the AB/Tü and SWT strains and correlate SNPs to Pxr function *in vivo*. This could be done by exposure of zebrafish with distinct *pxr* variants to a zfPxr agonist, for instance clotrimazole, and search for correlation between the variants and transcriptional activities. Measured endpoints could be sequencing and transcript levels of zfPxr target genes, for instance *cyp3a65*.

6.6 Identify the amino acid(s) responsible for the functional difference among zfPxr variants

PxrAB/Tü was the zfPxr variant that was most susceptible to activation by clotrimazole, while PxrUNK was the least susceptible to activation by 4BAB. To identify the amino acid substitution(s) that is/are responsible for the augmented CLO-induced response of PxrAB/Tü or the perturbed 4BAB-induced response of PxrUNK, the amino acids unique to these two variants could be altered. For instance, each of the four amino acids that were unique to PxrAB/Tü could be changed to the corresponding amino acid of one of he less responsive zfPxr variants. The effect of these mutations on the CLO-induces transcriptional activity of the mutant receptors could be evaluated in a reporter gene assay.

Source of data (references)

Ali, A. T., W. E. Hochfeld, R. Myburgh, and M. S. Pepper. 2013. Adipocyte and adipogenesis. *Eur J Cell Biol* 92 (6-7):229-36.

Aninat, C., A. Piton, D. Glaise, T. Le Charpentier, S. Langouet, F. Morel, C. Guguen-Guillouzo, and A. Guillouzo. 2006. Expression of cytochromes P450, conjugating enzymes and nuclear receptors in human hepatoma HepaRG cells. *Drug Metab Dispos* 34 (1):75-83.

Aprile, M., M. R. Ambrosio, V. D'Esposito, F. Beguinot, P. Formisano, V. Costa, and A. Ciccodicola. 2014. PPARG in Human Adipogenesis: Differential Contribution of Canonical Transcripts and Dominant Negative Isoforms. *PPAR Res* 2014:537865.

Atkinson, S. N., R. A. Nelson, and M. A. Ramsay. 1996. Changes in the body composition of fasting polar bears (Ursus maritimus): The effect of relative fatness on protein conservation. *Physiological Zoology* 69 (2):304-316.

Atkinson, S. N., and M. A. Ramsay. 1995. The Effects of Prolonged Fasting of the Body Composition and Reproductive Success of Female Polar Bears (*Ursus maritimus*). *Functional Ecology* 9 (4):559-567.

Bainy, A. C., A. Kubota, J. V. Goldstone, R. Lille-Langoy, S. I. Karchner, M. C. Celander, M. E. Hahn, A. Goksoyr, and J. J. Stegeman. 2013. Functional characterization of a full length pregnane X receptor, expression in vivo, and identification of PXR alleles, in zebrafish (Danio rerio). *Aquat Toxicol* 142-143:447-57.

Baker, K. D., L. M. Shewchuk, T. Kozlova, M. Makishima, A. Hassell, B. Wisely, J. A. Caravella, M. H. Lambert, J. L. Reinking, H. Krause, C. S. Thummel, T. M. Willson, and D. J. Mangelsdorf. 2003. The Drosophila orphan nuclear receptor DHR38 mediates an atypical ecdysteroid signaling pathway. *Cell* 113 (6):731-42.

Barak, Y., M. C. Nelson, E. S. Ong, Y. Z. Jones, P. Ruiz-Lozano, K. R. Chien, A. Koder, and R. M. Evans. 1999. PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol Cell* 4 (4):585-95.

Bastos Sales, L., J. H. Kamstra, P. H. Cenijn, L. S. van Rijt, T. Hamers, and J. Legler. 2013. Effects of endocrine disrupting chemicals on in vitro global DNA methylation and adipocyte differentiation. *Toxicol In Vitro* 27 (6):1634-43.

Baumann, H., K. Paulsen, H. Kovacs, H. Berglund, A. P. Wright, J. A. Gustafsson, and T. Hard. 1993. Refined solution structure of the glucocorticoid receptor DNA-binding domain. *Biochemistry* 32 (49):13463-71.

Beischlag, T. V., J. Luis Morales, B. D. Hollingshead, and G. H. Perdew. 2008. The aryl hydrocarbon receptor complex and the control of gene expression. *Crit Rev Eukaryot Gene Expr* 18 (3):207-50.

Bell-Parikh, L. C., T. Ide, J. A. Lawson, P. McNamara, M. Reilly, and G. A. FitzGerald. 2003. Biosynthesis of 15-deoxy-delta12,14-PGJ2 and the ligation of PPARgamma. *J Clin Invest* 112 (6):945-55.

Bentzen, T., D. Muir, S. Amstrup, and T. O'Hara. 2008. Organohalogen concentrations in blood and adipose tissue of Southern Beaufort Sea polar bears. *The Science of the total environment* 406 (1-2):352-367.

Beringer, P. M., and R. L. Slaughter. 2005. Transporters and their impact on drug disposition. *Ann Pharmacother* 39 (6):1097-108.

Bernhoft, A., J. Skaare, O. Wiig, A. Derocher, and H. Larsen. 2000. Possible immunotoxic effects of organochlorines in polar bears (Ursus maritimus) at Svalbard. *Journal of toxicology and environmental health. Part A* 59 (7):561-574.

Bertilsson, G., J. Heidrich, K. Svensson, M. Asman, L. Jendeberg, M. Sydow-Backman, R. Ohlsson, H. Postlind, P. Blomquist, and A. Berkenstam. 1998. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc Natl Acad Sci U S A* 95 (21):12208-13.

Bertrand, S., B. Thisse, R. Tavares, L. Sachs, A. Chaumot, P. L. Bardet, H. Escriva, M. Duffraisse, O. Marchand, R. Safi, C. Thisse, and V. Laudet. 2007. Unexpected novel relational links uncovered by extensive developmental profiling of nuclear receptor expression. *PLoS Genet* 3 (11):e188.

Biggs, J. S., J. Wan, N. S. Cutler, J. Hakkola, P. Uusimaki, H. Raunio, and G. S. Yost. 2007. Transcription factor binding to a putative double E-box motif represses CYP3A4 expression in human lung cells. *Mol Pharmacol* 72 (3):514-25.

Black, B. E., J. M. Holaska, F. Rastinejad, and B. M. Paschal. 2001. DNA binding domains in diverse nuclear receptors function as nuclear export signals. *Curr Biol* 11 (22):1749-58.

Blumberg, B., W. Sabbagh, Jr., H. Juguilon, J. Bolado, Jr., C. M. van Meter, E. S. Ong, and R. M. Evans. 1998. SXR, a novel steroid and xenobiotic-sensing nuclear receptor. *Genes Dev* 12 (20):3195-205.

Bookout, A. L., Y. Jeong, M. Downes, R. T. Yu, R. M. Evans, and D. J. Mangelsdorf. 2006. Anatomical profiling of nuclear receptor expression reveals a hierarchical transcriptional network. *Cell* 126 (4):789-99.

Bowers, R. R., and M. D. Lane. 2007. A role for bone morphogenetic protein-4 in adipocyte development. *Cell Cycle* 6 (4):385-9.

Braathen, Marte, Andrew Derocher, Øystein Wiig, Eugen Sørmo, Elisabeth Lie, Janneche Skaare, and Bjørn Jenssen. 2004. Relationships between PCBs and thyroid hormones and retinol in female and male polar bears. *Environmental health perspectives* 112 (8):826-833.

Bresolin, T., M. de Freitas Rebelo, and A. Celso Dias Bainy. 2005. Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol* 140 (3-4):403-7.

Brewer, C. T., and T. Chen. 2016. PXR variants: the impact on drug metabolism and therapeutic responses. *Acta Pharm Sin B* 6 (5):441-449.

Briancon, N., and M. C. Weiss. 2006. In vivo role of the HNF4alpha AF-1 activation domain revealed by exon swapping. *EMBO J* 25 (6):1253-62.

Bridgham, J. T., G. N. Eick, C. Larroux, K. Deshpande, M. J. Harms, M. E. Gauthier, E. A. Ortlund, B. M. Degnan, and J. W. Thornton. 2010. Protein evolution by molecular tinkering: diversification of the nuclear receptor superfamily from a ligand-dependent ancestor. *PLoS Biol* 8 (10).

Bruning, J. B., M. J. Chalmers, S. Prasad, S. A. Busby, T. M. Kamenecka, Y. He, K. W. Nettles, and P. R. Griffin. 2007. Partial agonists activate PPARgamma using a helix 12 independent mechanism. *Structure* 15 (10):1258-71.

Bruning, J. B., A. A. Parent, G. Gil, M. Zhao, J. Nowak, M. C. Pace, C. L. Smith, P. V. Afonine, P. D. Adams, J. A. Katzenellenbogen, and K. W. Nettles. 2010. Coupling of receptor conformation and ligand orientation determine graded activity. *Nat Chem Biol* 6 (11):837-43.

Bugel, S. M., L. A. White, and K. R. Cooper. 2013. Inhibition of vitellogenin gene induction by 2,3,7,8-tetrachlorodibenzo-p-dioxin is mediated by aryl hydrocarbon receptor 2 (AHR2) in zebrafish (Danio rerio). *Aquat Toxicol* 126:1-8.

Bugge, A., L. Grontved, M. M. Aagaard, R. Borup, and S. Mandrup. 2009. The PPARgamma2 A/B-domain plays a gene-specific role in transactivation and cofactor recruitment. *Mol Endocrinol* 23 (6):794-808.

Bühler, F, P Schmid, and C Schlatter. 1988. Kinetics of PCB elimination in man. *Chemosphere* 17 (9):1717-1726.

Bytingsvik, Jenny, Elisabeth Lie, Jon Aars, Andrew Derocher, Øystein Wiig, and Bjørn Jenssen. 2012. PCBs and OH-PCBs in polar bear mother-cub pairs: a comparative study based on plasma levels in 1998 and 2008. *The Science of the total environment* 417-418:117-128.

Campbell, R. N., M. K. Leverentz, L. A. Ryan, and R. J. Reece. 2008. Metabolic control of transcription: paradigms and lessons from Saccharomyces cerevisiae. *Biochem J* 414 (2):177-87.

Cannon, B., and J. Nedergaard. 2004. Brown adipose tissue: function and physiological significance. *Physiol Rev* 84 (1):277-359.

Carvan, M. J., 3rd, E. P. Gallagher, A. Goksoyr, M. E. Hahn, and D. G. Larsson. 2007. Fish models in toxicology. *Zebrafish* 4 (1):9-20.

Chamorro-Garcia, R., S. Kirchner, X. Li, A. Janesick, S. C. Casey, C. Chow, and B. Blumberg. 2012. Bisphenol A diglycidyl ether induces adipogenic differentiation of multipotent stromal stem cells through a peroxisome proliferator-activated receptor gamma-independent mechanism. *Environ Health Perspect* 120 (7):984-9.

Chandra, V., P. Huang, Y. Hamuro, S. Raghuram, Y. Wang, T. P. Burris, and F. Rastinejad. 2008. Structure of the intact PPAR-gamma-RXR- nuclear receptor complex on DNA. *Nature* 456 (7220):350-6.

Chrencik, J. E., J. Orans, L. B. Moore, Y. Xue, L. Peng, J. L. Collins, G. B. Wisely, M. H. Lambert, S. A. Kliewer, and M. R. Redinbo. 2005. Structural disorder in the complex of human pregnane X receptor and the macrolide antibiotic rifampicin. *Mol Endocrinol* 19 (5):1125-34.

Coe, T. S., P. B. Hamilton, A. M. Griffiths, D. J. Hodgson, M. A. Wahab, and C. R. Tyler. 2009. Genetic variation in strains of zebrafish (Danio rerio) and the implications for ecotoxicology studies. *Ecotoxicology* 18 (1):144-50.

Coleman, MD. 2010. Drug Biotransformational Systems - Origins and Aims. In *Human drug metabolsim*: Wiley-Blackwell.

Cristancho, A. G., and M. A. Lazar. 2011. Forming functional fat: a growing understanding of adipocyte differentiation. *Nat Rev Mol Cell Biol* 12 (11):722-34.

Cui, J. Y., S. S. Gunewardena, C. E. Rockwell, and C. D. Klaassen. 2010. ChIPing the cistrome of PXR in mouse liver. *Nucleic Acids Res* 38 (22):7943-63.

Dai, Y. J., Y. F. Jia, N. Chen, W. P. Bian, Q. K. Li, Y. B. Ma, Y. L. Chen, and D. S. Pei. 2014. Zebrafish as a model system to study toxicology. *Environ Toxicol Chem* 33 (1):11-7.

Davies, S. S., A. V. Pontsler, G. K. Marathe, K. A. Harrison, R. C. Murphy, J. C. Hinshaw, G. D. Prestwich, A. S. Hilaire, S. M. Prescott, G. A. Zimmerman, and T. M. McIntyre. 2001. Oxidized alkyl phospholipids are specific, high affinity peroxisome proliferator-activated receptor gamma ligands and agonists. *J Biol Chem* 276 (19):16015-23.

Debier, C., C. Chalon, B. J. Le Boeuf, T. de Tillesse, Y. Larondelle, and J. P. Thome. 2006. Mobilization of PCBs from blubber to blood in northern elephant seals (Mirounga angustirostris) during the post-weaning fast. *Aquat Toxicol* 80 (2):149-57.

Delfosse, V., B. Dendele, T. Huet, M. Grimaldi, A. Boulahtouf, S. Gerbal-Chaloin, B. Beucher, D. Roecklin, C. Muller, R. Rahmani, V. Cavailles, M. Daujat-Chavanieu, V. Vivat, J. M. Pascussi, P. Balaguer, and W. Bourguet. 2015. Synergistic activation of human pregnane X receptor by binary cocktails of pharmaceutical and environmental compounds. *Nat Commun* 6:8089.

Delfosse, V., A. L. Maire, P. Balaguer, and W. Bourguet. 2014. A structural perspective on nuclear receptors as targets of environmental compounds. *Acta Pharmacol Sin*.

Derocher, A. E., and I. Stirling. 1996. Aspects of survival in juvenile polar bears. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 74 (7):1246-1252.

Derocher, A. E., O. Wiig, and M. Andersen. 2002. Diet composition of polar bears in Svalbard and the western Barents Sea. *Polar Biology* 25 (6):448-452.

Donaldson, S., J Odland, B Adlard, P Ayotte, C. Bastien, C Behe, J Bell, I Bergdahl, J Berner, P Bjerregaard, E Bonefeld-Jørgensen, M Brubaker, A Carlsen, F Debes, E Dewailly, A Dudarev, P Egede, C Furgal, JC Gibson, A Gilman, P Grandjean, J Halling, S Hansen, J Jacobson, S Jacobson, EM Krümmel, AR Lager, T. Leech, M Long, S Meakin, G Muckle, G Mulvad, T Nost, K Olafsdottir, A Parkinson, M Skaalum Petersen, P Plusquellec, A Rautio, B Revitch, D Saint-Amour, ™ Sandanger, cheripanoff M, AS Veyhe, P Weihe, and M Wennberg. 2015. AMAP Assessment 2015: Human Health in the Arctic. In *Arctic Monitoring and Assessment Programme (AMAP)*. Oslo, Norway: Arctic Monitoring and Assessment Programme (AMAP).

Dotzlaw, H., E. Leygue, P. Watson, and L. C. Murphy. 1999. The human orphan receptor PXR messenger RNA is expressed in both normal and neoplastic breast tissue. *Clin Cancer Res* 5 (8):2103-7.

Dreyer, C., G. Krey, H. Keller, F. Givel, G. Helftenbein, and W. Wahli. 1992. Control of the peroxisomal beta-oxidation pathway by a novel family of nuclear hormone receptors. *Cell* 68 (5):879-87.

Duniec-Dmuchowski, Z., E. Ellis, S. C. Strom, and T. A. Kocarek. 2007. Regulation of CYP3A4 and CYP2B6 expression by liver X receptor agonists. *Biochem Pharmacol* 74 (10):1535-40.

Durner, G. M., D. C. Douglas, R. M. Nielson, S. C. Amstrup, T. L. McDonald, I. Stirling, M. Mauritzen, E. W. Born, O. Wiig, E. DeWeaver, M. C. Serreze, S. E. Belikov, M. M. Holland, J. Maslanik, J. Aars, D. A. Bailey, and A. E. Derocher. 2009. Predicting 21st-century polar bear habitat distribution from global climate models. *Ecological Monographs* 79 (1):25-58.

Ecotoxmodels.org. *Mechanistic effect models for ecotoxicology* [cited. Available from http://www.ecotoxmodels.org/hot-topics/toxicokinetic-toxicodynamic-models/.

Ekins, Sean, Erica Reschly, Lee Hagey, and Matthew Krasowski. 2008. Evolution of pharmacologic specificity in the pregnane X receptor. *BMC evolutionary biology* 8:103.

Elias, A., J. Wu, and T. Chen. 2013. Tumor suppressor protein p53 negatively regulates human pregnane X receptor activity. *Mol Pharmacol* 83 (6):1229-36.

Ellero, S., G. Chakhtoura, C. Barreau, S. Langouet, C. Benelli, L. Penicaud, P. Beaune, and I. de Waziers. 2010. Xenobiotic-metabolizing cytochromes p450 in human white adipose tissue: expression and induction. *Drug Metab Dispos* 38 (4):679-86.

Engeszer, R. E., L. B. Patterson, A. A. Rao, and D. M. Parichy. 2007. Zebrafish in the wild: a review of natural history and new notes from the field. *Zebrafish* 4 (1):21-40.

Escriva, H., M. C. Langlois, R. L. Mendonca, R. Pierce, and V. Laudet. 1998. Evolution and diversification of the nuclear receptor superfamily. *Ann N Y Acad Sci* 839:143-6.

Evans, R. M., and D. J. Mangelsdorf. 2014. Nuclear Receptors, RXR, and the Big Bang. *Cell* 157 (1):255-66.

Fahmi, O. A., J. L. Raucy, E. Ponce, S. Hassanali, and J. M. Lasker. 2012. Utility of DPX2 cells for predicting CYP3A induction-mediated drug-drug interactions and associated structure-activity relationships. *Drug Metab Dispos* 40 (11):2204-11.

Fain, J. N., A. K. Madan, M. L. Hiler, P. Cheema, and S. W. Bahouth. 2004. Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. *Endocrinology* 145 (5):2273-82.

Farmer, S. R. 2006. Transcriptional control of adipocyte formation. *Cell Metab* 4 (4):263-73.

Feige, J. N., L. Gelman, L. Michalik, B. Desvergne, and W. Wahli. 2006. From molecular action to physiological outputs: peroxisome proliferator-activated receptors are nuclear receptors at the crossroads of key cellular functions. *Prog Lipid Res* 45 (2):120-59.

Ferguson, S. S., Y. Chen, E. L. LeCluyse, M. Negishi, and J. A. Goldstein. 2005. Human CYP2C8 is transcriptionally regulated by the nuclear receptors constitutive androstane receptor, pregnane X receptor, glucocorticoid receptor, and hepatic nuclear factor 4alpha. *Mol Pharmacol* 68 (3):747-57.

Fleck, S. J. 1983. Body composition of elite American athletes. *Am J Sports Med* 11 (6):398-403.

Forman, B. M., P. Tontonoz, J. Chen, R. P. Brun, B. M. Spiegelman, and R. M. Evans. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83 (5):803-12.

Frayn, K. N., F. Karpe, B. A. Fielding, I. A. Macdonald, and S. W. Coppack. 2003. Integrative physiology of human adipose tissue. *Int J Obes Relat Metab Disord* 27 (8):875-88.

Freedman, L. P., B. F. Luisi, Z. R. Korszun, R. Basavappa, P. B. Sigler, and K. R. Yamamoto. 1988. The function and structure of the metal coordination sites within the glucocorticoid receptor DNA binding domain. *Nature* 334 (6182):543-6.

Fukuen, S., T. Fukuda, H. Matsuda, A. Sumida, I. Yamamoto, T. Inaba, and J. Azuma. 2002. Identification of the novel splicing variants for the hPXR in human livers. *Biochem Biophys Res Commun* 298 (3):433-8.

Gardner-Stephen, D., J. M. Heydel, A. Goyal, Y. Lu, W. Xie, T. Lindblom, P. Mackenzie, and A. Radominska-Pandya. 2004. Human PXR variants and their differential effects on the regulation of human UDP-glucuronosyltransferase gene expression. *Drug Metab Dispos* 32 (3):340-7.

Geick, A., M. Eichelbaum, and O. Burk. 2001. Nuclear receptor response elements mediate induction of intestinal MDR1 by rifampin. *J Biol Chem* 276 (18):14581-7.

Germain, P., C. Gaudon, V. Pogenberg, S. Sanglier, A. Van Dorsselaer, C. A. Royer, M. A. Lazar, W. Bourguet, and H. Gronemeyer. 2009. Differential action on coregulator interaction defines inverse retinoid agonists and neutral antagonists. *Chem Biol* 16 (5):479-89.

Germain, P., B. Staels, C. Dacquet, M. Spedding, and V. Laudet. 2006. Overview of nomenclature of nuclear receptors. *Pharmacol Rev* 58 (4):685-704.

Gianni, M., A. Tarrade, E. A. Nigro, E. Garattini, and C. Rochette-Egly. 2003. The AF-1 and AF-2 domains of RAR gamma 2 and RXR alpha cooperate for triggering the transactivation and the degradation of RAR gamma 2/RXR alpha heterodimers. *J Biol Chem* 278 (36):34458-66.

Gnerre, C., S. Blattler, M. R. Kaufmann, R. Looser, and U. A. Meyer. 2004. Regulation of CYP3A4 by the bile acid receptor FXR: evidence for functional binding sites in the CYP3A4 gene. *Pharmacogenetics* 14 (10):635-45.

Goldstone, J., A. Hamdoun, B. Cole, M. Howard-Ashby, D. Nebert, M. Scally, M. Dean, D. Epel, M. Hahn, and J. Stegeman. 2006. The chemical defensome: environmental sensing and response genes in the Strongylocentrotus purpuratus genome. *Developmental biology* 300 (1):366-384.

Goldstone, J. V. 2008. Environmental sensing and response genes in cnidaria: the chemical defensome in the sea anemone Nematostella vectensis. *Cell Biol Toxicol* 24 (6):483-502.

Goldstone, J. V., A. G. McArthur, A. Kubota, J. Zanette, T. Parente, M. E. Jonsson, D. R. Nelson, and J. J. Stegeman. 2010. Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *Bmc Genomics* 11.

Goncharov, Alexey, Marian Pavuk, Herman Foushee, and David Carpenter. 2011. Blood pressure in relation to concentrations of PCB congeners and chlorinated pesticides. *Environmental health perspectives* 119 (3):319-325.

Goodwin, B., K. C. Gauthier, M. Umetani, M. A. Watson, M. I. Lochansky, J. L. Collins, E. Leitersdorf, D. J. Mangelsdorf, S. A. Kliewer, and J. J. Repa. 2003. Identification of bile acid precursors as endogenous ligands for the nuclear xenobiotic pregnane X receptor. *Proc Natl Acad Sci U S A* 100 (1):223-8.

Goodwin, B., E. Hodgson, and C. Liddle. 1999. The orphan human pregnane X receptor mediates the transcriptional activation of CYP3A4 by rifampicin through a distal enhancer module. *Mol Pharmacol* 56 (6):1329-39.

Grasso, L. C., D. C. Hayward, J. W. Trueman, K. M. Hardie, P. A. Janssens, and E. E. Ball. 2001. The evolution of nuclear receptors: evidence from the coral Acropora. *Mol Phylogenet Evol* 21 (1):93-102.

Gray, J. P., J. W. Davis, 2nd, L. Gopinathan, T. L. Leas, C. A. Nugent, and J. P. Vanden Heuvel. 2006. The ribosomal protein rpL11 associates with and inhibits the transcriptional activity of peroxisome proliferator-activated receptor-alpha. *Toxicol Sci* 89 (2):535-46.

Gronemeyer, H., J. A. Gustafsson, and V. Laudet. 2004. Principles for modulation of the nuclear receptor superfamily. *Nat Rev Drug Discov* 3 (11):950-64.

Grun, F., and B. Blumberg. 2006. Environmental obesogens: organotins and endocrine disruption via nuclear receptor signaling. *Endocrinology* 147 (6 Suppl):S50-5.

Guengerich, F. P. 2008. Cytochrome p450 and chemical toxicology. *Chem Res Toxicol* 21 (1):70-83.

Guryev, Victor, Marco Koudijs, Eugene Berezikov, Stephen Johnson, Ronald Plasterk, Fredericus van Eeden, and Edwin Cuppen. 2006. Genetic variation in the zebrafish. *Genome research* 16 (4):491-497.

Guzelian, J., J. L. Barwick, L. Hunter, T. L. Phang, L. C. Quattrochi, and P. S. Guzelian. 2006. Identification of genes controlled by the pregnane X receptor by microarray analysis of mRNAs from pregnenolone 16alpha-carbonitrile-treated rats. *Toxicol Sci* 94 (2):379-87.

Haave, Marte, Erik Ropstad, Andrew Derocher, Elisabeth Lie, Ellen Dahl, Øystein Wiig, Janneche Skaare, and Bjørn Jenssen. 2003. Polychlorinated biphenyls and reproductive hormones in female polar bears at Svalbard. *Environmental health perspectives* 111 (4):431-436.

Hao, C., X. Cheng, H. Xia, and X. Ma. 2012. The endocrine disruptor mono-(2-ethylhexyl) phthalate promotes adipocyte differentiation and induces obesity in mice. *Biosci Rep* 32 (6):619-29.

Harms, M., and P. Seale. 2013. Brown and beige fat: development, function and therapeutic potential. *Nat Med* 19 (10):1252-63.

Harper, S. L., J. L. Carriere, J. M. Miller, J. E. Hutchison, B. L. Maddux, and R. L. Tanguay. 2011. Systematic evaluation of nanomaterial toxicity: utility of standardized materials and rapid assays. *ACS Nano* 5 (6):4688-97.

He, P., M. H. Court, D. J. Greenblatt, and L. L. von Moltke. 2006. Human pregnane X receptor: genetic polymorphisms, alternative mRNA splice variants, and cytochrome P450 3A metabolic activity. *J Clin Pharmacol* 46 (11):1356-69.

Helsen, C., S. Kerkhofs, L. Clinckemalie, L. Spans, M. Laurent, S. Boonen, D. Vanderschueren, and F. Claessens. 2012. Structural basis for nuclear hormone receptor DNA binding. *Mol Cell Endocrinol* 348 (2):411-7.

Henry, K. M., C. A. Loynes, M. K. Whyte, and S. A. Renshaw. 2013. Zebrafish as a model for the study of neutrophil biology. *J Leukoc Biol* 94 (4):633-42.

Hobson, K. A., and H. E. Welch. 1992. Determination of trophic relationships within a high Arctic marine food web using δ13C and δ15N analysis. *MARINE ECOLOGY PROGRESS SERIES* 84:9-18.

Hollenberg, A. N., T. Monden, J. P. Madura, K. Lee, and F. E. Wondisford. 1996. Function of nuclear co-repressor protein on thyroid hormone response elements is regulated by the receptor A/B domain. *J Biol Chem* 271 (45):28516-20.

Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K. McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J. C. Barrett, R. Koch, G. J. Rauch, S. White, W. Chow, B. Kilian, L. T. Quintais, J. A. Guerra-Assuncao, Y. Zhou, Y. Gu, J. Yen, J. H. Vogel, T. Eyre, S. Redmond, R. Banerjee, J. Chi, B. Fu, E. Langley, S. F. Maguire, G. K. Laird, D. Lloyd, E. Kenyon, S. Donaldson, H. Sehra, J. Almeida-King, J. Loveland, S. Trevanion, M. Jones, M. Quail, D. Willey, A. Hunt, J. Burton, S. Sims, K. McLay, B. Plumb, J. Davis, C. Clee, K. Oliver, R. Clark, C. Riddle, D. Elliot, G. Threadgold, G. Harden, D. Ware, S. Begum, B. Mortimore, G. Kerry, P. Heath, B. Phillimore, A. Tracey, N. Corby, M. Dunn, C. Johnson, J. Wood, S. Clark, S. Pelan, G. Griffiths, M.

Smith, R. Glithero, P. Howden, N. Barker, C. Lloyd, C. Stevens, J. Harley, K. Holt, G. Panagiotidis, J. Lovell, H. Beasley, C. Henderson, D. Gordon, K. Auger, D. Wright, J. Collins, C. Raisen, L. Dyer, K. Leung, L. Robertson, K. Ambridge, D. Leongamornlert, S. McGuire, R. Gilderthorp, C. Griffiths, D. Manthravadi, S. Nichol, G. Barker, S. Whitehead, M. Kay, J. Brown, C. Murnane, E. Gray, M. Humphries, N. Sycamore, D. Barker, D. Saunders, J. Wallis, A. Babbage, S. Hammond, M. Mashreghi-Mohammadi, L. Barr, S. Martin, P. Wray, A. Ellington, N. Matthews, M. Ellwood, R. Woodmansey, G. Clark, J. Cooper, A. Tromans, D. Grafham, C. Skuce, R. Pandian, R. Andrews, E. Harrison, A. Kimberley, J. Garnett, N. Fosker, R. Hall, P. Garner, D. Kelly, C. Bird, S. Palmer, I. Gehring, A. Berger, C. M. Dooley, Z. Ersan-Urun, C. Eser, H. Geiger, M. Geisler, L. Karotki, A. Kirn, J. Konantz, M. Konantz, M. Oberlander, S. Rudolph-Geiger, M. Teucke, C. Lanz, G. Raddatz, K. Osoegawa, B. Zhu, A. Rapp, S. Widaa, C. Langford, F. Yang, S. C. Schuster, N. P. Carter, J. Harrow, Z. Ning, J. Herrero, S. M. Searle, A. Enright, R. Geisler, R. H. Plasterk, C. Lee, M. Westerfield, P. J. de Jong, L. I. Zon, J. H. Postlethwait, C. Nusslein-Volhard, T. J. Hubbard, H. Roest Crollius, J. Rogers, and D. L. Stemple. 2013. The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496 (7446):498-503.

Hsu, C. H., Z. H. Wen, C. S. Lin, and C. Chakraborty. 2007. The zebrafish model: use in studying cellular mechanisms for a spectrum of clinical disease entities. *Curr Neurovasc Res* 4 (2):111-20.

Hustert, E., A. Zibat, E. Presecan-Siedel, R. Eiselt, R. Mueller, C. Fuss, I. Brehm, U. Brinkmann, M. Eichelbaum, L. Wojnowski, and O. Burk. 2001. Natural protein variants of pregnane X receptor with altered transactivation activity toward CYP3A4. *Drug Metab Dispos* 29 (11):1454-9.

Igarashi, M., Y. Yogiashi, M. Mihara, I. Takada, H. Kitagawa, and S. Kato. 2007. Vitamin K induces osteoblast differentiation through pregnane X receptor-mediated transcriptional control of the Msx2 gene. *Mol Cell Biol* 27 (22):7947-54.

Ihunnah, C. A., M. Jiang, and W. Xie. 2011. Nuclear receptor PXR, transcriptional circuits and metabolic relevance. *Biochim Biophys Acta* 1812 (8):956-63.

Information, National Center for Biotechnology. 2015. dsSNP - Short Genetic Variations.

Issemann, I., and S. Green. 1990. Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. *Nature* 347 (6294):645-50.

Ito, Y., and T. Nakajima. 2008. PPARalpha- and DEHP-Induced Cancers. *PPAR Res* 2008:759716.

Jones, S., L. Moore, J. Shenk, G. Wisely, G. Hamilton, D. McKee, N. Tomkinson, E. LeCluyse, M. Lambert, T. Willson, S. Kliewer, and J. Moore. 2000. The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology (Baltimore, Md.)* 14 (1):27-66.

Jover, R., M. Moya, and M. J. Gomez-Lechon. 2009. Transcriptional regulation of cytochrome p450 genes by the nuclear receptor hepatocyte nuclear factor 4-alpha. *Curr Drug Metab* 10 (5):508-19.

Kaduce, TL, AA Spector, and GE Jr Folk. 1981. Characterization of the plasma lipids and lipoproteins of the polar bear.

Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology Part B: Comparative Biochemistry (3):541-545.

Kamstra, J. H., P. Alestrom, J. M. Kooter, and J. Legler. 2014. Zebrafish as a model to study the role of DNA methylation in environmental toxicology. *Environ Sci Pollut Res Int*.

Kamstra, J. H., E. Hruba, B. Blumberg, A. Janesick, S. Mandrup, T. Hamers, and J. Legler. 2014. Transcriptional and epigenetic mechanisms underlying enhanced in vitro adipocyte differentiation by the brominated flame retardant BDE-47. *Environ Sci Technol* 48 (7):4110-9.

Kannan, Kurunthachalam, Se Yun, and Thomas Evans. 2005. Chlorinated, brominated, and perfluorinated contaminants in livers of polar bears from Alaska. *Environmental science & technology* 39 (23):9057-9063.

Kim, M. J., P. Marchand, C. Henegar, J. P. Antignac, R. Alili, C. Poitou, J. L. Bouillot, A. Basdevant, B. Le Bizec, R. Barouki, and K. Clement. 2011. Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. *Environ Health Perspect* 119 (3):377-83.

Kim, Min-Ji, Philippe Marchand, Corneliu Henegar, Jean-Philippe Antignac, Rohia Alili, Christine Poitou, Jean-Luc Bouillot, Arnaud Basdevant, Bruno Le Bizec, Robert Barouki, and Karine Clément. 2011. Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. *Environmental health perspectives* 119 (3):377-383.

King Heiden, T. C., J. Spitsbergen, W. Heideman, and R. E. Peterson. 2009. Persistent adverse effects on health and reproduction caused by exposure of zebrafish to 2,3,7,8-tetrachlorodibenzo-p-dioxin during early development and gonad differentiation. *Toxicol Sci* 109 (1):75-87.

King-Jones, K., and C. S. Thummel. 2005. Nuclear receptors--a perspective from Drosophila. *Nat Rev Genet* 6 (4):311-23.

Kiyose, C., H. Saito, K. Kaneko, K. Hamamura, M. Tomioka, T. Ueda, and O. Igarashi. 2001. Alpha-tocopherol affects the urinary and biliary excretion of 2,7,8-trimethyl-2 (2'-carboxyethyl)-6-hydroxychroman, gamma-tocopherol metabolite, in rats. *Lipids* 36 (5):467-72.

Kliewer, S. A., B. M. Forman, B. Blumberg, E. S. Ong, U. Borgmeyer, D. J. Mangelsdorf, K. Umesono, and R. M. Evans. 1994. Differential expression and activation of a family of murine peroxisome proliferator-activated receptors. *Proc Natl Acad Sci U S A* 91 (15):7355-9.

Kliewer, S. A., J. T. Moore, L. Wade, J. L. Staudinger, M. A. Watson, S. A. Jones, D. D. McKee, B. B. Oliver, T. M. Willson, R. H. Zetterstrom, T. Perlmann, and J. M. Lehmann. 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92 (1):73-82.

Kliewer, S., J. Moore, L. Wade, J. Staudinger, M. Watson, S. Jones, D. McKee, B. Oliver, T. Willson, R. Zetterström, T. Perlmann, and J. Lehmann. 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. *Cell* 92 (1):73-82.

Kliewer, Steven, Bryan Goodwin, and Timothy Willson. 2002. The nuclear pregnane X receptor: a key regulator of xenobiotic metabolism. *Endocrine reviews* 23 (5):687-702.

Knobel, M., F. J. Busser, A. Rico-Rico, N. I. Kramer, J. L. Hermens, C. Hafner, K. Tanneberger, K. Schirmer, and S. Scholz. 2012. Predicting adult fish acute

lethality with the zebrafish embryo: relevance of test duration, endpoints, compound properties, and exposure concentration analysis. *Environ Sci Technol* 46 (17):9690-700.

Kodama, S., R. Moore, Y. Yamamoto, and M. Negishi. 2007. Human nuclear pregnane X receptor cross-talk with CREB to repress cAMP activation of the glucose-6-phosphatase gene. *Biochem J* 407 (3):373-81.

Kodama, S., and M. Negishi. 2011. Pregnane X receptor PXR activates the GADD45beta gene, eliciting the p38 MAPK signal and cell migration. *J Biol Chem* 286 (5):3570-8.

Kojetin, D. J., and T. P. Burris. 2013. Small molecule modulation of nuclear receptor conformational dynamics: implications for function and drug discovery. *Mol Pharmacol* 83 (1):1-8.

Kojima, H., F. Sata, S. Takeuchi, T. Sueyoshi, and T. Nagai. 2011. Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays. *Toxicology* 280 (3):77-87.

Konig, J., F. Muller, and M. F. Fromm. 2013. Transporters and drug-drug interactions: important determinants of drug disposition and effects. *Pharmacol Rev* 65 (3):944-66.

Koyano, S., K. Kurose, S. Ozawa, M. Saeki, Y. Nakajima, R. Hasegawa, K. Komamura, K. Ueno, S. Kamakura, T. Nakajima, H. Saito, H. Kimura, Y. Goto, O. Saitoh, M. Katoh, T. Ohnuma, M. Kawai, K. Sugai, T. Ohtsuki, C. Suzuki, N. Minami, Y. Saito, and J. Sawada. 2002. Eleven novel single nucleotide polymorphisms in the NR1I2 (PXR) gene, four of which induce non-synonymous amino acid alterations. *Drug Metab Pharmacokinet* 17 (6):561-5.

Krasowski, M. D., A. Ni, L. R. Hagey, and S. Ekins. 2011. Evolution of promiscuous nuclear hormone receptors: LXR, FXR, VDR, PXR, and CAR. *Mol Cell Endocrinol* 334 (1-2):39-48.

Krasowski, Matthew, Kazuto Yasuda, Lee Hagey, and Erin Schuetz. 2005. Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts. *Molecular endocrinology (Baltimore, Md.)* 19 (7):1720-1739.

Krasowski, M., Yasuda, K., Hagey, L., and E. Schuetz. 2005. Evolutionary selection across the nuclear hormone receptor superfamily with a focus on the NR1I subfamily (vitamin D, pregnane X, and constitutive androstane receptors). *Nuclear receptor* 3:2.

Kubota, N., Y. Terauchi, H. Miki, H. Tamemoto, T. Yamauchi, K. Komeda, S. Satoh, R. Nakano, C. Ishii, T. Sugiyama, K. Eto, Y. Tsubamoto, A. Okuno, K. Murakami, H. Sekihara, G. Hasegawa, M. Naito, Y. Toyoshima, S. Tanaka, K. Shiota, T. Kitamura, T. Fujita, O. Ezaki, S. Aizawa, T. Kadowaki, and et al. 1999. PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol Cell* 4 (4):597-609.

Kurose, K., S. Koyano, S. Ikeda, M. Tohkin, R. Hasegawa, and J. Sawada. 2005. 5' diversity of human hepatic PXR (NR1I2) transcripts and identification of the major transcription initiation site. *Mol Cell Biochem* 273 (1-2):79-85.

Lamba, J., V. Lamba, S. Strom, R. Venkataramanan, and E. Schuetz. 2008. Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos* 36 (1):169-81.

Lamba, V., K. Yasuda, J. K. Lamba, M. Assem, J. Davila, S. Strom, and E. G. Schuetz. 2004. PXR (NR1I2): splice variants in human tissues, including

brain, and identification of neurosteroids and nicotine as PXR activators. *Toxicol Appl Pharmacol* 199 (3):251-65.

Landes, N., P. Pfluger, D. Kluth, M. Birringer, R. Ruhl, G. F. Bol, H. Glatt, and R. Brigelius-Flohe. 2003. Vitamin E activates gene expression via the pregnane X receptor. *Biochem Pharmacol* 65 (2):269-73.

Laudet, V. 1997. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. *J Mol Endocrinol* 19 (3):207-26.

Lefterova, M. I., D. J. Steger, D. Zhuo, M. Qatanani, S. E. Mullican, G. Tuteja, E. Manduchi, G. R. Grant, and M. A. Lazar. 2010. Cell-specific determinants of peroxisome proliferator-activated receptor gamma function in adipocytes and macrophages. *Mol Cell Biol* 30 (9):2078-89.

Letcher, R. J., W. A. Gebbink, C. Sonne, E. W. Born, M. A. McKinney, and R. Dietz. 2009. Bioaccumulation and biotransformation of brominated and chlorinated contaminants and their metabolites in ringed seals (Pusa hispida) and polar bears (Ursus maritimus) from East Greenland. *Environ Int* 35 (8):1118-24.

Letcher, R. J.; Klasson-Wehler, E.; Bergman, Å. 2000. Methyl sulfone and hydroxylated metabolites of polychlorinated biphenyls. In *The Handbook of Environmental Chemistry*, edited by O. Hutzinger. Ottawa: Springer.

Li, J., V. Papadopoulos, and V. Vihma. 2015. Steroid biosynthesis in adipose tissue. *Steroids* 103:89-104.

Li, T., W. Chen, and J. Y. Chiang. 2007. PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine. *J Lipid Res* 48 (2):373-84.

Li, Y., M. Choi, G. Cavey, J. Daugherty, K. Suino, A. Kovach, N. C. Bingham, S. A. Kliewer, and H. E. Xu. 2005. Crystallographic identification and functional characterization of phospholipids as ligands for the orphan nuclear receptor steroidogenic factor-1. *Mol Cell* 17 (4):491-502.

Lie, E., H. J. Larsen, S. Larsen, G. M. Johansen, A. E. Derocher, N. J. Lunn, R. J. Norstrom, O. Wiig, and J. U. Skaare. 2004. Does high organochlorine (OC) exposure impair the resistance to infection in polar bears (Ursus maritimus)? Part I: Effect of OCs on the humoral immunity. *J Toxicol Environ Health A* 67 (7):555-82.

Lie, Elisabeth, Hans Larsen, Stig Larsen, Grethe Johansen, Andrew Derocher, Nicholas Lunn, Ross Norstrom, Oystein Wiig, and Janneche Skaare. 2005. Does high organochlorine (OC) exposure impair the resistance to infection in polar bears (Ursus maritimus)? Part II: Possible effect of OCs on mitogen- and antigen-induced lymphocyte proliferation. *Journal of toxicology and environmental health. Part A* 68 (6):457-484.

Lieschke, G. J., and P. D. Currie. 2007. Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8 (5):353-67.

Lille-Langoy, R., J. V. Goldstone, M. Rusten, M. R. Milnes, R. Male, J. J. Stegeman, B. Blumberg, and A. Goksoyr. 2015. Environmental contaminants activate human and polar bear (Ursus maritimus) pregnane X receptors (PXR, NR1I2) differently. *Toxicol Appl Pharmacol* 284 (1):54-64.

Lim, Y. P., C. H. Liu, L. J. Shyu, and J. D. Huang. 2005. Functional characterization of a novel polymorphism of pregnane X receptor, Q158K, in Chinese subjects. *Pharmacogenet Genomics* 15 (5):337-41.

Lin, W., J. Wu, H. Dong, D. Bouck, F. Y. Zeng, and T. Chen. 2008. Cyclin-dependent kinase 2 negatively regulates human pregnane X receptor-

mediated CYP3A4 gene expression in HepG2 liver carcinoma cells. *J Biol Chem* 283 (45):30650-7.

Lin, Y. S., K. Yasuda, M. Assem, C. Cline, J. Barber, C. W. Li, V. Kholodovych, N. Ai, J. D. Chen, W. J. Welsh, S. Ekins, and E. G. Schuetz. 2009. The major human pregnane X receptor (PXR) splice variant, PXR.2, exhibits significantly diminished ligand-activated transcriptional regulation. *Drug Metab Dispos* 37 (6):1295-304.

Lind, P., Bert van Bavel, Samira Salihovic, and Lars Lind. 2012. Circulating levels of persistent organic pollutants (POPs) and carotid atherosclerosis in the elderly. *Environmental health perspectives* 120 (1):38-43.

Liu, M. J., Y. Takahashi, T. Wada, J. He, J. Gao, Y. Tian, S. Li, and W. Xie. 2009. The aldo-keto reductase Akr1b7 gene is a common transcriptional target of xenobiotic receptors pregnane X receptor and constitutive androstane receptor. *Mol Pharmacol* 76 (3):604-11.

Liu, S., E. D. Lorenzen, M. Fumagalli, B. Li, K. Harris, Z. Xiong, L. Zhou, T. S. Korneliussen, M. Somel, C. Babbitt, G. Wray, J. Li, W. He, Z. Wang, W. Fu, X. Xiang, C. C. Morgan, A. Doherty, M. J. O'Connell, J. O. McInerney, E. W. Born, L. Dalen, R. Dietz, L. Orlando, C. Sonne, G. Zhang, R. Nielsen, E. Willerslev, and J. Wang. 2014. Population genomics reveal recent speciation and rapid evolutionary adaptation in polar bears. *Cell* 157 (4):785-94.

Liu, Y., W. Ji, Y. Yin, L. Fan, J. Zhang, H. Yun, N. Wang, Q. Li, Z. Wei, D. Ouyang, and H. H. Zhou. 2009. The effects of splicing variant of PXR PAR-2 on CYP3A4 and MDR1 mRNA expressions. *Clin Chim Acta* 403 (1-2):142-4.

Luisi, B. F., W. X. Xu, Z. Otwinowski, L. P. Freedman, K. R. Yamamoto, and P. B. Sigler. 1991. Crystallographic analysis of the interaction of the glucocorticoid receptor with DNA. *Nature* 352 (6335):497-505.

Luo, G., M. Cunningham, S. Kim, T. Burn, J. Lin, M. Sinz, G. Hamilton, C. Rizzo, S. Jolley, D. Gilbert, A. Downey, D. Mudra, R. Graham, K. Carroll, J. Xie, A. Madan, A. Parkinson, D. Christ, B. Selling, E. LeCluyse, and L. S. Gan. 2002. CYP3A4 induction by drugs: correlation between a pregnane X receptor reporter gene assay and CYP3A4 expression in human hepatocytes. *Drug Metab Dispos* 30 (7):795-804.

Luscombe, N. M., S. E. Austin, H. M. Berman, and J. M. Thornton. 2000. An overview of the structures of protein-DNA complexes. *Genome Biol* 1 (1):REVIEWS001.

Ma, Q. 2008. Xenobiotic-activated receptors: from transcription to drug metabolism to disease. *Chem Res Toxicol* 21 (9):1651-71.

Malhotra, D., E. Portales-Casamar, A. Singh, S. Srivastava, D. Arenillas, C. Happel, C. Shyr, N. Wakabayashi, T. W. Kensler, W. W. Wasserman, and S. Biswal. 2010. Global mapping of binding sites for Nrf2 identifies novel targets in cell survival response through ChIP-Seq profiling and network analysis. *Nucleic Acids Res* 38 (17):5718-34.

Mandard, S., M. Muller, and S. Kersten. 2004. Peroxisome proliferator-activated receptor alpha target genes. *Cell Mol Life Sci* 61 (4):393-416.

Mangelsdorf, D. J., and R. M. Evans. 1995. The RXR heterodimers and orphan receptors. *Cell* 83 (6):841-50.

Martinez-Jimenez, C. P., M. J. Gomez-Lechon, J. V. Castell, and R. Jover. 2005. Transcriptional regulation of the human hepatic CYP3A4: identification of a new distal enhancer region responsive to CCAAT/enhancer-binding protein

beta isoforms (liver activating protein and liver inhibitory protein). *Mol Pharmacol* 67 (6):2088-101.

Matsubara, T., K. Yoshinari, K. Aoyama, M. Sugawara, Y. Sekiya, K. Nagata, and Y. Yamazoe. 2008. Role of vitamin D receptor in the lithocholic acid-mediated CYP3A induction in vitro and in vivo. *Drug Metab Dispos* 36 (10):2058-63.

Matsumura, K., T. Saito, Y. Takahashi, T. Ozeki, K. Kiyotani, M. Fujieda, H. Yamazaki, H. Kunitoh, and T. Kamataki. 2004. Identification of a novel polymorphic enhancer of the human CYP3A4 gene. *Mol Pharmacol* 65 (2):326-34.

Mauritzen, M., A. E. Derocher, and O. Wiig. 2001. Space-use strategies of female polar bears in a dynamic sea ice habitat. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* 79 (9):1704-1713.

McGrath, P., and C. Q. Li. 2008. Zebrafish: a predictive model for assessing drug-induced toxicity. *Drug Discov Today* 13 (9-10):394-401.

McIntyre, T. M., A. V. Pontsler, A. R. Silva, A. St Hilaire, Y. Xu, J. C. Hinshaw, G. A. Zimmerman, K. Hama, J. Aoki, H. Arai, and G. D. Prestwich. 2003. Identification of an intracellular receptor for lysophosphatidic acid (LPA): LPA is a transcellular PPARgamma agonist. *Proc Natl Acad Sci U S A* 100 (1):131-6.

McKinney, M. A., T. Atwood, R. Dietz, C. Sonne, S. J. Iverson, and E. Peacock. 2014. Validation of adipose lipid content as a body condition index for polar bears. *Ecol Evol* 4 (4):516-27.

McKinney, M. A., R. Dietz, C. Sonne, S. De Guise, K. Skirnisson, K. Karlsson, E. Steingrimsson, and R. J. Letcher. 2011. Comparative hepatic microsomal biotransformation of selected PBDEs, including decabromodiphenyl ether, and decabromodiphenyl ethane flame retardants in Arctic marine-feeding mammals. *Environ Toxicol Chem* 30 (7):1506-14.

Medina-Gomez, G., S. L. Gray, L. Yetukuri, K. Shimomura, S. Virtue, M. Campbell, R. K. Curtis, M. Jimenez-Linan, M. Blount, G. S. Yeo, M. Lopez, T. Seppanen-Laakso, F. M. Ashcroft, M. Oresic, and A. Vidal-Puig. 2007. PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. *PLoS Genet* 3 (4):e64.

Meinke, G., and P. B. Sigler. 1999. DNA-binding mechanism of the monomeric orphan nuclear receptor NGFI-B. *Nat Struct Biol* 6 (5):471-7.

Mensah-Osman, E. J., D. G. Thomas, M. M. Tabb, J. M. Larios, D. P. Hughes, T. J. Giordano, M. L. Lizyness, J. M. Rae, B. Blumberg, P. F. Hollenberg, and L. H. Baker. 2007. Expression levels and activation of a PXR variant are directly related to drug resistance in osteosarcoma cell lines. *Cancer* 109 (5):957-65.

Milan, D. J., T. A. Peterson, J. N. Ruskin, R. T. Peterson, and C. A. MacRae. 2003. Drugs that induce repolarization abnormalities cause bradycardia in zebrafish. *Circulation* 107 (10):1355-8.

Milnes, M. R., A. Garcia, E. Grossman, F. Grun, J. Shiotsugu, M. M. Tabb, Y. Kawashima, Y. Katsu, H. Watanabe, T. Iguchi, and B. Blumberg. 2008. Activation of steroid and xenobiotic receptor (SXR, NR1I2) and its orthologs in laboratory, toxicologic, and genome model species. *Environ Health Perspect* 116 (7):880-5.

Moore, David, Shigeaki Kato, Wen Xie, David Mangelsdorf, Daniel Schmidt, Rui Xiao, and Steven Kliewer. 2006. International Union of Pharmacology. LXII.

The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. *Pharmacological reviews* 58 (4):742-759.

Moore, L. B., B. Goodwin, S. A. Jones, G. B. Wisely, C. J. Serabjit-Singh, T. M. Willson, J. L. Collins, and S. A. Kliewer. 2000. St. John's wort induces hepatic drug metabolism through activation of the pregnane X receptor. *Proc Natl Acad Sci U S A* 97 (13):7500-2.

Moore, L. B., J. M. Maglich, D. D. McKee, B. Wisely, T. M. Willson, S. A. Kliewer, M. H. Lambert, and J. T. Moore. 2002. Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* 16 (5):977-86.

Moore, L., D. Parks, S. Jones, R. Bledsoe, T. Consler, J. Stimmel, B. Goodwin, C. Liddle, S. Blanchard, T. Willson, J. Collins, and S. Kliewer. 2000. Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *The Journal of biological chemistry* 275 (20):15122-15129.

Mueller, E., S. Drori, A. Aiyer, J. Yie, P. Sarraf, H. Chen, S. Hauser, E. D. Rosen, K. Ge, R. G. Roeder, and B. M. Spiegelman. 2002. Genetic analysis of adipogenesis through peroxisome proliferator-activated receptor gamma isoforms. *J Biol Chem* 277 (44):41925-30.

Muir, D., R. Norstrom, and M. Simon. 1988. Organochlorine contaminants in arctic marine food chains: accumulation of specific polychlorinated biphenyls and chlordane-related compounds. *Environmental science & technology* 22 (9):1071-1079.

Mukherjee, R., L. Jow, G. E. Croston, and J. R. Paterniti, Jr. 1997. Identification, characterization, and tissue distribution of human peroxisome proliferator-activated receptor (PPAR) isoforms PPARgamma2 versus PPARgamma1 and activation with retinoid X receptor agonists and antagonists. *J Biol Chem* 272 (12):8071-6.

Nagy, L., and J. W. Schwabe. 2004. Mechanism of the nuclear receptor molecular switch. *Trends Biochem Sci* 29 (6):317-24.

Nagy, L., P. Tontonoz, J. G. Alvarez, H. Chen, and R. M. Evans. 1998. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARgamma. *Cell* 93 (2):229-40.

Nahoum, V., E. Perez, P. Germain, F. Rodriguez-Barrios, F. Manzo, S. Kammerer, G. Lemaire, O. Hirsch, C. A. Royer, H. Gronemeyer, A. R. de Lera, and W. Bourguet. 2007. Modulators of the structural dynamics of the retinoid X receptor to reveal receptor function. *Proc Natl Acad Sci U S A* 104 (44):17323-8.

Naspinski, C., X. Gu, G. D. Zhou, S. U. Mertens-Talcott, K. C. Donnelly, and Y. Tian. 2008. Pregnane X receptor protects HepG2 cells from BaP-induced DNA damage. *Toxicol Sci* 104 (1):67-73.

National Center for Biotechnology Information, NCBI. . PubChem Compound Database.

Ni, H., B. Su, L. Pan, X. Li, X. Zhu, and X. Chen. 2015. Functional variants inPXRare associated with colorectal cancer susceptibility in Chinese populations. *Cancer Epidemiol* 39 (6):972-7.

Nishimura, M., S. Naito, and T. Yokoi. 2004. Tissue-specific mRNA expression profiles of human nuclear receptor subfamilies. *Drug Metab Pharmacokinet* 19 (2):135-49.

Niwa, T., Y. Yabusaki, K. Honma, N. Matsuo, K. Tatsuta, F. Ishibashi, and M. Katagiri. 1998. Contribution of human hepatic cytochrome P450 isoforms to regioselective hydroxylation of steroid hormones. *Xenobiotica* 28 (6):539-47.

Nolte, R. T., G. B. Wisely, S. Westin, J. E. Cobb, M. H. Lambert, R. Kurokawa, M. G. Rosenfeld, T. M. Willson, C. K. Glass, and M. V. Milburn. 1998. Ligand binding and co-activator assembly of the peroxisome proliferator-activated receptor-gamma. *Nature* 395 (6698):137-43.

Norén, K., C. Weistrand, and F. Karpe. 1999. Distribution of PCB congeners, DDE, hexachlorobenzene, and methylsulfonyl metabolites of PCB and DDE among various fractions of human blood plasma. *Archives of environmental contamination and toxicology* 37 (3):408-414.

Norheim, G., J. Skaare, and Ø Wiig. 1992. Some heavy metals, essential elements, and chlorinated hydrocarbons in polar bear (Ursus maritimus) at Svalbard. *Environmental pollution (Barking, Essex : 1987)* 77 (1):51-57.

Norstrom, R., S. Belikov, E. Born, G. Garner, B. Malone, S. Olpinski, M. Ramsay, S. Schliebe, I. Stirling, M. Stishov, M. Taylor, and O. Wiig. 1998. Chlorinated hydrocarbon contaminants in polar bears from eastern Russia, North America, Greenland, and Svalbard: biomonitoring of Arctic pollution. *Archives of environmental contamination and toxicology* 35 (2):354-367.

Nuclear Receptors Nomenclature, Committee. 1999. A unified nomenclature system for the nuclear receptor superfamily. *Cell* 97 (2):161-3.

OECD. *OECD Guidelines for the Testing of Chemicals, Section 2, Effects on Biotic Systems*. OECD Publishing [cited. Available from http://www.oecd-ilibrary.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-2-effects-on-biotic-systems_20745761.

Orans, Jillian, Denise Teotico, and Matthew Redinbo. 2005. The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges. *Molecular endocrinology (Baltimore, Md.)* 19 (12):2891-2900.

Ormbostad, I. 2012. Relationships Between Persistent Organic Pollutants (POPs) and Plasma Clinical-Chemical parameters in Polar Bears (Ursus maritimus) from Svalbard, Norway, Norwegian University of Science and Technology, Department of Biology, Trondheim, Norway.

Ortega, F. J., D. Mayas, J. M. Moreno-Navarrete, V. Catalan, J. Gomez-Ambrosi, E. Esteve, J. I. Rodriguez-Hermosa, B. Ruiz, W. Ricart, B. Peral, G. Fruhbeck, F. J. Tinahones, and J. M. Fernandez-Real. 2010. The gene expression of the main lipogenic enzymes is downregulated in visceral adipose tissue of obese subjects. *Obesity (Silver Spring)* 18 (1):13-20.

Paguio, Aileen, Pete Stecha, Keith Wood, and Frank Fan. 2010. Improved dual-luciferase reporter assays for nuclear receptors. *Current chemical genomics* 4:43-49.

Park, Y. C., S. Lee, and M. H. Cho. 2014. The Simplest Flowchart Stating the Mechanisms for Organic Xenobiotics-induced Toxicity: Can it Possibly be Accepted as a "Central Dogma" for Toxic Mechanisms? *Toxicol Res* 30 (3):179-84.

Pascussi, J. M., A. Robert, M. Nguyen, O. Walrant-Debray, M. Garabedian, P. Martin, T. Pineau, J. Saric, F. Navarro, P. Maurel, and M. J. Vilarem. 2005.

Possible involvement of pregnane X receptor-enhanced CYP24 expression in drug-induced osteomalacia. *J Clin Invest* 115 (1):177-86.

Pawlak, M., P. Lefebvre, and B. Staels. 2012. General molecular biology and architecture of nuclear receptors. *Curr Top Med Chem* 12 (6):486-504.

Peirce, V., S. Carobbio, and A. Vidal-Puig. 2014. The different shades of fat. *Nature* 510 (7503):76-83.

Pellegrinelli, V., S. Carobbio, and A. Vidal-Puig. 2016. Adipose tissue plasticity: how fat depots respond differently to pathophysiological cues. *Diabetologia* 59 (6):1075-88.

Pinne, M., and J. L. Raucy. 2014. Advantages of cell-based high-volume screening assays to assess nuclear receptor activation during drug discovery. *Expert Opin Drug Discov* 9 (6):669-86.

Planchart, A., C. J. Mattingly, D. Allen, P. Ceger, W. Casey, D. Hinton, J. Kanungo, S. W. Kullman, T. Tal, M. Bondesson, S. M. Burgess, C. Sullivan, C. Kim, M. Behl, S. Padilla, D. M. Reif, R. L. Tanguay, and J. Hamm. 2016. Advancing toxicology research using in vivo high throughput toxicology with small fish models. *ALTEX*.

Polischu, S. C., R. J. Norstrom, and M. A. Ramsay. 2002. Body burdens and tissue concentrations of organochlorines in polar bears (Ursus maritimus) vary during seasonal fasts. *Environ Pollut* 118 (1):29-39.

Puigserver, P., Z. Wu, C. W. Park, R. Graves, M. Wright, and B. M. Spiegelman. 1998. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92 (6):829-39.

Quaife, N. M., O. Watson, and T. J. Chico. 2012. Zebrafish: an emerging model of vascular development and remodelling. *Curr Opin Pharmacol* 12 (5):608-14.

Rae, J. M., M. D. Johnson, M. E. Lippman, and D. A. Flockhart. 2001. Rifampin is a selective, pleiotropic inducer of drug metabolism genes in human hepatocytes: studies with cDNA and oligonucleotide expression arrays. *J Pharmacol Exp Ther* 299 (3):849-57.

Rakhshandehroo, M., G. Hooiveld, M. Muller, and S. Kersten. 2009. Comparative analysis of gene regulation by the transcription factor PPARalpha between mouse and human. *PLoS One* 4 (8):e6796.

Raucy, J. L., and J. M. Lasker. 2013. Cell-based systems to assess nuclear receptor activation and their use in drug development. *Drug Metab Rev* 45 (1):101-9.

Remerowski, M. L., E. Kellenbach, R. Boelens, G. A. van der Marel, J. H. van Boom, B. A. Maler, K. R. Yamamoto, and R. Kaptein. 1991. 1H NMR studies of DNA recognition by the glucocorticoid receptor: complex of the DNA binding domain with a half-site response element. *Biochemistry* 30 (50):11620-4.

Ren, D., T. N. Collingwood, E. J. Rebar, A. P. Wolffe, and H. S. Camp. 2002. PPARgamma knockdown by engineered transcription factors: exogenous PPARgamma2 but not PPARgamma1 reactivates adipogenesis. *Genes Dev* 16 (1):27-32.

Reschly, E. J., and M. D. Krasowski. 2006. Evolution and function of the NR1I nuclear hormone receptor subfamily (VDR, PXR, and CAR) with respect to metabolism of xenobiotics and endogenous compounds. *Curr Drug Metab* 7 (4):349-65.

Ring, B. J., B. E. Patterson, M. I. Mitchell, M. Vandenbranden, J. Gillespie, A. W. Bedding, H. Jewell, C. D. Payne, S. T. Forgue, J. Eckstein, S. A. Wrighton, and D. L. Phillips. 2005. Effect of tadalafil on cytochrome P450 3A4-

mediated clearance: studies in vitro and in vivo. *Clin Pharmacol Ther* 77 (1):63-75.

Robinson-Rechavi, M., A. S. Carpentier, M. Duffraisse, and V. Laudet. 2001. How many nuclear hormone receptors are there in the human genome? *Trends Genet* 17 (10):554-6.

Rode, K. D., S. C. Amstrup, and E. V. Regehr. 2010. Reduced body size and cub recruitment in polar bears associated with sea ice decline. *Ecol Appl* 20 (3):768-82.

Rodriguez-Antona, C., R. Bort, R. Jover, N. Tindberg, M. Ingelman-Sundberg, M. J. Gomez-Lechon, and J. V. Castell. 2003. Transcriptional regulation of human CYP3A4 basal expression by CCAAT enhancer-binding protein alpha and hepatocyte nuclear factor-3 gamma. *Mol Pharmacol* 63 (5):1180-9.

Rose, R. L. and Levi, P. E. 2004. Reactive Metabolites. In *A textbook of Modern Toxicology*, edited by E. Hodgson. New Jersey: John Wiley and Sons, Inc.

Rosen, E. D., C. H. Hsu, X. Wang, S. Sakai, M. W. Freeman, F. J. Gonzalez, and B. M. Spiegelman. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev* 16 (1):22-6.

Rosen, E. D., P. Sarraf, A. E. Troy, G. Bradwin, K. Moore, D. S. Milstone, B. M. Spiegelman, and R. M. Mortensen. 1999. PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol Cell* 4 (4):611-7.

Rosen, E. D., and B. M. Spiegelman. 2006. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444 (7121):847-53.

Rosen, E. D., and B. M. Spiegelman. 2014. What we talk about when we talk about fat. *Cell* 156 (1-2):20-44.

Rosenfeld, J. M., R. Vargas, Jr., W. Xie, and R. M. Evans. 2003. Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor. *Mol Endocrinol* 17 (7):1268-82.

Routti, H., R. Lille-Langoy, M. K. Berg, T. Fink, M. Harju, K. Kristiansen, P. Rostkowski, M. Rusten, I. Sylte, L. Oygarden, and A. Goksoyr. 2016. Environmental Chemicals Modulate Polar Bear (Ursus maritimus) Peroxisome Proliferator-Activated Receptor Gamma (PPARG) and Adipogenesis in Vitro. *Environ Sci Technol* 50 (19):10708-10720.

Russell, D. W., and K. D. Setchell. 1992. Bile acid biosynthesis. *Biochemistry* 31 (20):4737-49.

Salihovic, Samira, Erik Lampa, Gunilla Lindström, Lars Lind, P. Lind, and Bert van Bavel. 2012. Circulating levels of persistent organic pollutants (POPs) among elderly men and women from Sweden: results from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). *Environment international* 44:59-67.

Sartor, M. A., M. Schnekenburger, J. L. Marlowe, J. F. Reichard, Y. Wang, Y. Fan, C. Ma, S. Karyala, D. Halbleib, X. Liu, M. Medvedovic, and A. Puga. 2009. Genomewide analysis of aryl hydrocarbon receptor binding targets reveals an extensive array of gene clusters that control morphogenetic and developmental programs. *Environ Health Perspect* 117 (7):1139-46.

Schmidt, J. S., K. Schaedlich, N. Fiandanese, P. Pocar, and B. Fischer. 2012. Effects of di(2-ethylhexyl) phthalate (DEHP) on female fertility and adipogenesis in C3H/N mice. *Environ Health Perspect* 120 (8):1123-9.

Schopfer, F. J., Y. Lin, P. R. Baker, T. Cui, M. Garcia-Barrio, J. Zhang, K. Chen, Y. E. Chen, and B. A. Freeman. 2005. Nitrolinoleic acid: an endogenous

peroxisome proliferator-activated receptor gamma ligand. *Proc Natl Acad Sci U S A* 102 (7):2340-5.

Schupp, M., and M. A. Lazar. 2010. Endogenous ligands for nuclear receptors: digging deeper. *J Biol Chem* 285 (52):40409-15.

Shukla, S. J., S. Sakamuru, R. Huang, T. A. Moeller, P. Shinn, D. Vanleer, D. S. Auld, C. P. Austin, and M. Xia. 2011. Identification of clinically used drugs that activate pregnane X receptors. *Drug Metab Dispos* 39 (1):151-9.

Siccardi, M., A. D'Avolio, L. Baietto, S. Gibbons, M. Sciandra, D. Colucci, S. Bonora, S. Khoo, D. J. Back, G. Di Perri, and A. Owen. 2008. Association of a single-nucleotide polymorphism in the pregnane X receptor (PXR 63396C-->T) with reduced concentrations of unboosted atazanavir. *Clin Infect Dis* 47 (9):1222-5.

Sinz, M., S. Kim, Z. Zhu, T. Chen, M. Anthony, K. Dickinson, and A. D. Rodrigues. 2006. Evaluation of 170 xenobiotics as transactivators of human pregnane X receptor (hPXR) and correlation to known CYP3A4 drug interactions. *Curr Drug Metab* 7 (4):375-88.

Skaare, J., A. Bernhoft, A. Derocher, G. Gabrielsen, A. Goksøyr, E. Henriksen, H. Larsen, E. Lie, and Wiig. 2000. Organochlorines in top predators at Svalbard--occurrence, levels and effects. *Toxicology letters* 112-113:103-109.

Sladek, F. M. 2011. What are nuclear receptor ligands? *Mol Cell Endocrinol* 334 (1-2):3-13.

Smit-McBride, Z., and M. L. Privalsky. 1994. DNA sequence specificity of the v-erb A oncoprotein/thyroid hormone receptor: role of the P-box and its interaction with more N-terminal determinants of DNA recognition. *Mol Endocrinol* 8 (7):819-28.

Smith, R. P., W. L. Eckalbar, K. M. Morrissey, M. R. Luizon, T. J. Hoffmann, X. Sun, S. L. Jones, S. Force Aldred, A. Ramamoorthy, Z. Desta, Y. Liu, T. C. Skaar, N. D. Trinklein, K. M. Giacomini, and N. Ahituv. 2014. Genome-wide discovery of drug-dependent human liver regulatory elements. *PLoS Genet* 10 (10):e1004648.

Smutny, T., M. Bitman, M. Urban, M. Dubecka, R. Vrzal, Z. Dvorak, and P. Pavek. 2014. U0126, a mitogen-activated protein kinase kinase 1 and 2 (MEK1 and 2) inhibitor, selectively up-regulates main isoforms of CYP3A subfamily via a pregnane X receptor (PXR) in HepG2 cells. *Arch Toxicol* 88 (12):2243-59.

Sonne, C., R. J. Letcher, TØ Bechshøft, F. F. Rigét, D. C. G. Muir, P. S. Leifsson, E. W. Born, L. Hyldstrup, N. Basu, and M. Kirkegaard. 2012. Two decades of biomonitoring polar bear health in Greenland: a review. *Acta Veterinaria Scandinavica* 54:1-7.

Sonne, Christian. 2010. Health effects from long-range transported contaminants in Arctic top predators: An integrated review based on studies of polar bears and relevant model species. *Environment international* 36 (5):461-491.

Sookoian, S., G. O. Castano, A. L. Burgueno, T. F. Gianotti, M. S. Rosselli, and C. J. Pirola. 2010. The nuclear receptor PXR gene variants are associated with liver injury in nonalcoholic fatty liver disease. *Pharmacogenet Genomics* 20 (1):1-8.

Spinella-Jaegle, S., G. Rawadi, S. Kawai, S. Gallea, C. Faucheu, P. Mollat, B. Courtois, B. Bergaud, V. Ramez, A. M. Blanchet, G. Adelmant, R. Baron, and S. Roman-Roman. 2001. Sonic hedgehog increases the commitment of pluripotent mesenchymal cells into the osteoblastic lineage and abolishes adipocytic differentiation. *J Cell Sci* 114 (Pt 11):2085-94.

Srivastava, M., E. Begovic, J. Chapman, N. H. Putnam, U. Hellsten, T. Kawashima, A. Kuo, T. Mitros, A. Salamov, M. L. Carpenter, A. Y. Signorovitch, M. A. Moreno, K. Kamm, J. Grimwood, J. Schmutz, H. Shapiro, I. V. Grigoriev, L. W. Buss, B. Schierwater, S. L. Dellaporta, and D. S. Rokhsar. 2008. The Trichoplax genome and the nature of placozoans. *Nature* 454 (7207):955-60.

Staudinger, J. L., B. Goodwin, S. A. Jones, D. Hawkins-Brown, K. I. MacKenzie, A. LaTour, Y. Liu, C. D. Klaassen, K. K. Brown, J. Reinhard, T. M. Willson, B. H. Koller, and S. A. Kliewer. 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc Natl Acad Sci U S A* 98 (6):3369-74.

Stegeman, J., JV. Goldstone, and ME. Hahn. 2010. Perspectives on zebrafish as a model in environmental toxicology. In *Fish Physiology*, edited by A. Farrell and C. Brauner: Elsevier.

Sugatani, J., S. Nishitani, K. Yamakawa, K. Yoshinari, T. Sueyoshi, M. Negishi, and M. Miwa. 2005. Transcriptional regulation of human UGT1A1 gene expression: activated glucocorticoid receptor enhances constitutive androstane receptor/pregnane X receptor-mediated UDP-glucuronosyltransferase 1A1 regulation with glucocorticoid receptor-interacting protein 1. *Mol Pharmacol* 67 (3):845-55.

Swanson, J. E., R. N. Ben, G. W. Burton, and R. S. Parker. 1999. Urinary excretion of 2,7, 8-trimethyl-2-(beta-carboxyethyl)-6-hydroxychroman is a major route of elimination of gamma-tocopherol in humans. *J Lipid Res* 40 (4):665-71.

Swart, M., H. Whitehorn, Y. Ren, P. Smith, R. S. Ramesar, and C. Dandara. 2012. PXR and CAR single nucleotide polymorphisms influence plasma efavirenz levels in South African HIV/AIDS patients. *BMC Med Genet* 13:112.

Symonds, M. E., A. Mostyn, S. Pearce, H. Budge, and T. Stephenson. 2003. Endocrine and nutritional regulation of fetal adipose tissue development. *J Endocrinol* 179 (3):293-9.

Tabb, Michelle, Vladyslav Kholodovych, Felix Gr√ºn, Changcheng Zhou, William Welsh, and Bruce Blumberg. 2004. Highly chlorinated PCBs inhibit the human xenobiotic response mediated by the steroid and xenobiotic receptor (SXR). *Environmental health perspectives* 112 (2):163-172.

Tachibana, K., D. Yamasaki, K. Ishimoto, and T. Doi. 2008. The Role of PPARs in Cancer. *PPAR Res* 2008:102737.

Takashima, K., Y. Ito, F. J. Gonzalez, and T. Nakajima. 2008. Different mechanisms of DEHP-induced hepatocellular adenoma tumorigenesis in wild-type and Ppar alpha-null mice. *J Occup Health* 50 (2):169-80.

Takeuchi, S., T. Matsuda, S. Kobayashi, T. Takahashi, and H. Kojima. 2006. In vitro screening of 200 pesticides for agonistic activity via mouse peroxisome proliferator-activated receptor (PPAR)alpha and PPARgamma and quantitative analysis of in vivo induction pathway. *Toxicol Appl Pharmacol* 217 (3):235-44.

Tan, N. S., L. Michalik, N. Noy, R. Yasmin, C. Pacot, M. Heim, B. Fluhmann, B. Desvergne, and W. Wahli. 2001. Critical roles of PPAR beta/delta in keratinocyte response to inflammation. *Genes Dev* 15 (24):3263-77.

Tanaka, T., J. Yamamoto, S. Iwasaki, H. Asaba, H. Hamura, Y. Ikeda, M. Watanabe, K. Magoori, R. X. Ioka, K. Tachibana, Y. Watanabe, Y. Uchiyama, K. Sumi, H. Iguchi, S. Ito, T. Doi, T. Hamakubo, M. Naito, J. Auwerx, M. Yanagisawa, T. Kodama, and J. Sakai. 2003. Activation of peroxisome proliferator-activated receptor delta induces fatty acid beta-oxidation in

skeletal muscle and attenuates metabolic syndrome. *Proc Natl Acad Sci U S A* 100 (26):15924-9.

Tang, Q. Q., T. C. Otto, and M. D. Lane. 2004. Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. *Proc Natl Acad Sci U S A* 101 (26):9607-11.

Taxvig, C., K. Dreisig, J. Boberg, C. Nellemann, A. B. Schelde, D. Pedersen, M. Boergesen, S. Mandrup, and A. M. Vinggaard. 2012. Differential effects of environmental chemicals and food contaminants on adipogenesis, biomarker release and PPARgamma activation. *Mol Cell Endocrinol* 361 (1-2):106-15.

Teotico, D. G., J. J. Bischof, L. Peng, S. A. Kliewer, and M. R. Redinbo. 2008. Structural basis of human pregnane X receptor activation by the hops constituent colupulone. *Mol Pharmacol* 74 (6):1512-20.

Thiemann, G. W., S. J. Iverson, and I. Stirling. 2008. POLAR BEAR DIETS AND ARCTIC MARINE FOOD WEBS: INSIGHTS FROM FATTY ACID ANALYSIS. *Ecological Monographs* 78 (4):591-613.

Thiemann, GW, SJ Iverson, and I. Stirling. 2006. Seasonal, sexual and anatomical variability in the adipose tissue of polar bears (Ursus maritimus). *Journal of Zoology* (269):65-76.

Tirona, R. G., B. F. Leake, L. M. Podust, and R. B. Kim. 2004. Identification of amino acids in rat pregnane X receptor that determine species-specific activation. *Mol Pharmacol* 65 (1):36-44.

Tolson, A. H., and H. Wang. 2010. Regulation of drug-metabolizing enzymes by xenobiotic receptors: PXR and CAR. *Adv Drug Deliv Rev* 62 (13):1238-49.

Tomura, H., J. Lazar, M. Phyillaier, and V. M. Nikodem. 1995. The N-terminal region (A/B) of rat thyroid hormone receptors alpha 1, beta 1, but not beta 2 contains a strong thyroid hormone-dependent transactivation function. *Proc Natl Acad Sci U S A* 92 (12):5600-4.

Tontonoz, P., R. A. Graves, A. I. Budavari, H. Erdjument-Bromage, M. Lui, E. Hu, P. Tempst, and B. M. Spiegelman. 1994. Adipocyte-specific transcription factor ARF6 is a heterodimeric complex of two nuclear hormone receptors, PPAR gamma and RXR alpha. *Nucleic Acids Res* 22 (25):5628-34.

Tontonoz, P., and B. M. Spiegelman. 2008. Fat and beyond: the diverse biology of PPARgamma. *Annu Rev Biochem* 77:289-312.

Tsukahara, T., R. Tsukahara, Y. Fujiwara, J. Yue, Y. Cheng, H. Guo, A. Bolen, C. Zhang, L. Balazs, F. Re, G. Du, M. A. Frohman, D. L. Baker, A. L. Parrill, A. Uchiyama, T. Kobayashi, K. Murakami-Murofushi, and G. Tigyi. 2010. Phospholipase D2-dependent inhibition of the nuclear hormone receptor PPARgamma by cyclic phosphatidic acid. *Mol Cell* 39 (3):421-32.

Uppal, H., D. Toma, S. P. Saini, S. Ren, T. J. Jones, and W. Xie. 2005. Combined loss of orphan receptors PXR and CAR heightens sensitivity to toxic bile acids in mice. *Hepatology* 41 (1):168-76.

Verreault, J., F. Maisonneuve, R. Dietz, C. Sonne, and R. J. Letcher. 2009. Comparative hepatic activity of xenobiotic-metabolizing enzymes and concentrations of organohalogens and their hydroxylated analogues in captive Greenland sledge dogs (Canis familiaris). *Environ Toxicol Chem* 28 (1):162-72.

Verreault, J., R. J. Norstrom, M. A. Ramsay, M. Mulvihill, and R. J. Letcher. 2006. Composition of chlorinated hydrocarbon contaminants among major adipose tissue depots of polar bears (Ursus maritimus) from the Canadian high Arctic. *Sci Total Environ* 370 (2-3):580-7.

Verreault, Jonathan, Rune Dietz, Christian Sonne, Wouter Gebbink, Soheila Shahmiri, and Robert Letcher. 2008. Comparative fate of organohalogen contaminants in two top carnivores in Greenland: captive sledge dogs and wild polar bears. *Comparative biochemistry and physiology. Toxicology & pharmacology : CBP* 147 (3):306-315.

Wager, K., and C. Russell. 2013. Mitophagy and neurodegeneration: the zebrafish model system. *Autophagy* 9 (11):1693-709.

Wagner, R. L., J. W. Apriletti, M. E. McGrath, B. L. West, J. D. Baxter, and R. J. Fletterick. 1995. A structural role for hormone in the thyroid hormone receptor. *Nature* 378 (6558):690-7.

Waku, T., T. Shiraki, T. Oyama, K. Maebara, R. Nakamori, and K. Morikawa. 2010. The nuclear receptor PPARgamma individually responds to serotonin- and fatty acid-metabolites. *EMBO J* 29 (19):3395-407.

Walogorsky, M., R. Mongeon, H. Wen, N. R. Nelson, J. M. Urban, F. Ono, G. Mandel, and P. Brehm. 2012. Zebrafish model for congenital myasthenic syndrome reveals mechanisms causal to developmental recovery. *Proc Natl Acad Sci U S A* 109 (43):17711-6.

Wang, H., S. Faucette, T. Sueyoshi, R. Moore, S. Ferguson, M. Negishi, and E. L. LeCluyse. 2003. A novel distal enhancer module regulated by pregnane X receptor/constitutive androstane receptor is essential for the maximal induction of CYP2B6 gene expression. *J Biol Chem* 278 (16):14146-52.

Wang, T., X. Ma, K. W. Krausz, J. R. Idle, and F. J. Gonzalez. 2008. Role of pregnane X receptor in control of all-trans retinoic acid (ATRA) metabolism and its potential contribution to ATRA resistance. *J Pharmacol Exp Ther* 324 (2):674-84.

Wang, Z., G. Benoit, J. Liu, S. Prasad, P. Aarnisalo, X. Liu, H. Xu, N. P. Walker, and T. Perlmann. 2003. Structure and function of Nurr1 identifies a class of ligand-independent nuclear receptors. *Nature* 423 (6939):555-60.

Watkins, R. E., J. M. Maglich, L. B. Moore, G. B. Wisely, S. M. Noble, P. R. Davis-Searles, M. H. Lambert, S. A. Kliewer, and M. R. Redinbo. 2003. 2.1 A crystal structure of human PXR in complex with the St. John's wort compound hyperforin. *Biochemistry* 42 (6):1430-8.

Watkins, R. E., S. M. Noble, and M. R. Redinbo. 2002. Structural insights into the promiscuity and function of the human pregnane X receptor. *Curr Opin Drug Discov Devel* 5 (1):150-8.

Watkins, R. E., G. B. Wisely, L. B. Moore, J. L. Collins, M. H. Lambert, S. P. Williams, T. M. Willson, S. A. Kliewer, and M. R. Redinbo. 2001. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. *Science* 292 (5525):2329-33.

Watkins, Ryan, Paula Davis-Searles, Mill Lambert, and Matthew Redinbo. 2003. Coactivator binding promotes the specific interaction between ligand and the pregnane X receptor. *Journal of molecular biology* 331 (4):815-828.

Werman, A., A. Hollenberg, G. Solanes, C. Bjorbaek, A. J. Vidal-Puig, and J. S. Flier. 1997. Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor gamma (PPARgamma). Differential activity of PPARgamma1 and -2 isoforms and influence of insulin. *J Biol Chem* 272 (32):20230-5.

Williams, J. A., R. Hyland, B. C. Jones, D. A. Smith, S. Hurst, T. C. Goosen, V. Peterkin, J. R. Koup, and S. E. Ball. 2004. Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for

typically observed low exposure (AUCi/AUC) ratios. *Drug Metab Dispos* 32 (11):1201-8.

Wisely, G. B., A. B. Miller, R. G. Davis, A. D. Thornquest, Jr., R. Johnson, T. Spitzer, A. Sefler, B. Shearer, J. T. Moore, A. B. Miller, T. M. Willson, and S. P. Williams. 2002. Hepatocyte nuclear factor 4 is a transcription factor that constitutively binds fatty acids. *Structure* 10 (9):1225-34.

Wu, Z., E. D. Rosen, R. Brun, S. Hauser, G. Adelmant, A. E. Troy, C. McKeon, G. J. Darlington, and B. M. Spiegelman. 1999. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol Cell* 3 (2):151-8.

Wurtz, J. M., W. Bourguet, J. P. Renaud, V. Vivat, P. Chambon, D. Moras, and H. Gronemeyer. 1996. A canonical structure for the ligand-binding domain of nuclear receptors. *Nat Struct Biol* 3 (1):87-94.

Xie, W., A. Radominska-Pandya, Y. Shi, C. M. Simon, M. C. Nelson, E. S. Ong, D. J. Waxman, and R. M. Evans. 2001. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc Natl Acad Sci U S A* 98 (6):3375-80.

Xin, X., S. Yang, J. Kowalski, and M. E. Gerritsen. 1999. Peroxisome proliferator-activated receptor gamma ligands are potent inhibitors of angiogenesis in vitro and in vivo. *J Biol Chem* 274 (13):9116-21.

Xu, C., X. Wang, and J. L. Staudinger. 2009. Regulation of tissue-specific carboxylesterase expression by pregnane x receptor and constitutive androstane receptor. *Drug Metab Dispos* 37 (7):1539-47.

Xu, H. E., M. H. Lambert, V. G. Montana, D. J. Parks, S. G. Blanchard, P. J. Brown, D. D. Sternbach, J. M. Lehmann, G. B. Wisely, T. M. Willson, S. A. Kliewer, and M. V. Milburn. 1999. Molecular recognition of fatty acids by peroxisome proliferator-activated receptors. *Mol Cell* 3 (3):397-403.

Xue, Y., L. B. Moore, J. Orans, L. Peng, S. Bencharit, S. A. Kliewer, and M. R. Redinbo. 2007. Crystal structure of the pregnane X receptor-estradiol complex provides insights into endobiotic recognition. *Mol Endocrinol* 21 (5):1028-38.

Yagi, K., Y. Satou, F. Mazet, S. M. Shimeld, B. Degnan, D. Rokhsar, M. Levine, Y. Kohara, and N. Satoh. 2003. A genomewide survey of developmentally relevant genes in Ciona intestinalis. III. Genes for Fox, ETS, nuclear receptors and NFkappaB. *Dev Genes Evol* 213 (5-6):235-44.

Yang, J. Y., M. A. Della-Fera, S. Rayalam, S. Ambati, D. L. Hartzell, H. J. Park, and C. A. Baile. 2008. Enhanced inhibition of adipogenesis and induction of apoptosis in 3T3-L1 adipocytes with combinations of resveratrol and quercetin. *Life Sci* 82 (19-20):1032-9.

Yang, X., M. Downes, R. T. Yu, A. L. Bookout, W. He, M. Straume, D. J. Mangelsdorf, and R. M. Evans. 2006. Nuclear receptor expression links the circadian clock to metabolism. *Cell* 126 (4):801-10.

Yin, L., N. Wu, J. C. Curtin, M. Qatanani, N. R. Szwergold, R. A. Reid, G. M. Waitt, D. J. Parks, K. H. Pearce, G. B. Wisely, and M. A. Lazar. 2007. Rev-erbalpha, a heme sensor that coordinates metabolic and circadian pathways. *Science* 318 (5857):1786-9.

Yin, L., K. S. Yu, K. Lu, and X. Yu. 2016. Benzyl butyl phthalate promotes adipogenesis in 3T3-L1 preadipocytes: A High Content Cellomics and metabolomic analysis. *Toxicol In Vitro* 32:297-309.

You, L., S. K. Chan, J. M. Bruce, S. Archibeque-Engle, M. Casanova, J. C. Corton, and H. Heck. 1999. Modulation of testosterone-metabolizing hepatic cytochrome P-450 enzymes in developing Sprague-Dawley rats following in utero exposure to p,p'-DDE. *Toxicol Appl Pharmacol* 158 (2):197-205.

Zhai, Y., H. V. Pai, J. Zhou, J. A. Amico, R. R. Vollmer, and W. Xie. 2007. Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. *Mol Endocrinol* 21 (1):138-47.

Zhang, H. Y., W. Y. Xue, Y. Y. Li, Y. Ma, Y. S. Zhu, W. Q. Huo, B. Xu, W. Xia, and S. Q. Xu. 2014. Perinatal exposure to 4-nonylphenol affects adipogenesis in first and second generation rats offspring. *Toxicol Lett* 225 (2):325-32.

Zhang, J., P. Kuehl, E. D. Green, J. W. Touchman, P. B. Watkins, A. Daly, S. D. Hall, P. Maurel, M. Relling, C. Brimer, K. Yasuda, S. A. Wrighton, M. Hancock, R. B. Kim, S. Strom, K. Thummel, C. G. Russell, J. R. Hudson, Jr., E. G. Schuetz, and M. S. Boguski. 2001. The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* 11 (7):555-72.

Zhang, Z., P. E. Burch, A. J. Cooney, R. B. Lanz, F. A. Pereira, J. Wu, R. A. Gibbs, G. Weinstock, and D. A. Wheeler. 2004. Genomic analysis of the nuclear receptor family: new insights into structure, regulation, and evolution from the rat genome. *Genome Res* 14 (4):580-90.

Zhou, C., M. M. Tabb, E. L. Nelson, F. Grun, S. Verma, A. Sadatrafiei, M. Lin, S. Mallick, B. M. Forman, K. E. Thummel, and B. Blumberg. 2006. Mutual repression between steroid and xenobiotic receptor and NF-kappaB signaling pathways links xenobiotic metabolism and inflammation. *J Clin Invest* 116 (8):2280-2289.

Zhou, J., Y. Zhai, Y. Mu, H. Gong, H. Uppal, D. Toma, S. Ren, R. M. Evans, and W. Xie. 2006. A novel pregnane X receptor-mediated and sterol regulatory element-binding protein-independent lipogenic pathway. *J Biol Chem* 281 (21):15013-20.

Zilliacus, J., A. P. Wright, J. Carlstedt-Duke, and J. A. Gustafsson. 1995. Structural determinants of DNA-binding specificity by steroid receptors. *Mol Endocrinol* 9 (4):389-400.

Zwart, W., R. de Leeuw, M. Rondaij, J. Neefjes, M. A. Mancini, and R. Michalides. 2010. The hinge region of the human estrogen receptor determines functional synergy between AF-1 and AF-2 in the quantitative response to estradiol and tamoxifen. *J Cell Sci* 123 (Pt 8):1253-61.

Paper I

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Environmental contaminants activate human and polar bear (*Ursus maritimus*) pregnane X receptors (PXR, NR1I2) differently

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ABSTRACT

Background: Many persistent organic pollutants (POPs) accumulate readily in polar bears because of their position as apex predators in Arctic food webs. The pregnane X receptor (PXR, formally NR1I2, here proposed to be named promiscuous xenobiotic receptor) is a xenobiotic sensor that is directly involved in metabolizing pathways of a wide range of environmental contaminants.
Objectives: In the present study, we comparably assess the ability of 51 selected pharmaceuticals, pesticides and emerging contaminants to activate PXRs from polar bears and humans using an in vitro luciferase reporter gene assay.
Results: We found that polar bear PXR is activated by a wide range of our test compounds (68%) but has a slightly more narrow ligand specificity than human PXR that was activated by 86% of the 51 test compounds. The majority of the agonists identified (70%) produces a stronger induction of the reporter gene via human PXR than via polar bear PXR, however with some notable and environmentally relevant exceptions.
Conclusions: Due to the observed differences in activation of polar bear and human PXRs, exposure of each species to environmental agents is likely to induce biotransformation differently in the two species. Bioinformatics analyses and structural modeling studies suggest that amino acids that are not part of the ligand-binding domain and do not interact with the ligand can modulate receptor activation.

Introduction

As a top predator in the Arctic, polar bears (*Ursus maritimus*) accumulate environmental pollutants efficiently through their diet, and carry some of the highest concentrations of manmade chemicals seen in mammals (Norén et al., 1999; Sonne et al., 2012; Verreault et al., 2006). The populations of polar bears in Russia, East Greenland and Svalbard bear the highest burdens of persistent organic pollutants (POPs) compared to polar bears from other areas (Norstrom et al., 1998; Verreault et al., 2005) and the concentrations are significantly higher in polar bears than in humans (Kim et al., 2011; Verreault et al., 2006). In both species, the most prevalent POPs in blood are polychlorinated biphenyls (PCBs) (Bytingsvik et al., 2012; Kim et al., 2011; Salihovic et al., 2012; Skaare et al., 2000), while brominated flame retardants (BFRs) and pesticides (except chlordanes) appear to be less prevalent in both species (Bentzen et al., 2008; Goncharov et al., 2011; Lind et al., 2012; Salihovic et al., 2012; Verreault et al., 2008). Multiple studies have observed correlations between concentrations of organohalogen compounds (OHCs) and adverse effects, including repression of humoral and cellular immunity (Bernhoft et al., 2000; Lie et al., 2004, 2005), disruption of endocrine function (Braathen et al., 2004; Haave et al., 2003; Verreault et al., 2009), and tissue pathology [reviewed in (Sonne, 2010)] in polar bears. Polar bears possess the capacity to metabolize OHCs, such as certain PCBs and organochlorine pesticides (OCPs) like chlordane and dichlorodiphenyltrichloroethane (DDT). Evidence for this capacity is the low bioaccumulation factors from seal to bear seen for some OHCs, differences in PCB chlorination pattern observed in bears and prey, relatively high liver cytochrome P450 monooxygenase (CYP) activities and the depletion of hexabromocyclododecane (HBCD) in polar bear hepatic microsomes

Abbreviations: CMV, cytomegalovirus; DBD, DNA-binding domain; GAL4, yeast regulatory protein GAL4; hPXR, human PXR; LBD, ligand-binding domain; NR1I2, nuclear receptor subfamily 1 group I member 2, a. k. a pregnane X receptor; pbPXR, polar bear PXR; PXR, pregnane X receptor; REC_{h_20}, the concentration of agonist that produces a response equal to 20% of the maximum hPXR induced luciferase response to rifampicin; SXR, steroid and xenobiotic receptor; UAS, up-stream activation sequence.

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(Kannan et al., 2005; Letcher et al., 2009; Muir et al., 1988). In contrast, other studies have reported that polar bears appear to be limited in their capacity to metabolize polybrominated diphenyl ethers (PBDEs) (Letcher et al., 2009; McKinney et al., 2011).

Although xenobiotic metabolism is generally protective, some chemicals that undergo biotransformation are converted into more toxic compounds. Relevant examples of this are hydroxylated and methyl sulfone metabolites of PCBs (OH- and Me-PCBs) that have been shown to have anti-estrogenic effects in vitro (Letcher et al., 2002) and to affect thyroid hormone homeostasis in polar bears (Brouwer et al., 1990; Sandau et al., 2000). Positive correlation between the concentration of PCBs and the expression and activity of CYP1A- and CYP2B-like proteins in polar bear liver suggest that exposure to xenobiotics induces biotransformation in polar bears (Bandiera et al., 1997; Letcher et al., 1996).

The induction of biotransformation enzymes is largely mediated by three transcription factors, all of which act as xenosensors: the aryl hydrocarbon receptor (AHR), the pregnane X receptor (aka steroid and xenobiotic receptor: PXR/SXR, formally NR1I2) and the constitutive androstane receptor (CAR, formally NR1I3) [reviewed in (Kohle and Bock, 2009)]. Of these, PXR has the highest number of ligands and the greatest number of target genes, including numerous genes involved in the initial redox-reactions, conjugations and eventually excretion (Orans et al., 2005; Rosenfeld et al., 2003). Changes in the composition of endogenous ligands such as bile acids and/or differing exposure to exogenous compounds have been suggested as driving forces for the unusually large divergence among PXR orthologs, especially in the ligand binding domain (Krasowski et al., 2005b). This large sequence divergence has been linked to species-specific ligand-dependent activation that is evident among PXR orthologs (as reported by e.g. (Ekins et al., 2008; Krasowski et al., 2005a; Milnes et al., 2008)).

The ability to extrapolate toxicological responses in model species to other species is highly desirable; however, most data are of limited value for this purpose without a better understanding of species-specific nuances in the response of interest. The identification of molecular response pathways (or adverse effect pathways) and detailed understanding of similarity and differences in protein function have been emphasized (Celander et al., 2011). Knowledge about how divergence in PXR amino acid composition may affect ligand preference and activation, and possibly molecular response pathways, is needed to perform meaningful extrapolations. Several different classes of environmental pollutants bind and activate human PXR (Al-Salman and Plant, 2012; Kojima et al., 2011; Milnes et al., 2008). To link this knowledge to the activation of polar bear PXR, we compared the ligand activation of the PXR orthologs from humans and polar bears by selected environmental pollutants, and assessed functional differences on the basis of sequence and structural homology of human and polar bear PXRs.

Methods

Pharmaceuticals and environmental pollutants as PXR agonists. Fifty-one compounds were surveyed for their ability to activate human and polar bear PXRs, including pharmaceutical drugs, PCBs, BFRs, siloxanes, OCPs and other environmentally relevant compounds (Table 1). With the exception of two coplanar congeners (CB118 and CB190), all of the 15 polychlorinated biphenyls used were non-dioxin-like (NDL, CB28, −47, −52, −60, −97, −99, −101, −138, −151, −153, −170, −180, −183 and −184). Nine of the PCBs used (CB28, −47, −52, −101, −118, −138, −170, −180 and −190) had been highly purified as previously described (Danielsson et al., 2008) and were kindly provided by Krister Halldin and Helen Håkansson (ATHON project, Karolinska Institute, Stockholm, Sweden). Five NDL-PCBs, CB60, −97, −151, −183 and −184, were purchased from AccuStandard Inc. (≥99% purity, New Haven, USA) and CB153 from ChemService Inc. (98.3% purity, West Chester, USA). Individual PBDEs (BDE28, −47, −99, −100, −153), a DE-71 pentaBDE mixture and a technical mixture of HBCD, all purified to >99% purity (Hamers et al., 2006), were gifts from Åke Bergman (FIRE project, Stockholm University, Sweden). The main constituents and composition of the purified DE-71 pentaBDE mixture was BDE47 (42%), −99 (34%), −100 (9%), −153 (2%) and −154 (2%) (van der Ven et al., 2008), somewhat different than reported for the commercial DE-71 BDE47 (28%), −99 (43%), −100 (8%), −153 (6%) and −154 (4%) (Pohl et al., 2004; van der Ven et al., 2008). BDE209 was purchased from Chiron AS (>99.5% purity, Trondheim, Norway). All other compounds were purchased from Sigma Aldrich Inc. (St. Louis, USA). The endosulfan tested contained α- and β-endosulfan in the ratio 2:1. All chemicals were dissolved in dimethyl sulfoxide (DMSO) supplied by Sigma Aldrich Inc. (Cat. No D2650).

Cloning of polar bear PXR. The polar bear *PXR* was cloned from liver total RNA kindly provided by Dr Robert J. Letcher (National Wildlife Research Center, Carleton University, Canada). Complementary DNA (cDNA) was synthesized from 0.5 μg RNA (Superscript II RT, Invitrogen) and used as template in degenerate PCR to amplify a part of the polar bear *PXR* flanked by regions highly conserved between mammalian *PXR* orthologs. The 5′- and 3′-sequences missing in the partially amplified cDNA were obtained by rapid amplification of cDNA ends (RACE) (SMART RACE cDNA amplification kit, Clontech Laboratories, Mountain View, CA).

Sequence homology and similarity analysis. To assess evolutionary conservation of the cloned cDNA from a polar bear, known full-length NR1I2 amino acid sequences and the predicted polar bear PXR (pbPXR) candidate were aligned using ClustalW2 (Larkin et al., 2007) and a phylogenetic tree was constructed by maximum likelihood using RAxML (v7.2.6) with the PROTWAGCAT model of amino acid substitution (Stamatakis, 2006). The accession numbers of the nuclear receptor LBD-sequences used in the phylogenetic analysis are listed in Supplemental Table S1. Alignments were edited and visualized in Jalview (Waterhouse et al., 2009) and Bioedit (Hall, 1999). Similarity and identity analyses were performed using the Sequence identity and similarity (SIAS) resource utilizing a BLOSUM62 matrix (Reche, 2008).

Luciferase reporter transactivation assays. In vitro transactivation assays were performed in COS-7 cells co-transfected with a luciferase reporter plasmid regulated by a thymidine kinase promoter with a Gal4 upstream activation sequence (Gal4-UAS) (tk(MH100)x4 luc; (Forman et al., 1995)), a CMV-promoter based plasmid constitutively expressing β-galactosidase to monitor toxicity and transfection efficiencies (pCMV-β-galactosidase; (Blumberg et al., 1998)) and an effector plasmid expressing a chimeric protein of yeast Gal4-DNA-binding domain (DBD; AA1-147; NM_001184062) and PXR ligand-binding domain (LBD), also driven by a CMV-promoter. While an effector plasmid expressing Gal4-DBD and human PXR-LBD was available to us (Blumberg et al., 1998), a plasmid encoding the yeast Gal4-DBD and polar bear PXR LBD was constructed by replacing the human PXR (AA107-434; NP_003880.3) reading frame with a polar bear PXR (AA107-434; GenBank: KM067117) reading frame using existing EcoRI and BamHI sites in the effector plasmid.

COS-7 simian kidney cells were maintained in phenol red Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamate, 1 mM sodium pyruvate at 37 °C with 5% carbon dioxide (CO_2). Microbial contamination in the growth media was prevented by adding penicillin and streptomycin to the medium to concentrations of 100 U/mL.

Transactivation assays for each compound were performed in triplicates for each concentration in at least three independent experiments. Cells were harvested at approximately 70–80% confluence, seeded in 96-well plates at a density of $5 * 10^3$ cells/well and cultivated for 24 h prior to transfection. The cells were then co-transfected with 500 ng of the effector plasmid encoding the PXR-LBD-Gal4-DBD chimeric

Table 1
Overview of test panel. Overview of the 51-compound panel used to test for agonistic activity on human and polar bear PXRs in an in vitro ligand activation assay.

Compound name	Supplier	Product number	Cas no	Mol. mass (g/mol)	Formula
Pharmaceuticals					
Rifampicin	Sigma Aldrich	R3501	13292-46-1	822.94	$C_{43}H_{58}N_4O_{12}$
SR12813[a]	Sigma Aldrich	S4194	126411-39-0	504.53	$C_{24}H_{42}O_7P_2$
Carbamazepine	Sigma Aldrich	C4024	298-46-4	236.27	$C_{15}H_{12}N_2O$
Clotrimazole	Sigma Aldrich	C6014	23593-75-1	344.84	$C_{22}H_{17}ClN_2$
Ketoconazole	Sigma Aldrich	K1003	65277-42-1	531.43	$C_{26}H_{28}Cl_2N_4O_4$
Omeprazole	Sigma Aldrich	O104	73590-58-6	345.42	$C_{17}H_{19}N_3O_3S$
Pesticides					
Methoxychlor	Sigma Aldrich	M1501	72-43-5	345.65	$C_{16}H_{15}Cl_3O_2$
Dieldrin	Sigma Aldrich	33491	60-57-1	380.91	$C_{12}H_8Cl_6O$
Chlordane	Sigma Aldrich	45378	12789-03-6	409.78	$C_{10}H_6Cl_8$
Pentachlorophenol	Sigma Aldrich	P2604	87-86-5	266.34	C_6HCl_5O
Toxaphene	Sigma Aldrich	PS79	8001-35-2	411.79	$C_{10}H_8Cl_8$
Endosulfan (α + β ~ 2:1)	Sigma Aldrich	32015	115-29-7	406.93	$C_9H_6Cl_6O_3S$
α-hexachlorocyclohexane (α-HCH)	Sigma Aldrich	33856	319-84-6	290.83	$C_6H_6Cl_6$
Lindane (γ-HCH)	Sigma Aldrich	45548	58-89-9	290.83	$C_6H_6Cl_6$
Vinclozolin	Sigma Aldrich	45705	50471-44-8	286.11	$C_{12}H_9Cl_2NO_3$
4,4′-DDT[b]	Sigma Aldrich	31041	50-29-3	354.49	$C_{14}H_9Cl_5$
4,4′-DDE[c]	Sigma Aldrich	35487	72-55-9	318.03	$C_{14}H_8Cl_4$
1,2,3-trichlorobenzene (1,2,3-TCB)	Sigma Aldrich	36742	87-61-6	181.45	$C_6H_3Cl_3$
1,2,4-trichlorobenzene (1,2,4-TCB)	Sigma Aldrich	36627	120-82-1	181.45	$C_6H_3Cl_3$
Polychlorinated biphenyls					
PCB 28	ATHON project		7012-37-5	257.54	$C_{12}H_7Cl_3$
PCB 47	ATHON project		2437-79-8	291.99	$C_{12}H_6Cl_4$
PCB 52	ATHON project		35693-99-3	291.99	$C_{12}H_6Cl_4$
PCB 60	AccuStandard	C060N	33025-41-1	291.10	$C_{12}H_6Cl_4$
PCB 97	AccuStandard	C097N	41464-51-1	326.43	$C_{12}H_5Cl_5$
PCB 101	ATHON project		37680-73-2	326.43	$C_{12}H_5Cl_5$
PCB 118	ATHON project		57465-28-8	326.43	$C_{12}H_5Cl_5$
PCB 138	ATHON project		35065-28-2	360.88	$C_{12}H_4Cl_6$
PCB 151	AccuStandard	C151N	52663-63-5	360.88	$C_{12}H_4Cl_6$
PCB 153	ChemService	5019C	35065-27-1	360.88	$C_{12}H_4Cl_6$
PCB 170	ATHON project		35065-30-6	395.32	$C_{12}H_3Cl_7$
PCB 180	ATHON project		35065-29-3	395.32	$C_{12}H_3Cl_7$
PCB 183	AccuStandard	C183N	52663-69-1	395.32	$C_{12}H_3Cl_7$
PCB 184	AccuStandard	C184N	74472-48-3	395.32	$C_{12}H_3Cl_7$
PCB 190	ATHON project		41411-64-7	395.32	$C_{12}H_3Cl_7$
Brominated flame retardants					
BDE 28	FIRE project		2050-47-7	328.00	$C_{12}H_8Br_2O$
BDE 47	FIRE project		5436-43-1	485.79	$C_{12}H_6Br_4O$
BDE 99	FIRE project		60348-60-9	564.69	$C_{12}H_5Br_5O$
BDE 100	FIRE project		189084-64-8	564.69	$C_{12}H_5Br_5O$
BDE 153	FIRE project		68631-49-2	643.58	$C_{12}H_4Br_6O$
BDE 209	Chiron AS	1811.12	68631-49-2	959.17	$C_{12}Br_{10}O$
PentaBDE mix	FIRE project				
BDE47 (42%)			5436-43-1	485.79	$C_{12}H_6Br_4O$
BDE99 (34%)			60348-60-9	564.69	$C_{12}H_5Br_5O$
BDE100 (9%)			189084-64-8	564.69	$C_{12}H_5Br_5O$
BDE153 (2%)			68631-49-2	643.58	$C_{12}H_4Br_6O$
BDE154 (2%)			207122-15-4	643.58	$C_{12}H_4Br_6O$
HBCD[d] technical mixture	FIRE project				
α-HBCD (10%)			134237-50-6	641.70	$C_{12}H_{18}Br_6$
β-HBCD (9%)			134237-51-7	641.70	$C_{12}H_{18}Br_6$
γ-HBCD (81%)			134237-52-8	641.70	$C_{12}H_{18}Br_6$
Tetrabromobisphenol A (TBBPA)	Sigma Aldrich	330396	79-94-7	543.87	$C_{15}H_{12}Br_4O_2$
Siloxanes					
Hexamethylcyclotrisiloxane (D3)	Sigma Aldrich	235687	541-05-9	222.46	$C_6H_{18}O_3Si_3$
Octamethylcyclotetrasiloxane (D4)	Sigma Aldrich	235695	556-67-2	296.62	$C_8H_{24}O_4Si_4$
Decamethylcyclopentasiloxane (D5)	Sigma Aldrich	444278	541-02-6	370.77	$C_{10}H_{30}O_5Si_5$
Miscellaneous					
β-naphtoflavone (BNF)	Sigma Aldrich	N3633	6051-87-2	272.30	$C_{19}H_{12}O_2$
4-nonylphenol	Fluka	74430	104-40-5	220.35	$C_{15}H_{24}O$
4-octylphenol	Sigma Aldrich	384445	1806-26-4	206.32	$C_{14}H_{22}O$
Bisphenol A (BPA)	Sigma Aldrich	239658	80-05-7	228.29	$C_{15}H_{16}O_2$
Perfluorononanoic acid (PFNA)	Sigma Aldrich	77282	375-95-1	464.08	$C_9HF_{17}O_2$

[a] 4-[2,2-Bis(diethoxyphosphoryl)ethenyl]-2,6-ditert-butylphenol.
[b] Dichlorodiphenyltrichloroethane.
[c] Dichlorodiphenyldichloroethylene.
[d] Hexabromocyclododecane.

Table 2
Summary of in vitro ligand activation of human and polar bear PXR by 51 pharmaceuticals and environmental pollutants. In vitro ligand activations in a COS-7-based luciferase reporter gene assay were expressed as maximum fold change of luciferase activity in lysates from exposed cells compared to the activity in DMSO control cells. Relative activation represents maximum responses as percentage of the maximum response resulting from activation of hPXR or pbPXR by rifampicin, while REC_{h20} represent the concentration required to induce a response equal to 20% of the maximum hPXR-mediated response induced by rifampicin. Students T-test was used to test for statistically significant differences in luciferase activities in exposed and DMSO-treated cells (*, $p < 0.05$).

Compound name	Human PXR			Polar bear PXR			Relative max induction (polar bear vs human)
	Fold induction	Relative activation (%)	REC_{h20} (M)	Fold induction	Relative activation (%)	REC_{h20} (M)	
Pharmaceuticals							
Rifampicin	9.8 ± 0.9*	100%	4.6E−7	7.4 ± 1.5*	76%	1.1E−6	0.8
SR12813[a]	7.0 ± 1.1*	72%	3.8E−8	9.4 ± 0.9*	96%	3.2E−7	1.3
Carbamazepine	2.3 ± 0.3*	23%	1.3E−5	1.4 ± 0.3*	14%	N/D	0.6
Clotrimazole	8.3 ± 1.3*	84%	3.4E−7	5.9 ± 2.0*	61%	6.3E−7	0.7
Ketoconazole	2.7 ± 0.5*	28%	1.3E−5	0.9 ± 0.1	9%	N/D	0.3
Omeprazole	4.3 ± 0.3*	44%	9.1E−6	1.3 ± 0.3	13%	N/D	0.3
Pesticides							
Methoxychlor	7.3 ± 0.7*	75%	2.7E−6	3.9 ± 0.5*	40%	1.7E−5	0.5
Dieldrin	4.8 ± 0.4*	49%	1.7E−6	2.2 ± 0.4*	22%	2.5E−5	0.5
Chlordane	6.9 ± 1.5*	70%	1.7E−6	2.5 ± 0.5*	25%	1.6E−5	0.4
Pentachlorophenol	1.0 ± 0.2	11%	N/D	0.9 ± 0.3	9%	N/D	0.9
Toxaphene	4.6 ± 0.3*	47%	7.8E−7	7.0 ± 0.8*	71%	1.9E−6	1.5
Endosulfan (α + β ~ 2:1)	4.1 ± 1.1*	42%	7.8E−6	4.6 ± 0.3*	47%	4.5E−6	1.1
α-hexachlorocyclohexane (α-HCH)	5.6 ± 1.5*	57%	3.8E−6	1.3 ± 0.8	13%	N/D	0.2
Lindane (γ-HCH)	9.3 ± 0.6*	94%	7.9E−7	9.0 ± 2.9*	92%	6.0E−6	1.0
Vinclozolin	3.7 ± 0.2*	38%	4.4E−5	1.1 ± 0.2	11%	N/D	0.3
4,4′-DDT[b]	5.2 ± 0.6*	53%	9.3E−6	4.1 ± 0.6*	42%	1.1E−5	0.8
4,4′-DDE[c]	4.2 ± 0.4*	43%	9.1E−6	2.2 ± 0.4*	22%	3.6E−5	0.5
1,2,3-trichlorobenzene (1,2,3-TCB)	0.9 ± 0.1	9%	N/D	0.9 ± 0.3	9%	N/D	0.9
1,2,4-trichlorobenzene (1,2,4-TCB)	1.0 ± 0.1	10%	N/D	0.9 ± 0.3	9%	N/D	0.9
Polychlorinated biphenyls							
PCB 28	1.9 ± 0.2*	19%	N/D	1.1 ± 0.1	12%	N/D	0.6
PCB 47	2.6 ± 0.1*	27%	3.0E−5	1.4 ± 0.4	14%	N/D	0.5
PCB 52	1.1 ± 0.2	11%	N/D	1.0 ± 0.2	10%	N/D	0.9
PCB 60	1.0 ± 0.2	10%	N/D	0.8 ± 0.1	8%	N/D	0.7
PCB 97	2.4 ± 0.4*	25%	2.8E−5	2.7 ± 0.6*	28%	2.5E−5	1.1
PCB 101	3.3 ± 05*	34%	2.0E−5	1.8 ± 0.4*	19%	N/D	0.6
PCB 118	2.4 ± 0.2*	25%	3.4E−5	1.4 ± 0.1*	14%	N/D	0.6
PCB 138	1.1 ± 0.1	12%	N/D	1.1 ± 0.1	11%	N/D	0.9
PCB 151	7.1 ± 1.0*	72%	8.1E−6	1.5 ± 0.2*	15%	N/D	0.2
PCB 153	3.2 ± 0.3*	33%	2.2E−5	3.0 ± 0.2*	31%	2.5E−5	0.9
PCB 170	2.3 ± 0.3*	24%	3.5E−5	1.5 ± 0.3*	15%	N/D	0.6
PCB 180	1.3 ± 0.1*	14%	N/D	1.6 ± 0.3*	16%	N/D	1.2
PCB 183	2.9 ± 0.5*	28%	2.3E−5	3.0 ± 0.6*	31%	1.3E−5	1.1
PCB 184	3.2 ± 0.5*	33%	5.4E−6	1.8 ± 0.6*	19%	N/D	0.6
PCB 190	4.5 ± 0.5*	46%	9.8E−6	1.8 ± 0.3*	18%	N/D	0.4
Brominated flame retardants							
BDE 28	3.1 ± 0.5*	31%	1.1E−5	0.9 ± 0.2	9%	N/D	0.3
BDE 47	5.8 ± 0.6*	59%	5.0E−6	1.5 ± 0.5*	16%	N/D	0.3
BDE 99	5.1 ± 0.7*	52%	4.8E−6	1.6 ± 0.3*	16%	N/D	0.3
BDE 100	3.8 ± 0.3*	39%	6.6E−6	1.3 ± 0.2*	14%	N/D	0.4
BDE 153	4.4 ± 0.2*	45%	3.9E−6	1.7 ± 0.1*	17%	N/D	0.4
BDE 209	2.5 ± 0.2*	26%	1.6E−5	1.9 ± 0.3*	19%	N/D	0.7
PentaBDE mix	5.7 ± 0.2*	58%	5.1E−6	1.8 ± 0.3*	18%	N/D	0.3
BDE47 (42%)							
BDE99 (34%)							
BDE100 (9%)							
BDE153 (2%)							
BDE154 (2%)							
HBCD[d] technical mixture	4.9 ± 0.3*	41%	2.6E−6	9.4 ± 1.6*	96%	1.7E−6	2.3
α-HBCD (10%)							
β-HBCD (9%)							
γ-HBCD (81%)							
Tetrabromobisphenol A (TBBPA)	3.1 ± 1.1*	32%	2.9E−5	3.8 ± 0.5*	39%	3.5E−5	1.2
Siloxanes							
Hexamethylcyclotrisiloxane (D3)	1.7 ± 0.2*	17%	N/D	1.1 ± 0.2	11%	N/D	0.6
Octamethylcyclotetrasiloxane (D4)	4.3 ± 0.3*	44%	1.4E−5	1.1 ± 0.4	11%	N/D	0.3
Decamethylcyclopentasiloxane (D5)	2.2 ± 0.2*	22%	3.5E−5	1.6 ± 0.2*	16%	N/D	0.7
Miscellaneous							
β-naphtoflavone (BNF)	2.8 ± 0.4*	28%	6.9E−8	2.1 ± 0.4*	21%	6.9E−7	0.8
4-nonylphenol	4.9 ± 0.5*	50%	1.9E−6	7.0 ± 0.8*	71%	4.8E−6	1.4
4-octylphenol	1.7 ± 0.2*	17%	N/D	1.2 ± 0.3	12%	N/D	0.7
Bisphenol A (BPA)	5.7 ± 0.4*	58%	6.6E−6	5.6 ± 0.9*	57%	2.1E−5	1.0
Perfluorononanoic acid (PFNA)	1.1 ± 0.2	11%	N/D	0.9 ± 0.3	10%	N/D	0.9

fusion protein (pCMX_Gal4_humPXR or pCMX_Gal4_pbPXR) and 5 μg each of the luciferase reporter and the β-galactosidase plasmids using calcium phosphate ($CaPO_4$) methodology as previously described (Grun et al., 2002). Twenty-four hours post-transfection, the cells were exposed to the test compounds diluted in DMSO ranging in final concentration from 10^{-7} to 10^{-4} M in phenol red-free DMEM supplemented 10% heat-inactivated, charcoal-resin stripped FBS. Twenty-four hours after treatments, enzyme activities of luciferase and β-galactosidase were assayed in cell lysates as previously described (Grun et al., 2002). Luciferase activities were measured as luminescence and reflect the level of transactivation induced by the test compounds via the different PXR orthologs in the transfected cells. The enzyme activity of β-galactosidase resulting from the constitutive expression of the control plasmid was used to correct for differences in transfection efficiencies between wells. Activation of the PXRs was expressed as fold induction of luciferase activity in cells exposed to test compound relative to cells exposed to solvent (DMSO). Dose-response curves were fitted by non-linear regression using Prism (GraphPad Software, LaJolla, CA). In addition to reporting the maximum responses with ±95% confidence intervals, responses induced by test compounds were also reported as a percentage relative to the maximum luciferase response induced by rifampicin, a known PXR agonist, via hPXR or pbPXR. The concentrations that resulted in luciferase activity corresponding to 20% of the maximum hPXR response to rifampicin (REC_{h20}) were determined from the fitted dose-response curves.

Modeling. Homology models of the ligand binding domain of polar bear PXR were created using Modeller (v9.11) (Sali and Blundell, 1993), based on multiple crystal structures of human PXR co-crystallized with a variety of ligands, or unligated (PDB: 1M13, 1ILG, 1SKX, 4J5W, 3CTB, 2O9I). Multiple models were generated based on these templates. Homology modeling was carried out by satisfaction of spatial restraints using the automodel function of Modeller, with very thorough variable target function method (VTFM), thorough molecular dynamics (MD), and two repeat cycles of minimization. The best model from the generated structures was selected based on the Discrete Optimized Protein Energy (DOPE) score (Eramian et al., 2006; Shen and Sali, 2006), and further assessed using Procheck (Laskowski et al., 1993). Computational solvent mapping was performed using FTMAP (Brenke et al., 2009; Kozakov et al., 2011). Multiple human PXR crystal structures were mapped, and overlapping and novel clusters were retained.

Pharmacophore generation was performed using PharmaGist (Inbar et al., 2007; Schneidman-Duhovny et al., 2008). Initial ligand models were minimized using the PM6 method in MOPAC2009 (Steward, 2008).

Results

Cloning of polar bear PXR and evolutionary conservation

The polar bear *PXR* amplified from polar bear liver cDNA (Supplement Fig. S2) predicts a 434 AA protein translated from a non-AUG translation initiation codon (CUG) in accordance to hPXR1A (NM_003889). The phylogenetic association of the predicted polar bear PXR was as expected, based on the evolutionary relationships among the species represented in the analyses (Supplement Fig. S3). The inferred amino acid sequence similarity of the polar bear and human PXRs (97% identity in the DNA binding domain, 87% identity in the ligand binding domain) was comparable to the sequence similarity between human and the other caniforms, giant panda and dog (Supplement Fig. S4).

Qualitative and quantitative differences in the activation of polar bear and human PXRs

To evaluate the ligand selectivity of polar bear PXR, we tested a panel consisting of 51 compounds that were examined for their ability to activate human and polar bear PXRs (Table 1). We defined agonistic activity as a significant change in luciferase activity in cells treated with test compounds compared to DMSO-treated cells (Students T-test <0.05). Monitoring of β-galactosidase activities during exposures was used to assess the cytotoxicity of the test compounds, and the ranges of concentrations were adjusted to avoid toxicity. Members of all the six classes of compounds tested activated both hPXR and pbPXR, indicating that both orthologs have a broad ligand affinity (Table 2). However, qualitative and quantitative differences in the activation of hPXR and pbPXR were observed. Qualitatively, hPXR appears to be more susceptible to activation than pbPXR as 86% of the test compounds activated hPXR compared to 68% for pbPXR (Table 2, Fig. 1, Supplement Fig. S6).

Quantitatively, exposure to equivalent concentrations of ligand typically resulted in greater luciferase activity in cells expressing hPXR compared to cells expressing pbPXR (Fig. 2, Supplement Fig. S6). Five structurally diverse compounds, hexabromocyclododecane, SR12813, toxaphene, 4-nonylphenol and tetrabromobisphenol A, induced stronger responses via pbPXR than via hPXR (Fig. 2, Supplement Fig. S6).

Sequential and structural differences between hPXR and pbPXR

To explain the qualitative and quantitative differences in ligand activation of hPXR and pbPXR, we compared primary, secondary and tertiary protein structures of the orthologs. We found the degree of conservation of the ligand binding domains (AA205-434) of hPXR and pbPXR to be comparable to that of the LBDs from other caniforms (appr. 90%), including dogs and giant panda (Supplement Figs. S4 and S5). None of the 25 amino acid substitutions between hPXR and pbPXR, correspond to residues known to participate in ligand binding, in interaction with co-activators or the formation of internal salt-bridges (Fig. 3, Supplement Fig. S7). Almost three quarters of the substitutions involved amino acids participating in secondary structures (72%) and about half of these secondary structure substitutions could be classified as radical in terms of change in charge, polarity and volume (Supplement table S8). However, the secondary structure properties of replacement amino acids were comparable to those of the replaced amino acids.

Homology modeling of the pbPXR based on multiple human PXR crystal structures shows that no steric conflicts have emerged because of amino acid replacements between the orthologs. Thus, radical changes in the pbPXR model compared to hPXR structure were not found (Fig. 4A and B). No polar bear amino acid substitutions (highlighted in orange) were found in the regions known to bind the steroid coactivator SRC1 in human PXR (red helix, Fig. 4A and B), nor in the helix interacting with the heterodimeric receptor partner, RXR (cyan). Computational solvent mapping (Brenke et al., 2009; Kozakov et al., 2011), in which small molecule probe fragments are mapped onto structures to identify molecular binding 'hot spots,' reveals that the bulk of the binding sites is in the ligand binding domain (Fig. 4C). However, some binding sites outside the LBD (possible allosteric sites) differ between humans and polar bears, perhaps contributing to some of the observed activation differences.

Pharmacophores generated for the most potent binding compounds (activation > 5-fold) suggest that there may be some subtle alterations to the ligand binding site that are not captured in the homology models. The polar bear pharmacophore generated from 5 compounds (Supplemental Fig. S9) is approximately tripodal, with a hydrophobic end

* Statistically significant difference between maximum luciferase activity induced via hPXR and pbPXR (T-test < 0.05).
[a] 4-[2,2-Bis(diethoxyphosphoryl)ethenyl]-2,6-ditert-butylphenol.
[b] Dichlorodiphenyltrichloroethane.
[c] Dichlorodiphenyldichloroethylene.
[d] Hexabromocyclododecane.

and a tripod of hydrophilic donor/acceptor sites. In contrast, the best human pharmacophore, while still roughly pyramidal, has one corner that is aromatic, rather than hydrophilic (Fig. 5).

Discussion

Human and polar bear PXRs are promiscuous xenosensors

The role of human PXR as a xenobiotic sensor has been supported by the structurally diverse compounds shown to bind and activate human PXR, including pharmaceuticals and several classes of persistent organic pollutants (e.g. Al-Salman and Plant, 2012; Kojima et al., 2011; Milnes et al., 2008; Moore et al., 2002). This role is further supported by the abundant expression of hPXRs in metabolically active tissues (Bertilsson et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998). The cloning of polar bear *PXR* demonstrates that in polar bears it is also transcribed in metabolically active tissue, namely the liver. We show that the polar bear PXR is a ligand-dependent transcription factor that can be activated by pharmaceuticals and several different classes of persistent organic pollutants, suggesting that PXR also serves as a

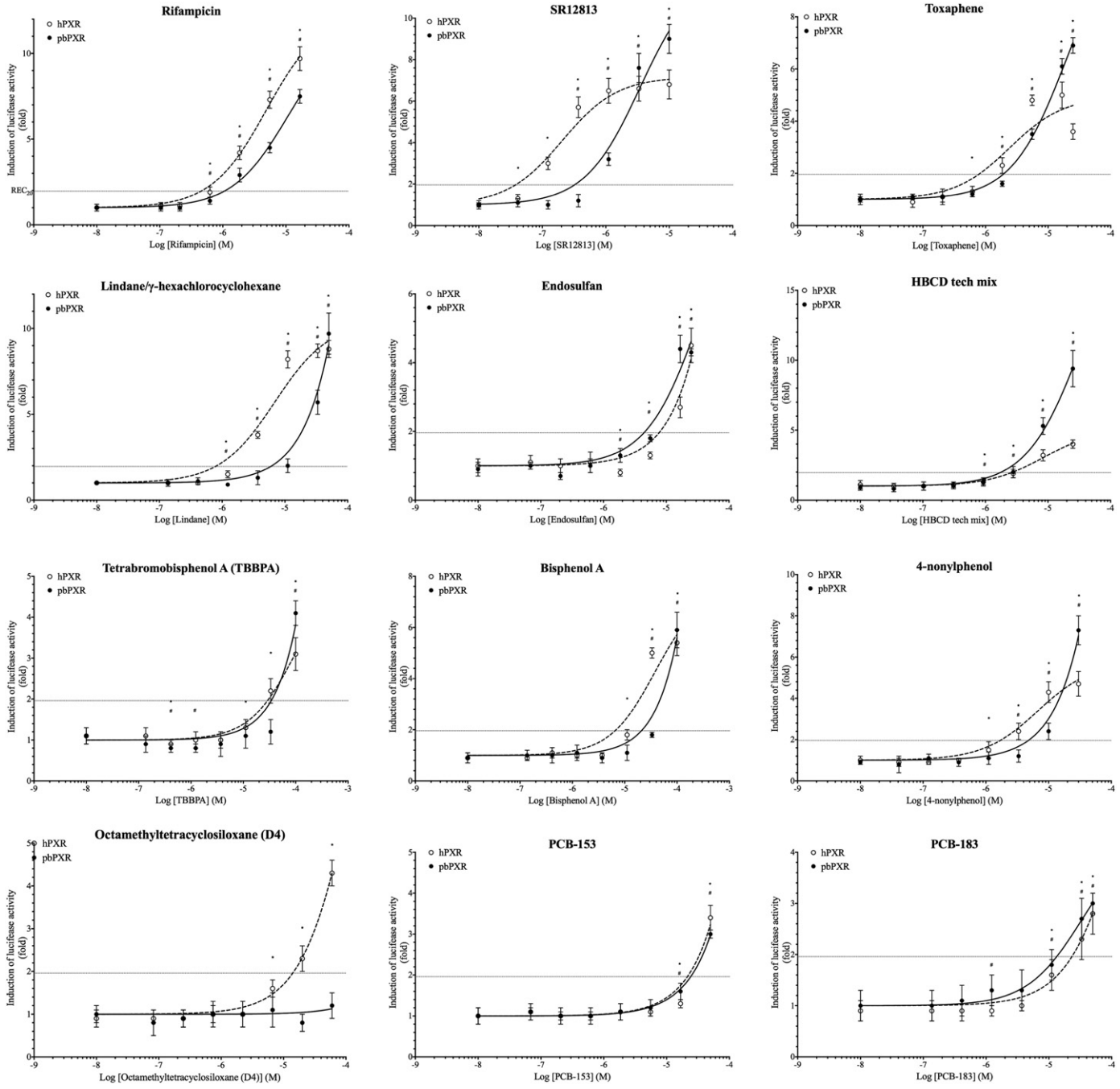

Fig. 1. Ligand activation dose-response curves of human and polar bear PXR by a 51-compound test panel. Ligand activation of PXRs by selected test compounds was reported as fold increase in luciferase activity in cells exposed to the test compound over cells exposed to solvent. Dose-response-curves were fitted by non-linear regression (GraphPad Prism). Dotted curves with hollow circles and solid lines with solid circles represent human and polar bear PXRs respectively. Students T-test was used to test for statistically significant differences in luciferase activities in exposed and DMSO-treated cells ($p < 0.05$, * and # represent hPXR and pbPXR, respectively). Horizontal dotted line represents 20% of maximum luciferase activity induced by rifampicin in COS-7 cells expressing hPXR (REC_{h20}).

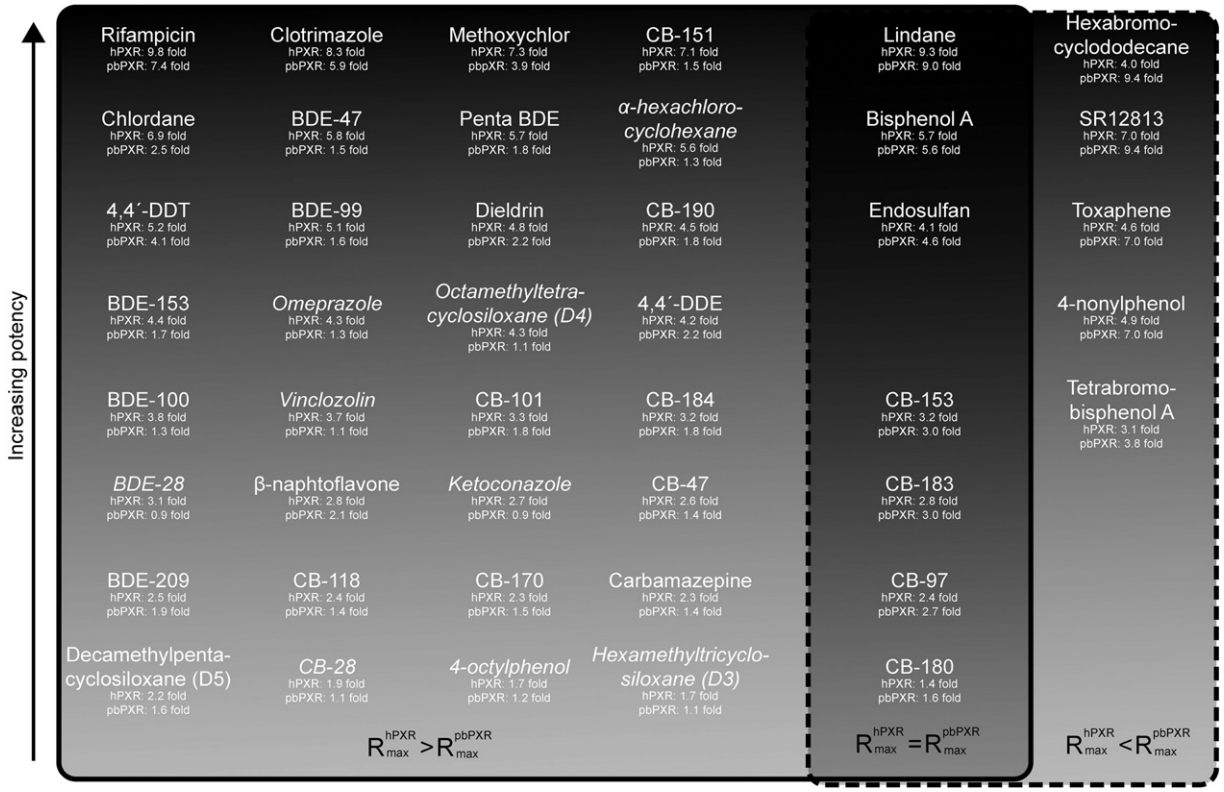

Fig. 2. Comparison of agonistic potential of PXR ligands via hPXR or pbPXR presented as a Venn diagram. Forty-two agonists were grouped according to their potential for transactivation via hPXR and/or pbPXR. The 29 compounds grouped within the solid box had higher potential for transactivation via hPXR (Students T-test $p < 0.05$), while the compounds in the dashed box induced strongly via pbPXR. Compounds enclosed by both boxes exerted comparative transactivation potential via hPXR and pbPXR. Compounds in italic activate hPXR but not pbPXR.

xenosensor in polar bears. To resolve the issue of different and non-consistent naming of PXR across species, we propose the novel naming "promiscuous xenobiotic receptor" for NR1I2 receptors in all species.

Qualitative and quantitative differences in the activation of human and polar bear PXRs by environmental pollutants

Although human and polar bear PXRs both appear to be promiscuous nuclear receptors, the activation profiles of the two orthologs were quite different. Qualitatively, a broad range of compounds in our test panel activated both orthologs. This panel consisted of 51 compounds, including pharmaceuticals, pesticides, polychlorinated biphenyls, brominated flame-retardants, and siloxanes. Approximately 86% of the test compounds had the ability to induce transcription via hPXR, while approximately 68% of the compounds had agonistic activity on pbPXR. These results confirm that hPXR is highly promiscuous, as is pbPXR.

Quantitative differences in the activation of PXR orthologs from multiple species have been described in several studies (Al-Salman and Plant, 2012; Kojima et al., 2011; Milnes et al., 2008; Moore et al., 2002; Tabb et al., 2004), and here we show that quantitative differences exist in the activation of the human and polar bear PXR orthologs. Less than a fifth of the agonists among our test compounds (18%) produced similar responses via hPXR as via pbPXR. The majority of the compounds were stronger agonists of hPXR than of pbPXR (70%), while only five compounds induced a stronger response via pbPXR than via hPXR (appr. 10%), indicating that hPXR is more susceptible to activation by this panel of test compounds than pbPXR is. Among the test compounds that induced relatively strong activation of hPXR (>4-fold) we found representatives of all classes of compounds included in this study, indicating that the relationship between chemical structure and the inductive potential of the ligands is complex. Likewise, the five test compounds that induced quantitatively greater responses via pbPXR than via hPXR represent four different classes of compounds, indicating that the ability of a ligand to exert strong agonistic activity on pbPXR is similarly difficult to predict without a functional characterization such as the LBD-luciferase reporter assay reported here.

Responses measured with different systems for measuring ligand activation often differ quantitatively. Rifampicin-induced responses in the range of 8- to 100-fold have been reported in previous studies and this complicated direct comparison of results (Al-Salman and Plant, 2012; Jacobs et al., 2005; Kojima et al., 2011; Tabb et al., 2004). However, in general relative activation of PXR in our study and other studies correlates well, including with the several pesticides in this study (Coumoul et al., 2002; Kojima et al., 2011; Milnes et al., 2008). While several studies have addressed PCBs as ligands for PXR (Al-Salman and Plant, 2012; Jacobs et al., 2005; Tabb et al., 2004), their reports are inconsistent. Thus, while Al-Salman and Plant found PCB-153 to induce a strong response that exceeded the response from rifampicin, this study and Jacobs et al. (Jacobs et al., 2005) found a weak response (relative activation of 25–30%), and others found no response (relative activation appr. 10%, Tabb et al., 2004).

Structural determinants for qualitative and quantitative differences in ligand activation

Compared to the hPXR ligand-binding domain, pbPXR has 25 variable sites, a degree of divergence consistent with findings for other related species, e.g. dog and pigs (Milnes et al., 2008; Moore et al., 2002). To investigate whether the LBD-sequence variations could explain observed differences in ligand activity, we examined primary, secondary and tertiary protein structures. We constructed structural models of pbPXR based on the known structures of hPXR. Mapping the substituted amino acids in the polar bear PXR model on human

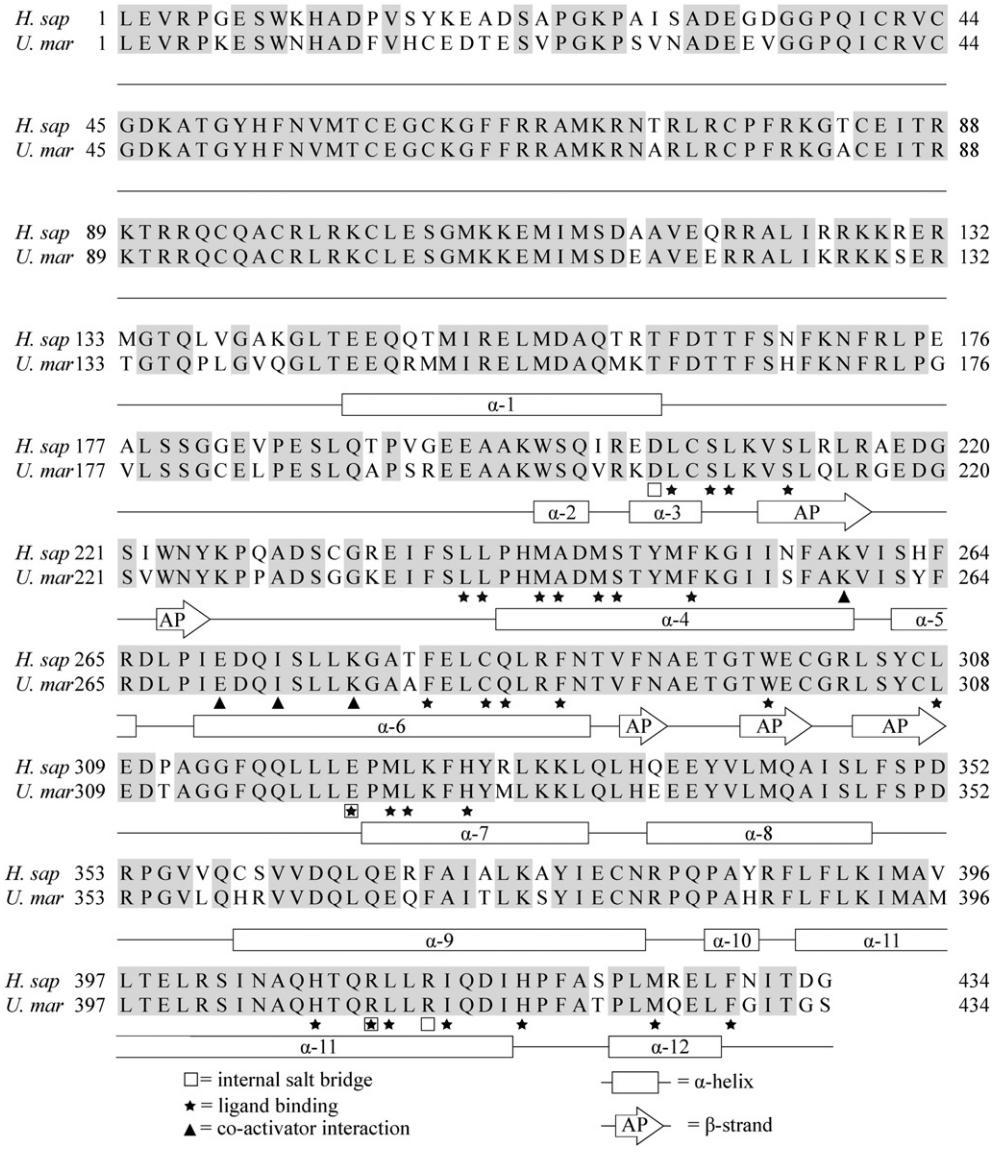

Fig. 3. Alignment of hPXR and pbPXR supplemented with known secondary structure and ligand-binding residues. Human PXR structures (Chrencik et al., 2005; Teotico et al., 2008; Watkins et al., 2001, 2003; Xue et al., 2007a, 2007b) was used to supplement a hPRX and pbPXR amino acid sequence comparison with information of residues known to be involved in ligand binding, secondary structures, salt bridge and SRC-1 interaction. Rectangular boxes indicate α-helices (α) and arrows anti β-strands that form an antiparallel (AP) sheet. Stars indicate ligand-binding residues, squares residues involved in salt-bridges and triangles residues interacting with co-activator (SRC-1).

PXR showed that the substituted amino acids in general were located on the surface of the protein structure, consistent with studies showing that residues in the core of PXR are better conserved (Chrencik et al., 2005). While the analyses revealed that substitutions occurred in several secondary structures, disruption of tertiary structures seem unlikely due to the chemical nature of the substitutions and the orientation of the side-chains of the amino acids involved, a conclusion borne out by the robustness of the pbPXR homology models.

Structures of hPXR co-crystallized with various ligands have identified amino acids that contribute to ligand binding (Chrencik et al., 2005; Teotico et al., 2008; Watkins et al., 2001, 2003; Xue et al., 2007a, 2007b). The LBD of human, pig, dog, mouse and rat PXRs all differ in multiple positions and only 71% of the amino acids are conserved in these species (Chrencik et al., 2005). This variation has been suggested to explain qualitative and quantitative differences in the activation of PXR orthologs from distantly related species. In eleven non-human primate PXRs, only two LBD substitutions were found, of which one was a conservative substitution while the other substitution was found in only two of the eleven species (data not shown). Thus, similar binding and activation properties of hPXR and non-human primate PXRs could be expected. And indeed, the activation of human PXR has been shown to qualitatively represent PXR-activation in non-human primates (Milnes et al., 2008). All LBD-residues in pbPXR are conserved compared to hPXR, including residues shown to participate in the binding of ligands. Consequently, differences in amino acids within the ligand binding pocket cannot readily explain the observed qualitative and quantitative differences in ligand activation between hPXR and pbPXR. Thus the differences in activation of hPXR and pbPXR indicate that amino acids that are not part of the binding pocket or those that do not interact directly with ligand could modulate binding of ligand and/or receptor activation. Solvent mapping of the homology model highlighted some potential regions of allosteric binding that differ between humans and polar bears, but does not provide an obvious answer.

The toxicological relevance of data from in vitro PXR activation

Interpreting the toxicological relevance of PXR activation measured in vitro is complicated. Any in vitro method is likely to represent a

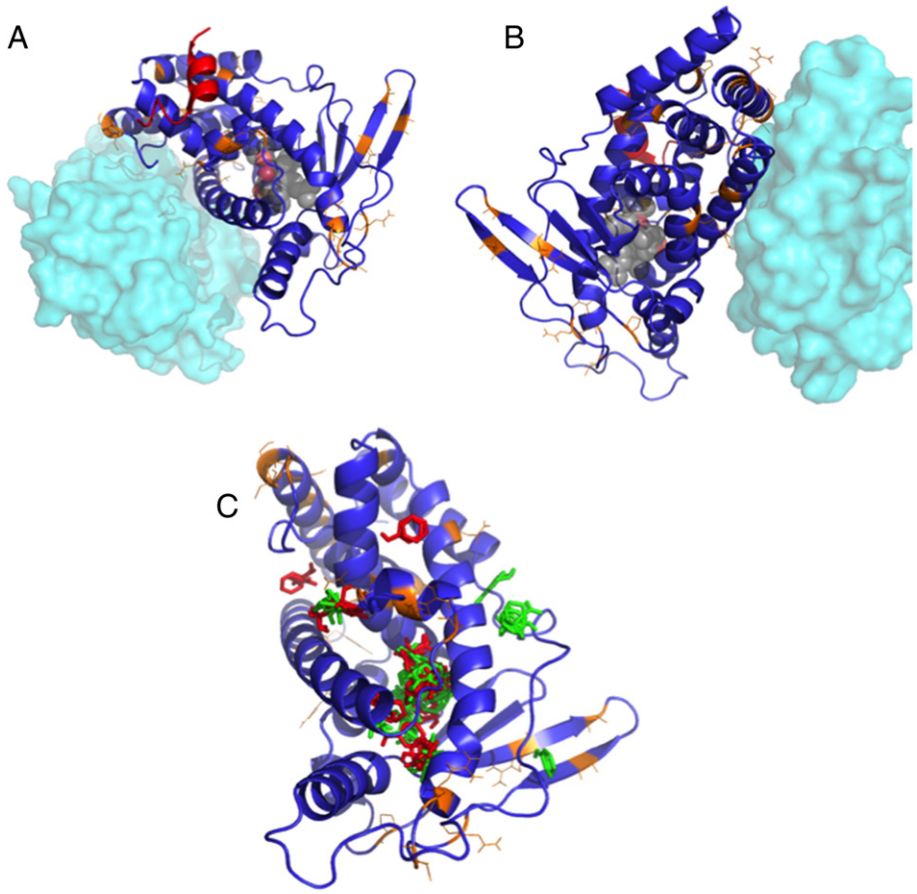

Fig. 4. Homology model for pbPXR based on multiple hPXR structures showing residue substitutions between human and polar bear. (A and B) Positions of residues differing between hPXR and pbPXR are shown in orange. Also shown is the relative position of the heterodimerization partner, RXR, from PDB 4J5W in cyan, and the position of the coactivator protein SRC1 from PDB 2O9I in red. (C) Positions of solvent-mapped small molecule clusters from FTMAP are shown in red (pbPXR) and green (hPXR). Note that most clusters fall into the known ligand binding pocket, but there are significant clusters found outside the pocket, which differ between hPXR and pbPXR. (Figure size: Double column).

simplification of the biological processes of interest, thus caution must be taken in the interpretation of the results. The Gal4-LBD method used here indicates binding of ligand to the transcription factor LBD, but it cannot reflect gene, tissue or species-specific differences in promoters, or the function of transcriptional repressors, dimerization partners or other proteins involved in the transcription factor action in vivo. Cell based assays also do not necessarily relate exposure concentrations to those relevant in the environment.

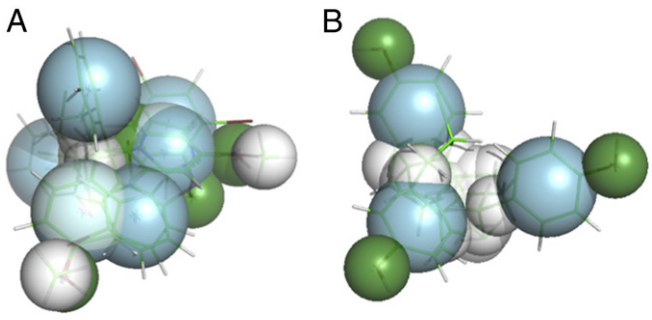

Fig. 5. Pharmacophores generated from the best-binding ligands for hPXR (A) and pbPXR (B). Aromatic overlaps are shown in blue, hydrophobic regions in white, and hydrophilic donor/acceptors are shown in green. Note the pyramidal shape for both pharmacophores, fitting the shape of the known PXR binding pocket. However, the human pharmacophore differs in the positioning of aromatic residues at one pyramid apex, in contrast to the pbPXR. Direct residue substitutions are not observed in the binding pocket, suggesting that longer range differences in the tertiary structure e.g. via A281T may play a role in the different agonist profiles between human and polar bear PXRs. (Figure size: Single column).

Despite the role of PXR in regulation of the xenobiotic biotransformation system, the toxicological effects of PXR activation are not fully understood. Depending on the products of biotransformation, PXR can contribute to both detoxification (Staudinger et al., 2001; Xie et al., 2000), or to enhancement of toxicity of certain compounds (Cheng et al., 2009), and chemicals can perturb physiological functions by interfering with the homeostasis of endogenous compounds such as steroid hormones (Mikamo et al., 2003; Xie et al., 2003; Zhai et al., 2007; Zhou et al., 2009). That CAR and PXR have partially overlapping target genes and common ligands (Kliewer et al., 2002; Xie et al., 2000), gives the biotransformation system a redundancy that complicates the prediction of toxicological effects of PXR activation. Moreover, while *ortho*-PCBs activate both CAR and PXR, it was recently shown that CAR contributes much more to the expression of CYP3A1 in mice than PXR (Gahrs et al., 2013). Thus, the toxic effects of a compound may be difficult to evaluate from the activation of PXR alone. However, the observed differences in activation of PXR in polar bears and humans suggest that biotransformation is less inducible in polar bears than in humans, and that PXR-mediated enhancement of toxicity and disruption of the homeostasis of endogenous compounds might be less likely to occur in polar bears than in humans at similar exposure levels.

Importantly, although these data indicate activation of both hPXR and pbPXR by high concentrations of some ligands, like PCBs and PBDEs, other studies have shown several of these compounds to be antagonistic at lower concentrations (Tabb et al., 2004). The biological significance of such non-monotonic effects, and whether such compounds might be competitive inhibitors or partial agonists, should not be overlooked. Future studies should investigate such possibilities. It will be informative to determine whether compounds that do not activate PXR in our studies might bind to the receptor, and act as antagonists. Molecular docking studies might provide such information, and will be undertaken in the future.

Our data shows that despite similarities in activation profiles, predictions of activation of polar bear PXR based on a hPXR model, will likely lead to over-estimation of activation both qualitatively and quantitatively. However, the validity of this assumption may depend on determining the contribution of polar bear CAR to responses to the chemicals in question, particularly as in humans CAR has been reported to mediate the majority of the *ortho*-PCB effects on gene regulation (Gahrs et al., 2013).

Conclusions

This study demonstrates that the polar bear PXR is a promiscuous nuclear receptor capable of being activated by structurally diverse compounds. Both qualitative and quantitative differences in ligand activation of pbPXR and hPXR were observed. The polar bear PXR is less promiscuous than its human counterpart and with a few but environmentally relevant exceptions, our test compounds generally induced quantitatively lower responses via pbPXR than via hPXR. Among these exceptions were the environmental pollutants HBCD, toxaphene, 4-nonylphenol and TBBPA, which all induced greater agonistic response via pbPXR than via hPXR, indicating that these compounds may have different toxic effects in polar bears than in humans.

Conflict of interest

B.B. is a named inventor on U.S. patents 6,756,491, 6,809,178, 7,214,482 and 6,984,773 related to human PXR. The authors declare they have no actual or potential conflicts of interest.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.taap.2015.02.001.

References

Al-Salman, F., Plant, N., 2012. Non-coplanar polychlorinated biphenyls (pcbs) are direct agonists for the human pregnane-X receptor and constitutive androstane receptor, and activate target gene expression in a tissue-specific manner. Toxicol. Appl. Pharmacol. 263, 7–13.

Bandiera, S., Torok, S., Letcher, R., Norstrom, R., 1997. Immunoquantitation of cytochromes p450 1a and p450 2b and comparison with chlorinated hydrocarbon levels in archived polar bear liver samples. Chemosphere 34, 1469–1479.

Bentzen, T., Muir, D., Amstrup, S., O'Hara, T., 2008. Organohalogen concentrations in blood and adipose tissue of Southern Beaufort Sea polar bears. Sci. Total Environ. 406, 352–367.

Bernhoft, A., Skaare, J., Wiig, O., Derocher, A., Larsen, H., 2000. Possible immunotoxic effects of organochlorines in polar bears (*Ursus maritimus*) at Svalbard. J. Toxic. Environ. Health A 59, 561–574.

Bertilsson, G., Heidrich, J., Svensson, K., Asman, M., Jendeberg, L., Sydow-Backman, M., et al., 1998. Identification of a human nuclear receptor defines a new signaling pathway for cyp3a induction. Proc. Natl. Acad. Sci. U. S. A. 95, 12208–12213.

Blumberg, B., Sabbagh Jr., W., Juguilon, H., Bolado Jr., J., van Meter, C.M., Ong, E.S., et al., 1998. Sxr, a novel steroid and xenobiotic-sensing nuclear receptor. Genes Dev. 12, 3195–3205.

Braathen, M., Derocher, A., Wiig, Ø., Sørmo, E., Lie, E., Skaare, J., et al., 2004. Relationships between PCBS and thyroid hormones and retinol in female and male polar bears. Environ. Health Perspect. 112, 826–833.

Brenke, R., Kozakov, D., Chuang, G.Y., Beglov, D., Hall, D., Landon, M.R., et al., 2009. Fragment-based identification of druggable 'hot spots' of proteins using Fourier domain correlation techniques. Bioinformatics 25, 621–627.

Brouwer, A., Klasson-Wehler, E., Bokdam, M., Morse, D.C., Traag, W.A., 1990. Competitive inhibition of thyroxin binding of transthyretin by monohydroxy metabolites of 3,4,3′,4′-tetrachlorobiphenyl. Chemosphere 20, 1257–1262.

Bytingsvik, J., Lie, E., Aars, J., Derocher, A., Wiig, Ø., Jenssen, B., 2012. PCBS and OH-PCBS in polar bear mother-cub pairs: a comparative study based on plasma levels in 1998 and 2008. Sci. Total Environ. 417–418, 117–128.

Celander, M., Goldstone, J., Denslow, N., Iguchi, T., Kille, P., Meyerhoff, R., et al., 2011. Species extrapolation for the 21st century. Environ. Toxicol. Chem. 30, 52–63.

Cheng, J., Ma, X., Krausz, K.W., Idle, J.R., Gonzalez, F.J., 2009. Rifampicin-activated human pregnane X receptor and cyp3a4 induction enhance acetaminophen-induced toxicity. Drug Metab. Dispos. 37, 1611–1621.

Chrencik, J.E., Orans, J., Moore, L.B., Xue, Y., Peng, L., Collins, J.L., et al., 2005. Structural disorder in the complex of human pregnane X receptor and the macrolide antibiotic rifampicin. Mol. Endocrinol. 19, 1125–1134.

Coumoul, X., Diry, M., Barouki, R., 2002. PXR-dependent induction of human CYP3A4 gene expression by organochlorine pesticides. Bichem. Pharmacol. http://dx.doi.org/10.1016/S0006-2952(02)01298-4.

Danielsson, C., Harju, M., Halldin, K., Tysklind, M., Andersson, P., 2008. Comparison of levels of PCDD/Fs and non-ortho PCBS in PCB 153 from seven different suppliers. Organohalogen Compd. 70, 1201–1204.

Ekins, S., Reschly, E., Hagey, L., Krasowski, M., 2008. Evolution of pharmacologic specificity in the pregnane X receptor. BMC Evol. Biol. 8, 103.

Eramian, D., Shen, M.Y., Devos, D., Melo, F., Sali, A., Marti-Renom, M.A., 2006. A composite score for predicting errors in protein structure models. Protein Sci. 15, 1653–1666.

Forman, B.M., Umesono, K., Chen, J., Evans, R.M., 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81, 541–550.

Gahrs, M., Roos, R., Andersson, P.L., Schrenk, D., 2013. Role of the nuclear xenobiotic receptors CAR and PXR in induction of cytochromes p450 by non-dioxinlike polychlorinated biphenyls in cultured rat hepatocytes. Toxicol. Appl. Pharmacol. 272, 77–85.

Goncharov, A., Pavuk, M., Foushee, H., Carpenter, D., 2011. Blood pressure in relation to concentrations of PCB congeners and chlorinated pesticides. Environ. Health Perspect. 119, 319–325.

Grun, F., Venkatesan, R.N., Tabb, M.M., Zhou, C., Cao, J., Hemmati, D., et al., 2002. Benzoate X receptors alpha and beta are pharmacologically distinct and do not function as xenobiotic receptors. J. Biol. Chem. 277, 43691–43697.

Haave, M., Ropstad, E., Derocher, A., Lie, E., Dahl, E., Wiig, Ø., et al., 2003. Polychlorinated biphenyls and reproductive hormones in female polar bears at Svalbard. Environ. Health Perspect. 111, 431–436.

Hall, T., 1999. Bioedit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acid Symposium Series, pp. 95–98.

Hamers, T., Kamstra, J., Sonneveld, E., Murk, A., Kester, M., Andersson, P., et al., 2006. In vitro profiling of the endocrine-disrupting potency of brominated flame retardants. Toxicol. Sci. 92, 157–230.

Pohl, H.R., Bosch, S., Amata, R.J., Eisenmann, C.J., 2004. Toxicological Profile for Polybrominated Biphenyls and Polybrominated Diphenyl Ethers. Agency for Toxic Substances and Disease Registry (ATSDR), Atlanta, GA, United States.

Inbar, Y., Schneidman-Duhovny, D., Dror, O., Nussinov, R., Wolfson, H.J., 2007. Deterministic pharmacophore detection via multiple flexible alignment of drug-like molecules. Lect. Notes Comput. Sci 4453, 412–429.

Jacobs, M.N., Nolan, G.T., Hood, S.R., 2005. Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicol. Appl. Pharmacol. 209 (2), 123–133.

Kannan, K., Yun, S., Evans, T., 2005. Chlorinated, brominated, and perfluorinated contaminants in livers of polar bears from Alaska. Environ. Sci. Technol. 39, 9057–9063.

Kim, M.-J., Marchand, P., Henegar, C., Antignac, J.-P., Alili, R., Poitou, C., et al., 2011. Fate and complex pathogenic effects of dioxins and polychlorinated biphenyls in obese subjects before and after drastic weight loss. Environ. Health Perspect. 119, 377–383.

Kliewer, S.A., Moore, J.T., Wade, L., Staudinger, J.L., Watson, M.A., Jones, S.A., et al., 1998. An orphan nuclear receptor activated by pregnanes defines a novel steroid signaling pathway. Cell 92, 73–82.

Kliewer, S.A., Goodwin, B., Willson, T.M., 2002. The nuclear pregnane x receptor: a key regulator of xenobiotic metabolism. Endocr. Rev. 23, 687–702.

Kohle, C., Bock, K.W., 2009. Coordinate regulation of human drug-metabolizing enzymes, and conjugate transporters by the AH receptor, pregnane X receptor and constitutive androstane receptor. Biochem. Pharmacol. 77, 689–699.

Kojima, H., Sata, F., Takeuchi, S., Sueyoshi, T., Nagai, T., 2011. Comparative study of human and mouse pregnane X receptor agonistic activity in 200 pesticides using in vitro reporter gene assays. Toxicology 280, 77–87.
Kozakov, D., Hall, D.R., Chuang, G.Y., Cencic, R., Brenke, R., Grove, L.E., et al., 2011. Structural conservation of druggable hot spots in protein–protein interfaces. Proc. Natl. Acad. Sci. U. S. A. 108, 13528–13533.
Krasowski, M., Yasuda, K., Hagey, L., Schuetz, E., 2005a. Evolutionary selection across the nuclear hormone receptor superfamily with a focus on the NR1I subfamily (vitamin D, pregnane X, and constitutive androstane receptors). Nucl. Recept. 3, 2.
Krasowski, M., Yasuda, K., Hagey, L., Schuetz, E., 2005b. Evolution of the pregnane X receptor: adaptation to cross-species differences in biliary bile salts. Mol. Endocrinol. 19, 1720–1739.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., et al., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.
Laskowski, R.A., MacArthur, M.W., Moss, D.S., Thornton, J.M., 1993. PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst. 26, 283–291.
Letcher, R., Norstrom, R., Lin, S., Ramsay, M., Bandiera, S., 1996. Immunoquantitation and microsomal monooxygenase activities of hepatic cytochromes p4501a and p4502b and chlorinated hydrocarbon contaminant levels in polar bear (*Ursus maritimus*). Toxicol. Appl. Pharmacol. 137, 127–140.
Letcher, R., Lemmen, J., van der Burg, B., Brouwer, A., Bergman, A., Giesy, J., et al., 2002. In vitro antiestrogenic effects of aryl methyl sulfone metabolites of polychlorinated biphenyls and 2,2-bis(4-chlorophenyl)-1,1-dichloroethene on 17beta-estradiol-induced gene expression in several bioassay systems. Toxicol. Sci. 69, 362–372.
Letcher, R.J., Gebbink, W.A., Sonne, C., Born, E.W., McKinney, M.A., Dietz, R., 2009. Bioaccumulation and biotransformation of brominated and chlorinated contaminants and their metabolites in ringed seals (*Pusa hispida*) and polar bears (*Ursus maritimus*) from East Greenland. Environ. Int. 35, 1118–1124.
Lie, E., Larsen, H.J., Larsen, S., Johansen, G.M., Derocher, A.E., Lunn, N.J., et al., 2004. Does high organochlorine (OC) exposure impair the resistance to infection in polar bears (*Ursus maritimus*)? Part I: effect of OCS on the humoral immunity. J. Toxicol. Environ. Health A 67, 555–582.
Lie, E., Larsen, H., Larsen, S., Johansen, G., Derocher, A., Lunn, N., et al., 2005. Does high organochlorine (OC) exposure impair the resistance to infection in polar bears (*Ursus maritimus*)? Part II: possible effect of OCS on mitogen- and antigen-induced lymphocyte proliferation. J. Toxic. Environ. Health A 68, 457–484.
Lind, P., van Bavel, B., Salihovic, S., Lind, L., 2012. Circulating levels of persistent organic pollutants (pops) and carotid atherosclerosis in the elderly. Environ. Health Perspect. 120, 38–43.
McKinney, M.A., Dietz, R., Sonne, C., De Guise, S., Skirnisson, K., Karlsson, K., et al., 2011. Comparative hepatic microsomal biotransformation of selected pbdes, including decabromodiphenyl ether, and decabromodiphenyl ethane flame retardants in arctic marine-feeding mammals. Environ. Toxicol. Chem. 30, 1506–1514.
Mikamo, E., Harada, S., Nishikawa, J., Nishihara, T., 2003. Endocrine disruptors induce cytochrome p450 by affecting transcriptional regulation via pregnane X receptor. Toxicol. Appl. Pharmacol. 193, 66–72.
Milnes, M.R., Garcia, A., Grossman, E., Grun, F., Shiotsugu, J., Tabb, M.M., et al., 2008. Activation of steroid and xenobiotic receptor (SXR, NR1I2) and its orthologs in laboratory, toxicologic, and genome model species. Environ. Health Perspect. 116, 880–885.
Moore, L.B., Maglich, J.M., McKee, D.D., Wisely, B., Willson, T.M., Kliewer, S.A., et al., 2002. Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. Mol. Endocrinol. 16, 977–986.
Muir, D., Norstrom, R., Simon, M., 1988. Organochlorine contaminants in arctic marine food chains: accumulation of specific polychlorinated biphenyls and chlordane-related compounds. Environ. Sci. Technol. 22, 1071–1079.
Norén, K., Weistrand, C., Karpe, F., 1999. Distribution of PCB congeners, DDE, hexachlorobenzene, and methylsulfonyl metabolites of PCB and DDE among various fractions of human blood plasma. Arch. Environ. Contam. Toxicol. 37, 408–414.
Norstrom, R., Belikov, S., Born, E., Garner, G., Malone, B., Olpinski, S., et al., 1998. Chlorinated hydrocarbon contaminants in polar bears from Eastern Russia, North America, Greenland, and Svalbard: biomonitoring of arctic pollution. Arch. Environ. Contam. Toxicol. 35, 354–367.
Orans, J., Teotico, D., Redinbo, M., 2005. The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges. Mol. Endocrinol. 19, 2891–2900.
Reche, P., 2008. Sequence Identity and Similarity Tool. Available: http://imed.med.ucm.es/Tools/sias.html.
Rosenfeld, J., Vargas, R., Xie, W., Evans, R., 2003. Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor. Mol. Endocrinol. 17, 1268–1282.
Sali, A., Blundell, T.L., 1993. Comparative protein modeling by satisfaction of spatial restraints. J. Mol. Biol. 234, 779–815.
Salihovic, S., Lampa, E., Lindström, G., Lind, L., Lind, P., van Bavel, B., 2012. Circulating levels of persistent organic pollutants (POPs) among elderly men and women from Sweden: results from the Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS). Environ. Int. 44, 59–67.
Sandau, C., Ayotte, P., Dewailly, E., Duffe, J., Norstrom, R., 2000. Analysis of hydroxylated metabolites of PCBS (OH-PCBS) and other chlorinated phenolic compounds in whole blood from Canadian Inuit. Environ. Health Perspect. 108, 611–616.
Schneidman-Duhovny, D., Dror, O., Inbar, Y., Nussinov, R., Wolfson, H.J., 2008. Pharmagist: a webserver for ligand-based pharmacophore detection. Nucleic Acids Res. 36, W223–W228.
Shen, M.Y., Sali, A., 2006. Statistical potential for assessment and prediction of protein structures. Protein Sci. 15, 2507–2524.
Skaare, J., Bernhoft, A., Derocher, A., Gabrielsen, G., Goksøyr, A., Henriksen, E., et al., 2000. Organochlorines in top predators at Svalbard—occurrence, levels and effects. Toxicol. Lett. 112–113, 103–109.
Sonne, C., 2010. Health effects from long-range transported contaminants in arctic top predators: an integrated review based on studies of polar bears and relevant model species. Environ. Int. 36, 461–491.
Sonne, C., Letcher, R.J., Bechshøft, T., Rigét, F.F., Muir, D.C.G., Leifsson, P.S., et al., 2012. Two decades of biomonitoring polar bear health in Greenland: a review. Acta Vet. Scand. 54, 1–7.
Stamatakis, A., 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22, 2688–2690.
Staudinger, J.L., Goodwin, B., Jones, S.A., Hawkins-Brown, D., MacKenzie, K.I., LaTour, A., et al., 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. Proc. Natl. Acad. Sci. U. S. A. 98, 3369–3374.
Steward, J., 2008. Mopac2009. Available: http://openmopac.net.
Tabb, M.M., Kholodovych, V., Grun, F., Zhou, C., Welsh, W.J., Blumberg, B., 2004. Highly chlorinated pcbs inhibit the human xenobiotic response mediated by the steroid and xenobiotic receptor (SXR). Environ. Health Perspect. 112, 163–169.
Teotico, D.G., Bischof, J.J., Peng, L., Kliewer, S.A., Redinbo, M.R., 2008. Structural basis of human pregnane X receptor activation by the hops constituent colupulone. Mol. Pharmacol. 74, 1512–1520.
van der Ven, L., van de Kuil, T., Verhoef, A., Leonards, P., Slob, W., Cantón, R.F., et al., 2008. A 28-day oral dose toxicity study enhanced to detect endocrine effects of a purified technical pentabromodiphenyl ether (pentabde) mixture in Wistar rats. Toxicology 245, 109–131.
Verreault, J., Muir, D., Norstrom, R., Stirling, I., Fisk, A., Gabrielsen, G., et al., 2005. Chlorinated hydrocarbon contaminants and metabolites in polar bears (*Ursus maritimus*) from Alaska, Canada, East Greenland, and Svalbard: 1996–2002. Sci. Total Environ. 351–352, 369–390.
Verreault, J., Norstrom, R.J., Ramsay, M.A., Mulvihill, M., Letcher, R.J., 2006. Composition of chlorinated hydrocarbon contaminants among major adipose tissue depots of polar bears (*Ursus maritimus*) from the Canadian High Arctic. Sci. Total Environ. 370, 580–587.
Verreault, J., Dietz, R., Sonne, C., Gebbink, W., Shahmiri, S., Letcher, R., 2008. Comparative fate of organohalogen contaminants in two top carnivores in Greenland: captive sledge dogs and wild polar bears. Comp. Biochem. Physiol. Toxicol. Pharmacol. 147, 306–315.
Verreault, J., Maisonneuve, F., Dietz, R., Sonne, C., Letcher, R.J., 2009. Comparative hepatic activity of xenobiotic-metabolizing enzymes and concentrations of organohalogens and their hydroxylated analogues in captive Greenland sledge dogs (*Canis familiaris*). Environ. Toxicol. Chem. 28, 162–172.
Waterhouse, A., Procter, J., Martin, D., Clamp, Ml, Barton, G., 2009. Jalview version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics 25, 1189–1191.
Watkins, R.E., Wisely, G.B., Moore, L.B., Collins, J.L., Lambert, M.H., Williams, S.P., et al., 2001. The human nuclear xenobiotic receptor PXR: structural determinants of directed promiscuity. Science 292, 2329–2333.
Watkins, R.E., Maglich, J.M., Moore, L.B., Wisely, G.B., Noble, S.M., Davis-Searles, P.R., et al., 2003. 2.1 a crystal structure of human PXR in complex with the St. John's wort compound hyperforin. Biochemistry 42, 1430–1438.
Xie, W., Barwick, J.L., Simon, C.M., Pierce, A.M., Safe, S., Blumberg, B., et al., 2000. Reciprocal activation of xenobiotic response genes by nuclear receptors SXR/PXR and CAR. Genes Dev. 14, 3014–3023.
Xie, W., Yeuh, M.-F., Radominska-Pandya, A., Saini, S., Negishi, Y., Bottroff, B., et al., 2003. Control of steroid, heme, and carcinogen metabolism by nuclear pregnane X receptor and constitutive androstane receptor. Proc. Natl. Acad. Sci. U. S. A. 100, 4150–4155.
Xue, Y., Chao, E., Zuercher, W.J., Willson, T.M., Collins, J.L., Redinbo, M.R., 2007a. Crystal structure of the PXR-T1317 complex provides a scaffold to examine the potential for receptor antagonism. Bioorg. Med. Chem. 15, 2156–2166.
Xue, Y., Moore, L.B., Orans, J., Peng, L., Bencharit, S., Kliewer, S.A., et al., 2007b. Crystal structure of the pregnane X receptor–estradiol complex provides insights into endobiotic recognition. Mol. Endocrinol. 21, 1028–1038.
Zhai, Y., Pai, H.V., Zhou, J., Amico, J.A., Vollmer, R.R., Xie, W., 2007. Activation of pregnane X receptor disrupts glucocorticoid and mineralocorticoid homeostasis. Mol. Endocrinol. 21, 138–147.
Zhou, C., Verma, S., Blumberg, B., 2009. The steroid and xenobiotic receptor (SXR), beyond xenobiotic metabolism. Nucl. Recept. Signal. 7, e001.

Paper II

II

Routti H, Lille-Langøy R, Berg MK, Fink T, Harju M, Kristiansen K, Rostkowski P, Rusten M, Sylte I, Øygarden L and Goksøyr A.
Environmental chemicals modulates polar bear (*Ursus maritimus*) peroxisome proliferator-activated receptor gamma (PPARG) and adipogenesis *in vitro*.
Environmental Science and Technology

Paper III

III

Lille-Langøy R, Karlsen OA, Myklebust LM, Goldstone JV, Mork-Jansson A, Male R, Stegeman JJ, Blumberg B and Goksøyr A.
Sequence variations in *pxr* from zebrafish (*Danio rerio*) affect nuclear receptor function.
In preparation.

Sequence variations in *pxr* from zebrafish (*Danio rerio*) affect nuclear receptor function

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Abstract

The strong relationship between the metabolism and disposition of chemicals and their toxicity makes regulators of biotransformation are of particular interest in pharmacology and toxicology. As a xenosensor and regulator of biotransformation, the pregnane X receptor (PXR/NR1I2/SXR) has a central role in the detoxification and elimination of both endogenous and exogenous compounds. Zebrafish (*Danio rerio*) is a key test organism in various fields of research, including risk assessment in environmental toxicology. As the genetic background of zebrafish is more varied than in commonly used rodent model organisms, it is likely that sequence variation occurs in the *pxr* gene of zebrafish. In the present work, we describe sequence variation in *pxr* from four different strains of zebrafish, and demonstrate that these Pxr variants differ in their ability to bind three structurally diverse ligands and to induce transcription *in vitro*. Importantly, these functional differences have the potential to cause strain-dependent biotransformation of xenobiotics in zebrafish, and further suggest that the choice of zebrafish strain could affect the outcome of downstream toxicological studies and risk assessments.

Introduction

Due to the strong relationship between the metabolism and disposition of chemicals and their toxicity regulators of xenobiotic biotransformation are of particular importance in pharmacology and toxicology. The pregnane X receptor, or steroid and xenobiotic receptor (PXR, nuclear receptor 1I2 (NR1I2), aka steroid and xenobiotic receptor (SXR) is a ligand-modulated transcription factor with exceptionally broad ligand-specificity and a high number of target genes involved in xenobiotic metabolism (1, 2). Thus, PXR is a key regulator of metabolism and excretion of both endogenous and exogenous compounds.

Zebrafish (*Danio rerio*) is a model organism used in various fields of biological research, including genetics, developmental biology, medicine, pharmacology and toxicology, including effects of pollutants (reviewed in (3-8)). The widespread use of zebrafish as a model species is due to its well-studied and annotated genome, rapid early development and transparent embryos, relatively short live cycle, and established methods and tools for manipulation of gene expression and gene knock downs. Furthermore, with predicted orthologs for at least 70% of all human genes in the zebrafish genome (9), as well as similarities in gene structure and regulation, early development, and organs and organ systems (reviewed in (5)), mechanistic findings in zebrafish may translate to or guide studies in humans and other mammalian species quite well. Ease of handling, low cost of husbandry, high fecundity and a rapid reproductive cycle that allows for high fish production, also contribute to the popularity of zebrafish as a model organism.

In the field of toxicology, adverse effects mechanisms and the chemical defensome are still being described on a molecular level in zebrafish (10). Interestingly, there are both notable similarities and differences in the defensome of zebrafish and humans. Broad similarities between zebrafish and human occur in the cytochrome P450 (CYP) complement, conjugating enzymes, and other xenobiotic response pathways (10), although there are some significant dissimilarities (e.g. zebrafish have 96 CYP genes, as opposed

to 57 in humans (11). Moreover, CYP families that are known to be important in the biotransformation of xenobiotic compounds (CYP1-4) have less conservation of sequence between zebrafish and humans than genes of CYPs that principally have more narrow endogenous functions (11). Factors influencing the activation of PXR and other NRs by xenobiotics can determine the functional levels of the biotransformation enzymes. How do functional properties of PXR vary within zebrafish and between zebrafish and other species?

Treatment of zebrafish with the mammalian PXR agonists clotrimazole and pregnenolone-16α-carbonitrile, resulted in a coordinated induction of defensome genes, including *cyp3a* and *mdr1*, and *pxr* itself, indicating similarities in xenobiotic response pathways in zebrafish and mammals (12). We recently described the sequencing and functional characterization of zebrafish Pxr (13), including regulatory targeting and a crosstalk between Pxr and the aryl hydrocarbon receptor (14). A key finding was the high degree of allelic variation in Pxr sequence discovered in various strains of zebrafish.

*A*llelic variation is known in *PXR* of mammals and thousands of polymorphisms, both in coding or non-coding regions, have been reported for the human *PXR* gene (15). Some of these polymorphisms have been shown to affect the levels of PXR expression, the regulation of PXR target gene *CYP3A4*, as well as rates of drug clearance (16-19). Single nucleotide polymorphisms (SNPs) have also been described in *PXR* of other species, including mice and pigs. In zebrafish, two allelic variants of *pxr*, denoted *pxr***1* and *pxr***2,* have recently been described in the Tupfel long fin (TL) strain. These two allelic variants are distinguished by differences in the amino acids at positions 184, 218 and 385 in the zebrafish Pxr sequence (13).

Functional differences among Pxr variants in zebrafish could have important implications for the use of zebrafish as a model species in toxicology and risk assessment. In this study we determined whether functional properties of Pxr are related to sequence variation in *pxr* from four strains of zebrafish. This study includes Pxr from three commonly used laboratory strains,

AB/Tüebingen (AB/Tü), Tupfel long fin (TL) and Singapore wild type (SWT), as well as a strain of unknown origin (hereafter denoted UNK). We have studied ligand activation of the zebrafish Pxr variants as well as ligand-receptor interactions, using a luciferase reporter gene assay and surface plasmon resonance (SPR), respectively.

Methods

Strains of zebrafish

Three commonly used strains of zebrafish were used in this study, including a hybrid of the Tüebingen and AB strains (AB/Tü), the Singapore wild type (SWT), the Tupfel long fin (TL), as well as a strain of unknown origin (UNK).

Cloning of pxr from zebrafish

The cloning of zebrafish *pxr* from the Tupfel strain and from the unknown strain have previously been described elsewhere (13, 20). Here we cloned *pxr* from the AB/Tü and SWT strains. Total RNA was extracted from liver tissues by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol) and cDNA was synthesized using Superscript III reverse transcriptase (both from Invitrogen). *Pxr* open reading frames were amplified by PCR (Takara ExTaq, Clonetech, Forward primer: 5´-GTCACCATGGCAATGTCCCGCTTATATGAC-3´, Reverse primer: 5´-TTGTGGATCCGAGGACTTAGGTGTCTTTGC-3´)), subcloned in pSC-A (Agilent technologies) and subsequently sequenced by Sanger sequencing (Big Dye 3.1, ThermoFisher Scientific, University of Bergen Sequencing Facility).

In vitro ligand activation of zebrafish PXR variants

Ligand induced transcriptional activity of four zebrafish Pxr variants was measured *in vitro* in COS7 cells using a GAL4-DBD/GAL4-UAS-based luciferase reporter gene assay (13, 21). Briefly, COS7 cells were transiently transfected to express one of the Pxr variants, and exposed to a rage of concentrations of clotrimazole (CLO, 0.04-4.5 μM) or butyl-4-aminobenzoate (4BAB, 0.14-50 μM) for 24 hours. Ligand activation is reported as β-galactosidase normalized luciferase activities in lysates from exposed cells over activities in cells exposed to solvent control alone (dimethylsulfoxide, DMSO, 0,5%). Maximum effect (E_{max}) and effective concentration 50 (EC_{50}) were

calculated from dose-response curves fitted by non-linear regression (GraphPad Prism).

Recombinant expression and purification of zebrafish Pxrs

The hinge region and ligand-binding domain of the different zebrafish Pxr variants (amino acids 111-430/431) were recombinantly expressed in *E. coli* BL21 as N-terminally 6Xhistidine tagged fusion-proteins with maltose-binding protein (pETM-41, EMBL). Lysogenic broth expression cultures were cultivated at 37C until entering the exponential growth phase (OD_{600nm} = 0.5-0.6). While at exponential growth, protein expression was induced by addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration 0.5 mM, and subsequently, the cultures were incubated at 18C for approximately 16 hours. Cells were harvested by centrifugation (5000xg, 10 minutes) and stored at -20C awaiting protein purification. Frozen cell pastes were resuspended in Ni-NTA binding buffer (phosphate buffered saline, pH 7,4, 500 mM sodium chloride (NaCl), 30 mM imidazole, EDTA-free protease inhibitors (Roche cOmplete)) and lysed by sonication. Soluble protein fractions were collected by centrifugation (35000xg, 30 minutes) and filtered (0.45μm), prior to further purification by immobilized metal ion affinity chromatography (IMAC) (5mL HisTrap HP, GE Healthcare). Selected IMAC fractions were pooled, concentrated by selective centrifugation (Amicon Ultra-15, 30k MWCO), and subjected to size exclusion chromatography (Sephadex 75 16/60, GE Healthcare; 1xPBS pH 7.4, 500 mM NaCl).

Analysis of receptor-ligand interactions

Receptor-ligand interactions were analysed by surface plasmon resonance (BIACORE T-200; CM5 chip; GE Healthcare) to establish kinetic constants and binding strength. The flow cells were preconditioned by injections of running buffer (1XHBS-P: 10 mM HEPES, pH 7.4, 0.15 M NaCl, 0.05% surfactant P20 and 2% DMSO; 3x10 seconds), of 100 mM hydrogen chloride (2x10 seconds), of 50 mM sodium hydroxide (2x10 seconds), and

finally of 0.5% sodium dodecyl sulphate (2x10 seconds), all with a flow of 100 µL/min. An 1:1 mixture of N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride and N-hydroxysuccinimide (ThermoFisher Scientific) was injected (420 seconds, 5 µL/minute) to allow covalent immobilization of the MBP-zfPxr-LBD fusion proteins by amino coupling to the CM5 chip. Typically, purified recombinant MBP-Pxr LBD fusion proteins (10 µg/mL in 10 mM sodium acetate, pH 4.5) were amino coupled to the CM5 chip at a flow rate of 5 µL/minute to densities of 2300 RU on channels FC-2 and FC-4. No MBP-Pxr LBD was coupled to the surfaces of channels FC-1 and FC-3 and these were used as references. After immobilization, unreacted esters of the receptors were blocked by an injection of ethanolamine-hydrochloride (420 seconds).

Kinetic analyses were performed with CLO and 4BAB in concentrations ranging from 0-50 µM. The test compounds were injected over measurement and reference surfaces with a contact time of 60 seconds and flow rate of 60 µL/min to avoid mass transport. The chip surfaces were regenerated by injection of running buffer (60 µL/min for 10 minutes) prior to change of test compound. The interaction analysis between the MBP-Pxr LBD and ligand was performed with both multi- and single cycle kinetics at the same conditions (25°C). Surface plasmon resonance sensorgrams for the receptor-ligand interactions were fitted globally using a 1:1 Langmuir interaction model.

Sequence alignments, amino acid identity analysis, and functional prediction of amino acid substitutions

Sequence alignments were conducted in Clustal Omega (22) and visualized using Jalview (23). Amino acid identity analysis was performed using the "Sequence identity and similarity" (SIAS) resource with a BLOSUM62 matrix (24). Percentage of amino acid identity was calculated by accounting for gaps in the sequences using the entire length of the alignment**.** Functional predictions of substitutions were performed using SIFT (25) and PolyPhen2 (26).

Results

Sequence analysis of zebrafish pxr variants

As a first step in assessing the divergence of the zebrafish Pxr variants, the overall number of identical amino acids in the variants was compared. The Pxr sequence similarities were very high, and 94-96% of the amino acids in the zebrafish Pxr variants were positionally conserved (Table 1). As expected, the intra-species conservation of Pxrs from zebrafish (97-98%) was much greater than the conservation between the zebrafish Pxrs and the human PXR (45-46%). Interestingly, the portion of positionally conserved amino acids between zebrafish and medaka (*Oryzias latipes*) Pxrs (51%), were only slightly higher than between zebrafish and human (*Homo sapiens*) PXRs (44%), indicating that large sequence variance in Pxr also exist within teleost species.

When comparing the sequences of the zebrafish Pxr variants from AB/Tü, SWT and TL, we found that the DNA-binding domains (1-110) were identical. However, two indels occur in the hinge region and in the ligand-binding domain (Figure 1), and as a result the zebrafish Pxr variants vary in length (PxrAB and PxrTL: 430 AAs, PxrAB/Tü and PxrSWT: 431 AAs) (Table 1). To ease the amino acid numbering of the Pxr sequences, a consensus sequence of zebrafish Pxr was constructed by combining the four individual sequences. The resulting sequence, PxrCON, consisted of 432 amino acids. In the following, references to amino acid positions is based on the PxrCON numbering.

Two allelic variants of *pxr* denoted *pxr*1* and *pxr*2* were recently discovered among zebrafish of the TL strain (13). These variants are characterized by nonsynonymous substitutions leading to difference in amino acids in positions 184, 218 and 385. Of the variants from this study, PxrTL and PxrAB/Tü correspond to the Pxr*1 variant (S184, Y218 and H385), while PxrUNK corresponds to Pxr*2 (I184, C218 and N385). PxrSWT possess two of three characteristics that correspond to Pxr*1, but a phenylalanine (F) occupies position 218, which is in contrast to a tyrosine (Y) found in the Pxr*1 allelic variant (Table 4, Figure 1).

We found a total of 18 positions in which the amino acid varied between the zebrafish Pxr variants (Supplement 1). PxrAB/Tü and PxrUNK were the most divergent variants, with 16 of 18 variable positions occupied by different amino acids. The most similar variants were PxrTL and PxrUNK that had different amino acids in only five positions.

Ligand activation of Pxr variants in zebrafish

A luciferase reporter gene assay was used to investigate if the variation in the amino acid sequences of the zebrafish Pxr variants had functional effects. We measured the ability of the variants to be activated into forms that induce transcription of the luciferase reporter gen *in vitro* by a local anaesthetic butyl-4-aminobenzoate (4BAB) and an antifungal drug clotrimazole (CLO). CLO activated all four variants and induced a strong transcription of the luciferase reporter gene. The maximal response (E_{max}) to exposure to CLO in cells expressing PxrAB/Tü was a 22-fold increase in the luciferase activity, while E_{max} in cells expressing PxrTL, PxrSWT or PxrUNK were in the range of 12-16-fold increase in luciferase activities (Figure 2A, Table 2). 4BAB induced a moderate increase in the luciferase activities in cells expressing PxrAB/Tü, PxrTL or PxrSWT (4-5-fold), while the luciferase activity in cells expressing PxrUNK did not increase significantly (Fig. 2B, Table 2).

PxrAB/Tü and PxrTL were most susceptible to activation by CLO, according to estimated EC50s, while PxrUNK was least responsive (Table 2). Typically, the EC_{50}s of responses induced by CLO was 100-fold lower than for responses induced by 4BAB, indicating that CLO is a more potent agonist for zebrafish. The larger magnitude of response induced by CLO than by 4BAB, demonstrates also that CLO is an agonist of higher efficacy for zebrafish Pxrs than 4BAB.

Receptor-ligand interactions

After demonstrating differences in transactivation by zebrafish Pxr variants activated by CLO or 4BAB, we wanted to investigate if the sequence

variations affected the receptor-ligand interactions. The hinge and ligand binding domains (AA111-430) of Pxr from TL, AB/Tü, SWT and the unknown strain (UNK) were recombinantly expressed in *E. coli* BL21 as 6Xhistidine-tagged fusion proteins with maltose binding protein (MBP). The different variants of MBP-Pxr LBD fusion proteins were purified to near homogeneity (Supplement 2A-E).

The ligand-receptor interaction of the ligand CLO and the four purified zebrafish Pxr variants was explored with surface plasmon resonance (SPR). While the dissociation constant (K_d) of the interaction between CLO and PxrAB/Tü was in the nanomolar range (40 nM), the K_d of the CLO-PxrTL interaction was approximately 100 fold higher in the low micromolar range (4 μM, Table 2). The K_d of the dissociation constant for the interactions between CLO, PxrSWT and PxrUNK were 260 and 180 μM, respectively, demonstrating that PxrAB/Tü has higher affinity for CLO than PxrTL, PxrSWT and PxrUNK. Based on the K_d determined, CLO interacts with PxrSWT and PxrUNK with significantly less affinity (Table 2 and Figure 3). While interaction between 4BAB and the zebrafish Pxr variants could be demonstrated by SPR, reliable quantification of the strength of interactions could not be made.

Predicting functional effects of amino acid variation

To explain the observed difference in dose-response dynamics, the amino acid sequence of the most responsive variants, PxrAB/Tü, was compared to the sequences of less responsive variants. PxrAB/Tü is most similar to PxrSWT (98.4%) and least similar to PxrUNK (96.3%). In four positions PxrAB/Tü holds a unique amino acid compared to the other variants, including one position in the hinge region (L186V) and three in the ligand binding domain (S235T, L417P and L421P). Two different tools, the SIFT platform (25) and the PolyPhen2 (26), were used to predict functional effects of the four substitutions that were unique to PxrAB/Tü. Similarly, the substitutions not

unique to PxrUNK were evaluated and PolyPhen2 characterized the S184I and H385N substitutions to be possibly damaging.

Discussion

We have described variation in *pxr* from several different strains of zebrafish, and observed differences between these resulting Pxr variants in their interaction with agonists and in their ligand-induced transcriptional activities. We also demonstrate that the efficacy of clotrimazole as an agonist for zebrafish Pxrs is strongly variant dependent.

Intraspecies variation in *pxr* is not unique to zebrafish. Numerous polymorphisms in human *pxr* have been reported, and to date (March 2016), approximately 2220 non-coding region single nucleotide polymorphisms (SNPs) and 349 coding region SNPs have been reported in human *PXR*, including 12 indels (15). Some polymorphisms in coding and non-coding regions of human *pxr* have been shown to affect the levels of PXR, and of its target gene *CYP3A4*, as well as rates of drug clearance (16-19). SNPs have also been reported in the *PXR* of other species, including the domestic pig (*Sus scrofa*: 699 SNPs) and in mice (*Mus musculus*: 1484 SNPs).

Compared to other model organisms, such as mice, zebrafish has a more diverse genetic background. To explain interspecies difference in the diversity of the genetic backgrounds, difference between the species in the methods used to generate the lines has been mentioned (27). Although the laboratory strains of zebrafish have a diverse genetic background, the genetic variation of commonly used laboratory strains of zebrafish is generally lower than in wild zebrafish (28). Thus, it is not unlikely to find considerable variation in the *pxr* of zebrafish.

Strain differences in activation of zebrafish Pxr

Clotrimazole (CLO) activates PXRs from different organisms, including zebrafish, mouse, polar bear and human (13, 20, 21, 29, 30). We found that CLO induced the transcriptional activity of zebrafish Pxr variants differently *in vitro*. PxrAB/Tü was significantly more sensitive to activation (lower EC_{50}) by

CLO, compared to the other zfPxr variants. Additionally, CLO induced a greater transcriptional activity (higher E_{max}) in PxrAB/Tü than in zfPxrs variants from the TL, SWT, and UNK strains. Interestingly, previous reports of the ability of CLO to activate zebrafish Pxr are somewhat conflicting. Some reports indicate that CLO is an agonist for zebrafish Pxr, both *in vivo* and *in vitro* (13, 20, 31). However, others have found no activation of zebrafish Pxr *in vitro* by CLO (32) and furthermore, treatment of zebrafish with CLO did not significantly increase levels of putative Pxr target genes, including *cyp3a65* and *mdr1* (12).

Discrepancies in ligand activation of zebrafish Pxr, such as those seen for CLO, have been observed with other potential agonists, including 4BAB and nifedipine. 4BAB activates several mammalian PXRs *in vitro*, CXR from chicken (31) and the zebrafish Pxr (31, 33). We found that 4BAB was an agonist of moderate efficacy that activated three of four variants of the zebrafish Pxr. Nifedipine (NIF) has been reported to be a relatively potent agonist of zfPxr that induce a transcriptional activity corresponding to about 50% of the activity induced by CLO equal (31, 32). However, in zebrafish *in vivo* exposed to NIF no increase in the transcript levels of Pxr target genes, such as *cyp3a65* and *mdr*, were seen (12). In our experiments, NIF was a weak agonist of PxrAB/Tü, PxrSWT and PxrTL producing a maximum response corresponding to about 10% of that induced by CLO (Data not shown). The discrepancies in reported activation of zebrafish Pxr by several compounds we suggest may be the result of allelic differences in the *pxr*. However, as detailed information of the origin of the zebrafish used in studies is often scarce, we have not managed to identify the origin of the zebrafish used in all of these studies that possibly could explain these discrepancies.

PxrAB/Tü binds clotrimazole more strongly

The dissociation constants for nuclear receptor-ligand interactions typically range from low nanomolar to micromolar concentrations. The strongest nuclear receptor-ligand interactions, as measured by K_d, are those

between hormone receptors and their endogenous ligands (reviewed in (34). In contrast to hormone receptors, PXRs bind a variety of different ligands, and each interaction has unique properties and dose-response dynamics. Interestingly, the dissociation constant for the interactions between human PXR and SR12813, a cholesterol lowering drug, is comparable to that of the PxrAB/Tü-CLO interaction (Table 3) (35). Additionally, the dynamics of activation of the hPXR by SR12813 and PxrAB/Tü-CLO in ligand activation assays are similar.

The K_d of the PxrTL-CLO interaction was approximately 100-times higher than for the PxrAB/Tü-CLO interaction (Table 2). Interestingly, CLO induced a stronger transcriptional activity in PxrAB/Tü than in PxrTL, which may suggest a relationship between the strength of the interaction between receptor and the maximum transcriptional activity induced. However, this relationship appears not to extend to cover the potency of ligand activation. Despite large differences in K_ds of interactions PxrAB/Tü-CLO, PxrTL-CLO and PxrSWT-CLO, no difference were seen in potency of CLO as an agonist (EC_{50}) for these three zfPxr variants in the ligand activation assay (Table 2). Neither maximal response seems to be predictive from receptor-ligand interaction. Although, the K_d of the PxrSWT-CLO interaction is approximately 65-times higher than the K_d of the PxrTL-CLO interaction, CLO induces similar maximal response via the two zfPxr variants in the luciferase reporter assay. Hence, if the strength of the receptor-ligand interaction is a determinant for the ligand activation response, it is probably not the only factor that determines induction of transcriptional activity.

Binding of ligand has been shown to stabilize the nuclear receptors by increasing dimerization with partner receptors (36). This in turn, increases the affinity for DNA response elements (37) and the recruitment of coactivators (38). Thus, our finding of greater response for the high affinity interaction is in accordance with that, at any concentration of ligand, the fraction of ligand-bound receptors is greater for a high affinity interaction than for a low affinity interaction. Which in turn result in more activated receptors available to

heterodimerize, to bind response elements and to induce transactivation. Interestingly, interactions with quite different dissociation constants (Kd of CLO-PxrTL >> Kd of CLO-PxrSWT and PxrUNK) produced similar maximal activations 12-15-fold) (Table 2).

The EC_{50}s of the dose-response resulting from exposure of COS7 cells expressing different zebrafish Pxr variants to 4BAB were typically 100-fold lower than for exposures of CLO. However, we were not able to obtain information on the Pxr-4BAB interaction by surface plasmon resonance (SPR), possible due to the sensitivity of the SPR method. Although we were unable to determine the K_d of Pxr-4BAB interactions, the dose-response dynamics for exposure of COS7 cells expressing different zebrafish Pxr variants to 4BAB indicated differences in ligand-receptor interactions. While 4BAB had agonistic effect on three of the zebrafish Pxr variants, the PxrUNK was not activated by 4BAB.

Explaining differences in receptor function by variation in amino acid sequence

CLO showed both higher efficacy (greater E_{max}) and greater potency (lowest EC_{50}) as an agonist for the PxrAB/Tü variant than for the other variants. The amino acid sequence of PxrAB/Tü was therefore compared to the other Pxr variants to determine whether specific residue differences might be associated with the observed differences in dose-response dynamics. PxrAB/Tü differed from all other variants by having unique amino acid substitutions in four positions, one in the hinge region (L186V) and three in the ligand-binding domain (S335T, L416P and L420P). Based on the degree of conservation of amino acids in sequence alignments from related sequences (SIFT) and physical and comparable consideration comparisons (PolyPhen2), none of these four substitutions was predicted to have a deleterious effect on receptor function. Thus, the decreased efficacy and potency of PxrTL, PxrSWT and PxrUNK due to substitution in amino acids 186, 335, 416 or 420 do not appear

to explain the higher affinity of the PxrAB/Tü-CLO interaction and the greater efficacy of CLO as agonist for PxrAB/Tü.

While 4BAB is an agonist of moderate efficacy for PxrAB/Tü, PxrTL and PxrSWT, it showed no agonistic activity on PxrUNK. One amino acid substitution in the hinge region (S184I) and three LBD substitutions (Y/F218C, M305K and H385N) are unique to the PxrUNK variant. In addition PxrUNK has an insertion of isoleucine in position N428, close to the C-terminal. SIFT and PolyPhen2 predicted no functional effects of the PxrUNK specific substitutions, with the exception of H385N, which that was predicted to be deleterious. Possibly, the replacement of the positively charged histidine by the negatively charged asparagine in position 385 impairs the ability of PxrUNK to be activated by 4BAB. To prove this, the N385 of PxrUNK could be mutated *in vitro* to histidine and the mutant´s function could be evaluated in a luciferase reporter assay. However, no difference in either efficacy or potency of CLO as an agonist was observed for PxrUNK and PxrTL that have asparagine and histidine in position 385, respectively. This indicates that the effects of the H385N substitution could be compound specific, and that histidine in position 385 is more important for the interaction of zebrafish Pxr with 4BAB than with CLO. Since clotrimazole and 4BAB are very different both in size and structure (Supplement 4), it is not unlikely that different amino acids could be involved in docking and coordinating these compounds in the ligand binding pocket of zebrafish Pxr. Identifying functional differences among the alleles *in vivo* would be important to confirm the inferences from the studies in vitro.

Implications of sequence variation for the use of zebrafish as model

According to ZFIN, there are approximately 30 lines of zebrafish (March 2016). Not all of these lines are widely used and laboratories currently use several different strains of zebrafish, globally. As previously mentioned, some discrepancies exist between different reports on activation of zebrafish Pxr *in vitro* (13, 31, 32). The occurrence of different Pxr variants of zebrafish

in the zebrafish strains used could provide an explanation for the observed discrepancies.

Although laboratory strains of zebrafish are much less genetically variable than wild zebrafish (28), more SNPs were found in publicly available ESTs and mRNA sequences from zebrafish compared to rodents (27). Thus, the number of SNPs in zebrafish *pxr* could be greater than the 1484 SNPs currently reported for the *PXR* of mice (15). The high degree of genetic variance could result from the origin of laboratory strains of zebrafish from outbred stocks (27). However, due to crossbreeding and differences in the degree of outbreeding, the genetic background of some strains of zebrafish is more diverse than others. In a study by Coe and co-workers, the SWT strain was found to have the most diverse genetic background, while the variation in genetic background was the least in the TL and AB/Tü strains (SWT>AB>WIK=TL=AB/Tü)(28).

To be able to evaluate how intraspecies variation in *pxr* sequences affects the usefulness of zebrafish as a model species, more information is needed. The prevalence of different *pxr* variants in zebrafish both within and between strains, and the link between variation and phenotypic outcomes *in vivo*, should be explored. This information could be obtained by a large-scale screening of commonly used strains of zebrafish to link *pxr* genotype to functions in gene expression and xenobiotic biotransformation.

Conclusion

Here we describe sequence variation in *pxr* obtained from three commonly used laboratory strains of zebrafish and one pet store source, and demonstrate with a luciferase reporter gene assay that the sequence variation is associated with the ability of the zebrafish Pxrs to be activated by clotrimazole and butyl-4-aminobenzoate. Furthermore, SPR analyses of the purified zebrafish Pxr variants and clotrimazole revealed significant differences in the strength of the receptor-ligand interactions, with K_D values ranging from 0.04 to 260 μM. Our findings indicate that intraspecies differences in the ability of zebrafish to sense foreign compounds, and to initiate the biotransformation of

xenobiotics, may arise from sequence variation in the *pxr* gene. Consequently, the choice of zebrafish has the potential of significantly influencing the outcome any toxicological study.

References

1. Orans J, Teotico D, & Redinbo M (2005) The nuclear xenobiotic receptor pregnane X receptor: recent insights and new challenges. *Molecular endocrinology (Baltimore, Md.)* 19(12):2891-2900.
2. Rosenfeld J, Vargas R, Xie W, & Evans R (2003) Genetic profiling defines the xenobiotic gene network controlled by the nuclear receptor pregnane X receptor. *Molecular endocrinology (Baltimore, Md.)* 17(7):1268-1282.
3. Kamstra JH, Alestrom P, Kooter JM, & Legler J (2014) Zebrafish as a model to study the role of DNA methylation in environmental toxicology. *Environmental science and pollution research international*.
4. Carvan MJ, 3rd, Gallagher EP, Goksoyr A, Hahn ME, & Larsson DG (2007) Fish models in toxicology. *Zebrafish* 4(1):9-20.
5. Lieschke GJ & Currie PD (2007) Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8(5):353-367.
6. Quaife NM, Watson O, & Chico TJ (2012) Zebrafish: an emerging model of vascular development and remodelling. *Curr Opin Pharmacol* 12(5):608-614.
7. Dai YJ*, et al.* (2014) Zebrafish as a model system to study toxicology. *Environ Toxicol Chem* 33(1):11-17.
8. Walogorsky M*, et al.* (2012) Zebrafish model for congenital myasthenic syndrome reveals mechanisms causal to developmental recovery. *Proc Natl Acad Sci U S A* 109(43):17711-17716.
9. Howe K*, et al.* (2013) The zebrafish reference genome sequence and its relationship to the human genome. *Nature* 496(7446):498-503.
10. Stegeman J, Goldstone J, & Hahn M (2010) Perspectives on zebrafish as a model in environmental toxicology. *Fish Physiology*, eds Farrell A & Brauner C (Elsevier), Vol 29, pp 367-439.
11. Goldstone JV*, et al.* (2010) Identification and developmental expression of the full complement of Cytochrome P450 genes in Zebrafish. *Bmc Genomics* 11.
12. Bresolin T, de Freitas Rebelo M, & Celso Dias Bainy A (2005) Expression of PXR, CYP3A and MDR1 genes in liver of zebrafish. *Comp Biochem Physiol C Toxicol Pharmacol* 140(3-4):403-407.
13. Bainy AC*, et al.* (2013) Functional characterization of a full length pregnane X receptor, expression in vivo, and identification of PXR alleles, in zebrafish (Danio rerio). *Aquat Toxicol* 142-143:447-457.
14. Kubota A*, et al.* (2015) Role of pregnane X receptor and aryl hydrocarbon receptor in transcriptional regulation of pxr, CYP2, and CYP3 genes in developing zebrafish. *Toxicol Sci* 143(2):398-407.
15. Information NCfB (2015) dsSNP - Short Genetic Variations.
16. Lamba J, Lamba V, Strom S, Venkataramanan R, & Schuetz E (2008) Novel single nucleotide polymorphisms in the promoter and intron 1 of human pregnane X receptor/NR1I2 and their association with CYP3A4 expression. *Drug Metab Dispos* 36(1):169-181.
17. Swart M*, et al.* (2012) PXR and CAR single nucleotide polymorphisms influence plasma efavirenz levels in South African HIV/AIDS patients. *BMC Med Genet* 13:112.
18. Zhang J*, et al.* (2001) The human pregnane X receptor: genomic structure and identification and functional characterization of natural allelic variants. *Pharmacogenetics* 11(7):555-572.

19. Siccardi M, *et al.* (2008) Association of a single-nucleotide polymorphism in the pregnane X receptor (PXR 63396C-->T) with reduced concentrations of unboosted atazanavir. *Clin Infect Dis* 47(9):1222-1225.
20. Milnes MR, *et al.* (2008) Activation of steroid and xenobiotic receptor (SXR, NR1I2) and its orthologs in laboratory, toxicologic, and genome model species. *Environ Health Perspect* 116(7):880-885.
21. Lille-Langoy R, *et al.* (2015) Environmental contaminants activate human and polar bear (Ursus maritimus) pregnane X receptors (PXR, NR1I2) differently. *Toxicol Appl Pharmacol* 284(1):54-64.
22. Sievers F, *et al.* (2011) Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 7:539.
23. Waterhouse A, Procter J, Martin D, Clamp Ml, & Barton G (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics (Oxford, England)* 25(9):1189-1191.
24. Reche P (2008) Sequence Identity and Similarity tool. (Universitad Complutense Madrid).
25. Kumar P, Henikoff S, & Ng PC (2009) Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm. *Nat Protoc* 4(7):1073-1081.
26. Adzhubei IA, *et al.* (2010) A method and server for predicting damaging missense mutations. *Nat Methods* 7(4):248-249.
27. Guryev V, *et al.* (2006) Genetic variation in the zebrafish. *Genome research* 16(4):491-497.
28. Coe TS, *et al.* (2009) Genetic variation in strains of zebrafish (Danio rerio) and the implications for ecotoxicology studies. *Ecotoxicology* 18(1):144-150.
29. Moore L, *et al.* (2000) Orphan nuclear receptors constitutive androstane receptor and pregnane X receptor share xenobiotic and steroid ligands. *The Journal of biological chemistry* 275(20):15122-15129.
30. Ekins S, *et al.* (2007) Human pregnane X receptor antagonists and agonists define molecular requirements for different binding sites. *Mol Pharmacol* 72(3):592-603.
31. Moore LB, *et al.* (2002) Pregnane X receptor (PXR), constitutive androstane receptor (CAR), and benzoate X receptor (BXR) define three pharmacologically distinct classes of nuclear receptors. *Mol Endocrinol* 16(5):977-986.
32. Ekins S, Reschly E, Hagey L, & Krasowski M (2008) Evolution of pharmacologic specificity in the pregnane X receptor. *BMC evolutionary biology* 8:103.
33. Krasowski M, Yasuda K, Hagey L, & Schuetz E (2005) Evolution of the pregnane x receptor: adaptation to cross-species differences in biliary bile salts. *Molecular endocrinology (Baltimore, Md.)* 19(7):1720-1739.
34. Escriva H, Delaunay F, & Laudet V (2000) Ligand binding and nuclear receptor evolution. *Bioessays* 22(8):717-727.
35. Jones S, *et al.* (2000) The pregnane X receptor: a promiscuous xenobiotic receptor that has diverged during evolution. *Molecular endocrinology (Baltimore, Md.)* 14(1):27-66.
36. Forman BM, Chen J, & Evans RM (1997) Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome

proliferator-activated receptors alpha and delta. *Proc Natl Acad Sci U S A* 94(9):4312-4317.
37. Kuntz MA & Shapiro DJ (1997) Dimerizing the estrogen receptor DNA binding domain enhances binding to estrogen response elements. *J Biol Chem* 272(44):27949-27956.
38. Delfosse V*, et al.* (2015) Synergistic activation of human pregnane X receptor by binary cocktails of pharmaceutical and environmental compounds. *Nat Commun* 6:8089.
39. Reche P (2008) SIAS - Sequence Identity And Similarity.
40. Waterhouse AM, Procter JB, Martin DM, Clamp M, & Barton GJ (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9):1189-1191.
41. Lin K, Simossis VA, Taylor WR, & Heringa J (2005) A simple and fast secondary structure prediction method using hidden neural networks. *Bioinformatics* 21(2):152-159.

Figures, tables and supplemental information:

Table 1 – Amino acid identity analyses of PXRs/Pxrs from selected organisms. Identical amino acids in PXR/Pxrs were identified from multiple sequence alignments (Clustal Omega) of full-length receptors (no background) and alignments of hinge and ligand-binding regions (grey background). The amino acid identity analysis was performed using the SIAL tool and accounted for sequence gaps (39).

Ortholog /variant	PXR HUM	Pxr MED	Pxr TL	Pxr AB/Tü	Pxr SWT	Pxr UNK
	1-434	1-414	1-430	1-431	1-431	-
	107-434	87-414	111-430	111-431	111-431	111-431
PXR HUM	100%	42%	46%	45%	46%	
	100%	37%	44%	44%	44%	44%
Pxr MED	42%	100%	51%	51%	51%	
	37%	100%	51%	51%	51%	51%
Pxr TL	46%	51%	100%	98%	98%	
	44%	51%	100%	97%	97%	97%
Pxr AB/Tü	45%	51%	98%	100%	98%	
	44%	51%	97%	100%	98%	94%
Pxr SWT	46%	51%	98%	98%	100%	
	44%	51%	97%	98%	100%	95%
Pxr UNK						
	44%	51%	97%	94%	95%	100%

Table 2 – Dose-response dynamics for activation of zebrafish Pxrs by CLO and 4BAB and ligand-receptor interactions. The table describes the maximum activation (E_{max}) and effective concentration 50 (EC_{50}) of Pxr-mediated induction of luciferase activities in COS7 cells. E_{max} and EC_{50} were calculated from dose-response curves fitted by non-linear regression (GraphPad Prism). Statistical significance indicators (T-test < 0.05): (e): EC_{50}s for responses induced by same agonist via different receptor variants are significantly different, (f): EC_{50}s for responses induced by different agonist via same receptor variant, (g): E_{max} for response via PxrAB/Tü ≠ E_{max} for responses via PxrTL or PxrUNK and (h): E_{max} for response via PxrSWT and PxrTL ≠ E_{max} for responses via PxrUNK. Dissociation constants were determined by surface plasmon resonance.

Zebrafish Pxr variant	Clotrimazole (CLO)			Butyl 4-aminobenzoate (4BAB)	
	E_{max} (fold)	**EC_{50}** (M)	**Dissociation constant (K_d)** (μM)	**E_{max}** (fold)	**EC_{50}** (M)
PxrAB/Tü	22.2 (g)	$1.0*10^{-7}$ (e, f)	0.04	5.4	$1.3*10^{-5}$ (f)
PxrTL	12.5 (h)	$1.2*10^{-7}$ (f)	4.1	4.0	$1.0*10^{-5}$ (f)
PxrSWT	15.8 (h)	$2.1*10^{-7}$ (f)	260	5.2	$1.3*10^{-5}$ (f)
PxrUNK	11.6	$3.6*10^{-7}$ (e)	180	1.2	N/D

Table 3 – Overview of dose-response and receptor-ligand interaction properties for the interaction between human and zebrafish PXR/Pxr and their respective ligands SR12813 and clotrimazole.

Interaction Property	hPXR-SR12813	zfPxr-CLO
K_d (μM)	0.04	0.04
E_{max} (fold)	≈20	22.2
EC_{50} (μM)	0.2	0.1
Reference	(35)	This study

Table 4 – Classification of zebrafish Pxr variants according to previously described allelic variants. Zebrafish Pxr variants, PxrTL, PxrAB/Tü, PxrSWT, PxrUNK, were compared to allelic variants previously described by Bainy et. al. (13).

Variant	Reference	Position		
		184	218	385
Pxr*1	(13)	Ser (S)	Tyr (Y)	His (H)
Pxr*2	(13)	Ile (I)	Cys (C)	Asn (N)
PxrTL	This study	Ser (S)	Tyr (Y)	His (H)
PxrAB/Tü	This study	Ser (S)	Tyr (Y)	His (H)
PxrSWT	This study	Ser (S)	Phe (F)	His (H)
PxrUNK	This study	Ile (I)	Cys (C)	Asn (N)

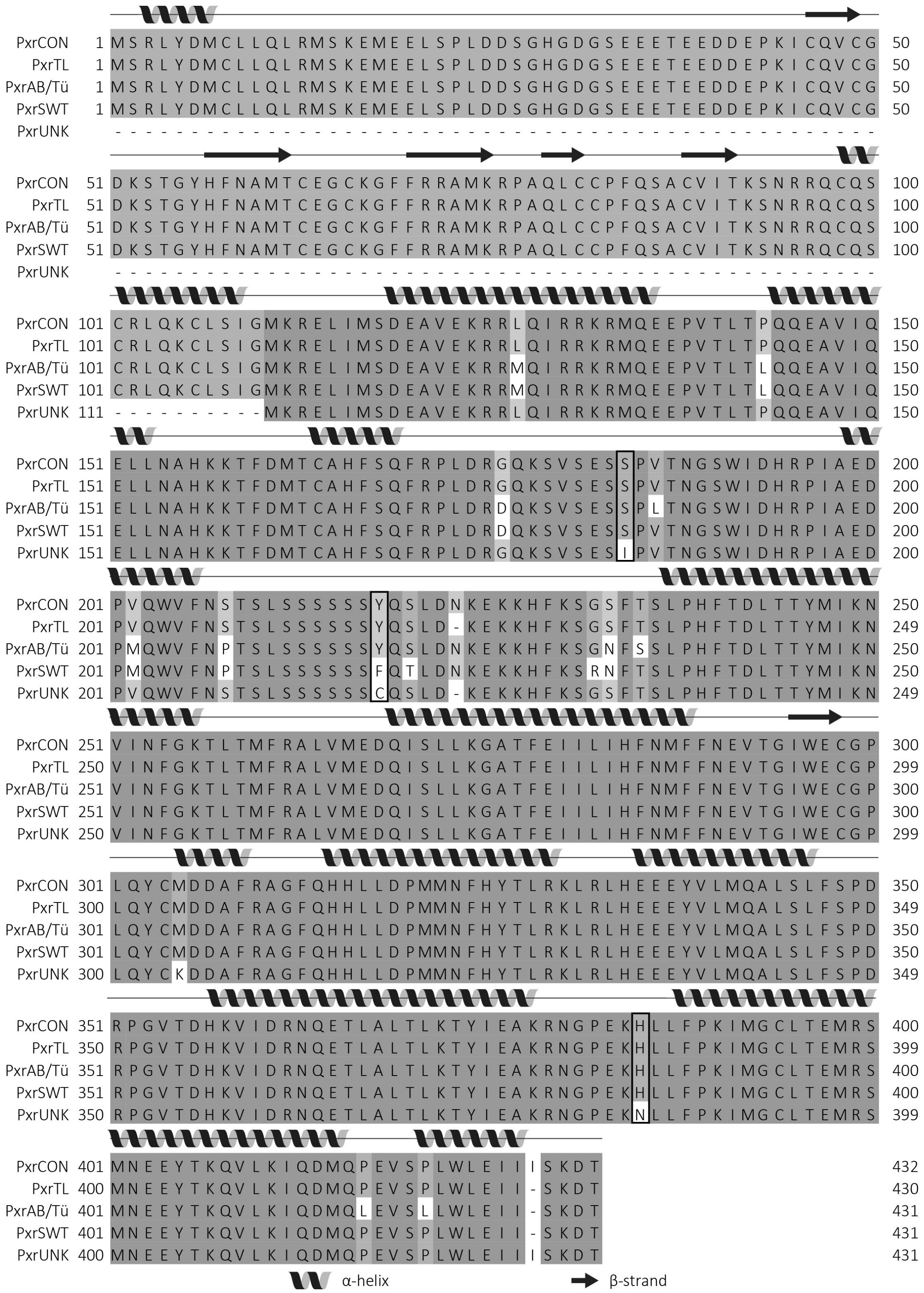


Figure 1 – Multiple sequence alignment of Pxr from five strains of zebrafish. Protein sequences were aligned using Clustal Omega (22) and visualised in Jalview (40). Darker scale of grey indicates higher degree of amino acid identity. Secondary structures were predicted using hidden neural networks (YAPIN (41) and were indicated by spirals (α-helices) and arrows (β-strands). Amino acid positions characteristic for Pxr*1 or Pxr*2 classification according to Bainy *et. al.* (13) are boxed.

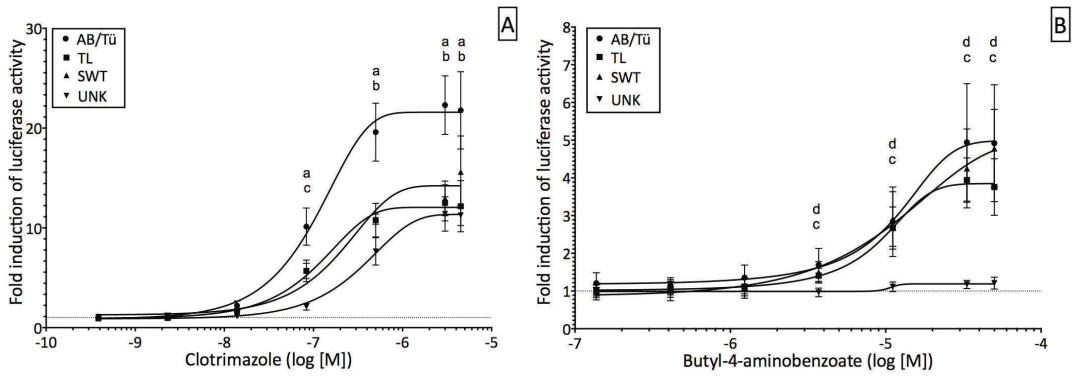


Figure 2 – Variant dependent activation of zebrafish Pxrs. *In vitro* activation of the zebrafish Pxr variants were measured in a luciferase reporter gene assay. Cells were exposed to clotrimazole (A, up to 4.5 µM) and butyl 4-aminobenzoate (B, up to 50 µM), and responses were reported as normalized luciferase activities in test cells related to unexposed control cells. Dose-response curves were fitted by non-linear regression (GraphPad Prism). Statistical significance indicators (T-test < 0.05): a: response via PxrAB/Tü ≠ response via PxrTL, PxrSWT and Pxr UNK, b: luciferase activity in exposed cells with PxrAB/Tü, PxrTL, PxrSWT or PxrUNK ≠ control/DMSO cells, c: luciferase activity in exposed cells with PxrAB/Tü, PxrTL or PxrSWT ≠ control/DMSO cells and d: response via PxrAB/Tü, PxrTL and PxrSWT ≠ response via Pxr UNK.

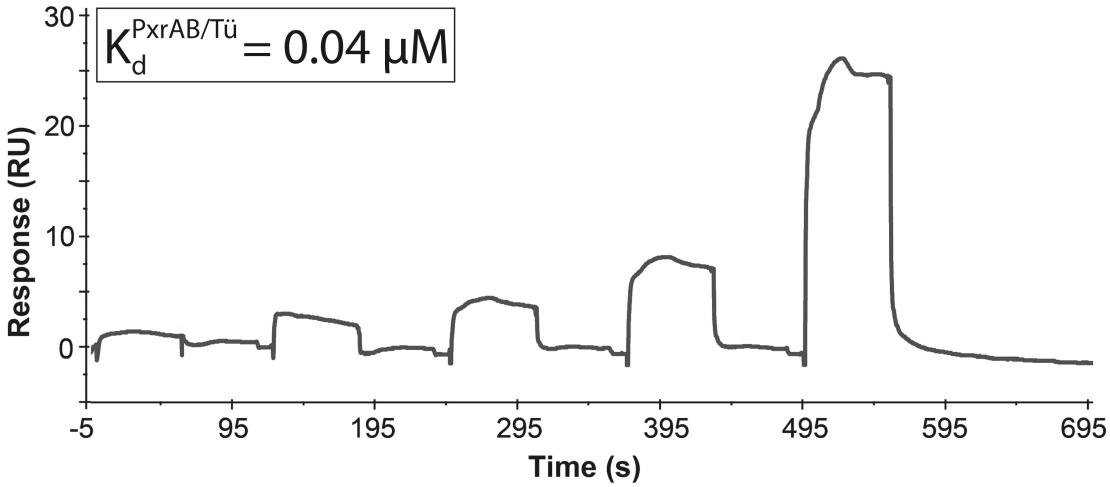
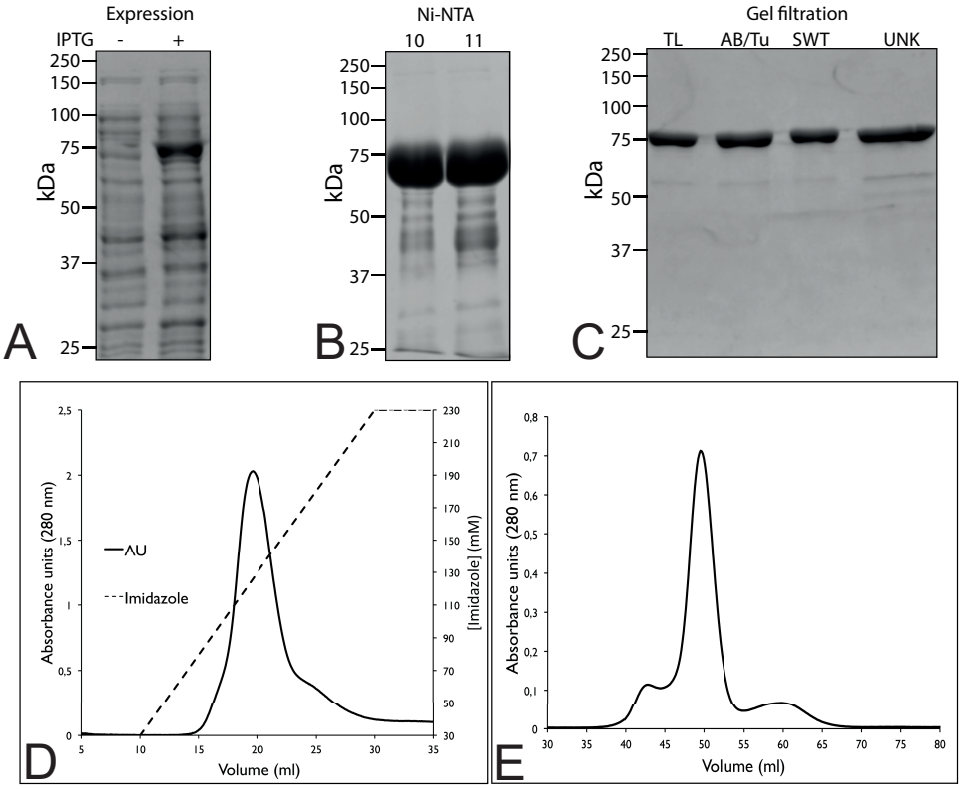


Figure 3 – Surface plasmon resonance dissociation curves for the interactions between clotrimazole and PxrAB/Tü. The interaction analysis between the MBP-Pxr LBD and ligand was performed with both multi- and single cycle kinetics at the same conditions (25°C). Surface plasmon resonance sensorgram for the receptor-ligand interaction was fitted globally using a 1:1 Langmuir interaction model.

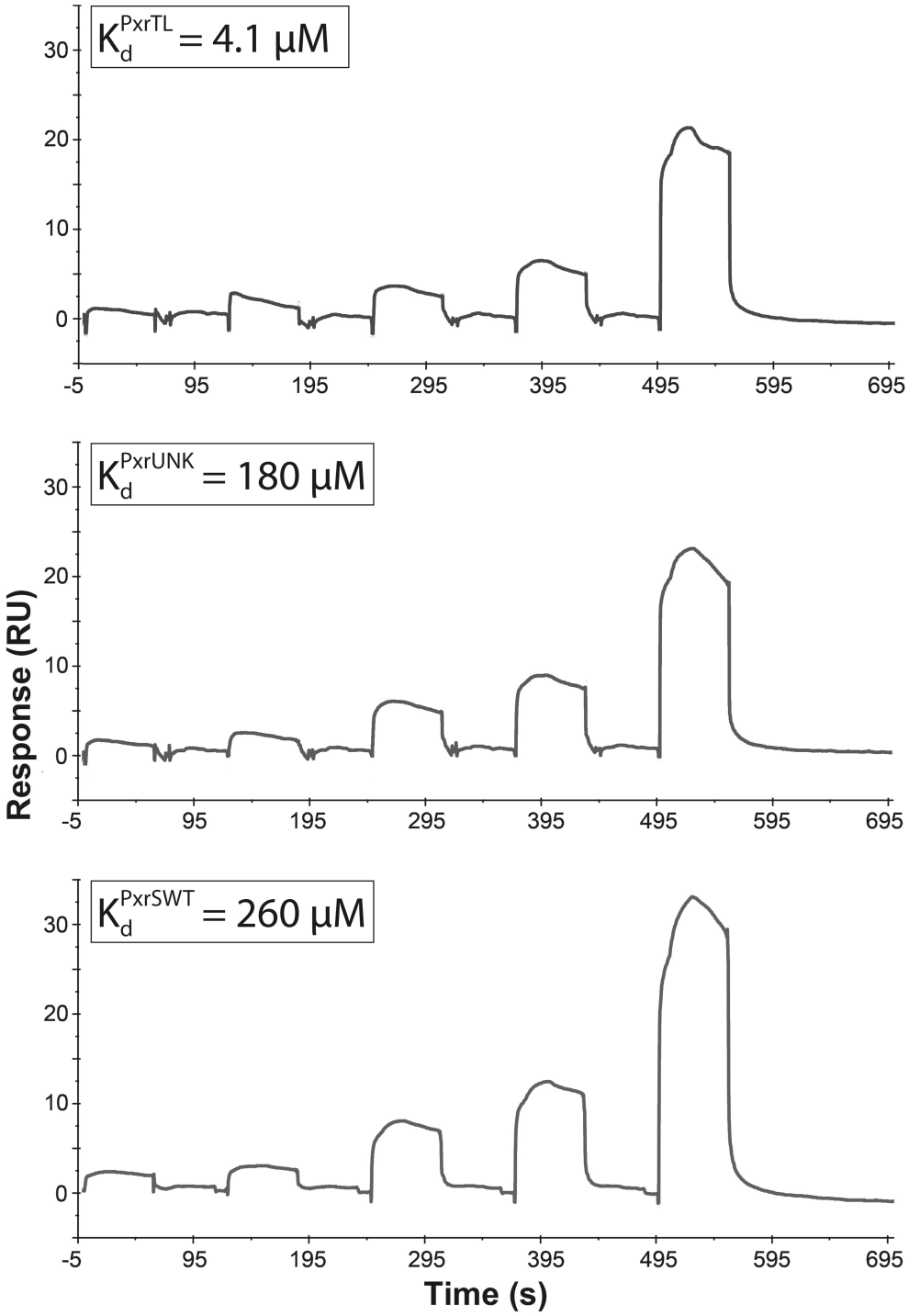
Position*	PxrCON	PxrTL	PxrAB/Tü	PxrSWT	PxrUNK
127	Leu (L)	Leu (L)	Met (M)	Met (M)	Leu (L)
143	Pro (P)	Pro (P)	Leu (L)	Leu (L)	Pro (P)
176	Gly (G)	Gly (G)	Asp (D)	Asp (D)	Gly (G)
184	Ser (S)	Ser (S)	Ser (S)	Ser (S)	Ile (I)
186	Val (V)	Val (V)	Leu (L)	Val (V)	Val (V)
202	Val (V)	Val (V)	Met (M)	Met (M)	Val (V)
208	Ser (S)	Ser (S)	Pro (P)	Pro (P)	Ser (S)
218	Tyr (Y)	Tyr (Y)	Tyr (Y)	Phe (F)	Cys (C)
220	Ser (S)	Ser (S)	Ser (S)	Thr (T)	Ser (S)
223	Asn (N)	-	Asn (N)	Asn (N)	-
232	Gly (G)	Gly (G)	Gly (G)	Arg (R)	Gly (G)
233	Ser (S)	Ser (S)	Asn (N)	Asn (N)	Ser (S)
235	Thr (T)	Thr (T)	Ser (S)	Thr (T)	Thr (T)
305	Met (M)	Met (M)	Met (M)	Met (M)	Lys (K)
385	His (H)	His (H)	His (H)	His (H)	Asn (N)
417	Pro (P)	Pro (P)	Leu (L)	Pro (P)	Pro (P)
421	Pro (P)	Pro (P)	Leu (L)	Pro (P)	Pro (P)
428	Ile (I)	-	-	-	Ile (I)

* based on PxrCON numbering

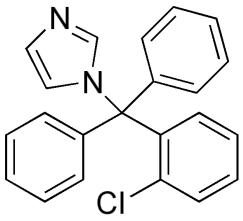
Supplement 1 – Overview of amino acid differences in four variants of zebrafish Pxr.



Supplement 2A-E – His-tagged fusion protein of maltose-binding protein (MBP) and zebrafish Pxr hinge and ligand-binding domains were purified to near homogeneity. Fusion proteins of MBP and the hinge region and LBD domain of zebrafish Pxrs were expressed recombinantly in *E. coli* BL21 cells. Coomassie stained SDS-PA gels demonstrated that addition of IPTG induced the production of the 75 kDa fusion protein (A), that was enriched by Ni-NTA (B and D), and further purified to near homogeneity by gel filtration (C and E). In Figures 3A, -B, -D and -E different steps in purification of PxrTL are shown and these were representative for the purification of PxrSWT, PxrAB/Tü and PxrUNK.



Supplement 3 – Surface plasmon resonance dissociation curves for the interaction between zebrafish Pxr variant and the agonist clotrimazole. The interaction analysis between the MBP-PxrTL, PxrSWT and PxrUNK and clotrimazole were performed with both multi- and single cycle kinetics at the same conditions (25°C). Surface plasmon resonance sensorgrams for the receptor-ligand interactions were fitted globally using a 1:1 Langmuir interaction model.

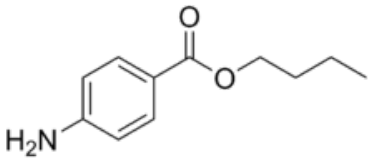


Clotrimazole

CAS NO: 23593-75-1

MW: 344,8 g/mole

Topological Polar Surface Area: 17.8 A^2



Butyl 4-aminobenzoate

CAS NO: 202-317-1

MW: 193.2 g/mole

Topological Polar Surface Area: 53.2 A^2

Supplement 4 – Chemical structure of test compounds.