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Homology modelling of the nuclear receptors: human oestrogen receptorβ (hERβ), the human pregnane-X-receptor (PXR), the Ah receptor (AhR) and the constitutive androstane receptor (CAR) ligand binding domains from the human oestrogen receptor α (hERα) crystal structure, and the human peroxisome proliferator activated receptor α (PPARα) ligand binding domain from the human PPARγ crystal structure☆

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Abstract

We have generated by homology the three-dimensional structures of the ligand binding domain (LBD) of several interrelated human steroid hormone receptors (SHRs).

These are the oestrogen receptor β (hERβ), the pregnane-X-receptor (PXR), the Ah receptor (AhR) and the constitutive androstane receptor (CAR). They were produced by homology modelling from the human oestrogen receptor α (hERα) crystallographic coordinates [Nature 389 (1997) 753] as a template together with the amino acid sequences for hERβ [FEBS Lett. 392 (1996) 49], PXR [J. Clin. Invest. 102 (1998) 1016], AhR [Proc. Natl. Acad. Sci. U.S.A. 89 (1992) 815] and CAR [Nature 395 (1998) 612; Mol. Cell. Biol. 14 (1994) 1544], respectively. The selective endogenous ligand, in each case, was docked interactively within the putative ligand binding site using the position of oestradiol in hERα as a guide, and the total energy was calculated. In each receptor model a number of different ligands known to fit closely within the ligand binding site were interactively docked and binding interactions noted. Specific binding interactions included combinations of hydrogen bonding and hydrophobic contacts with key amino acid sidechains, which varied depending on the nature of the ligand and receptor concerned. We also produced the human peroxisome proliferator activated receptor α (PPARα) by homology modelling using the human PPARγ (hPPARγ) LBD crystallographic coordinates summarised in [Toxicol. In Vitro 12 (1998) 619] as a template together with the amino acid sequence for hPPARα [Toxicol. In Vitro 12 (1998) 619; Nature 395 (1998) 137].

The models will provide a useful tool in unravelling the complexity in the physiologic response to xenobiotics by examining the ligand binding interactions and differences between the steroid hormone receptors activation or inactivation by their ligands.

Keywords: Steroid hormone receptors (SHR); Ligand–receptor interaction; Molecular modelling; Estrogen receptor β (hERβ); Pregnane-X-receptor (PXR); Ah receptor (AhR); Constitutive androstane receptor (CAR); Peroxisome proliferator activated receptor α (PPARα)

Abbreviations: CYP, cytochrome P450; TCPOBOP, 1,4-bis[2-(,5-dichloropyridyloxy)]benzene; hERα, human oestrogen receptorα; hERβ, human oestrogen receptorβ; CAR, constitutive androstane receptor; PXR, pregnane-X-receptor; AhR, aryl hydrocarbon receptor; PPARα, human peroxisome proliferator activated receptor α; PPARγ, human peroxisome proliferator activated receptor γ; LBD, ligand binding domain; PCN, pregnenolone 16α-carbonitrile; HRE, hormone response element; EDC, endocrine disrupting compound; DBD, DNA-binding domain; RXR, 9-*cis*-retinoic acid receptor; 3′UTR, 3′untranslated region of hERα mRNA; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PCB, polychlorinated biphenyl; TZDs, thiazolidinediones; SHR, steroid hormone receptors; ARNT, aryl hydrocarbon receptor nuclear transporter protein; Arg, arginine; Gln, glutamine; Glu, glutamate; Ser, Serine; His, histidine

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1. Introduction

Nuclear steroid hormone receptors are a large protein superfamily that are involved in a wide range of physiological functions including development, reproduction, differentiation and homeostasis. They are regulated by hormones and chemicals that can mimic hormones [9]. After a hormone is produced, circulating and intracellular binding proteins regulate the hormones bioavailability. Then the hormone triggers action by binding to a specific cellular receptor by docking into a ligand binding domain (LBD) which is a hydrophobic pocket, and binding with specific amino acids [10].

An important requirement for homeostasis is the detoxication and removal of endogenous hormones and xenobiotic compounds with biological activity. This crucial metabolic role is conducted by the cytochrome P450 (CYP) enzyme superfamily. The induction of specific CYPs via the adaptive increase of *CYP* gene expression commonly utilises the nuclear receptor pathway where exposure to xenobiotics and drugs activates specific members of the nuclear receptor superfamily which in turn bind to their cognate DNA elements and stimulate the CYP target gene transcription [11–14]. Knowledge of nuclear hormone–receptor activation and action upon the regulation of gene expression can aid the understanding of the progression of certain diseases, and facilitate the design of drugs with improved efficacy and fewer side effects. The super family is broadly divisible into three subclasses: the type I receptors for steroid hormones, including progestins (PR), estrogens (ER), androgens (AR), glucocrticoids (GR) and mineralocorticoids (MR); the type II receptors for thyroid hormone (TR), Vitamin D (VDR), 9-*cis* retinoic acid (RXRs), all-*trans* retinoic acid (RARs) PPAR and the orphan class, for which cognate ligands have not yet been characterised, such as the PXR, CAR, COUP-TFs, HNF4, Rev Erb [15–19].

The currently accepted theory of steroid hormone-binding suggests that in the absence of the hormone, each receptor is associated with certain 'chaperone' proteins [10]. Binding of the steroid hormone with the receptor protein causes a conformational change. This molecular switch results in the removal of the heat shock complex and allows the receptors to dimerise. Then binding to a hormone response element (HRE) on DNA occurs, to produce a complex that can trigger or suppress the transcription of a selected set of genes [10,20], see Fig. 1.

So far 48 nuclear receptors have been identified in the human genome [16], but most of these are 'orphan receptors', in that they are awaiting the recognition of specific ligands and functions, and it is likely that more receptors will be discovered in the future. Each type of receptor has the potential to regulate a distinct endocrine signalling pathway, of which we only have a rudimentary knowledge. Members of this receptor family are related to each other in terms of their amino acid sequence and their function within cells. They therefore have structural features in common. These include a central highly conserved DNA binding domain (DBD) that targets the receptor to specific DNA sequences, termed hormone response elements (HREs). This domain contains eight cysteines, which form a pair of tetra coordinate binding sites for zinc atoms. When the zinc atoms allow folding of the protein, an α-helix is placed into the major groove of the DNA double helix. The amino acids on this α-helix enable the receptor to recognise the DNA in a sequence specific fashion. A terminal portion of this receptor (COOH) includes the ligand binding domain (LBD) which interacts directly with the hormone. This part of the receptor is larger and more complex than the DNA-binding domain. It is composed of three layers of α-helices forming a pocket. Embedded within this pocket is a hormone dependent transcriptional activation domain, and this is where ligands are transported prior to binding [10].

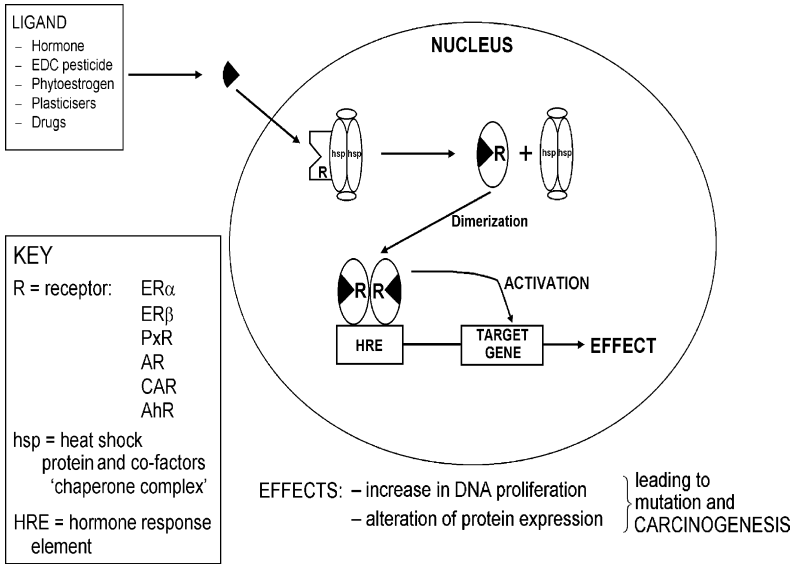

Fig. 1. Diagram showing the simplified mechanism of action of steroid hormone receptors.

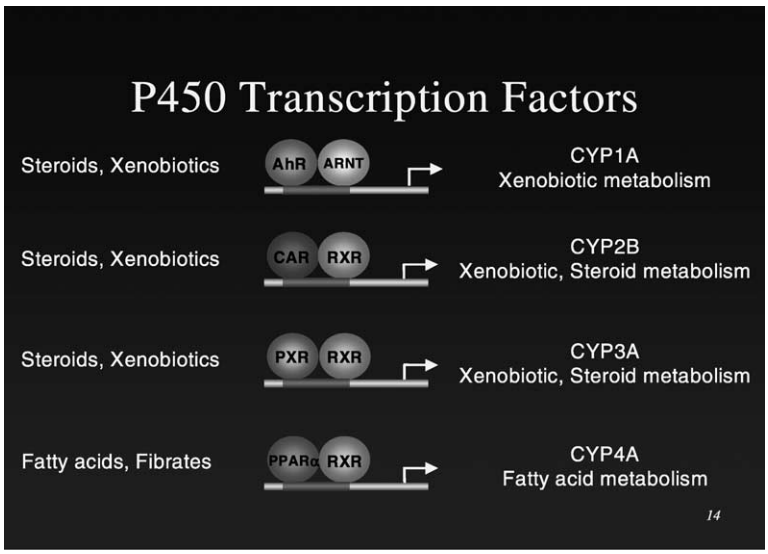

Fig. 2. P450 transcription factors.

In essence the LBD acts as a molecular switch that recruits co-activator proteins and activates the transcription of target genes when flipped into the active conformation by hormone-binding (Fig. 1). From a refinement of the characterisation of the role of the 3′ untranslated region (3′UTR) of hERα mRNA, the existence of another level in the control of the expression of the ligand-activated transcription factor hER in addition to transcriptional regulation has been determined [21]. Due to the general similarities in the structure and function of members of the steroid hormone family, it is likely that elements that influence the stability of mRNAs of other steroid hormones receptors should also be found in their 3′UTR [21]. The members of this family also have dimerisation receptor partners in common, particularly with the ubiquitous 9-*cis*-retinoic acid receptor (RXR) [12].

The ligand activated nuclear receptors CAR, PXR, and PPAR bind to their cognate DNA elements as heterodimers with RXR and thus activate the transcription of their *CYP2B*, *CYP3A*, and *CYP4A* genes (Fig. 2). This process of gene activation then leads to enhanced metabolism of the compound of exposure [12,14,16].

However, there are further complications as there may be competition between the receptors for RXR, as well as reduced RXR availability and activation in response to stress-signaling, triggered from a variety of environmental stimuli [22]. This suggests that stress-signaling will also indirectly affect all the receptors that dimerise with RXR, disabling gene activation even though ligand binding has occurred. Several receptors may be affected by the lack of availability of the dimerisation partner, including the ERs, PXR, CAR, the PPARs and other receptors. This will affect their ability to trigger or suppress gene transcription.

SHRs are subject to cross-talk interactions with other nuclear receptors, nuclear proteins, drug metabolizing enzymes (such as UGTs [23], and the transporter P-glycoprotein (Pgp) [24]) and with a broad range of other intracellular signaling pathways [12]. There may even be a cascade effect, where metabolites produced through the activities of one receptor are specific signaling molecules (and ligands) to modulate the next receptor, along the chain of a nuclear receptor intercommunication web.

Dependency upon interactions with other nuclear proteins or cofactors that are important tissue/cell specific mediators of nuclear receptor function introduces further regulation of the members of the SHR family. These may differ between receptors, or receptors may hold certain proteins or receptors in common with each other, such that part of the mechanism of action may be ascribable to competition between the receptor signalling pathways from the co activators or co repressors. This has been reported for the ER and AhR for example [25].

Fig. 3 provides a generalized schematic diagram of tissue distribution of steroid hormone receptors reported in the literature, representing potential sites of receptor action and the distribution of the selected steroid hormone receptors in humans, as reviewed in [26], and Table 1 summarises a number of activation compounds for these receptors, as reported in the literature.

1.1. The human oestrogen receptorα (hERα) and oestrogen receptorβ (hERβ)

The oestrogen receptors are known to exist as two subtypes, each one encoded by a separate gene. These are ERα [1], and the recently discovered ERβ [2] and its isoforms, of which a spliced isoform, ERβ/2 appears to be equally expressed in animal model tissue density studies [27]. The classical ERα subtype and ERβ receptors and isoforms apparently evolutionarily diverged over 450 million years ago, suggesting that although they have evolved in parallel, this

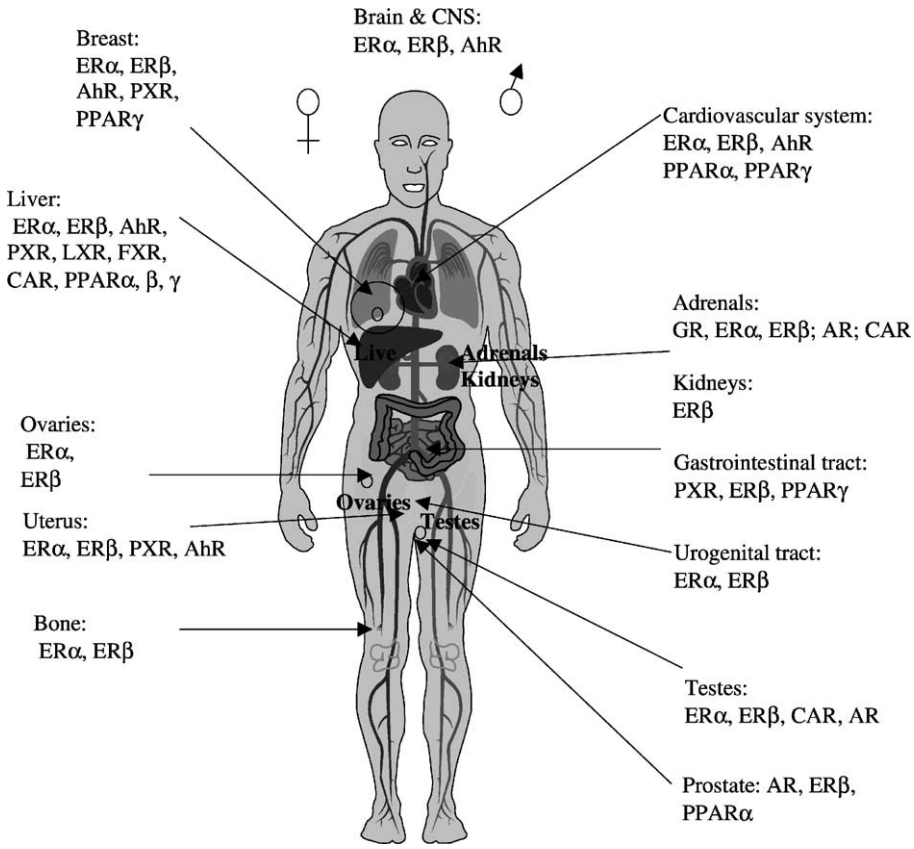

Fig. 3. Schematic diagram showing gender differences in tissue distribution of steroid hormone receptors reported in the literature, representing potential sites of receptor action.

ancient duplication was to facilitate unique roles in vertebrate physiology and reproduction [28]. The ERs differ in tissue distribution and relative ligand binding affinities for both endogenous and exogenous ligands (Table 2) [29,30], which may help explain the selective action of oestrogens and androgens in different tissues (Fig. 3) [31,32].

1.2. The pregnane-X-receptor (PXR)

The Pregnane-X Receptor (PXR), recently isolated and published by Lehmann [3] and Kliewer et al. [33], and later Blumberg et al. [34] is involved in activating the expression of several P450 detoxifying enzymes, including CYP3A4 in the adult and CYP3A7 in the foetus in response to xenobiotics and steroids [35]. CYP3A4 is the major human hepatic P450, and is involved in the metabolism of over 60% of drugs in clinical use [36].

PXR is highly divergent between species, with great differences in PXR activation profiles due to differences in the LBD [37].

The major site of PXR expression is in the liver hepatocytes and the gastrointestinal tissues, but they are also

Table 1
Selected receptors and activation compounds

Receptor	Activation compounds
ERβ	5α-Androstane diol and 3β-androstane diol [74], 17β-oestradiol, coumestrol, genistein, daidzein, *trans*-nonachlor, endosulphan, *o,p′*-DDT [29,30,73]
PXR	Rifampicin, RU 486, SR 12813, androstanol, coumestrol, PB, TCPOBOP, pregnenolone 16α-carbonitrile (PCN), hyperforin (active constituent of St Johns Wort), 17β oestradiol, β pregnane-3,20-dione [3,39,40]
CAR	Androstenol, androstanol, clotrimazole, TCPOBOP (in mCAR), PB, 5β pregnane-3,20-dione, PCN [40]
AhR	Halogenated aromatic hydrocarbons (HAHs), e.g. polychlorinated dibenzo-*p*-dioxins (dioxins), dibenzofurans, and biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) e.g. benzo(*a*)pyrene, 3-methylcholanthrene, benzoflavones, carbaryl [11,53] diaminotoluene, omeprazole, brevetoxin, indole carbinols (found in cruciferous vegetables) [50] endogenous ligands e.g. bilirubin, biliverdin [48,49], water soluble metabolites of tryptophan, tryptamine, indole acetic acid [51], retinoids [52]
PPARγ	Fatty acids, prostaglandins, antidiabetic thiazolidinedione (TZD) drugs [17,61,62,64,66]
PPARα	Long chain polyunsaturated fatty acids (LCPUFA), eicosanoids, fibrates, phthalate ester plasticisers pristinic acid, phytanic acid [17,62,65–68]

Table 2
Relative binding affinities of suspected endocrine disrupters for ERα and ERβ [29], adapted from solid phase competition experiments [30]

Compound	RBA	
	ERα	ERβ
17β Oestradiol	100	100
Isoflavones		
Coumestrol	20	140
Genistein	4	87
Daidzein	0.1	0.5
Pesticides		
o,p′-DDT	0.01	0.06
Chlordecone	<0.01	<0.01
Endosulfan	0.02	0.1
Methoxychlor[a]	<0.01	<0.01

RBA of each competitor was calculated as a ratio of concentrations of oestradiol and competitor required to reduce the specific radioligand binding by 50% = ratio of IC_{50} values. RBA value for oestradiol was arbitrarily set at 100.

[a] The metabolite of methoxychlor, 2,2-bis-(*p*-hydroxyphenyl)-1,1,1-trichloroethane (HPTE) is approximately 100-fold more active at ERα than methoxychlor, [73].

present in both normal and neoplastic breast tissue. Indeed a statistically inverse relationship between the level of PXR mRNA expression and ER status has been observed by ligand binding analysis [38]. PXR can be activated by a variety of chemically distinct ligands (Table 1), in a species dependent manner [37,39,40], including endogenous hormones such as pregnenolone, and progesterone and their synthetic derivatives such as pregnenolone 16α-carbonitrile (PCN), *trans*-nonachlor, rifampicin, dexamethasone, corticosterone, spironolactone, phenobarbital, and hyperforin (the active constituent of St. John's wort) [39].

It appears that there is a specific regulatory pathway where the accumulation of steroidal PXR ligands, including xenobiotics such as organochlorine pesticides, results in increased CYP3A transcription and steroid catabolism, possibly providing the route for excess steroids to be eliminated from the body. So not only is PXR a xenobiotic sensor, it is also a key player in the regulation of steroid homeostasis, steroid metabolism (by involvement in the expression of steroid hydroxylases [12]) and detoxication.

1.3. The constitutive androstane receptor (CAR)

The constitutive androstane receptor (CAR) is a member of the same nuclear receptor subfamily as PXR, sharing around 40% amino acid identity in their LBDs, with 70% similarity between hCAR and rodent CAR LBD regions [40]. In a pattern similar to that of the ERs, based upon phylogenetic analyses, it has been suggested that PXR and CAR are closely related to each other [40]. CAR is also present largely in the liver (and also the intestine, kidneys, lungs, heart, and muscle) [6] (Fig. 3), but it interacts with and is inhibited by two endogenous testosterone metabolites, androstanol and androstenol, via a mechanism that involves a widely expressed nuclear receptor coactivator, SRC-1 [5]. The hierarchy of ligand activation differs between the receptors as well as for receptors isolated from different species, and in many instances, as identified in CAR, molecules that were previously regarded as metabolic intermediates are in fact "intracrine" signalling molecules within tightly coupled metabolic pathways for altering gene expression.

Unlike most nuclear receptors, including PXR and ER, the steroidal ligand for CAR inhibits receptor-dependent gene transcription by way of a ligand-independent recruitment of transcriptional co-activators [5]. CAR functions in a manner opposite to that of the conventional nuclear receptor pathways and can be considered a 'repressed' nuclear receptor in the presence of androstane metabolites [41]. In cell based reporter gene assays, exogenously expressed CAR enters the nucleus and regulates the expression of target genes [5,6], it is not present in the nucleus but is sequestered in the cytoplasm, unlike the other receptors modelled and discussed here. There are significant sex differences in plasma androstane levels and it has been recently implicated as a transcriptional regulator of the gene governing the steroid hydroxylase CYP2B after binding with its cognate DNA response elements as a heterodimer with RXR [5]. There appear to be additional mechanisms for the regulation of CAR activity, including phosphorylation by phenobarbital (PB). The effects of PB on *CYP2B* expression are blocked by the phosphatase inhibitor okadaic acid [42] suggesting that dephosphorylation of CAR, rather than direct ligand binding, is involved in its translocation into the nucleus. This receptor suggests a new area of androgen physiology whose significance is unknown as yet [43]. A model of CAR can aid in the design of synthetic ligands that can help investigate the relevance of CAR to human metabolism and health.

1.4. The aryl hydrocarbon receptor (AhR)

The Ah receptor is a member of the Per-Arnt-Sim family of nuclear regulatory basic helix loop–helix proteins [4,44] that has been detected in nearly all vertebrate groups examined [45]. The Ah receptor binds to 2,3,7,8 TCDD and other structurally similar PAHs to activate the cognate xenobiotic response element of the *CYP 1A* and *1B1* genes [11,44,45].

However, the AhR is the only member of that regulatory family known to bind to a ligand prior to heterodimerisation and bind to DNA in upstream regulatory regions of target genes. Predominantly found in hepatocytes, but also in breast cancer cells [25], the AhR regulates the expression of a number of genes, including cytochrome P450 1A1, 1A2, 1B1, glutathione S transferase M (GSTM), DT-diaphorase, UGT and aldehyde dehydrogenase in a ligand dependent manner [46]. AhR is also up regulated during cell division and is expressed in a specific spatial and temporal pattern in the developing foetus in vivo [47]. The best-characterised high affinity AhR ligands include a variety of ubiquitous lipophilic environmental contaminants in

the polyhalogenated aromatic hydrocarbon family including dioxins, furans, coplanar biphenyls and polycyclic aromatic hydrocarbons [10]. Other lower affinity ligands can be found endogenously (e.g. biliverdin [48,49]) and in the diet [50,51]. Synthetic retinoids and pesticides have also been reported to activate the AhR pathway [52,53]. Exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent AhR ligand known, results in a wide variety of species- and tissue-specific toxic and biological responses [54]. They are associated with the disruption of almost every hormone system that has been examined and responses to activation include developmental and reproductive toxicity. Animals treated with 2,3,7,8 TCDD have developed abnormalities in several organs including the thyroid, thymus, lung and liver, immune and endocrine function. Wasting, lethality and induction of gene expression have also been shown to be AhR dependent [47,55–58].

Within the cytosol of the cell the AhR is associated with a heterodimeric transporter protein partner, termed the aryl hydrocarbon receptor nuclear transporter protein (ARNT). The unliganded AhR may also act through other mechanisms by being phosphorylated to key regulatory proteins such as HSP90, p37, AIP, XAP2, *src*, *rel*, and *Rb* [59].

1.5. The human peroxisome proliferator activated receptors (PPAR)

The peroxisome proliferator-activated receptors are a family of orphan receptors with fundamental roles in regulating energy balance [14,41,60–66]. A number of prevalent metabolic disorders such as obesity, atherosclerosis and type 2 diabetes are associated with a shift in this balance. The peroxisome proliferator activated receptors are activated by xenobiotics, which elicit increases in the number and size of peroxisomes when administered to rodents [12], and also induce hepatocellular carcinoma development by a non-genotoxic mechanism [63].

There are three known closely related receptors: PPARα β/δ and γ, found in the liver, kidney, heart, haematopoietic and adipose tissue, but having different expression patterns. PPARα is found in liver, kidney, heart and muscle, PPARδ is expressed in nearly all tissues and PPARγ is expressed in fat cells, the large intestine, and monocyte lineage cells [60]. They each play key roles in lipid metabolism and homeostasis; PPARα is responsible for CYP4A induction; peroxisomal enzyme induction and hepatic peroxisome proliferation. PPARα has a central role in hepatogenesis, and PPARγ, a central regulatory role in adipogenesis [17,63,64,66].

PPARα regulates key steps in lipid and fibrate metabolism. It is the molecular target for naturally occurring plant fatty acids (pristinic acid and phytanic acid) present at physiological concentrations [67], long chain polyunsaturated fatty acids (LCPUFA), eicosanoids [61,64], and peroxisome proliferators, which include drugs such as the fibrates, (used widely to lower high triglyceride levels, a risk factor in coronary heart disease), and synthetic chemicals such as the phthalate ester plasticisers, and pesticides [63]. PPARγ ligands include fatty acids, prostaglandins and the antidiabetic thiazolidinedione (TZD) drugs [17,66]. Pristinic acid and phytanic acid are branch chained fatty acids obtained through the diet from the chlorophyll in plants. Present at micromolar concentrations in healthy individuals, they can accumulate in a variety of inherited disorders. Potent binding of pristinic acid and phytanic acid in PPARα [67] indicates a primary mechanism for metabolising these dietary fatty acids.

Table 3
Comparison of nuclear receptor LBDs of known structure, adapted from Watkins et al. 2001 [69]

Receptor	Ligand binding cavity volume (Å^3)
ERα	476
PXR	1150
PPARγ	1619
Progesterone receptor	557
Vitamin D receptor	871

The LBD consists of 13 α-helices and a small 4-stranded β sheet forming a hydrophobic ligand binding pocket with a volume at least twice that of other receptors [68]. The PPARs have a far larger ligand binding pocket than the receptors so far discussed (Table 3) [69], and there are differences in the shape of each PPAR ligand binding pocket [68,70] giving broad ligand specificity on a structural basis. Rosiglitazone occupied a fraction of the available LBD space in PPARα, and less than that in PPARγ, particularly the rosiglitazone TZD head group, and thus comparatively reduced selectivity was observed. This has been observed for different ligands in the PPAR family, and is a clear descriptor for PPAR selectivity.

There may be expression of a dominant-negative inhibitory human PPARα variant (found in some individuals) [71] and human polymorphisms. It is possible that PPARγ and PPARδ (highly expressed in multiple human tissues) may be transactivated and consequently perturbed by a subset of peroxisome proliferating compounds, affecting the PPAR metabolic pathways, to elicit a pathophysiological response.

Another factor to be considered is modulation through cross-talk between PPAR and other nuclear receptors/signalling molecules. For example thyroid hormone suppresses hepatic peroxisome proliferation responses and exhibits inhibitory cross-talk with PPARα, due in part to competition between the thyroid receptor and PPAR for their common heterodimerization partner RXR [72]. (See Fig. 1). Indeed, all the receptors discussed here are interlinked not least by their requirement for RXR as the heterodimerisation partner (except AhR) (see Fig. 2).

In order to understand the role of ligand in receptor activation molecular models of the ERβ, hPXR, AhR and hCAR LBDs have been generated from a ligand bound hERα crystal structure [1]. Similarly, for the investigation of the role of ligand in hPPARα activation, a molecular model of the

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     298                                                 345
her  SKKNSLALSLTADQMVSAL.LD.AE.PPILYSEYDPTR.PFSEASMMGL
ahr  DRAEFQRQLHWALNPDSAQGVDEAHGPPQAAVYYTPDQLPPENASFMER
mer  TKKNSPALSLTADQMVSALLDAEPPM...IYSEYDPSRP.......FSEASMMGL
cer  NKKNSPALSLTAEQMVSALLEAEPPI...VYSEYDPNRP.......FNEASMMTL    LIGAND
herb RVRELLLDALSPEQLVLTLLEAEPPH....VLISRPSAP.......FTEASMMMS
hpxr EDPATWSQIMKDRVPMKISLQLRGEDG..SIWNYQPPSK....SDGKEIIPLLPH
car  IRTLLGAHTRHMGTMFEQFVQFRPPAH..LFIHHQPLPT....LAPVLP..LVTH
rxr  NEVESTS.SANEDMPVERILEAELAVEPKTETYVEANMG....LNPSSPNDPVTN
rar  GSPDSYELSPQLEELITKVSKAHQETFPSLCQLGKYTTNSSADHRVQLDLGLWDK    BINDING

     346                                                 395
her  LTNLADRELV HMINWAKRVP GFVDLTLHDQVH.LLECAWLE ILMIGLVWRS
ahr  CFRCRLRCLL DNSS...... GF....LAMNFQGRLKYLHG. ......QNKK
mer  LTNLADRELV HMINWAKRVP GFGDLNLHDQ VHLLECAWLE ILMIGLVWRS
cer  LTNLADRELV HMINWAKRVP GFVDLTLHDQ VHLLECAWLE ILMIGLVWRS     DOMAIN
herb LTKLADKELV HMISWAKKIP GFVELSLFDQ VRLLESCWME VLMMGLMWRS
hpxr LADVSTYMFK GVINFAKVIS YFRDLPIEDQ ISLLKGATFE MCILRFNT..
car  FADINTFMVL QVIKFTKDLP VFRSLPIEDQ ISLLKGAAVE ICHIVLNT..
rxr  ICQAADKQLF TLVEWAKRIP HFSELPLDDQ VILLRAGWNE LLIASFSHRS
rar  FSELATKCII KIVEFAKRLP GFTGLSIADQ ITLLKAACLD ILMLRICTRY

     396                                                 445
hera MEHPVKLLF APNLLLDRNQG KC..VEGMVEIFDMLLATSSRFRMMNLQGEE
ahr  .GKDGALLP PQLALFAIATP LQ..PPSILEIRTKNFIFRTKHK.LDFTPIG
mer  ME.HPGKLLF APNLLLDRNQG KC..VEGMVEIFDMLLATSSRFRMMNLQGEE
cer  ME.HPGKLLF APNLLLDRNQG KC..VEGMVEIFDMLLATAARFRMMNLQGEE     LIGAND
herb ID.HPGKLIF APDLVLDRDEG KC..VEGILEIFDMLLATTSRFRELKLQHKE
hpxr MF.DTETGTWECGRLAYCFED. PNGGFQKLLLDPLMKFHCMLK.K.LQLHKEE
car  TFCLQTQNFL.CGPLRYTIEDG ARVGFQVEFLELLFHFHGTLR.K.LQLQEPE
rxr  IAVKDGILLA TG.LHVHRNSA HSAGVGAIFDRVLTELVSKMRD..MQMDKTE
rar  TPEQDTMTFS DG.LTLNRTQM HNAGFGPLTDLVFAFAGQLLP...LEMDDTE

     446                                                 495
hera FVCLKSIILL NSGVYTFLSS TLKSLEEKDH IHRVLDKITD TLIHLMAKAG
ahr  CDAKGQLILG YTEVELCTRG S......... ..........  ..........
mer  FVCLKSIILL NSGVYTFLSS TLKSLEEKDH IHRVLDKITD TLIHLMAKAG
cer  FVCLKSIILL NSGVYTFLSS TLKSLEERDY IHRVLDKITD TLIHLMAKSG    BINDING
herb YLCVKAMILL NSSMYPLVTA TQDADS.SRK LAHLLNAVTD ALVWVIAKSG
hpxr YVLMQAISLF S.PDRPGVVQ .......RSV VDQLQERFAL TLKAYIECS.
car  YVLLAAMALF S.PDRPGVTQ .......RDE IDQLQEEMAL TLQSYIKGQ.
rxr  LGCLRAIVLF N.P....... DSKGLSNPAE VEALREKVYA SLEAYCKHK.
rar  TGLLSAICLI C.G....... DRMDLEEPEK VDKLQEPLLE ALRLYARRR.

     496                                                 545
hera LTLQQQHQRL AQLLLILSHI RHMSNKGMEH LYSMKCKNVV PLYDLLLEML
ahr  ..GYFIHAAD IILHCAESHI R.MIKTG.E. .SGMTVFRLL AKHSRWRWVQ
mer  LTLQQQHRRL AQLLLILSHI RHMSNKGMEH LYNMKCKNVV PLYDLLLEML
cer  LSLQQQHRRL AQLLLILSHI RHMSNKGMEH LYNMKCKNVV PLYDLLLEML    DOMAIN
herb ISSQQQSMRL ANLLMLLSHV RHASNKGMEH LLNMKCKNVV PVYDLLLEML
hpxr RPYPAHRFLFLKIMAVL.TELRSINAQQTQQLLRIQDSHPFA.TPLMQELFSST
car  QRRPRDRFLYAKLLGLL.AELRSINEAYGYQIQHIQG.LSAM.MPLLQEICS..
rxr  ..YPEQPGRF AKLLLRLPAL RSIGLKCLEH LFFFKLIGDT PIDTFLMEML
rar  ..RPSQPYMF PRMLMKITDL RGISAKGAER AITLKMEIPG PMPPLIREML

     546                                                   597
her  DAHRLHAPTS RGGASVEETD QSHLATAGST SSHSLQKYYI TG.EAEGFPATV
ahr  SRWRWVQ
mer  DAHRLHAPAS RMGVPPEEPS QTQLATTSST SAHSLQTYYI PP.EAEGFPNTI
cer  DAHRLHAPAA RSAAPMEEEN RNQL.TTAPA SSHSLQSFYI NSKEEESMQNTI
herb NAHVLRG..C KSSITGSECS PAEDSKSKEG SQNP
hpxr DG
car
rxr  EAPHQMT... .......... .......... .......... ............
rar  ENPEMFEDDS SQPGPHPNAS .SEDEVPGGQ GKGGLKSPA. ............
```

hera=human oestrogen receptor alpha;ahr=aryl hydrocarbon receptor; mer=mouse oestrogen receptor alpha; cer=chicken oestrogen receptor alpha; herb=human oestrogen receptor beta; hpxr=human pregnane X receptor; car=constitutive androstane receptor; rxr=retinoid X receptor; rar=retinoic acid receptor.
A=alanine; C=cysteine; D=aspartate; E=glutamate; F=phenylalanine; G=glycine; H=histidine; I=isoleucine; K=lysine; L=leucine; M=methionine; N=asparagine; P=proline; Q=glutamine; S=serine; T=threonine; V=valine; W=tryptophan; Y=tyrosine.
The numbering system refers to the human ERα crystal structure [1].

Fig. 4. A multiple sequence alignment between steroid hormone and retinoic acid receptors.

hPPARα ligand-bound hormone-binding domain (HBD) has been generated from a ligand bound hPPARγ crystal structure [7,8]. Using natural and synthetic ligands as chemical tools, the nature of receptor activation can be examined by assessing the structural mechanisms computationally, indicating highly probable modes and mechanisms of binding, together with the key amino acids involved. This can aid in the discovery of new hormone signalling pathways and cross-talk, and provide receptor specific insight into various disease scenarios, which in the case of hPPARα for example, would include the regulation and perturbation of lipid and TZD drug metabolism.

2. Materials and methods

The steroid hormone receptor super family shares a conserved primary sequence and it is likely that there three-dimensional structures are similar, so it is possible to model one member of the family from another.

The alignment was determined based upon previous alignments in the literature [1]. Domains of human ERα, retinoic acid receptor (RARα) and retinoid X receptor (RXRα), show conserved residues, these were used as the basis for modelling the HBD of hERβ, hPXR, AhR and CAR. The respective sequences were adjusted in relationship to the other proteins in order to match theoretical helices of the receptors to known helices of hERα (Fig. 4).

Similarly domains of human PPAR and retinoid X receptor (RXRα), show conserved residues, and these were used as the basis for modelling the hormone-binding domain (HBD) of the hPPARα. The sequence was adjusted in relationship to the other proteins in order to match theoretical helices of hPPARα to known helices of hPPARγ. The ligand binding domain sequences are shown in Fig. 5.

The homology models of hERβ (Fig. 6) hPXR (Fig. 7), CAR (Fig. 8) and AhR (Fig. 9a and b) were generated from the existing hERα structure (Fig. 6) using SYBYL biopolymer software from (Tripos associates, St Louis, Missouri) on a Silicon Graphics Indigo^2 IMPACT 10000 Unix

```
101                                                  150
 hrxr  VSSSEDIKPP LGLNGVLKVP AHPSGNMASF TKHICAICGD RSSGKHYGVY
mppar  ASTDESPGSA LNIE..... .......... ....CRICGD KASGYHYGVH
rppar  TSTDESPGNA LNIE..... .......... ....CRICGD KASGYHYGVH
hppar  GSVDESPSGA LNIE..... .......... ....CRICGD KASGYHYGVH    DNA
hpparg KPHEEPSNSL MAIE..... .......... ....CRVCGD KASGFHYGVH
hppard MGCDGASCGS LNME..... .......... ....CRVCGD KASGFHYGVH
       151                                           200    BINDING
 hrxr  SCEGCKGFFK RTVRKDLTYT.CRDNKDCLID KRQRNRCQYC RYQKCLAMGM
mppar  ACEGCKGFFR RTIRLKLVYDKC.D.RSCKIQ KKNRNKCQYC RFHKCLSVGM
rppar  ACEGCKGFFR RTIRLKLAYDKC.D.RSCKIQ KKNRNKCQYC RFHKCLSVGM
hppar  ACEGCKGFFR RTIRLKLVYDKC.D.RSCKIQ KKNRNKCQYC RFHKCLSVGM    DOMAIN
hpparg ACEGCKGFFR RTIRLKLIYDRC.D.LNCRIH KKSRNKCQYC RFQKCLAVGM
hppard ACEGCKGFFR RTIRMKLEYEKC.E.RSCKIQ KKNRNKCQYC RFQKCLALGM

       201                                  |
 hrxr  KREAVQEERQ RGK.DR.... .......... .......... ..........
mppar  SHNAIRFGRM P.RSEKAKLK AEILTCEHDL KDSETADLKS LGKRIHEAYL
rppar  SHNAIRFGRM P.RSEKAKLK AEILTCEHDL KDSETADLKS LAKRIHEAYL
hppar  SHNAIRFGRM P.RSEKAKLK AEILTCEHDI EDSETADLKS LAKRIYEAYL
hpparg SHNAIRFGRI A.QAEKEKLL AEISSDIDQL .NPESADLRA LAKHLYDSYI
hppard SHNAIRFGRM P.EAEKRKLV AGLTANEGSQ YNPQVADLKA FSKHIYNAYL

              220      |        <  helix 1  >    <helix2>
 hrxr  ...NENEVE. .STSSANE.D MPVERILEAE .LAV.EPKTETYVEANMGL.N
mppar  KNFNMNKVKA RVILAGKTSN NPPFVIHDME TLCMAE.KTLVAKMVANGV.E
rppar  KNFNMNKVKA RVILAGKTSN NPPFVIHDME TLCMAE.KTLVAKMVANGV.E
hppar  KNFNMNKVKA RVILSGKASN NPPFVIHDME TLCMAE.KTLVAKLVANGI.Q    LIGAND
hpparg KSFPLTKAKV RAILTGKTTD KSPFVIYDMN SLMMGE.DKIKFKHITPLQEQ
hppard KNFNMTKKKA RSILTGKASH TAPFVIHDIE TLWQAE.KGLVWKQLVNGP.P

         260 <        helix 3         >     290  <  helix4 ><
 hrxr  PSSPNDPVTN ICQAADKQLF T.LVEWAKRI PHFSELPLDD QVILLRAGWN    BINDING
mppar  DKEAEVRFFH CCQCMSVETV TELTEFAKAI PGFANLDLND QVTLLKYGVY
rppar  NKEAEVRFFH CCQCMSVETV TELTEFAKAI PGFANLDLND QVTLLKYGVY
hppar  NKEVEVRIFH CCQCTSVETV TELTEFAKAI PAFANLDLND QVTLLKYGVY
hpparg SKEVAIRIFQ GCQFRSVEAV QEITEYAKSI PGFVNLDLND QVTLLKYGVH
hppard YKEISVHVFY RCQCTTVETV RELTEFAKSI PSFSSLFLND QVTLLKYGVH    DOMAIN
```

The amino acid designations as the same as indicated in Figure 4.
The numbering system refers to the human PPARγ crystal structure [8]

Fig. 5. A multiple sequence alignment between PPAR and human RXR sequences.

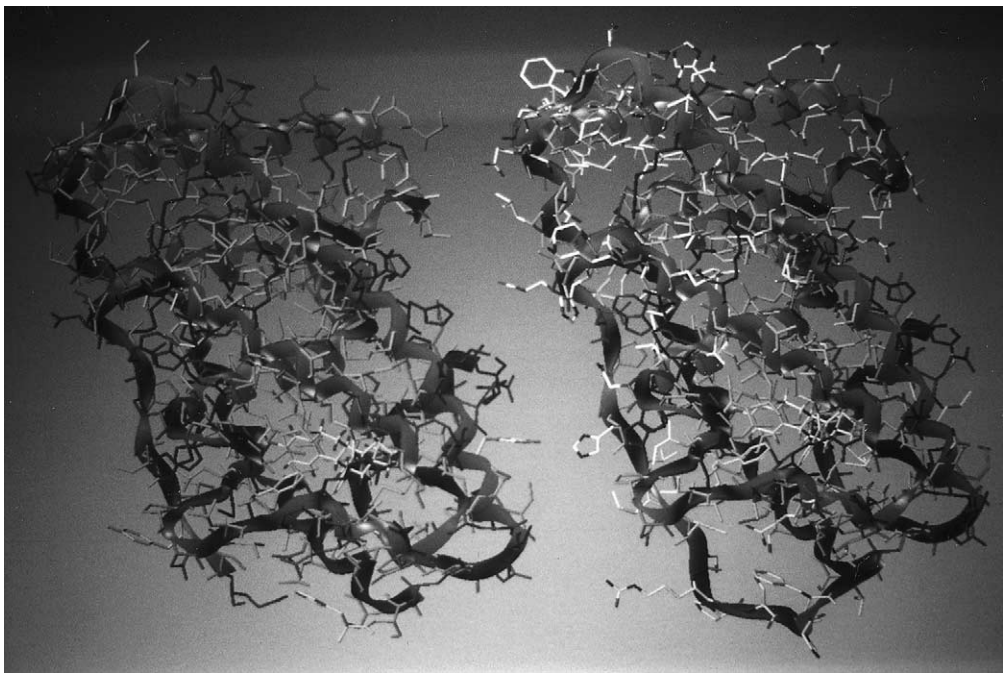

Fig. 6. A homology model of ERβ (left) with E2 docked in the ligand binding site, plotted from the crystal structure coordinates of ERα (right).

workstation. Loops were chosen from the Brookhaven Protein Databank to incur minimum interference with the core model. After energy minimisation with the Tripos force field the endogenous moiety was docked into the structure. Some adjustment was needed in order for hydrogen bonding to occur between ligand and receptor. Finally, the structures were energy minimised again to achieve self-consistency.

A number of compounds, with known receptor specific high, medium and low activation were individually docked in each receptor.

For ERβ these included the hormones 17β oestradiol, androstanes, phytoestrogens and synthetic chemicals such as triclosan. For hPXR these included pregnenolone, coumestrol, PCN (a low affinity hPXR ligand, but high affinity rPXR ligand), progesterone, corticosterone and triclosan. For the AhR these included 2,3,7,8 TCDD, and PCB 126. With the AhR little adjustment was needed in order for hydrogen bonding to occur.

For the hCAR these included the testosterone metabolites androstenol and androstanol, and clotrimazole and TCPOBOP (both high affinity mouse CAR ligands).

For both PPARα and PPARγ these included fatty acids, thiazolidinediones (TZDs) and other drugs, and lipophilic chemicals.

3. Results and discussion

Table 4 shows the results of the modelling of the receptors, including the energy before and after ligand binding, and indicates the main amino acid contacts between the ligands and receptors.

3.1. Promiscuity of ligands; cross-talk of receptors

Xenobiotics may act on some but not all of the receptors and their isoforms in the tissues of these organs, or act to different affinities, as methoxychlor and its analogue DDT do in ERα and ERβ [74]. Taking the ERs as a specific example, ERβ is dependent on pure agonists for the activation of transcription from its target promoters, while ERα can be activated by agonists, partial agonists (such as tamoxifen, which is used in the treatment of ER+ breast cancer) and ligand independent mechanisms.

3.1.1. Evidence of 'cross-talk'

Not only do receptors often have ligands in common (although with different binding affinities), and there is also a great deal of 'cross-talk' and 'ligand promiscuity'. For example, forms of the endogenous oestrogenic (oestradiol) and androgenic (androstanes) hormones are both ligands for ERβ. Oestradiol is less potent in ERβ than in ERα, whilst the natural ligands for ERβ may actually be androgens: 5α-androstane diol and 3β-androstane diol [74]. The organochlorine pesticides *trans*-nonachlor and chlordane are known to activate both known oestrogen receptors (ER), and the pregnane-X receptor (PXR), but with different affinities [30,63,75,76]. As these receptors are present in different ratios in different cell types and tissues, the response on a cellular, tissue and systemic level may be quantitatively very different, and may vary over time. Receptor modulation has been seen with lactation, when a form of ERβ has been observed to increase in the rat mammary glands [73,74], and in breast tissue hyperplasias where a frequent mutation in the ERα gene shows increased sensitivity to oestrogen

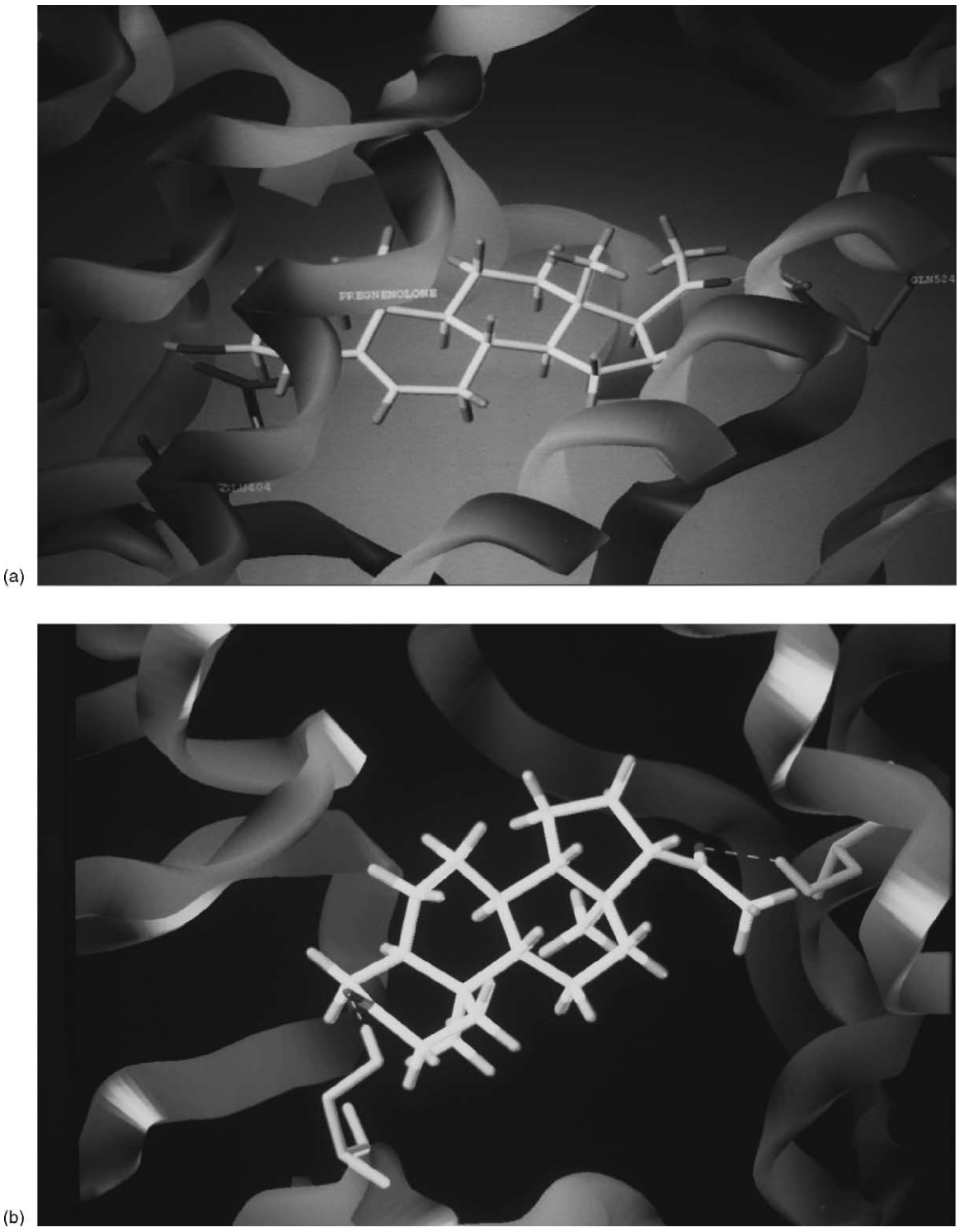

Fig. 7. (a) Molecular homology model of PXR with prenenolone docked in the LBD plotted from the crystal structure coordinates of ERα. (b) Crystal structure of PXR with prenenolone docked in the LBD.

compared with wild type ERα, by affecting the border of the hinge and hormone-binding domains (HBD) [77].

There is evidence that isoforms of different receptors modulate each other at a functional level attempting to retain a balance. The modulation is aided at low [31], and high hormone levels [27] by different ERβ isoforms,—this may enable a tissue to govern its own responsiveness to oestradiol, oestradiol metabolites and related hormones such as progestins, and oestrogen mimics or endocrine disrupting compounds (EDCs). This speculation is supported by what

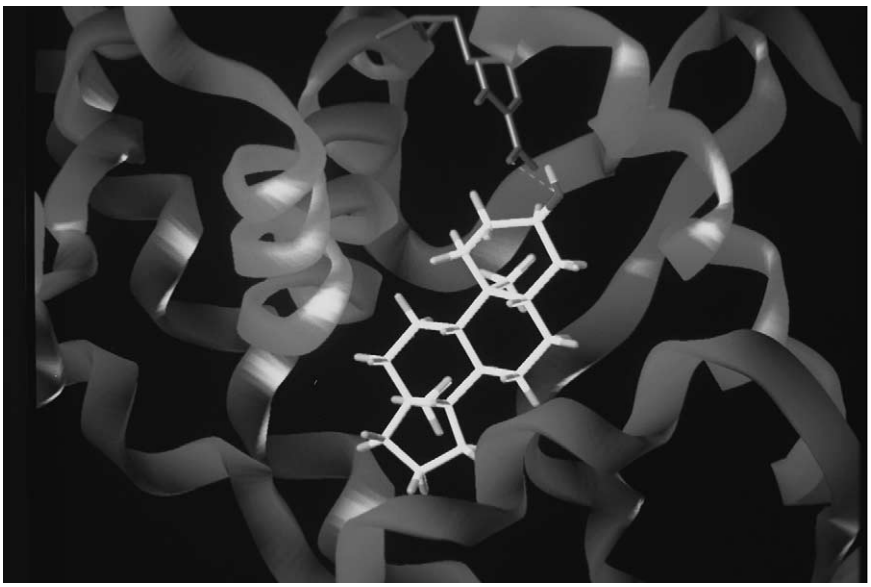

Fig. 8. Molecular homology model of CAR with androstanol docked in the LBD plotted from the crystal structure coordinates of ERα.

appears to be an emerging pattern in nuclear receptor signaling, as similar balancing acts have been observed in the α and β forms of the human glucocorticoid receptor, the progesterone receptor, and now also between PXR and CAR [14,69].

Phytoestrogens appear to have a greater affinity for ERβ than ERα [30], but they have an ERα selective efficacy [30], while the hydroxylated metabolites of methoxychlor appear to be an ERα specific agonist, and ERβ antagonist [73], but with about the same affinity for both isoforms [30].

3.2. The pregnane-X-receptor (PXR)

Examples of hydrogen bonding and hydrophobic contacts with key amino acid sidechains for pregnenolone, coumestrol and PCN were Gln 521, 524 and Glu 404. The steroids, progesterone and corticosterone, displayed hydrogen bonding with the same amino acids plus Ser 350. His 388 may well have a significant role to play with certain ligands such as tamoxifen, as it can both accept and donate H bonds, as well as change orientation to facilitate binding.

Thus, five key polar residues, including glutamine, glutamate, serine and histidine are important for ligand binding in this hPXR model, although not necessarily all of them for every ligand. The hPXR crystal structure was published recently, both alone and complexed with the high affinity ligand SR 12813, and here the five key polar residues critical for establishing the activation of PXR were Ser 208, Ser 247, His 407, Arg 410 and Gln 285. The ligand bind-

Table 4
Results for homology models of selected receptors with known ligand interactions

Receptor	Energy before binding (kcal/mol, 200 iterations)	Ligand	Energy after binding (kcal/mol, 200 iterations)	Main contacts between ligand and receptor (ERα alignment)
ERβ	−567.195	17β Oestradiol	−611.802	H bonding[a] with: histidine, arginine, glutamate
		Coumestrol	−626.966	
PXR	−558.873	Pregnenalone	−605.979	H bonding with: glutamines glutamate, serine, histidine
		Dexemethasone	−602.867[b]	
CAR	−542.440	Androstane 3β,11β-diol-17	−603.932	H bonding with: arginine, aspartate, gutamine, histidine. tyrosine may bond with longer structures or if androstane is moved further into the LBD hydrophoblic contacts: leucine, valine, cysteine, methionine
AhR	−334.982	2,3,7,8 TCDD	−364.120	H bonding with: arginines
		PCB 126	−368.301	π–π Stacking between phenylalanines
PPARα	−792.255	Arachidonic acid	−848.050	Ion bonding with: histidine, lysine, cysteines
		Bezafibrate	−836.033	H bonding with: serine

Note: The values given in this table are relative and not absolute. They are included to demonstrate the stable low energy conformations of the proteins.
[a] H bonding: hydrogen bonding.
[b] 400 iterations.

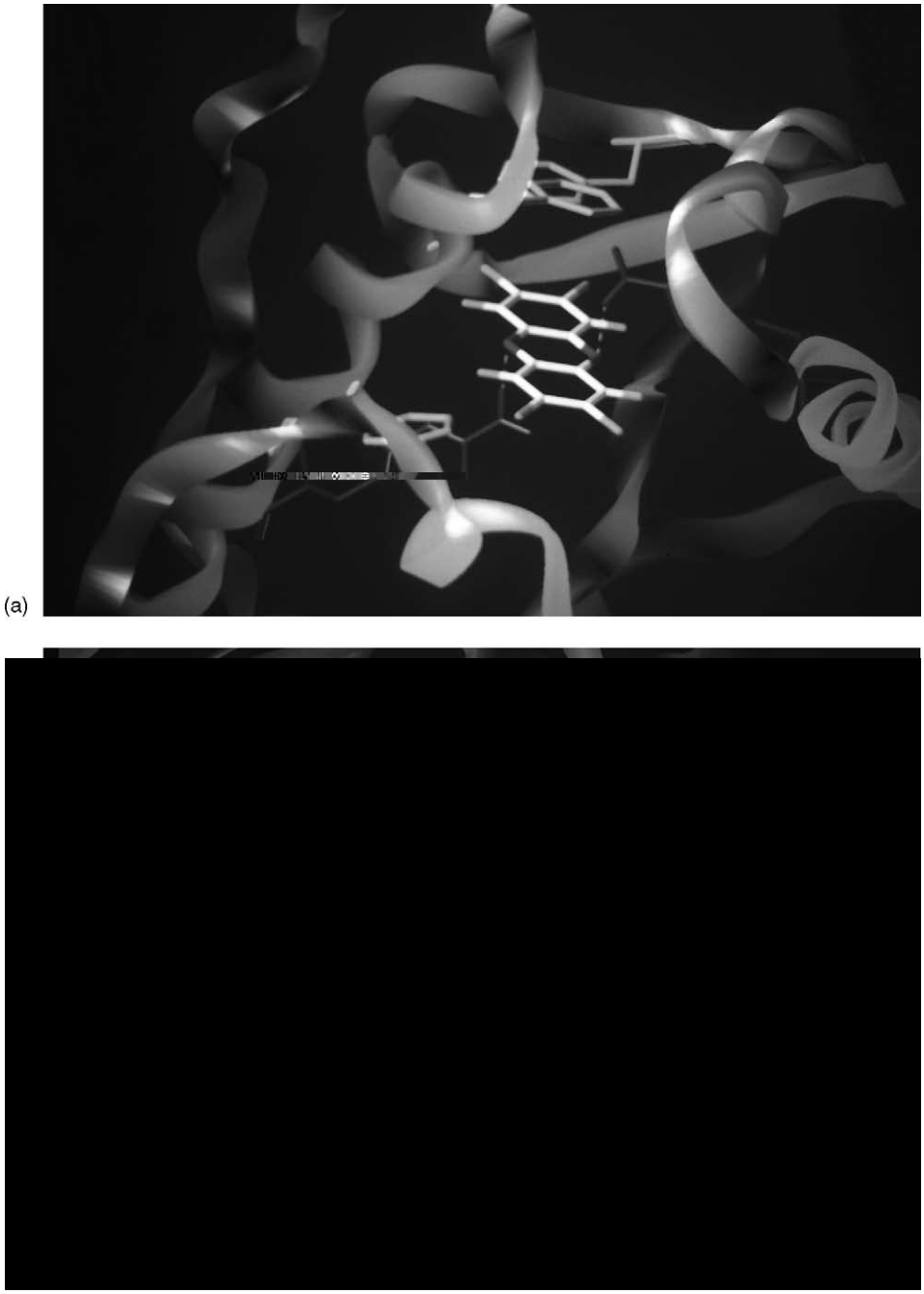

Fig. 9. (a) Molecular homology model of AhR with 2,3,7,8 TCDD docked in the LBD (showing hydrogen bonding) plotted from the crystal structure coordinates of ERα. (b) Molecular homology model of AhR with PCB 126 docked in the LBD (showing π–π stacking) plotted from the crystal structure coordinates of ERα.

ing pocket is highly flexible and may be able to expand the number of possible hydrophobic contacts by enlarging via a pore [69].

Comparisons between the homology model presented herein, and the recently published crystal structure of PXR [69] and pharmacophore [78] compare favourably, indicating that the use of homology modelling for receptors, as with enzymes, is a useful tool when crystal structure data is not available.

3.3. The constitutive androstane receptor (CAR)

There is clear evidence of cross-talk between PXR and CAR and marked pharmacological differences for the same ligands in the different receptors, as is being observed between the ERs. Thus, clotrimazole, for example, is a PXR activator, but a potent deactivator in CAR [40]. CAR has also been observed to be transactivated through the *CYP3A4* xenobiotic response element that serves as the PXR/RXR

binding site [79] and has been observed to be sequestered in the intact liver, in the absence of activators [42]. A ligand that affects androstanol and androstenol levels will also affect CAR and possibly the steroid hydroxylase CYP2B. CAR also has PB like PCB ligands in common with AhR.

Both CAR and PXR m RNAs are also markedly reduced by the cytokine interleukin-6 (IL-6), but this has not been observed for the AhR or glucocorticoid receptor (GR) [80].

3.4. The Ah receptor (AhR)

The 2,3,7,8 TCDD (Fig. 9a) and 3,3′,4,4′,5 PCB (IUPAC 126) (Fig. 9b), both high affinity ligands, were docked in the LBD. Differences in binding were observed. The HOMO energy appears to describe an important facet of the ligand binding process which could involve a π–π stacking interaction (see Fig. 9b) between benzene rings on the PCB molecule and one or more aromatic amino acid residues (e.g. phenylalanine) in the AhR ligand binding site itself. The ligand binding site preferentially accepts relatively planar aromatic molecules within a specific rectangular envelope. Hydrogen bonding does not appear to be as significant as it is with 2,3,7,8 TCDD.

For AhR ligands, QSAR studies indicate the HOMO energy to be an important descriptor with molecular planarity, together with the overall rectangularity as measured by the length/width ratio, planarity, length and energy of the highest occupied MO [81]. These descriptors have also been described for synthetic retinoids, with structural similarities to 2,3,7,8 TCDD and the coplanar PCBs, for the retinoic acid receptor (RAR) and the AhR/ARNT pathway [52].

3.5. The human peroxisome proliferator activated receptor (PPAR)

Rosiglitazone, a potent ligand in PPARγ but not PPARα, occupied a fraction of the available LBD space in PPARα, less than that in PPARγ, particularly the rosiglitazone TZD head group. Thus, comparatively reduced selectivity was observed in silico as reported experimentally for different ligands in the PPAR family, and is a clear descriptor for PPAR selectivity.

Nuclear receptors are important drug targets for intervention in disease processes. Exogenous compounds that target these receptors can therefore disrupt both normal and abnormal functioning of these key metabolic pathways. While environmental hormone mimics contribute to detrimental health effects by activating certain receptors and disturbing normal function, there are therapeutic uses from both dietary and pharmacological treatment for abnormal functioning of the hormone pathways and hormone dependent diseases.

4. Conclusion

The nuclear receptors modelled display a spectrum of ligand specificities, ranging from the highly specific, as seen in CAR which binds 5α-androstan-3α-ol (androstanol) but not 5α-androstan-3β-ol [40] and seen in PPAR selectivity, to the highly non-specific, such as hPXR which is very flexible, and can bind with a large number of wide ranging molecules, from rifampicin to steroidal structures.

They also display a spectrum of binding modes within the LBD, from hydrogen bonding with variable key amino acids (as observed in all the receptors) to π–π stacking, as seen in the AhR and also binding outside the LBD, as seen with antagonists such as tamoxifen in ERα.

The receptor models can be used to explore modes of binding of different ligands in the LBD, and conduct Quantitative Structure Activity Relationships in conjunction with experimental ligand binding data from both the literature and in house in vitro systems.

The identification and in silico assessment of the different ligands both in isolation and within the receptor models will add to a better knowledge of their specificity and may help explain the selective action of steroids and xenobiotics in different tissues. This information can be used to explore novel development compounds for therapeutic intervention in various functions that involve the PPARs, ERs, AhR, CAR and PXR, and their associated CYP enzymes, together with other chemicals of interest, such as industrial chemicals and dietary compounds. Since this work was conducted, the crystal structures of human Erβ [82], and PPARα [68,83] in addition to PXR [69] have become available, and they compare favourably with the models described herein, supporting the value of homology receptor protein models where crystal structures have not yet been determined.

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