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

(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 $5 \ast 10^3$ 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

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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}).
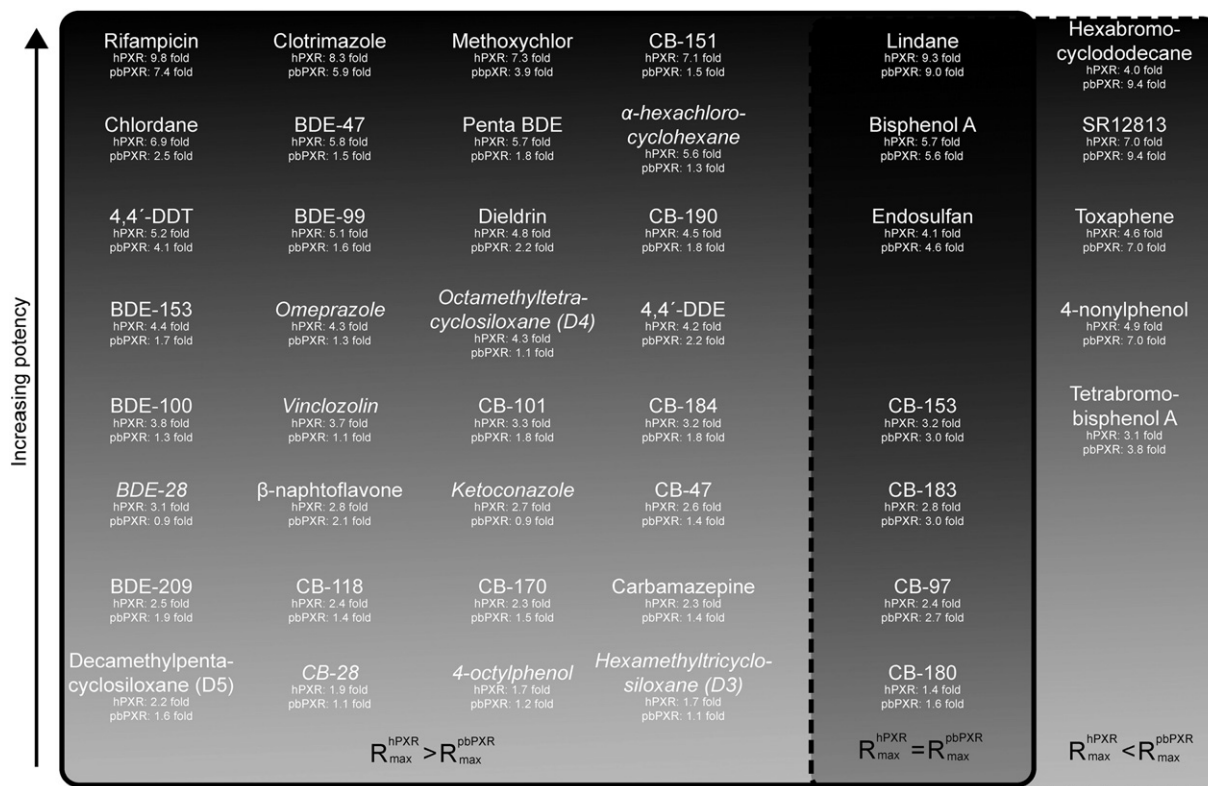
Fig. 2. Comparison of agonistic potential of PXR ligands via hPXR or pbPXR presented as a Venn diagram. Forty-two agonists were grouped according to their potential for transactivation via hPXR and/or pbPXR. The 29 compounds grouped within the solid box had higher potential for transactivation via hPXR (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
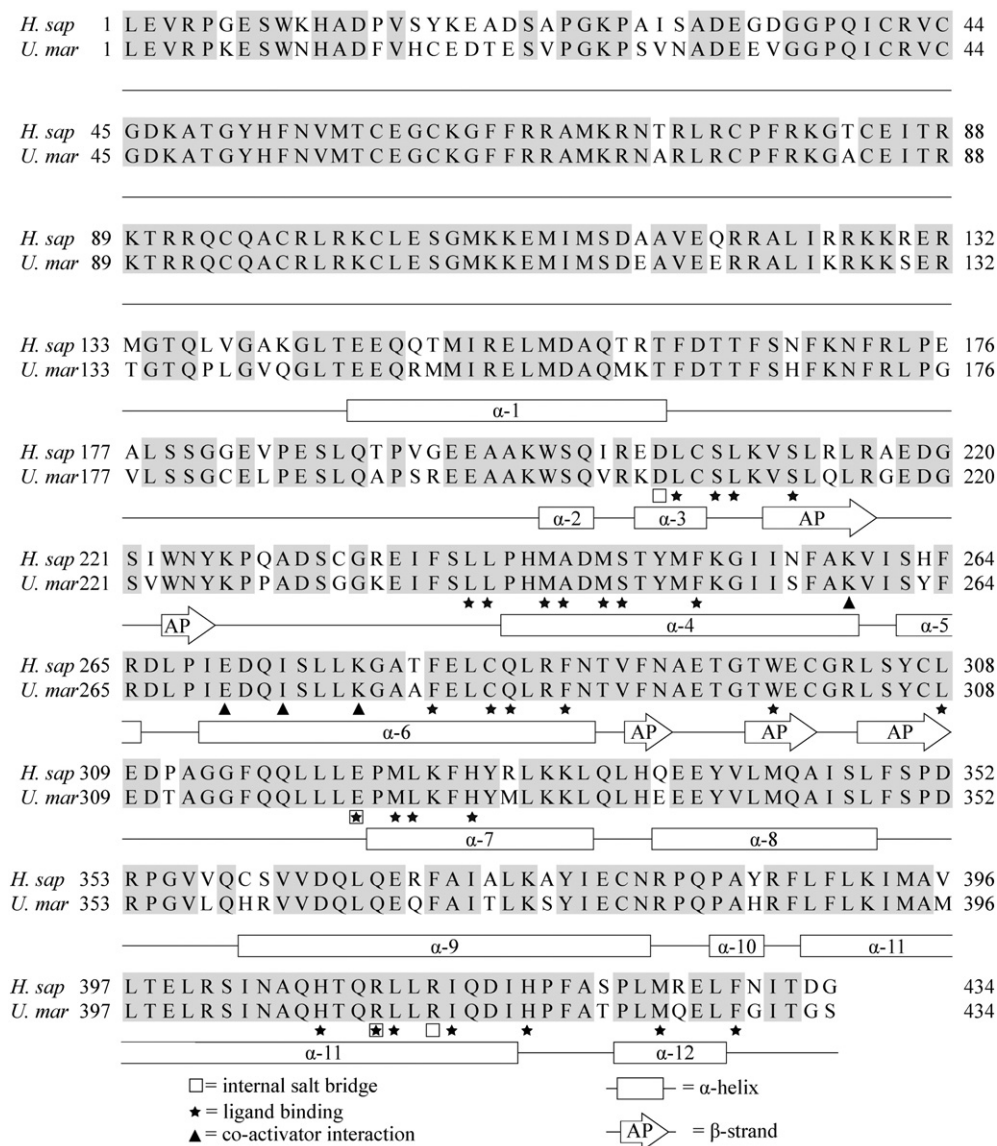
Fig. 3. Alignment of hPXR and pbPXR supplemented with known secondary structure and ligand-binding residues. Human PXR structures (Chrencik et al., 2005; Teotico et al., 2008; Watkins et al., 2001, 2003; Xue et al., 2007a, 2007b) was used to supplement a hPRX and pbPXR amino acid sequence comparison 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
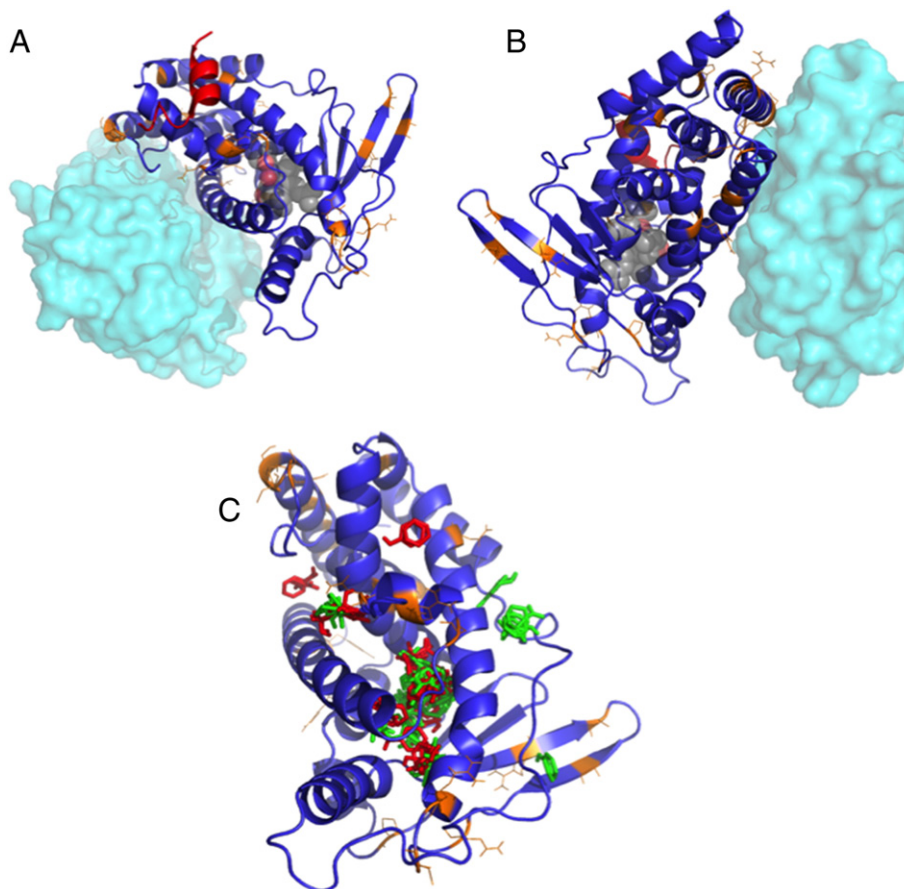
Fig. 4. Homology model for pbPXR based on multiple hPXR structures showing residue substitutions between human and polar bear. (A and B) Positions of residues differing between hPXR and pbPXR are shown in orange. Also shown is the relative position of the heterodimerization partner, RXR, from PDB 4J5W 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,

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).

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.

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.
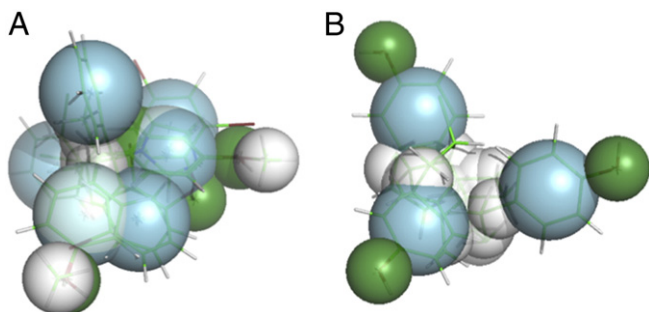
Importantly, although these data indicate activation of both hPXR and pbPXR by high concentrations of some ligands, like PCBs and PBDEs, other studies have shown several of these compounds to be antagonistic at lower concentrations (Tabb et al., 2004). The biological significance of such non-monotonic 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

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.

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