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Review Article

A Ligand-Based Approach to Understanding Selectivity of Nuclear Hormone Receptors PXR, CAR, FXR, LXRα, and LXRβ

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In recent years discussion of nuclear hormone receptors, transporters, and drug-metabolizing enzymes has begun to take place as our knowledge of the overlapping ligand specificity of each of these proteins has deepened. This ligand specificity is potentially valuable information for influencing future drug design, as it is important to avoid certain enzymes or transporters in order to circumvent potential drug–drug interactions. Similarly, it is critical that the induction of these same proteins via nuclear hormone receptors is avoided, as this can result in further toxicities. Using a ligand-based approach in this review we describe new and previously published computational models for PXR, CAR, FXR, LXRα, and LXRβ that may help in understanding the complexity of interactions between transporters and enzymes. The value of these types of models is that they may enable us to design molecules to selectively modulate pathways for therapeutic effect and in addition predict the potential for drug interactions more reliably. Simultaneously, we might learn which came first: the transporter, the enzyme, or the nuclear hormone receptor?

KEY WORDS: PXR; CAR; LXR; FXR; pharmacophore.

INTRODUCTION

It is widely understood that mammalian physiology has evolved an incredible armory of defense mechanisms to remove hydrophobic xenobiotic and endobiotic molecules to prevent their accumulation and eventual toxicity. This perhaps began as a functional response for regulation of intracellular signals, advanced with plant–animal warfare (1), and further evolved to remove the molecules humans have ingested from other organisms. Chief among the endobiotics are the abundant steroids and bile acids that have essential roles as hormones, signaling molecules, and detergents. However, we are now beginning to further understand how multiple xenobiotic, therapeutic, and environmental agents may interfere with the removal and metabolic mechanisms *in vivo* and potentially result in undesirable toxic consequences. These varied biologic defense mechanisms can be classified at least in the order that an orally administered pharmaceutical agent would reach them. First there is a growing list of numerous transporters in the intestine, such as the product of the MDR1 gene (P-glycoprotein, P-gp, ABCB1), which can efficiently expel hydrophobic molecules from the enterocyte back into the lumen (2–6). Second, if the molecule resides in the intestinal enterocyte long enough, it may be metabolized by a cytochrome P450 (CYP), primarily CYP3A4, which is highly expressed in this region (7), although other CYPs are also known to be expressed here. The metabolites will then reach the blood stream and then the liver, where the metabolites could be conjugated or metabolized further before active transport via P-glycoprotein or other transporters into the bile and eventual excretion. In the kidney there are also many drug-metabolizing enzymes and transporters that can serve dual functions with endogenous and exogenous molecules.

This simple case becomes exponentially more complex when one imagines that each of the proteins involved in this process may be transcriptionally regulated by one or more orphan nuclear receptors, so that many potential scenarios may emerge involving activation or repression. The possibility of having a functional polymorphism in both enzyme and transporter is slim, but it is possible that an overlapping regulatory system involving one or more nuclear receptors protects the organism from the failure of one route while the network of signaling, transport, and clearance pathways converge in response to a ligand to provide further levels of protection and organizational complexity. It is probably as important to remember that there are numerous uptake transporters such as the apical sodium-dependent bile acid transporter and the related ileal bile acid binding protein, which have fundamental physiologic roles and are also regu-

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ABBREVIATIONS: NHR, nuclear hormone receptor; LCA, lithocholic acid; PXR, pregnane X receptor; CAR, constitutive androstane receptor; FXR, farnesoid X receptor; LXR, liver X receptor.

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lated by nuclear hormone receptors (8–11). This complex situation *in vivo* involving uptake, export, metabolism, and regulatory pathways requires some discussion of the roles of the individual nuclear receptors, transporters, and enzymes from the perspective of the ligands involved. In this way we may be able to understand why a network of proteins with, in general, overlapping ligand affinities is able to deal with most physiologic molecules as well as those we continue to expose ourselves to.

In contrast to many of the excellent recent reviews that have discussed the nuclear receptors PXR, CAR, FXR, LXRα, and LXRβ (12–22), in this present review we use published data to generate pharmacophores to illustrate the likely important features of ligands of these key nuclear hormone receptor binding sites. We also discuss previous attempts at computational modeling of these receptors, transporters, and drug-metabolizing enzymes. The pharmacophore technique we use has been widely used previously, and the methodology (23,24) represents an inexpensive, insightful approach to highlight the important binding features of ligands for enzymes, receptors, transporters, and channels.

DRUG-METABOLIZING ENZYMES

The cytochrome P450 family of enzymes is probably the most important enzyme family in terms of endobiotic and xenobiotic metabolism and is expressed within many tissues such as the liver and intestine. Because of their affinity for hydrophobic molecules of varying sizes, these enzymes may be involved in drug–drug interactions. It is widely believed that an understanding of drug–drug interactions at the molecular level may lead to the development of more effective and safer therapeutics. One enzyme in particular, CYP3A4, has drawn considerable attention over the past decade because it metabolizes a large percentage of the molecules that are substrates for the CYPs (25). Computational modeling of ligands for this enzyme has defined hydrogen bonding and hydrophobicity as important determinants of substrates (26). Although computational inhibitor models of CYP3A4 differ slightly depending on the substrate probe used *in vitro,* they still continue this trend of highlighting hydrophobic and hydrogen-bonding features (27). More recently there has been a focus on understanding the regulation of human CYP3A4 as a way to understand the expression of this enzyme and variability in bioavailability and clearance of its substrates across the population (28) as well as drug–drug interactions (29,30). One of the few early QSAR studies of inducers of CYPs based on *in vitro* and *in vivo* data assessed the rat CYP3A family and described the electrostatic potential maps for 16-substituted pregnenolones. It was suggested that the length of the 16α substituent related to the *in vivo* and *in vitro* induction, whereas the steroidal skeleton of these molecules could be involved in lipophillic (hydrophobic) interactions (31). It was several years before we were able to potentially predict induction of human CYP3A4, and it took the discovery of a new nuclear hormone receptor to catalyze this development.

THE PREGNANE X RECEPTOR

Recently it was discovered that the pregnane X receptor (PXR, also known as SXR or PAR) is a regulator of CYP3A4 transcription (32–34) and is activated by many of the structurally diverse CYP3A4 inducers. This nuclear hormone receptor is just one member of a large family that acts as a transcription factor in order to regulate induction of numerous genes. The discovery of PXR provided some degree of understanding of the modulation of CYP3A expression in response to endobiotics and xenobiotics. Three years later the known roles of PXR expanded to include the regulation of expression of human MDR1 and CYP2C8/9 (35). These discoveries provided some insight as to how drugs could regulate not only their own metabolism but potentially their efflux too, as demonstrated for paclitaxel (36). Adding to the complexity of understanding the interindividual variability in the expression and functional response is the identification of 38 single nucleotide polymorphisms (SNPs). These SNPs were found not to be located in the ligand-binding site of human PXR but nonetheless could have important physiologic ramifications (37), as they suggest that PXR may have a crucial role in binding an endogenous ligand (38). PXR was also recently shown to regulate many more genes involved in the transport, metabolism, and biosynthesis of bile acids (39).

The impact of PXR on drug development is being felt in the pharmaceutical industry, where some advocate using high-throughput assays to eliminate likely CYP3A4 inducers early in discovery. Alternatively, molecules may progress through to development with this potential liability despite these results (14), although this is company dependent. PXR reporter and functional assays represent a surrogate for the *in vivo* animal assays of induction, with significantly higher throughput and without the complication of extrapolating animal *in vivo* data to the human *in vivo*. However, the availability of the PXR-null and SXR-transgenic mice represent *in vivo* models that incorporate human PXR with *in vivo* pharmacokinetics capabilities (40,41). Hence, species differences in induction may be avoided to some degree. The ligand-

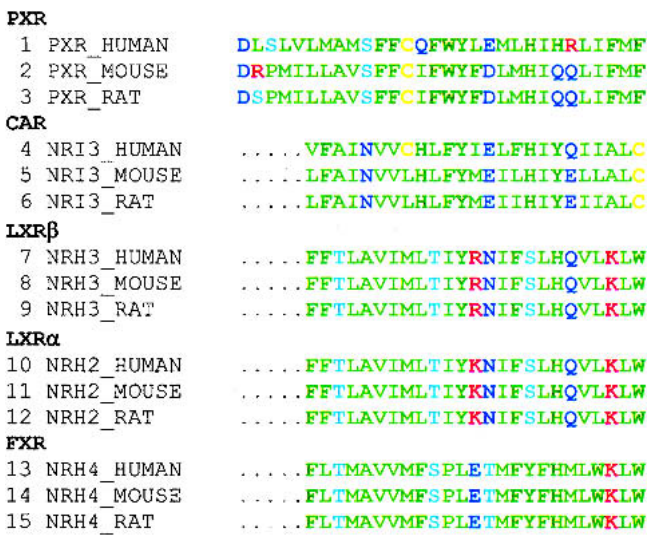

Fig. 1. Amino acids located in the ligand-binding pockets of the five nuclear receptors used in this study. These amino acids are located 5 Å or less from the ligand in the structure of PXR (PDB: 1ilh, chain A). Positions according to PXR (pdb 1ilh): 336, 337, 339, 340, 342, 371, 374, 375, 377, 378, 382, 412, 415, 416, 419, 430, 438, 440, 454, 456, 457, 460, 536, 540, 543, 544, 547, 553, 558. Amino acids are colored by type using MView (115). SwissProt names of the receptors are: PXR, PXR; CAR, NRI3; LXRα, NRH3; LXRβ, NRH2; and FXR, NRH4.

binding pocket of PXR contains 30 amino acids (Fig. 1 shows amino acids involved in ligand binding in the PXR as well other nuclear receptors in three species). The species differences between human and mouse PXR are significant, as 13 amino acids out of 30 are different. Although most of these substitutions are conservative (I-V, M-L, E-D), some of them are rather drastic: $L^{337}R$, $S^{339}P$, $Q^{416}I$, $H^{540}Q$, $R^{543}Q$. At this point it is important to understand the diversity of molecules that bind to human PXR *in vitro*. These ligands include a selection of bile acids (39,42), statins (43), components of herbal remedies such as hyperforin found in St. John's wort (44,45), HIV protease inhibitors (46), calcium channel modulators (47), steroids (48), plasticizers, and monomers (49–51) as well as a growing list of diverse xenobiotics (14,16,32,33,35,48,52–55). To an extent these *in vitro* findings explain *in vivo* drug–drug interactions that in some cases have been known for decades or others that may have only recently been recognized.

There is a growing list of known PXR ligands, and one might think that virtually any molecule could be added to this list, particularly because the ligand-binding domain is a large, flexible hydrophobic site with a few polar residues and three distinct binding sites for SR12813 [as described by X-ray crystallography (40)]. However, this structural information for the ligand-binding domain and ligands themselves can be used to select molecules that avoid binding to this protein. The suggestion that different molecules bind differently was illustrated when amino acids forming salt bridges adjacent to the binding cavity were mutated and CV-1 cells transfected with expression plasmids for PXR were then incubated with SR12813 or rifampicin. When Asp^{205} is mutated to Ala, rifampicin is a more potent PXR activator in the reporter gene assay. Similarly, targeted mutants to confer a human-like response to mouse PXR confirmed the importance of these residues in other species. It is likely that, rather than being responsible for direct binding with the ligand, these residues are altering the shape of the binding site significantly. Flexibility of the binding site probably enables the acceptance of more structurally diverse large ligands that may in turn act as antagonists (e.g., ecteinascidin). To date there have been several reports from the same laboratory describing PXR binding in terms of EC_{50} values (39,45,48). A study used these data for 12 molecules to generate a pharmacophore that may represent key features of ligands of the PXR binding site (52) and positioned this in the human PXR ligand-binding domain. This pharmacophore was further tested with other PXR ligands for which activation/deactivation data were available. The pharmacophore was also aligned to the structure of SR12813 (52) as presented in the three binding positions suggested by crystallization (40). The PXR pharmacophore provided a good correlation of observed and predicted training set data derived from a single laboratory. It also enabled a qualitative assessment of 28 other molecules, some of which were PXR ligands (CYP3A4 inducers), that had predicted EC_{50} data within the range of the training set. This pharmacophore possibly defined the determinants necessary for binding to this nuclear receptor, describing at least four hydrophobic features and at least one hydrogen-bonding feature that could be avoided in future molecules. A computational pattern recognition method such as this may be an additional approach to prescreening molecules for likely CYP3A4 induction. What may be difficult to understand from just a potential ligand structure alone is its binding orientation in the crystal structure. In addition to the three sites identified with SR12813, smaller ligands could have additional binding orientations, and multiple molecules may fit in a single receptor site simultaneously. Binding multiple molecules simultaneously begs the question of whether the extent of activation would be additive. With large molecules like paclitaxel and ecteinascidin, all binding contacts could be fulfilled, and this may result in the high-affinity antagonist response (low nanomolar) observed for the latter ligand (35). Therefore, for small molecules it may be difficult to determine *in silico,* using only the crystal structure, if a molecule is likely to be a potent or weak PXR ligand. Until cocrystals of the ligand bound to PXR are obtained for a selected number of molecules, *in vitro* reporter systems will continue to represent a valuable test system for evaluating computational predictions from docking in the structure. From such a combined *in vitro–in silico* approach we are more likely to determine the important binding site–ligand interactions as well as enable iterative improvement of the *in silico* models that result from the empirical data (56). Because both P-gp and CYP3A4 are coregulated, there must be features in common for these ligands that also translate into the binding site of PXR (4,35), and to some extent we have recently demonstrated this with multiple pharmacophores for inhibitors and substrates of P-gp, most of which contained multiple hydrophobic features and hydrogen bond acceptor features (57,58). CYP3A4 (as well as other CYPs) (59,60) and P-gp (61) appear to have multiple binding sites, so perhaps it is not surprising that PXR may behave in as complex a fashion, enabling these proteins to simultaneously handle diverse structures, some of which may result in up-regulation of transporters and enzymes to increase the rate of clearance.

The clinical implications of a complete understanding of PXR are obvious. It makes it possible to predict potential drug–drug interactions that might occur as a result of coadministration of xenobiotic PXR ligands with molecules known to be metabolized or transported by any of the genes upregulated at a particular moment in time. Subsequently, identification of further genes regulated by PXR has increased. CYP2B6, an enzyme involved in the metabolism of many diverse structural classes of drugs (62), has more recently been shown to be directly regulated by PXR (63). A further remarkable observation by these authors was the similar levels of induction for CYP2B6 and CYP3A4 for four out of five molecules. CYP2C8 and CYP2C9 are involved in the region- and stereoselective epoxidation of arachidonic acid in liver and kidney (64), and these enzymes have also been demonstrated to be induced by calcium channel antagonists via PXR (47). Induction of the intestinal transporter MDR1 by rifampicin and other xenobiotics has been shown to be mediated by PXR in the human colon carcinoma cell line LS174T as a model system (65). Human MRP1 (ABCC1) and MRP2 (ABCC2), responsible for eliminating conjugates of toxic molecules and biliary efflux of endogenous molecules, respectively, have been shown to be induced by redox-active compounds. However, it was unclear from one study what role PXR might play in the induction (66) of these proteins. The enzyme-inducible nitric oxide synthase (iNOS) is upregulated by PXR, which may explain how steroids affect inflammation (67). It is possible that genes previously thought to be regulated solely by alternative ligand-activated tran-

scription factors, such as the aryl hydrocarbon (Ah) receptor, may also be regulated partially via PXR. Endogenous hormones such as estradiol that are PXR ligands (32,33,68) can regulate their own metabolism in hepatic tissues via CYP3A4 and conceivably in extrahepatic tissues via CYP1B1 (69) or other CYPs simultaneously. There may also be a developmental role of nuclear receptors such as PXR in switching on genes at the appropriate times in response to endogenous ligands. This in turn may represent a point that can be interfered with by other molecules such as environmental pollutants that in turn act as teratogens under the right conditions (70).

THE CONSTITUTIVE ANDROSTANE RECEPTOR

A second orphan nuclear receptor, the constitutive androstane receptor (CAR), has approximately 40% identity with PXR in the ligand-binding domain (Fig. 1). Initially murine CAR was found to be inhibited (EC_{50} < 500 nM) by the androstane metabolites androstanol and androstenol (71), which are most likely the endogenous repressors (72). CAR seems to be important following exposure to phenobarbital, after which CAR accumulates in the nucleus, heterodimerizes with RXR, binds the two phenobarbital-responsive elements, and ultimately activates transcription of the CYP2B genes (73). Human CAR was found to be activated by the pesticide TCPOBOP, phenobarbital, chlorpromazine, *o,p′*-DDT, methoxychlor, and PCB, which corresponded well with the induction of the CYP2B6 gene (74). Other ligands for human CAR have been described, including clotrimazole and the progesterone metabolite 5β-pregnane-3,20-dione. Androstanol is a less potent activator of human CAR compared to mouse CAR (48). Murine CAR is also activated by metyrapone and clotrimazole (75). CAR seems to activate other genes besides CYP2B6, including CYP3A4 (74), similar to the way PXR can activate CYP2B6 as well as CYP3A4 (63). The induction of drug-metabolizing enzymes via CAR has also been demonstrated *in vivo* in mice, and CAR knockout mice have an altered sensitivity to inducers (76).

So far from the published data it appears that the human PXR ligand-binding domain binds very large molecules with the highest affinity [ecteinascidin, IC_{50} 3 nM (35)], whereas small planar molecules such as steroids and bile acids bind with a much lower affinity (39,48). In comparison, large molecules like TCPOBOP do not bind effectively to human CAR but do bind to murine CAR (48), whereas more compact molecules such as clotrimazole bind well to human CAR as a deactivator but not to mouse CAR (48). Interestingly, both the human PXR and CAR share only 70% amino acid identity with their homologs in other species (48,68,77) (Fig. 2A), and alignments to the human crystal structure for the PXR ligand binding domain (Fig. 1) show that there are some marked differences among the receptors. However, the amino acid differences between the species for each nuclear hormone receptor are much less pronounced than the differences between receptors (Fig. 2B), as only about 40 amino acids out of about 300 are conserved across all five nuclear receptors in the ligand-binding domain used in this comparison. None of the conserved amino acids are located in the ligand-binding pocket; hence, the ligand-binding pocket exhibits substantial differences between the receptors and between the species. Seven of 25 amino acids differ between the human CAR and the mouse one (see Fig. 2). Three of these seven substitutions are rather dramatic: Leu in mice is replaced with Cys in human, Leu with Phe, and Glu with Gln, suggesting somewhat different ligand specificity in human and mouse CAR. The difference between CAR and PXR binding pockets is very significant, with only seven identical and eight similar (I–L, etc.) amino acids out of 30. All other amino acids in the ligand-binding area are as different as Phe–Val, Gln–His, and Phe–Cys. Ultimately this suggests that although there might be some perceived overlap in the binding affinity of both human CAR and PXR for the same ligands, there may be some significant cross-receptor differences in binding affinity. Therefore, this might indicate that molecules like the bile acid metabolites, which activate PXR with low affinity, could have a higher affinity toward CAR or other nuclear hormone receptors. The ability of murine CAR to bind agonists and antagonists of differing size has not gone unnoticed (78), but the same facility for human CAR has not really been explored. This could indicate that the overlap between the receptors has an added functional consequence in sensing the level of ligand binding and possibly responding to a different extent. Hence, the body retains high-affinity nuclear hormone receptors and low-affinity nuclear hormone receptors for structurally similar molecules to more efficiently regulate metabolism and efflux or retain a steady state. The complexity comes in extrapolating to the *in vivo* state from the well-defined *in vitro* models. Does what we observe *in vitro* really translate to *in vivo?*

As more data are also generated for CAR involved in the induction of CYP2B (79), CYP3A4 (74), and possibly other genes, computational models will likely be generated for the ligands and inhibitors. At present a simple alignment of five molecules suggested to be mouse CAR activators [chlorpromazine, clotrimazole, metyrapone, TCPOBOP, and phenobarbital (75)] yield a pharmacophore (Fig. 3A) with two hydrophobic features and one hydrogen bond acceptor. This planar model could indicate that CAR is a less promiscuous receptor than PXR with ultimately less flexibility in the ligands involved in activation, as suggested by its high affinity for the rigid repressors, androstane metabolites, which appear also to fit well within the confines of this model (71) (Fig. 3B). To some extent this *in silico* finding may go some way toward answering one question as to whether androstanes bind directly to CAR (16). On the basis of this small pharmacophore, the answer may well be yes. When three molecules with EC_{50} values for human CAR [clotrimazole, androstanol, and 5β-pregnane-3,20-dione (48)] are used to build a pharmacophore, a model with three hydrophobes and one hydrogen bond acceptor is generated (Fig. 3C). This model represents a close approximation to a previously described human CYP2B6 substrate pharmacophore (80), which consists of the same features in a similar arrangement. When the human PXR pharmacophore (generated with *Catalyst* version 4.6 and fast conformer generation) and human CAR pharmacophores are merged together, there is some overlap in the hydrogen-bonding and hydrophobic features, but there seems to be one less hydrophobe in the CAR model (Fig. 3D). When the potent ligand 5β-pregnane-3,20-dione is fitted to this pharmacophore along with the bile acids identified as low-affinity PXR ligands (39), namely lithocholic acid and ketolithocholic acid, it can be seen there is some overlap between them (Fig. 3D). This may imply that these and related

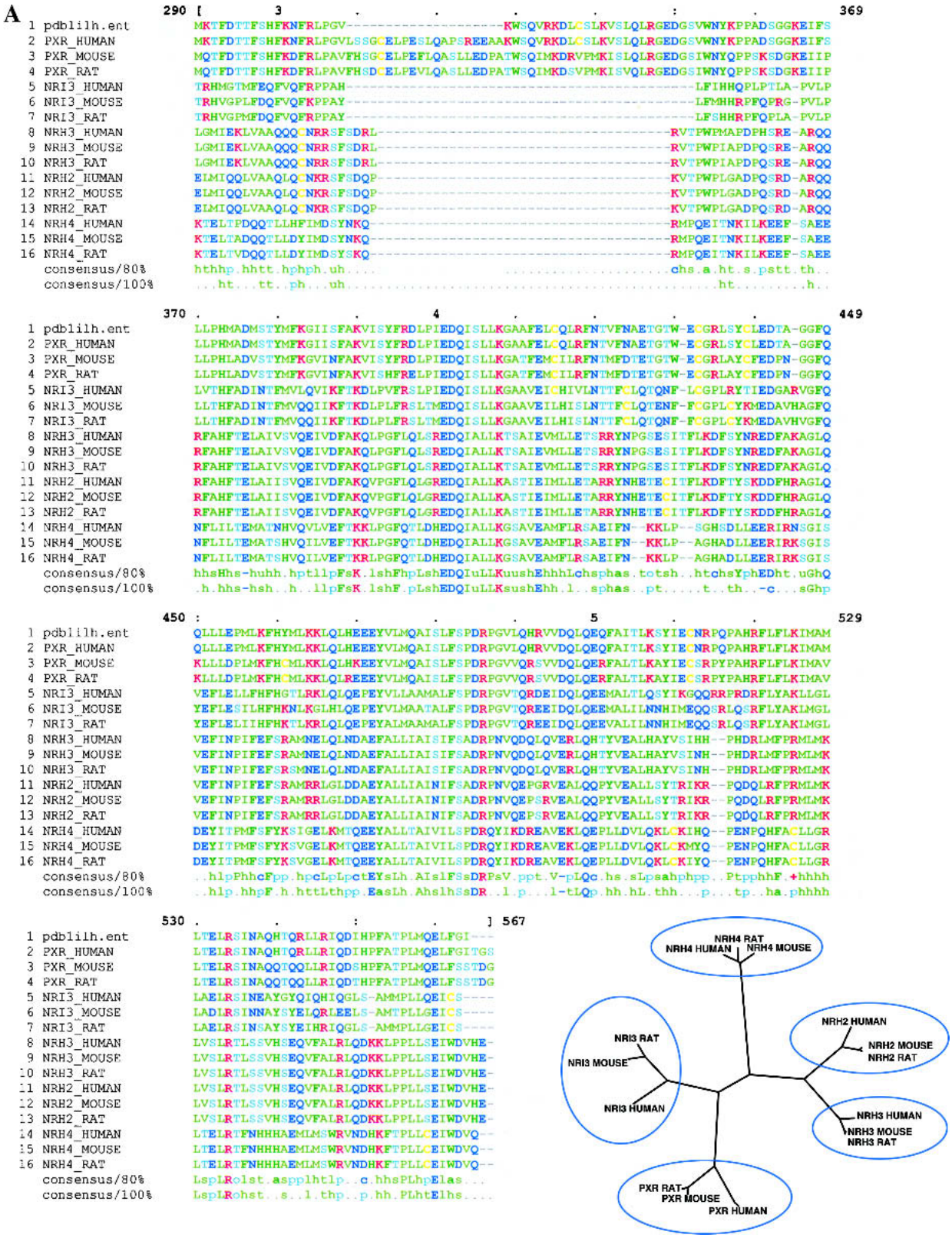

Fig. 2. Multiple sequence alignment (**A**) and phylogenetic tree (**B**) of the considered nuclear receptors from human, mouse, and rat. Amino acids are colored by type using MView (115). SwissProt names of the receptors are: PXR, PXR; CAR, NRI3; LXRα, NRH3; LXRβ, NRH2; and FXR, NRH4.. The alignment was built using ClustalW (116) with default parameters. The unrooted tree was built using the PHYLIP (117) package.

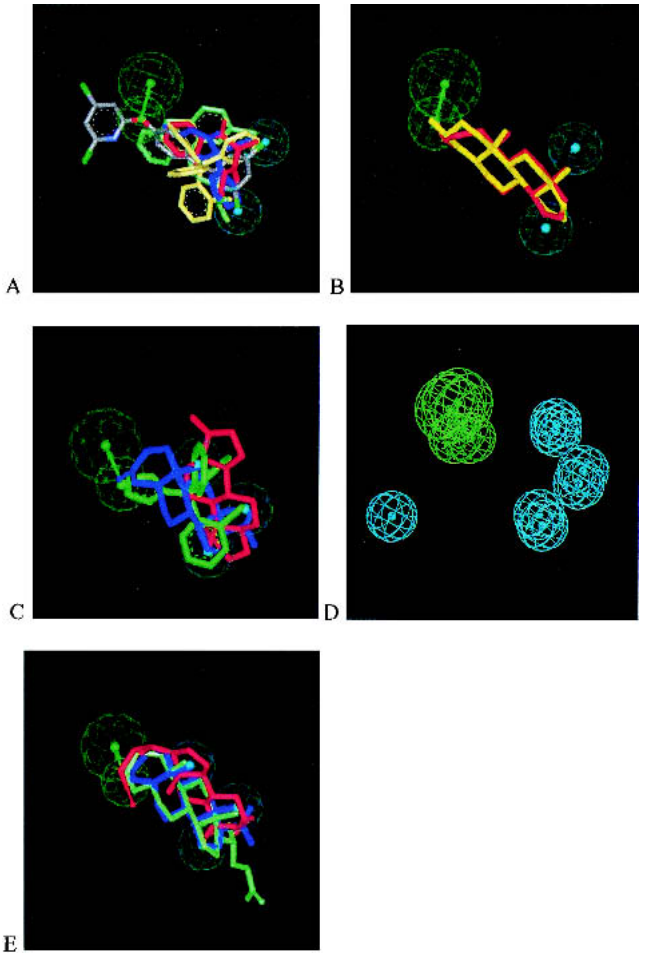

Fig. 3. CAR pharmacophores. **A,** Common feature alignment [HIPHOP function in *Catalyst,* (Accelrys, San Diego, CA)] of ligands for the murine constitutive androstane receptor (CAR). Murine CAR activators chlorpromazine (green), clotrimazole (yellow), metyrapone (red), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP, gray), and phenobarbital (blue) (75) used to generate an alignment in *Catalyst* to result in two hydrophobic features (cyan) and one hydrogen bond acceptor (green). TCPOBOP can be seen to partially fit outside of the pharmacophore to the left of the hydrogen bond acceptor. **B,** CAR-inhibiting steroids 16,5α-androsten-3α-ol (red) and 5α-androstan-3α-ol (yellow) fitted to the common features alignment generated with the structurally diverse murine CAR activators in Fig. 3A. **C,** Human CAR pharmacophore (observed vs. predicted EC_{50}, r = 0.99) derived from androstanol (red), clotrimazole (green), and 5β-pregnane-3,20-dione (blue) fitted to three hydrophobes (cyan) and one hydrogen bond acceptor (green) (48). **D,** Alignment of the human PXR pharmacophore (52) with the human CAR pharmacophore. Hydrophobic features (cyan) and one hydrogen bond acceptor (green) are shown to align well, leaving only one extra hydrophobe (bottom left) that is not present in CAR. **E,** Alignment of the bile acids [ketolithocholic acid (red) and lithocholic acid (green)] to the human CAR pharmacophore and the known CAR ligand 5β–pregnane-3,20-dione (blue).

bile acids could be higher-affinity ligands (activators or repressors) for CAR than PXR, which needs experimental verification. Perhaps it is appropriate to relate this observation back to the major genes that each orphan nuclear receptor regulates, namely CYP2B6 and CYP3A4, respectively. This may suggest that a molecule could potentially regulate both enzymes via different receptors, to differing extents, simultaneously. The similarity in the pharmacophore for CAR to that of human CYP2B6 (80) once again suggests that ligands for an enzyme (or transporter) would appear to have features in common with the ligands for the receptor responsible for regulating it as described for PXR (52,81). By using the human PXR crystal structure as a template to align human CAR too, we can show how a single amino acid, Phe^{161}, can essentially block SR12813 from binding, drastically reducing the volume of the CAR binding site compared with that of PXR in which Met^{243} is in the same position (Fig. 4). The steric interactions that are described for this molecule ultimately suggest that binding to CAR would be unfavorable as described *in vitro* (48).

A complication to our understanding of the roles of CAR and PXR comes in the form of submicromolar concentrations of glucocorticoids such as dexamethasone, which increase PXR, CAR, and RXR expression in human cultured hepatocytes and ultimately result in increased CYP expression and response (82–84). These authors suggest that glucocorticoids may control hepatic expression of PXR and CAR and be responsible for basal CYP3A4 expression in the absence of inducers, and on challenge with an inducer, these receptors are activated and increase CYP3A4 expression (82–84). The evidence they tender is that dexamethasone has been shown to be neither a CAR nor a PXR ligand at submicromolar levels, and they propose instead that it acts via the glucocorticoid receptor to up-regulate PXR and CAR, which then act on CYP3A4. This group has also shown that there is a strong correlation among the expression levels of PXR, CAR, and

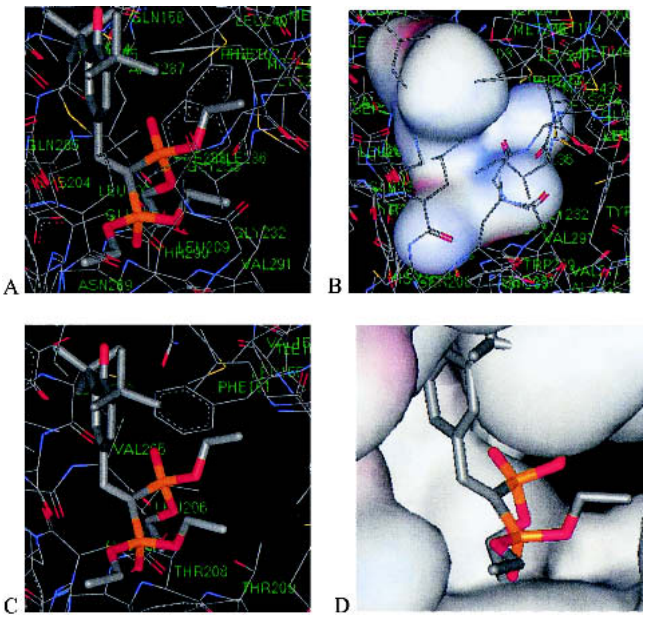

Fig. 4. SR12813 located in the crystal structure of PXR (**A,B**) and in the homology model of human CAR based on the structure of the human PXR ligand binding domain (**C**) showing the steric clashes with Phe^{161} (CAR represented as a Connolly surface) that would likely prevent it from binding in this orientation (**D**) if at all in this protein. The homology model was built using SwissModel (118) and further refined by removing the regions before Val^{154} and after Ile^{346}. Alignments of the central regions of CAR and PXR are unambiguous because of high sequence similarity. The unambiguous alignment is evidence in favor of the suggested homology model.

CYP3A4 in human liver microsomes (84), which contrasts with the experimental data derived from CAR and PXR knockout animals, which do not show any loss of CYP3A4 (19,39,41,76,85), indicative of no role in basal regulation for either of these receptors.

THE FARNESOID X RECEPTOR

Cholesterol homoeostasis is regulated by dietary uptake, biosynthesis, and conversion to bile acids, which in turn have important roles acting as detergents for absorption of dietary lipids and the regulation of gene transcription (10). Bile acids appear to be natural ligands for the farnesoid X receptor (FXR), expressed in the liver, kidney, and adrenals, which regulates expression of several genes including repression of cholesterol 7α-hydroxylase (CYP7α) and up-regulates the apical sodium-dependent bile acid transporter (ASBT, IBAT, SLC10A2). The cytosolic ileal bile acid binding protein (IBABP) binds bile acids in the enterocyte and may interact with ASBT to mediate transfer across the cell (11). Conjugated bile acids can also activate FXR, though these of course are actively transported into cells (10). The same authors evaluated a number of bile acids, oxysterols, and steroids for their ability to activate FXR at high concentrations. Chenodeoxycholic acid (CDCA) produced the highest level of activation, and the expression of IBAT in the same cells enabled glycine- and taurine-conjugated bile acids to be taken up and to activate FXR. This would suggest that there may be similarities between the computationally derived pharmacophores for the intestinal bile acid carrier derived for human (86) and rabbit (87) and a pharmacophore for FXR. Our attempts at modeling the available FXR data suggests a model with three hydrophobic features and one hydrogen bond acceptor (Fig. 5) that is similar to the models already derived for the rabbit and human bile acid transporters (86,87). The pharmacophore for FXR is similar to that of human CAR but has a unique arrangement of the hydrophobic features, although the ligand-binding sites of human FXR and human CAR possess only three common residues (V,L,F) out of 25. In view of potential ligand reciprocity with CAR, it may be interesting to test the diverse bile acid carrier ligands (87) against FXR to indicate non-bile-acid ligands.

The human bile salt excretory pump (BSEP, ABCB11) is expressed in the canalicular domain of hepatocytes where an ATP gradient enables a positive bile flow from blood to bile. This transporter has recently been shown to be transcriptionally regulated by the FXR/RXR heterodimer, with CDCA and 9-*cis*-retinoic acid resulting in activation of CAT activity (88), while CDCA and DCA activated the BSEP promoter cotransfected with an FXR expression vector in HepG2 cells (89). Neither ER/RXR, RAR/RXR, nor LXR/RXR induced transcription of the BSEP gene. It was also shown that there is a dramatic difference in the dose–response curves for the same bile acids to activate the target genes for FXR (BSEP) compared with PXR (CYP3A4), as they appear to have a higher affinity for FXR (89). Other genes regulated by FXR include the phospholipid transfer protein, phenylethanolamine-*N*-methyltransferase, carnitine palmitoyl-transferase II, and the basolateral bile acid transporter (sodium-dependent bile acid transporter, NTCP). Using a recent data set of inhibitors (bile acids and drugs) for the human NTCP (90) we have built a preliminary pharmacophore that highlights two hydrophobic and two hydrogen bond donor features in these ligands (Fig. 5B). This suggests that there is some overlap among the pharmacophores for FXR, ASBT, and NTCP as the requirement for hydrophobic features seems in all models; however, NTCP possesses only 37% homology and 48% similarity with ASBT (11). Thus, because nonbile acids appear to interact with NTCP (90) and ASBT (11), it is likely these same molecules potentially interact with FXR. To date it does not appear that anyone has investigated possible overlap in the regulation of these latter genes by either PXR or CAR, and this *in silico* hypothesis requires testing *in vitro*. The identification of the cholesterol-lowering natural product guggulsterone as an antagonist for FXR may

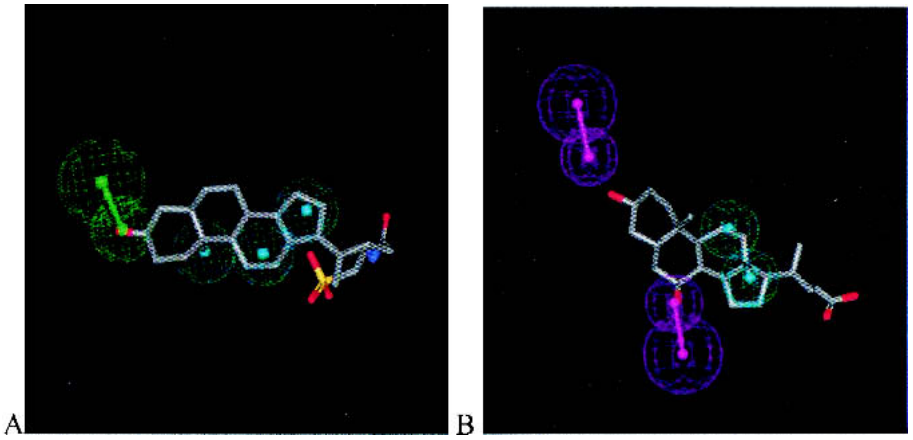

Fig. 5. (**A**) A pharmacophore for the farnesoid X receptor using 14 bile acids (10) and IC50 or EC_{50} values demonstrating a hydrogen-bonding feature (green) and three hydrophobic features (cyan). The observed vs. predicted data resulted in a correlation of r = 0.99. A training set member (taurine conjugate of lithocholic acid) is shown fitted to this pharmacophore. (**B**) A pharmacophore for the human hepatic sodium-dependent bile acid transporter using eight molecules (90) and IC_{50} values demonstrating two hydrophobic features (cyan) and two hydrogen bond donors (purple). The observed vs. predicted data resulted in a correlation r = 0.97. A training set member (ursodeoxycholic acid) is shown fitted to this pharmacophore.

suggest that a database of known natural products could be searched with a pharmacophore as described above in order to rapidly discover other molecules with this same activity (91).

THE LIVER X RECEPTOR

The liver X receptors (LXR) α and β are thought to have an important role in sensing the intracellular sterol level by regulating genes for controlling the absorption, storage, transport, and metabolism of cholesterol (22). LXRα and -β possess an amino acid sequence similarity of 77%, and both LXRα and -β bind to DNA at response elements (LXREs) after forming heterodimers with RXR (18). In mouse LXRα, mRNA seems to be highly expressed in liver, kidney, spleen, and intestine (92) as well as other organs (22). Both LXRα and -β can be activated by ligands binding to either or both LXR and RXR. The amino acids of the binding pockets of LXRs are conserved across human, mouse, and rat apart from a single residue (Figs. 1 and 2A: LXRα, Lys; LXRβ, Arg), suggesting similar if not identical ligand specificity of LXRs. Natural ligands for LXR have been suggested after screening various oxysterols, steroids, and fatty acids (93). Initially it was thought that 22(*R*)-hydroxycholesterol was the most potent activator for LXRα (93) until 24(*S*)-hydroxycholesterol, 24(*S*),25-epoxycholesterol, and other 24-oxo derivatives were assessed for LXR activation and were found to be potent activators of LXRα and LXRβ (94). Interestingly, some of these oxysterols are found at relatively high concentrations in the organs where LXRα and -β are expressed, such as the liver (94), or even in the circulation (95). It has been suggested that LXR activates CYP7α, which represents an important pathway in bile acid synthesis, whereas FXR downregulates this same gene (94). The structural requirements for both LXRα and -β were investigated by testing 23 molecules with a scintillation proximity assay (96). Stereochemistry was found to be important at the 24 and 22 positions on the oxysterols, and more potent ligands from both activation and binding perspectives were found in the dimethylhydroxycholenamide, and selectivity toward LXRα was obtained with further epoxidation to give 5,6-24(*S*),25-diepoxycholesterol (96). Potent LXR ligands were therefore suggested to require a single stereoselective oxygen on the sterol chain, which imparts hydrogen bond acceptor functionality. Recently a further analysis of LXRα (97) using a series of cholesterol derivatives, a homology model derived from RARγ, and site-directed mutagenesis suggested the same minimal pharmacophore for receptor activation as had been described previously. Trp^{443} was identified as a critical amino acid for hydrogen bonding to oxysterols (97). When oxysterols were modeled in the LXRα ligand-binding domain, they made a further hydrogen bond between the C3 position and Arg^{305}, while His^{421} was suggested to be a point for hydrogen bonding without promoting recruitment of the steroid receptor coactivator 1 (SRC), necessary for activation. It is unlikely the homology model this group generated (based on RARγ) will be applicable for other nuclear receptors, as the ligand-binding domains possess only 25% sequence similarity (33). These SAR studies and the limited homology modeling that has occurred to date suggest the viability of possibly developing further nonoxysterol agonists and antagonists for LXR.

The literature data for both LXRα and LXRβ have been used to build respective pharmacophores to which a nonsteroidal molecule T0901317 [demonstrated to be a selective LXRα and -β agonist (K_d and EC_{50} ~ 50 nM)] was fitted (Fig. 6A,B). When both pharmacophores are aligned, there are subtle differences in the positioning of the three hydrophobic and one hydrogen-bonding features (Fig. 6C). Both pharmacophores highlight a hydrophobic feature on the side chain as important for binding, which is clearly different from the pharmacophores derived for FXR. Using further ligands it may be possible to differentiate between both LXRs in terms of the molecules they bind, though that could be difficult because of their ligand-binding pocket similarity. Further nonsteroidal LXR agonists have been identified experimentally using parallel synthesis and both a cell-free-based ligand-sensing assay (LiSA) and a cell-based reporter assay (98). One of these molecules, GW3965, a nanomolar tertiary amine, seems promising as a potential therapeutic agent and also fits to the LXRα and -β pharmacophore models (Fig. 6A,B).

In mouse, the expression of the cholesterol transporter ABCA1 and hepatic CYP7A1 was found to be up-regulated by T0901317. The intestinal ABCA1 transporter acts to modulate cholesterol absorption by effluxing it into the lumen (99). The relevance of this to actual disease states has not gone unnoticed, as Tangier disease is characterized by a low level of HDL and macrophage accumulation of cholesterol. The latter is caused by a mutation in the human ABCA1 and hence results in the up-regulation of ABC1 and the choles-

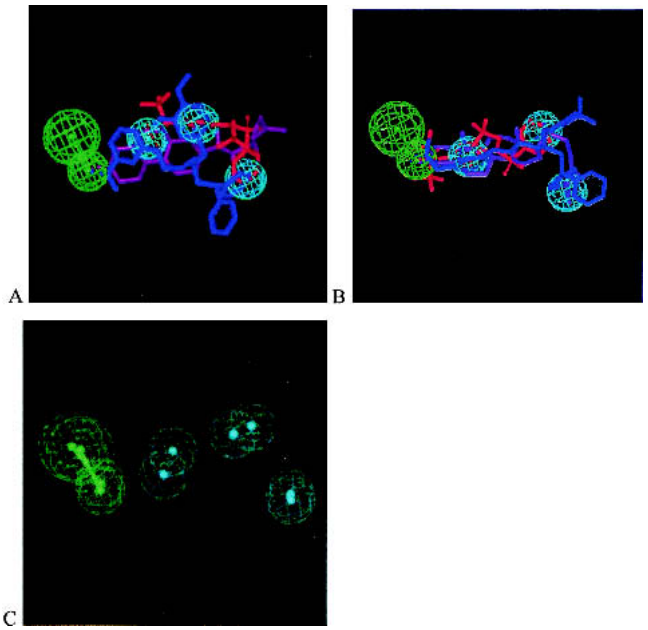

Fig. 6. A, Pharmacophore for the oxysterol liver X receptor (LXRα) built with 17 steroidal ligands with K_i values (96). The observed vs. predicted data resulted in a correlation r = 0.83. 24(*S*)-Hydroxycholesterol (purple) and the nonsteroidal molecules T0901317 (red) and GW3965 (blue) were fitted to this model. **B,** Pharmacophore for the oxysterol liver X receptor, (LXRβ) generated with 18 steroidal ligands. The observed vs. predicted data resulted in a correlation r = 0.83. 24(*S*)-Hydroxycholesterol (purple) and the nonsteroidal molecules T0901317 (red) and GW3965 (blue) were also fitted to this model. **C,** Alignment of pharmacophores for the oxysterol liver X receptor α and β demonstrating in both cases a hydrophobic feature (green) and three hydrophobic features (cyan).

terol ester transfer protein via ligands binding RXR/LXR. This could represent one area for therapeutic investigation (100). Increasingly ligands for nuclear hormone receptors may be valuable at modulating multiple steps in the cholesterol cascade rather than a single target (12). Other members of the ABC family of transporters found in human macrophages have also been found to be induced via LXR. These include human white (ABCG1)/murine ABC8 likely involved in sterol transport and homeostasis (101,102), which may also represent viable therapeutic targets for cholesterol control (18). This may be particularly important because it has been demonstrated that mutations in ABCG5 and ABCG8 occur in patients with sitosterolemia in which there is an increased absorption of dietary sterols, resulting in hypercholesterolemia and atherosclerosis (103), and that these transporters normally form a functional complex to efflux cholesterol. Recently it was confirmed that both of these latter genes are direct targets for both LXRα and -β in mouse (104). The recent identification of 27-hydroxycholesterol as an endogenous ligand for LXR following cholesterol loading of human macrophages suggests that even an oxysterol far removed from cholesterol biosynthesis can participate in controlling bile acid synthesis and cholesterol transport via this nuclear hormone receptor (95). This may itself have important implications for designing LXR agonists and treating metabolic disorders.

INSIGHT FROM NUCLEAR HORMONE RECEPTOR LIGANDS

The present study has taken a ligand-based approach using computational pharmacophore models to suggest features that may be important for binding to the nuclear hormone receptors, PXR, CAR, LXRα, LXRβ, and FXR, which are regulators of many genes that may be implicated in some drug interactions. In all pharmacophores generated for ligands for each receptor, multiple hydrophobic features are highlighted alongside a single hydrogen bond acceptor. It is highly likely that discrimination among the nuclear receptors is based on the positioning of amino acids within the ligand-binding domain that correspond with these features, particularly the hydrophobic features (see Fig. 1). Therefore, we should consider the diversity of receptors that a single ligand may bind to different extents, particularly because more than one receptor can act on the same cassette of genes (Fig. 7). We need to identify the diverse enzymes and transporters that may be affected by a ligand binding more than a single nuclear hormone receptor, so that protein transcription in, for example, the kidney and other organs may be affected simultaneously or separately, depending on the ligand and the receptors bound. Our own previous attempts at computational modeling of CYPs, P-gp, and PXR showed some similarities in the features essential for binding to these proteins. Computational modeling of human CAR, FXR, LXRα, and LXRβ has also been insightful, even though we are limited in the amount of data available at present in the literature for these receptors until much larger studies are performed. We have shown that the human CAR pharmacophore may be considerably smaller than that derived for PXR and that bile acid metabolites may have some affinity for CAR. We have suggested that the pharmacophore for FXR seems to resemble those for the ASBT and NTCP. Finally, we have indicated that the pharmacophores for LXRα and LXRβ are similar and could be valuable tools for finding further nonsteroidal ligands that may have a therapeutic role. These results are consistent with the similarities between the ligand-binding pockets of the receptors (Fig.7). Overall, these pharmacophore models share a great deal of similarity and build on the data from the literature suggesting functional overlaps among many receptors, transporters, and enzymes (Fig. 7) that have important endogenous roles in bile acid homeostasis as well as xenobiotic metabolism and efflux (3,4,35,89,105).

How can we use these ligand-based models to understand the functioning of these receptors, and how would we apply this information to drug discovery? To date the available data suggest that the human body possesses some receptors for small physiologic steroids or bile acids that can be bound with high affinity and some nuclear receptors with larger binding sites with a lower affinity toward these same molecules. Hence, we have a multiplicity of receptors that coexist with either specificity or promiscuity that can regulate

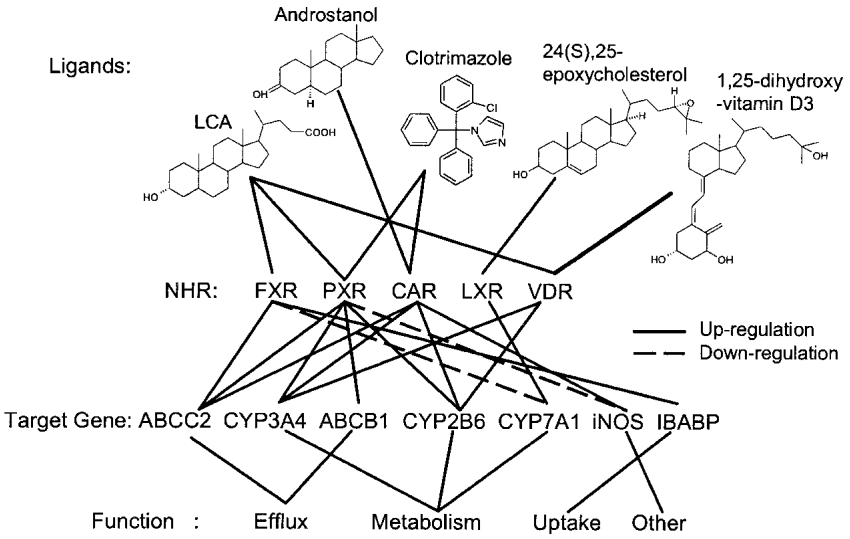

Fig. 7. Overlapping ligand recognition and nuclear receptor–mediated gene regulation.

the same gene products and probably deal with differing levels of ligand (Fig. 7). The ability for some of these receptors to down-regulate hepatic uptake transporters and up-regulate hepatic export pumps as well as to up- or down-regulate CYPs or other enzymes simultaneously represents a unique protective mechanism against toxicity that can probably be applied to other organs and tissues that are exposed to xenobiotics (106). The implications for drug discovery are that we need to be aware of more than just the PXR and CAR interactions of our molecules that have been predominantly highlighted to date, particularly if we are trying to pursue transporters, enzymes, or receptors as therapeutic targets that may ultimately possess a closely related pharmacophore to nuclear hormone receptors. Understanding the likely similarities of ligand-binding sites based on the sequence of a protein is important, but of more relevance is the protein–ligand interaction fingerprint (pharmacophore) for similarly regulated proteins. In this study we have described just one method by which we might capture this type of ligand-binding information in the future, but there will likely be many more. There has been some speculation about using the ligands for these receptors to modulate such diseases as cholestasis; however, it must also be recognized that as with any potential drug target, selectivity against other receptors is key in avoiding toxic interactions. It may be possible to modulate these nuclear receptors as part of a therapeutic strategy in combination with other coadministered molecules, though this needs considerable assessment because of the complex interaction pathways. Understanding the ligand-binding characteristics of these receptors is one step toward this goal and needs to be combined with similar data for the enzymes and transporters. Analysis of the convoluted ligand–nuclear hormone receptor–gene interconnections indicates some analogy with a neural network in which the molecule is the "input," the nuclear receptors and genes regulated represent "hidden neurons," and the function of the target gene is the "output" (Fig. 7).

As further new receptors are identified as having important regulatory roles for the same proteins, one would expect some degree of overlap in binding affinity (Fig. 7). For instance, the vitamin D receptor (VDR) has been recently suggested as a further regulator of intestinal CYP3A4 alongside PXR and CAR, with LCA binding to VDR with a higher affinity than for PXR (107). It has also been indicated that the VDR may regulate CYP2B6 and CYP2C9 (108). By using the crystal structure for VDR or similar receptors, it might be possible to explain how some bile acids (and possible other molecules) fit into the ligand-binding domains of some nuclear receptors and not others, and other bile acids do the opposite (109,110). The continual identification of new nuclear hormone ligands in natural products could lead us to more clearly define what it takes to be classified as a drug, particularly with the recent suggestion that phytosterols in corn oil reduce cholesterol absorption in humans (111).

Understanding further enzymes regulated by each nuclear hormone receptor is useful in trying to interpret clinical findings. For example, some endocrine and metabolic mechanisms have been linked to the development of polycystic ovary syndrome (PCOS), particularly hyperandrogenism related to induction of CYP17 (112). Insulin-sensitizing compounds, including thiazolidinedione ligands for PPARγ, have been shown to be useful in treating PCOS, possibly because these same individuals generally need treatment of insulin resistance (113). A further enzyme, aromatase (CYP19), is down-regulated by troglitazone in ovarian granulosa cells (113). Because some thiazolidinediones are ligands for PXR and possibly other nuclear receptors, there may be overlap with regulation of CYP17 and CYP19, and this may not necessarily be mediated solely by PPARγ. PCOS could also be drug-induced, where this molecule represents an agonist for the nuclear receptor responsible for regulation of CYP17 or CYP19. Understanding and treating complex diseases that may have one or more nuclear receptors as major components are clearly desirable and therefore require ligands optimized with affinity to multiple select nuclear receptors and not others.

Because transporters ultimately limit the exposure of nuclear receptors to an extracellular ligand, they represent the first gate that must be passed before a molecule reaches the enzymes for which it may also be a ligand. Few molecules may reach the nuclear receptors; however, it is unclear how many molecules that reach this point *in vivo* are actually ligands for nuclear receptors. For exogenous molecules, up-regulation of transcription of genes for metabolism and transport represents a way to rapidly remove a potential toxin that could interfere with an endogenous pathway such as those regulated by nuclear receptors. Obviously, the future development of computational models that can deal with the level of complexity that exists where multiple receptors overlap to regulate multiple genes in a ligand-dependent manner would be highly desirable, particularly as this impinges on our understanding of drug safety (114) and clinical findings with complex etiologies. In conclusion, perhaps nuclear receptors would not have such a major physiologic role without the apparently more rapidly evolving transporters and drug-metabolizing enzymes with which they are paired and which can possess a similarity in their recognition of the same ligands.

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