Ligand Recognition by Drug-Activated Nuclear Receptors PXR and CAR: Structural, Site-Directed Mutagenesis and Molecular Modeling Studies

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Abstract: Pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) are the principal regulators of drug/xenobiotic disposition and toxicity. These nuclear receptors display considerable cross-regulation of their target genes, and species-specific, yet promiscuous activation by a large number of structurally dissimilar ligands. Activation of PXR and/or CAR will frequently result in enhanced drug metabolism, disturbances in homeostasis of endogenous substances, and increased toxicity. Thus, understanding, measurement and prediction of ligand-elicited activation of PXR and CAR receptors is of utmost importance for the drug development process. In this mini-review, we will review the recent elucidation of structural properties of PXR and CAR, the molecular determinants of their ligand and species specificities and progress made in *in silico* models for identification of PXR and CAR activators.

1. INTRODUCTION

Pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3), two members of the nuclear receptor (NR) superfamily, have been clearly established as the principal regulators of drug/xenobiotic disposition and toxicity [1-4]. In addition to their role in drug disposition, both receptors coordinate some aspects of metabolism of physiologically important naturally occurring compounds such as cholesterol, bile acids, bilirubin, vitamins E and K2, and thyroid hormone [5-9]. It appears that drug-induced growth of liver mass, proliferation of endoplasmic reticulum and tumor promotion are controlled mainly by CAR [10,11]. Because of their overlapping DNA binding specificities, PXR and CAR are also capable of extensive cross-regulation of their target genes [2, 4, 12]. PXR and CAR are also promiscuous in the sense that they can recognize a large number of structurally dissimilar ligands. Furthermore, the activation profiles of CAR and PXR by diverse ligands are highly species-specific [2, 3, 13, 14]. Both these properties are in strong contrast with other NRs that appear more selective and conserved in recognition of their ligands.

Consequently, activation by drugs of PXR and/or CAR will frequently result in enhanced drug metabolism, disturbances in homeostasis of endogenous substances, increased toxicity and even tumor formation. To complicate matters, such undesired effects may not be extrapolated from animal studies to the human situation in any straight-forward manner. Therefore, understanding, measurement and prediction of ligand-elicited activation of PXR and CAR receptors is of utmost importance for the drug development process. We will review progress in this area by focusing on the recent elucidation of structural properties of PXR and CAR, the determinants of their ligand and species specificities and *in silico* models developed for identification of PXR and CAR activators.

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2. STRUCTURES OF PXR AND CAR LIGAND-BINDING DOMAINS

Several recent X-ray structures of both PXR and CAR ligand-binding domains (LBDs) have provided information on their distinctive, flexible organization that contributes to wide ligand binding specificities and functional properties, and distinguishes PXR and CAR from other NRs. The molecular details of how these receptors change from inactive to active state upon ligand binding are not entirely clear, and further studies are required before reliable models of ligand binding and activation can be generated.

2.1. Properties of PXR LBD

Human PXR LBD has been crystallized as a ligand-free apo-form and in complex with three activators SR12813, hyperforin and rifampicin [15-18]. The structure of the human PXR LBD conformed mostly to the three-layered α-helical sandwich as seen in other NR LBDs, but it had a unique five-stranded antiparallel β-sheet and a very large cavity (> 1,280 Å^3) that could expand to accommodate ligands of varying sizes. The molecular causes for this expansion appeared to reside in the flexibility of amino acids near regions 200-209, 229-235 and 310-317 (Fig. (**1A**)) to adopt new conformations, and movement of certain residues (L209 and H407) upon ligand binding [17, 18].

The human PXR ligand-binding pocket (LBP) was lined with 28 residues which are mostly hydrophobic. The remaining polar (S208, S247, C284, Q285) and potentially charged amino acids (E321, H327, H407, R410) were distributed rather evenly in the LBP [15]. Inspection of the various ligand complexes indicates that 14, 13 or 18 amino acid contacts were made by SR12813, hyperforin and rifampicin, respectively. Of these, hydrogen bonding with residues S247, Q285 and H407 and interactions with M243, W299 and F420 were shared by all three activators. These

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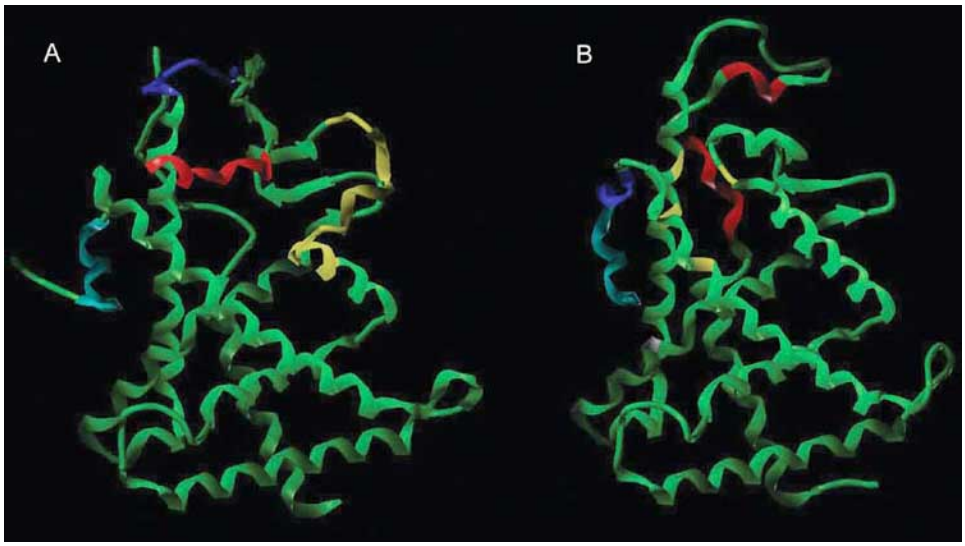

Fig. (1). Overall ribbon presentations of human PXR (**A**, PDB code 1M13) and human CAR (**B**, PDB code 1XV9) LBD folds. The backbone of PXR or CAR is depicted with green ribbon while the helix 12 is rendered cyan. The mobile regions of PXR (**A**) are shown in red (residues 200-209), blue (residues 229-235) and yellow (residues 310-327). In CAR structure (**B**), the helix X is shown in blue, the barrier residues (161, 165, 234, and 326) in yellow, K195 in white and residues forming the two 3_{10} helices (128-134 and 139-142) are in red.

data indicate that overlapping but distinct sets of LBP residues are sampled by structurally variable PXR ligands (Table **1**).

The ligand-induced reorientation of helix 12 is a central process that NRs employ to dissociate themselves from NR corepressors and to recruit NR coactivators in order to trigger transcription of their target genes [19, 20]. The finding that ligand-free PXR associates with NR corepressors which are released upon addition of an agonist [21] is consistent with this general mechanism. However, of all available PXR structures, only the complex with SR12813 displayed a direct ligand contact with the helix 12 [16], and notably, helix 12 was located in the active position also in the ligand-free apo-PXR crystal [15].

2.2. Properties of CAR LBD

Four CAR LBD structures became available in December 2004. Human CAR was crystallized with agonists 6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime (CITCO) and 5β-pregnanedione [22] while mouse CAR LBD was complexed with the inverse agonist androstenol [23] and the super-agonist 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) [24]. The unique features of both human and mouse CAR include the presence of two 3_{10} helices between helices 1 and 3, and a single-turn helix X (residues L336, S337, A338 and M339 in human CAR) that was suggested to restrict the movement of the short helix 12 (Fig. (**1B**)). The stabilization of human CAR helix 12 in the active conformation was further enhanced by its interactions with K195 and a "barrier" of LBP residues (F161, N165, F234, Y326 in human CAR), thus providing reasonable explanations to the known basal activity of CAR [22, 24]. Because the structure of the ligand-free CAR is not known at the moment, it is not clear whether the helix X is present in the apo-form or whether it is actually induced by the agonist ligand.

The CAR LBP cavity (525-675 $Å^3$) was much smaller than that of PXR, and consisted of 27 mostly hydrophobic residues. The polar (N165, Q215, C219, T225, N226) and potentially charged amino acids (H203, D228, H246) formed two regions that may contribute to hydrogen bonding to ligands [22-24] (Fig. (**3**)). In contrast to PXR, direct hydrogen bonding to CAR agonists was rarely observed: the C21 carbonyl of 5β-pregnanedione contacted H203 and the pyridine nitrogens of TCPOBOP were in contact with N175 *via* a water molecule. No clear hydrogen bonds were observed with CITCO, perhaps due to its undefined mode of binding [22, 24]. In the mouse CAR complexed with androstenol, the steroid had two hydrogen bonding interactions with its 3α-hydroxyl (N175 and *via* a water molecule with H213). Distinct, overlapping sets of LBP residues contacted the two human CAR agonists, as also seen with PXR. In the human CAR, no direct ligand contacts with helix 12 were observed while in the mouse CAR, the super-agonist TCPOBOP contacted both helix X (L346) and helix 12 (T350, L353).

The comparison of TCPOBOP- and androstenol-bound mouse CAR structures provided information to explain the inverse agonism in CAR [23]. Briefly, in the androstenol-bound complex, there is a distinct "kink" between helices 10 and 11 (reminiscent of other apo-NR structures) that may force helix 12 out of the active position. This "kink" is straightened by fusion of these two helices in the TCPOBOP-bound mouse CAR structure.

3. LBD RESIDUES IMPORTANT FOR LIGAND RECOGNITION AND ACTIVATION

As deduced from the X-ray structures, hydrophobic forces appear to predominate in ligand recognition by PXR

Table 1. Comparison of Common Agonist-Contacting Residues in Human PXR and CAR LBPs

Residue in human PXR	Residue in human CAR	Comments
L209	not present	L209 in PXR-specific loop between helices 1 and 3.
L240	*(V158)*	
M243	**F161**	
M246	**I164**	M246 contact only in SR12813-PXR complex.
S247*	**N165***	
(M250)	M168	
F251	*(V169)*	
F281	*(V199)*	V199 or corresponding residue contacts helix 12 in PXR, CAR and VDR structures.
Q285*	**H203***	
F288	**L206**	
W299	**F217**	
(Y306)	Y224	Y306 and F315 are located in or near a flexible loop structure in PXR.
(F315)	F234	
M323	**L242**	
L324	**F243**	L324 contact only in rifampicin-PXR complex.
H407*	**Y326***	Y326 forms a hydrogen bond with N165 as well.
L411	*(I330)*	
I414	*(I333)*	
F420	*(A338)*	A338 in CAR-specific helix X.

Residues contacting the agonist in at least two different PXR or CAR structures are listed. Shared contacts are in **boldface**. Hydrogen bonding contacts are indicated with asterisks (*). Residues *in italics & parenthesis* indicate that the ligand-residue interaction seen in one receptor is lacking from the corresponding residue of the other receptor.

and CAR and some of the key residues have already been identified in above sections. However, the variation of LBP residues in contact with different ligands (Table **1**), observed flexibility of the LBP and the wide species differences among PXR and CAR activators indicate that the relative importance of the residues should be confirmed experimentally. Collectively, the studies performed to date reinforce the idea that even though ligand recognition by PXR and CAR LBPs is promiscuous, there are certain critical residues governing ligand-selective contacts.

3.1. Site-directed Mutagenesis of PXR LBD and Species Differences

There are no systematic mutagenesis studies done on PXR LBDs; instead, studies have focused on LBP residues in contact with human PXR ligands and/or divergent between species. The basal activity of PXR tended to increase upon mutation of ligand-contacting residues (S247W, W299A, H407A, R410A) while the opposite was true with residues forming salt bridges (D205A/R413A, E321A/R410A) [15, 18, 25]. One possibility is that upon mutation, other LBP residues are rearranged which results in changes of the helix 12 position and subsequent co-activator recruitment. Mutation of ligand-contacting residues often results in subtle effects on the ligand selectivity: for example, F288A and W299M mutants displayed 2-3-fold but opposite changes in apparent EC50 values for hyperforin and SR12813 [16].

It is known that rifampicin and SR12813 activate the human PXR and that pregnenolone 16α-carbonitrile (PCN) is a specific activator of the rodent PXRs [2]. Such species-specific ligand recognition has naturally formed a basis for several investigations. First, human PXR residues in contact with SR12813 were used to replace the corresponding mouse residues (R203L, P205S, Q404H, Q407R). The resulting mutant mouse PXR gained activation by SR12813 and lost responsiveness to PCN [15]. Human/rat PXR chimeric receptors and mutagenesis studies helped to identify L308 as the key residue responsible for activation by rifampicin [18, 26], paclitaxel, hyperforin [26] and a novel cyclopropyl alkylamide [27]. Other regions which map at or close to the flexible loops of PXR LBD were also needed for full efficacy of rifampicin [26]. Even though Q285 and H407 form hydrogen bonds with rifampicin and SR12813, their

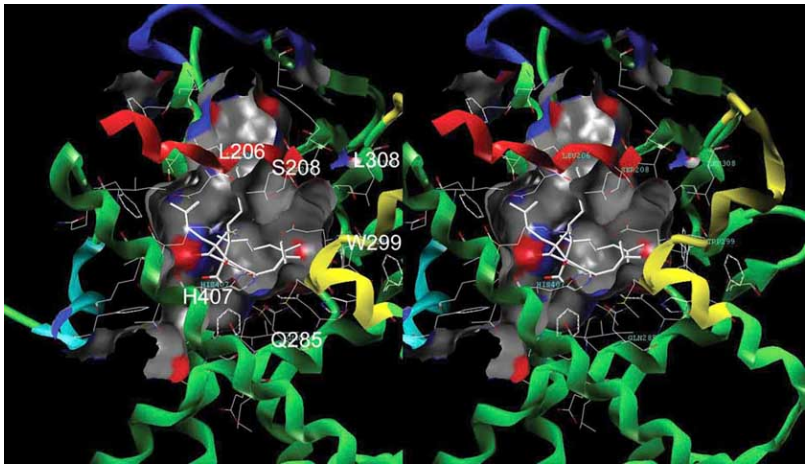

Fig. (2). Schematic stereoview representation of human PXR LBP in complex with hyperforin (licorice model). The surface represents the Connolly surface of the binding cavity, with red indicating hydrogen bond donor, blue for hydrogen bond acceptor and grey hydrophobic residues, as calculated by Molcad. Residues within 6 Å of agonist are shown, and selected residues cited in the text are indicated for clarity by labels on the left panel only. Features present in Figure 1A are encoded by that color scheme.

mutation to corresponding rodent residues (Q285I and H407Q) had surprisingly little [16, 25] or no effect [18] on the affinity of these human-selective activators.

3.2. Site-directed Mutagenesis of CAR LBD and Species Differences

Several studies have concentrated on the residues responsible for both mouse and human CAR constitutive activity. According to various homology models, helix 12 residues L342, L343, I346, C347 and S348 contribute to basal activity of CAR due to their stabilizing interactions with helices 11, 5 and 4 [28-30]. An alanine scanning study showed dramatic decreases in basal activity upon mutation of most LBP residues [30]. Molecular dynamics simulations suggested that these decreases are typically due to rearrangement of Y326 and N165 within the LBP and subsequent destabilization of helix 12, and that movement of F161 accompanies ligand binding [30, 31]. All these findings emphasize the importance of the "barrier" residues (F161, N165, F234, Y326) in maintaining the helix 12 in active position [22].

Only one systematic study of CAR ligand specificity has been conducted [30]. In human CAR, F161 is important for activation by both tri-(*p*-methylphenyl) phosphate (TMPP) and clotrimazole while other residues are ligand-selective (I164 and N165 for TMPP; H203, F234, F238 for clotrimazole). Similarly, H203 is required for inhibition by both androstenol and ethinylestradiol while C202 and F243 are crucial for the former and the latter steroid, respectively. About half of the critical residues in CAR correspond to those in PXR (Table **1**) although their relative importance varies.

Mouse/human CAR chimeras and mutagenesis were used to show that sensitivity of mouse CAR to progesterone and TCPOBOP was governed by residue 350: substitution of T350 by the corresponding human residue methionine abolished TCPOBOP activation [32] and progesterone inhibition [33]. In contrast, androstenol inhibition and ethinylestradiol activation took place regardless of the residue at position 350 [32]. The species-specific response to ethinylestradiol is determined essentially by one residue: mutation of F243 in human CAR into the corresponding mouse residue leucine converted ethinylestradiol from an inhibitor to an activator [30]. These two residues map to opposite ends of the mouse CAR LBP, where inclusion of methionine at position 350 would cause a steric clash with the TCPOBOP ligand [24].

3.3. Measurement of PXR and CAR Ligand Binding and Activation

Because PXR and CAR regulate gene transcription of drug-metabolizing enzymes, measurement of their ligand binding and/or activation *in vitro* would provide an early indication on whether the drug candidate is likely to cause drug/drug interactions *via* induction of CYP enzymes. Accordingly, counter screens have been devised to detect PXR ligands [34-37]. Intriguingly, the binding affinity of many ligands to PXR or CAR does not correlate too well with their activation potential [37] or CYP mRNA expression [38]. However, the activation of PXR seems to be a reasonable surrogate marker for CYP3A mRNA induction [35]. Because CAR ligands can elicit both activating or inhibiting responses, CAR binding assays are worthless in prediction of CYP induction [39]. Due to above reasons, activation of reporter gene has often been the method of choice, although fluorescence resonance energy transfer-based methods that rely on ligand-dependent co-activator recruitment have also been used [38, 40]. Because of its high constitutive activity, activation assays for CAR in mammalian cell lines appear to require modifications to be able to detect weak-to-moderate CYP2B inducers [30, 41]. Distinction between CAR agonists, mixed agonists and inverse agonists can be made by the use or yeast [30, 41] or mammalian [28] two-hybrid systems. Ligand-elicited nuclear translocation of CAR in primary hepatocytes could form an

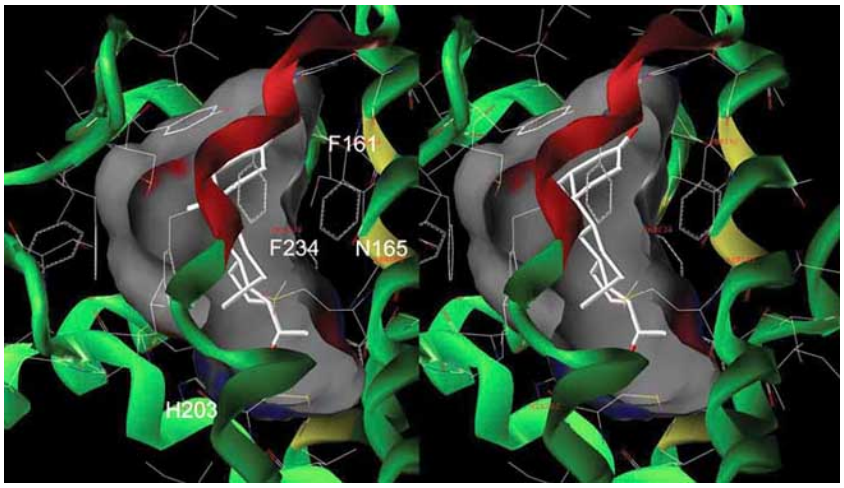

Fig. (3). Schematic stereoview representation of human CAR LBP in complex with 5β-pregnanedione (licorice model). The surface represents the Connolly surface of the binding cavity, with red indicating hydrogen bond donor, blue for hydrogen bond acceptor and grey hydrophobic residues, as calculated by Molcad. Residues within 6 Å of agonist are shown in line presentation, and selected residues cited in the text are indicated for clarity by labels on the left panel only. Features present in Fig. **1B** are encoded by that color scheme.

alternative assay for CAR-dependent CYP inducers [38, 42]. Because of the above complexities and the confounding crosstalk effects by PXR on CYP expression, activation assays for CAR have not yet been properly validated.

4. PREDICTION OF LIGANDS FOR PXR AND CAR

The prediction of potential PXR and/or CAR ligands is not an easy task. The recent publication of X-ray structures should allow the use of structure-based (direct) methods, utilizing the detailed 3D structural information of the PXR [15-17] and CAR [22] receptors. Prior to the publication of PXR and CAR crystal structures, there were several attempts to model the 3D-structures of LBD using different approaches [28, 30, 31, 43]. On the other hand, several attempts were also made with indirect, mainly pharmacophore-based approaches [25, 32, 44-49], either with or without the help of PXR or CAR X-ray structures. By October 2005, not a single direct use of crystal structures for the prediction of PXR or CAR ligands has been published.

4.1. Prediction of PXR Ligands

A pharmacophore model for human PXR ligands have been published [45], and this model has been later used to predict the activity of three PXR ligands [47]. The model was constructed using the Catalyst approach (for a review on Catalyst, see [50, 51]) and 12 compounds selected from the literature. The generated pharmacophore consisted of a hydrogen bond acceptor and four hydrophobic regions. The evaluation of the pharmacophore was carried out by predicting ligand properties of 28 compounds in PXR activation assay. The generated model was also compared with the first crystal structure of PXR [15].

In another paper [48], the crystal structure of PXR in complex with a co-activator peptide [16] was used to construct human PXR pharmacophore models and also to limit the size of the potential PXR ligands. As a test set, 30 PXR ligands and two non-ligand structures were used. Models based solely on ligand-based information were also constructed, using an undefined number of test set molecules. However, all created pharmacophore models were further validated by searching a database consisting of 53 known PXR ligands. The structure-based approach resulted several different pharmacophore models; the simplest included one hydrogen bond acceptor, three hydrophobic regions and 15 excluded regions (extracted from the X-ray structure). This model was unfortunately unable to differentiate between non-active and active compounds and so the authors decided to construct a more complicated model that consisted of one hydrogen bond acceptor, six hydrophobic regions of which three were "one-is-optional", 15 forbidden areas and a combined shape of two known ligands. Even this complicated model enables fast evaluation of PXR ligands if the compound fits the pharmacophore hypothesis, but it does not directly dock the compound into the LBP. The ligand-based approach resulted also several different pharmacophores [48]. The most general pharmacophore consisted of two hydrogen bonding acceptors and a hydrophobic region. However, the suggested need for Q285 hydrogen bonding in these models was not borne out by mutagenesis data (see 3.1 above).

In addition to pharmacophore approaches described above, molecular docking [49] and QSAR/Volsurf [52] modeling have been performed for PXR. The Volsurf method calculates alignment-independent surface properties using the GRID force field [53]. These parameters have been used to model several ADMET-type properties [52, 54-57] and are reasonable fast to calculate if the 3D structure of the ligand is available. The more detailed analysis of this model [52] is unfortunately not possible for two reasons. First, Volsurf descriptors are not easy to understand because they are scalar variables describing the surface properties of the molecules, and as such Catalyst and Volsurf methods are not comparable. The second problem is the lacking validation of the model, since no validation tests results such as cross-

validated Q^2 were mentioned in the case of PXR model [52]. The same problem is also affecting the docking procedure [49], since no detailed information is available concerning the methods used or gained results. One should also be aware that Volsurf parameters are thought to be better suited for pharmacokinetic models [56], not for pharmacodynamic models such as PXR binding. However, different results are also available [57].

It is surprising that the number of published *in silico* studies is so low in the case of PXR. Especially the lack of published and well-documented molecular docking or QSAR studies is indicative. It may well be that even the several X-ray structures of PXR are not revealing enough to allow reliable prediction of ligand binding and/or activation. If we analyze the common feature of all these different pharmacophores (one hydrogen bonding acceptor and at least one, usually 3-4, hydrophobic regions) it appears that the models are quite simple. However, this simplicity is only an artefact, since the promiscuity of PXR is hampering detailed analysis of the binding properties.

4.2. Prediction of CAR Ligands

In case of CAR, *in silico* methods for ligand prediction have been used even less than in case of PXR. There have been several attempts to predict the 3D structure of CAR LBD [30-32, 39, 43, 58, 59], but the only studies to actually predict CAR activity originate from our laboratory [30-32]. The ligand-based approach [32] was based on 3D-QSAR method GRID/GOLPE [53], comparing the molecular fields of 43 different steroid structures and their responses in mouse CAR activity assay. In addition, this study included truly external validation sets, since three other steroids were predicted using the 3D-QSAR model with rather good accuracy (residuals were 0.6 pIC_{50} units or less). As human and mouse CAR receptors do not respond identically to steroids [32, 33], these results can not be directly utilized to human CAR. Indeed, the number of published human CAR QSAR studies is still zero, and the only *in silico* studies were carried out using both molecular dynamics and molecular docking [30,31]. The problem with both of these studies is the fact that they were based on the modelled human CAR structure, not on the recent X-ray structure. While the modelled LBP in practically was identical with that of the crystallised human CAR [22], the model lacks helix X which might limit the applicability of the molecular dynamics results. Nevertheless, our recent data indicate that the model could reproduce the binding modes of CITCO and 5β-pregnanedione present in the human CAR crystal structures [60].

5. FUTURE DIRECTIONS

It is clear that there is an urgent need for more accurate and faster *in silico* prediction tools for PXR and CAR activators. How this can be reached? We believe that several aspects should be considered before *in silico* prediction of PXR/CAR activators is accurate and fast enough. In case of PXR, the flexibility of the binding cavity should be taken into account. Even though additional crystal structures of liganded PXR may reveal aspects of this flexibility [16], they are too time-consuming to produce and yield only snapshots of the real situation. On the other hand, the true meaning of helix X in CAR and the contacts between the ligand and helix 12 are still unresolved. With both receptors, the changes occurring in LBP are somehow transformed into the enhanced stability of helix 12 in the active position. All these problems are connected with dynamics of the receptor structure. One possible solution to these questions may come from long (up to 100 ns) molecular dynamics studies complemented by careful mutagenesis and functional studies. While these methods are time consuming, they offer us the "insight" view of receptor movements and flexibility. It remains an open question whether or not this insight is enough to create accurate models – hopefully so only for a short time.

ACKNOWLEDGMENTS

We wish to Academy of Finland and National Technology Agency (Drug2000 Program) for providing financial support, and our students and collaborators for their contributions to our research.

ABBREVIATIONS

CAR	=	Constitutive androstane receptor
CITCO	=	6-(4-chlorophenyl)imidazo[2,1-*b*][1,3]thiazole-5-carbaldehyde O-(3,4-dichlorobenzyl)oxime
LBD	=	Ligand-binding domain
LBP	=	Ligand-binding pocket
NR	=	Nuclear receptor
PCN	=	Pregnenolone 16α-carbonitrile
PXR	=	Pregnane X receptor
QSAR	=	Quantitative structure-activity relationship
TCPOBOP	=	1,4-bis[2-(3,5-dichloropyridyloxy)]benzene
TMPP	=	tri-(*p*-methylphenyl) phosphate

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Received: December 05, 2005 Revised: January 31, 2006 Accepted: February 01, 2006

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