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Drug Discov Today. Author manuscript; available in PMC 2010 May 01.

Published in final edited form as:
Drug Discov Today. 2009 May ; 14(0): 486–494. doi:10.1016/j.drudis.2009.03.003.

Understanding Nuclear Receptors Using Computational Methods

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Abstract

Nuclear receptors (NRs) are important targets for therapeutic drugs. NRs regulate transcriptional activities through binding to ligands and interacting with a number of regulating proteins. Computational methods can provide insights into essential ligand-receptor and protein-protein interactions. These in turn have facilitated the discovery of novel agonists and antagonists with high affinity and specificity as well as aiding in the prediction of toxic side effects of drugs by identifying possible off-target interactions. Here, we review the application of computational methods towards several clinically important NRs (with special emphasis on PXR) and discuss their use for screening and predicting the toxic side effects of xenobiotics.

Introduction

Nuclear receptors (NRs) are ligand-dependent transcription factors that regulate the expression of a variety of important target genes involved in a wide spectrum of developmental and physiological processes[1]. In addition to ligand binding, the transcriptional activities of NRs are also modulated through a range of regulating proteins termed a coactivator and a corepressor[2–4]. The ligand binding domain (LBD) of NRs is responsible for both ligand recognition and regulation of protein-protein interactions (Figure 1A) [5]. Upon agonist binding, conformational changes are induced in the LBD, particularly the AF-2 region, which leads to the dissociation of a corepressor and recruitment of a coactivator (Figure 1B) (reviewed in [6]). This contributes to downstream gene activation.

NRs represent one of the most important targets for therapeutic interventions for multiple diseases, including cancer, inflammation and metabolic diseases (such as metabolic syndrome)[7]. Understanding xenobiotic interactions with NRs is also important in the

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Teaser Sentence: Computational methods have been used with nuclear receptors for identification of novel agonists and antagonists and represent rapid approaches for lead discovery alongside *in vitro* methods.

Conflicts of Interest Sean Ekins is a consultant for Collaborations In Chemistry. Ni Ai, Matthew D. Krasowski and William J. Welsh have no conflicts of interest.

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context of endocrine disruptors and environmental toxicity assessment [8]. It is therefore important to identify synthetic compounds that mimic the cognate NR ligand activity, but also to develop synthetic compounds that selectively modulate the pharmacology of NRs in a cell-type and/or tissue-selective manner to exert the desired therapeutic effects while avoiding potentially undesirable off-target effects (reviewed in [9–15]).

Different computational methods have emerged aimed at understanding and modeling the functional activities of NR modulators at the molecular level. Generally these computational approaches fall into two categories, ligand-based and receptor-based approaches, although more recently there have been efforts to combine these usually distinct approaches [16]. Ligand-based methods essentially focus on molecular similarity, which implies molecules with similar features exhibit similar biological responses. It is a particularly valuable approach to identify compounds if structural information for a receptor is unavailable. In contrast, receptor-based (also synonymous with target-based) methods require the three-dimensional structure of the protein targets predominantly generated from X-ray crystallography, NMR structures or homology modeling, in order to address the fundamental question of how a potential ligand might bind to the receptor. Both ligand- and receptor-based strategies have been widely applied to advance the understanding of various aspects of pharmacology in NRs [17,18]. In this review, we focus on several key NRs including the androgen receptor (AR; NR3C4), estrogen receptor α and β (ERα and ERβ; NR3A1 and NR3A2), pregnane X receptor (PXR; NR1I2), farnesoid X receptor (FXR; NR1H4), liver X receptors α and β (LXRα and LXRβ; NR1H3 and NR1H2) and vitamin D receptor (VDR; NR1I1). We will describe the process of method development and optimization to accommodate distinct receptor features of NRs, detail the success of computational methods and finally discuss the application of computational strategies to examine adverse effects of drugs.

Computational methods to understand NR pharmacology and evolution

NR ligands typically occupy a hydrophobic pocket that lies within the core of the NR LBD (reviewed in [19]). In contrast to the extensively studied ligand-protein recognition inside the ligand binding pocket (LBP), the ligand entry or exit mechanisms to and from the binding site of NRs are poorly understood since there is not an obvious entry or exit route on the surface. Molecular dynamics (MD) simulations have emerged as a powerful approach to elucidate various potential dissociation routes of endogenous ligands and synthetic modulators for several NRs, such as the retinoic acid receptors (RARs; NR1B1, NR1B2, and NR1B3), thyroid receptor α and β (TRα and TRβ; NR1A1 and NR1A2), VDR and ERs[20–26]. Results from these computational simulations have revealed that significant conformational rearrangements of the receptor occur upon ligand binding and release. In addition to specific receptor and ligand types, computational simulations have indicated that the quaternary state of the receptor is one of the key factors that influences the prevalence of a particular pathway, implying that dimerization of NRs plays a role in ligand dissociation[20]. Several groups have suggested that it may be possible to develop pharmaceutically relevant ligands that could interact preferentially with NRs in specific oligomeric states, representing a new type of NR modulator[20].

Transcriptional activities of NRs are regulated by complex functional interactions of NRs with ligand, DNA, coactivators, and corepressors. Recently a comparison of multiple crystal structures of LBDs revealed distinct receptor conformations under different ligand conditions (ligand-free, agonist-bound, or antagonist bound) and coactivator/corepressor binding [27]. These structures make it possible to understand the ligand specificity of individual NRs at the atomic level. In the process they also identified helix 12 (H12) in the AF-2 site of the LBD as the molecular switch for NR activation. H12 is allosterically

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controlled by the binding of different ligands as it plays a structural role through its localization in the receptor, forming a corresponding site on the protein surface for cooperative coactivator/corepressor binding, in turn modulating the transcriptional effects of NRs. Several pieces of evidence suggest that receptor-specific recruitment or interaction with different transcriptional cofactors is necessary for gene activation in different cell types and leads to distinct physiological consequences [28–30].

Computational methods have been employed to understand the structural basis for recognition and specificity among interacting partners that are required for precise regulation of transcriptional activities of NRs. Wang *et al.*, constructed a structural model for the PXR LBD with two different interacting domains (ID1 and ID2) from corepressors using homology modeling[31]. MD simulations on these two models predicted preferential binding toward ID2 over ID1 by PXR and revealed the key interactions, which was further supported and validated experimentally. These studies provide insights into the molecular interactions that direct the assembly of PXR and the corepressor. This may in turn have important implications for understanding the role of corepressors in regulating the biological activities of PXR, facilitating the design of therapeutic modulators promoting corepressor binding. Recently, similar MD simulations were performed on various oligomerization states of PXR to understand how the AF-2 region rearranges to form an “active-capable” conformation on the receptor surface for obligate contacts with transcriptional coactivators[32]. These results revealed highly correlated motions by helices that comprise the AF-2 region, and indicated a path transmitting long-range motions from the PXR ligand scaffold to the surface (AF-2 region). Based on observations from computational simulations, it was also suggested that PXR formed a heterotetramer with RXR, instead of a heterodimer, to interact with coactivators [32]. Increased motion or flexibility/distortion of H12 on the AF-2 region was also demonstrated by MD performed with ERα after coactivator binding [32], as well as with AR and mutations associated with androgen insensitivity syndrome [33].

Computational methods have also shown their applicability to study adaptive evolutionary changes of the LBP of different NRs that correspond to varying ligand specificities across species. Reschly *et al.* recently combined computational approaches with *in vitro* studies to explore the structural basis of ligand preference across vertebrate species for NRs involved in the cholesterol metabolism pathway, identifying three patterns of co-evolution with bile salts, the major elimination metabolites of cholesterol[34,35]. FXRs were suggested to have different specificities for primary bile salts across species which was achieved by altering the shape and size of the LBP. Human FXR has a curved binding pocket best suited for the bent steroid ring configuration typical of evolutionarily more recent bile acids. VDRs have recently acquired sensitivity to lithocholic acid, a toxic secondary bile salt, by changing the entrance to the LBP. PXRs have expanded their specificity for bile salts, from a narrow selectivity for planar bile salts in zebrafish to broad specificity for a wide range of bile salt structures in human, by substantial increases in the volume of the LBP [36]. Analysis of crystal structures of these three human NRs and LXR show substantial differences in the volume of the ligand-binding cavity (PXR>>LXR>FXR> VDR) and distances between a critical arginine residue (Arg-328 in human FXR) and a key glutamine or histamine residue (His-444 in human FXR) (FXR> LXR> VDR>PXR)[34]. Using evidence from homology modeling and docking, Reschly *et al.* proposed that FXR, VDR, and PXR each acquired sensitivity to bile salts at separate points in vertebrate evolution and then adapted in different ways to the evolutionary changes in bile salt structure and metabolism[34]. In total, these combined *in vitro-in silico* observations present a new integrated picture of co-evolution of a biochemical pathway and protein structure.

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Computational analysis of three-dimensional coordinates of protein structures has helped to identify evolutionary important residues and correlate them with important NR functions, such as dimerization and DNA binding[37]. Recently a new functional site located on the receptor surface was identified for steroid receptors in the NR3 family using the evolutionary trace computational method, which integrates evolution of protein sequence, structures and functions[38]. As shown in Figure 2, this new site is different from amino residues in the AF-2 region, dimerization domains, or LBP and comprises residues specific for steroid receptors. Five of nine residues were found to correlate with certain human diseases, which suggests a biological role *in vivo* [39–43]. The biological relevance of the new functional site was further confirmed by experimental mutations of these residues in ER, showing they disrupted signaling by allosterically influencing estradiol binding and coactivator recruitment [38]. Results from this computational study have also expanded our knowledge of protein-protein interactions involved in NR signaling.

Virtual Screening methods to identify NR modulators

Ligand specificity of NRs is crucial in terms of both cellular transcription and therapeutic applications. In order to understand the essential interactions that determine specificity, it is important to identify the key ligand structural features and critical receptor residues that are involved. Computational methods have been widely applied to derive knowledge of such ligand-protein interactions and identify novel NR ligands, such as endocrine disrupting compounds (EDCs). EDCs are xenobiotics that activate NRs and disrupt crucial physiological functions by mimicking cognate ligands, posing a potential risk to wildlife and humans. Quantitative Structure-Activity Relationship (QSAR) and machine learning models are useful tools to rapidly extract the structural characteristics for binding from a set of active ligands using statistical techniques and specific molecular descriptors that may represent physicochemical properties, molecular shape or other properties. Multiple QSAR and machine learning models have been published for several NRs, including ER, AR, and PXR, primarily to address the increasing need for high throughput endocrine disruptor risk assessment[44–46], but also for toxicological screening [47] in combination with crystal structures and other *in silico* methods. A recent analysis of 74 natural or synthetic estrogens by a QSAR model yielded useful information on structural features for preferential activation of the ERα and ERβ subtypes[48]. In addition to QSAR models, non-linear statistical machine learning methods have been successfully applied to separate NR activators from non-activators[49].

Activation of PXR regulates the expression of metabolizing enzymes such as cytochrome P450 enzymes (CYP3A4, CYP2B6, and CYP2C8/9) and glutathione-S-transferases, as well as important drug transporters (P-glycoprotein, multidrug resistance protein as well as others)[47]. Since the CYP enzymes metabolize the majority of clinically important drugs, inadvertent up-regulation by PXR agonists may increase metabolism and excretion of other coadministered therapeutic agents and cause undesirable drug-drug interactions or the generation of toxic levels of a drug metabolite. It is important to identify molecules that interact with PXR early in the drug development process, while some environmental compounds may also activate PXR[50]. Computational methods continue to play a significant role in identification of PXR activators[45,51,52] and assist in avoiding unexpected drug-drug interactions before human clinical testing. For example a recent study used docking to propose candidate molecules that reduced PXR activity by forming destabilizing interactions [53]. Ung *et al.*, explored the application of three machine learning methods for predicting PXR activators[52]. Their results indicated that the support vector machine (SVM) method had the best performance with overall accuracy around 80%. A more recent machine learning analysis has used the same training set to create Tree and SVM models which were then tested with a large external test set, providing the most

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exhaustive evaluation of PXR models to date [54]. The selected descriptors in these models were in good accord with those used in previous pharmacophore and QSAR modeling, as well as interactions suggested in X-ray crystallography studies.

Some NRs display broad ligand selectivity such that they can promiscuously bind and be activated by an array of structurally diverse ligands. This characteristic is correlated with their biological functions as xenobiotic sensors (e.g. PXR) or lipid sensors (e.g., peroxisome-proliferator activated receptor, PPARs, NR1C1, NR1C2, NR1C3) [55]. Many proteins have been found to possess intrinsic disorder in some part of their sequence [56]. These disordered areas lack a rigid three-dimensional structure and frequently are not resolved in X-ray or NMR structures. These disorded regions can perform various important biological functions [57] such as protein-protein interactions [58], DNA binding, cell signaling [59], and protein-ligand interactions [60]. It was recently found that there is considerable variability in predicted intrinsic disorder within NRs, particularly in the D-domain (a region of NRs known to be involved in DNA recognition and heterodimerization, coactivator/corepressor interactions and protein-protein interactions) and LBD, which is likely an additional important evolutionary force in shaping protein-protein interactions, promiscuity and NR function [61].

Analysis of available crystal structures of PXR co-crystallized with different ligands indicated the LBP of PXR is a very large and flexible hydrophobic site, which may account for its promiscuity in binding structurally diverse ligands [62–68]. Receptor flexibility observed in the PXR LBD represents a considerable challenge for binding pose prediction and evaluation by docking methods and is an important consideration for applying receptor-based approaches to PXR. The majority of computational studies on PXR have therefore focused on ligand-based approaches to distinguish PXR activators and non-activators, such as pharmacophores and descriptor-based statistical models (as described above). Pharmacophores represent the geometric arrangement of essential structural features of ligands for binding to protein targets. The first pharmacophore study of PXR used 12 published ligands to develop a model that revealed the structural determinants necessary for binding and suggested its potential application as a structural filter before *in vitro* determination.[51] (Figure 3). As more three-dimensional receptor structures co-crystallized with various ligands became available, new structural information on the LBP was integrated to develop a receptor-based pharmacophore model that labeled the area prohibiting the ligand binding (`excluded volumes') and important residues involved in the binding[69]. To date, a consensus pharmacophore suggests that multiple hydrophobic features and one hydrogen bond acceptor are common for PXR agonists [70]. Recently we have evaluated a hybrid method combining ligand-based SVM models with molecular docking in an attempt to improve predictions [16]. A two-dimensional pharmacophore model has been used by Lemaire et al. to filter a commercial compound database for previously unknown PXR activators[71]. The resulting compounds were docked into a PXR crystal structure and scored for ligand-receptor interactions. Experimental data from *in vitro* and *in vivo* studies corroborated their predictions and identified more active compounds. It is important to note that across species, PXR shows a high degree of sequence diversity in the ligand binding domain (LBD), resulting in marked differences in ligand selectivity, which likely also correlates with the evolutionary pressure of adaption to toxic compounds throughout development. A pharmacophore analysis performed on the same 16 ligands for mammalian (human, mouse, rat), chicken, frog and zebrafish PXRs also helped to understand the evolutionary history influencing ligand specificity[72] as mammals had similar pharmacophore arrangements while other species differed widely.

Virtual screening (VS) of large compound databases, which incorporates molecular docking methods and mathematical scoring functions, has been widely adopted to predict ligand-

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protein interactions and identify new drug leads for many NRs, such as ER, progesterone receptor (PR, NR3C3), glucocorticoid receptor (GR, NR3C1), TR, PPARs, and LXR [73–80]. Since specificity for a given subtype of NR is of considerable importance for therapeutic applications, it is important to tailor the VS scheme with target-specific structural information, e.g. key hydrogen bonding residues, distance cutoff for important interactions and ligand-receptor complementarities in order to discover subtype-selective compounds. Using a VS protocol customized with ERβ specific parameters, Zhao *et al.*, identified ERβ specific ligands from a plant product-based database [81]. Binding affinity and selectivity of the 12 candidates from VS were evaluated by a fluorescence polarization binding assay. Three of twelve compounds displayed over 100-fold selectivity to ERβ over ERα. Similarly, Knox *et al.* proposed a target specific VS scheme optimized for ERα. 19 known ERα inhibitors were used to calibrate the scoring functions for specific ERα activity. Three of seven tested compounds bound to ERα and exhibited good selectivity of ERα over ERβ[78,82]. A similar strategy (Figure 4) can be utilized to discover novel compounds that interact with other NRs with high specificities and affinity such as the agonists and antagonists shown in Figure 5 and 6.

The LBP has been the predominant focus for identification of NR modulators. Computational methods, combined with experimental approaches, have shown that there are other sites on the receptor surface possible for small molecule binding to alter NR biological activities. The AF-2 site is a conserved region among NRs for cofactor protein binding, and is therefore a potential site for designing novel NR modulators which directly interfere with essential protein-protein interactions. Recently a VS scheme was integrated with a cell-based assay to identify novel ERα antagonists that may disrupt coactivator binding[83]. Through a molecular docking study, compounds from the VS were found to selectively fit into the AF-2 site on the ER surface due to their unique shape and charge properties [83]. A mammalian two-hydrid assay also confirmed that selected compounds disrupted the receptor co-activator interactions without displacing estradiol binding to ER. The best compound was not structurally similar to known anti-estrogens, which suggested a novel class of ERα modulators as an alternative to the current anti-estrogen therapy.

Ketoconazole and miconazole have been shown as GR antagonists of dexamethasone binding (while fluconazole had no effect), repressing PXR, CAR and downstream gene expression[84]. Mutagenesis data, has indicated that the AF-2 region is a potential binding site for azole antifungals (ketoconazole, enilconazole and fluconazole) acting as PXR antagonists [85]. Computational docking results revealed these PXR antagonists partially occupied the same hydrophobic groove where the coactivator motif binds to receptor, antagonizing the essential protein-protein interaction ([70] and references therein). Based on these PXR antagonists, a pharmacophore model was developed to elucidate the important structural features for binding. When this model was combined with docking studies and biological testing, it enabled discovery of several more potent non-azole PXR antagonists (included in Figure 6) which included commercially available synthetic compounds and the FDA approved prodrug leflunomide (Figure 6), confirmed experimentally *in vitro* [86]. These small molecules also had good ligand efficiencies (as they were more potent on a per heavy atom basis) compared with ketoconazole when determined using the published approaches (Figure 7) [87]. This suggests that smaller molecules could also be effective antagonists and with optimal protein-ligand interactions. It is also important to consider that antagonism of PXR or other NRs could occur via interactions with other proteins that interact with PXR or at other surface sites beyond those currently known. Novel PXR antagonists provide a possible small molecule intervention to control drug metabolism and transport by reducing the activation of these genes during therapeutic treatment.

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Computational methods to study toxicity profiles related to NR modulators

Selective estrogen receptor modulators (SERMs) such as tamoxifen have been developed to treat breast cancers and other diseases. However, several adverse effects are associated with treatment using these drugs, including cardiac abnormalities, thromoboembolic disorders, and ocular toxicity. In order to understand the molecular mechanism of adverse effects, considerable efforts have been taken to identify off-target interactions using computational approaches such as docking studies with structural similarity comparison methods for binding sites. The protein target with the highest similarity to the ERα LBP was sarcoplasmic reticulum (SR) Ca^{2+} ion channel ATPase protein (SERCA), which is a key mediator of cytosolic calcium levels by accumulating calcium in the lumen. Since the gradient concentration of calcium in the SR is important for muscle contraction, it is possible that inhibition of SERCA by SERMs may cause the loss of calcium homeostasis in platelets and lead to the reported adverse effects, such as cardiac abnormalities[88].

Similar *in silico* approaches could therefore be used with other NRs and incorporated into the drug discovery process for early identification of off-target adverse effects. Retrospectively, computational approaches are also able to discover endocrine effects for marketed drugs. These results can help to explain the observed side effects and provide an opportunity to optimize the pharmacological profiles of drugs to eliminate activity at the original target and ultimately enhance endocrine activities for related therapeutic indications (an example of drug repurposing[89]). This latter approach takes advantage of the presumably favorable bioavailability and toxicity profiles for marketed drugs to save time and cost during the drug development process. Bisson *et al.*, demonstrated the application of this computational approach on a library of marketed oral drugs. Their study led to a nonsteroidal antiandrogen with improved AR antagonistic activity and markedly reduced antipsychotic effects[90].

Conclusions

NRs are important transcriptional factors that regulate a number of essential physiological processes involved in metabolism, development and systemic homeostasis. Transcriptional activities of NRs are guided by interactions with ligands and multiple cofactor proteins. In recent years, computational modeling of NRs has proved increasingly valuable to advance the understanding of NR pharmacology. Detailed insights about how ligand-protein, protein-protein, protein-corepressor and protein-coactivator interactions occur make it possible to predict potential off-target effects. We have shown that a number agonists and antagonists were computationally discovered for NRs (Figures 5 and 6), which indicates their potential utility for discovering potential therapeutics to treat NR-related diseases in the future.

Acknowledgments

The authors kindly acknowledge the collaborations and the many valuable discussions with Dr's. Erica J. Reschly, Lee R. Hagey, Alan F. Hofmann, Sridhar Mani, Erin Schuetz, Ken Bachmann, Peter W. Swaan and our colleagues at UMDNJ.

SE also thanks Accelrys, San Diego, CA for making Discovery Studio Catalyst available. Support for this work has been provided by the USEPA-funded Environmental Bioinformatics and Computational Toxicology Center (ebCTC), under STAR Grant number GAD R 832721-010, and by the Defense Threat Reduction Agency, under contract number HDTRA- BB07TAS020. This work was also funded by NIH - GM081394 from the National Institute of General Medical Sciences (to WJW). MDK is supported by NIH K08-GM074238. This work has not been reviewed by and does not represent the opinions of the funding agencies.

We thank the reviewers for their very useful comments.

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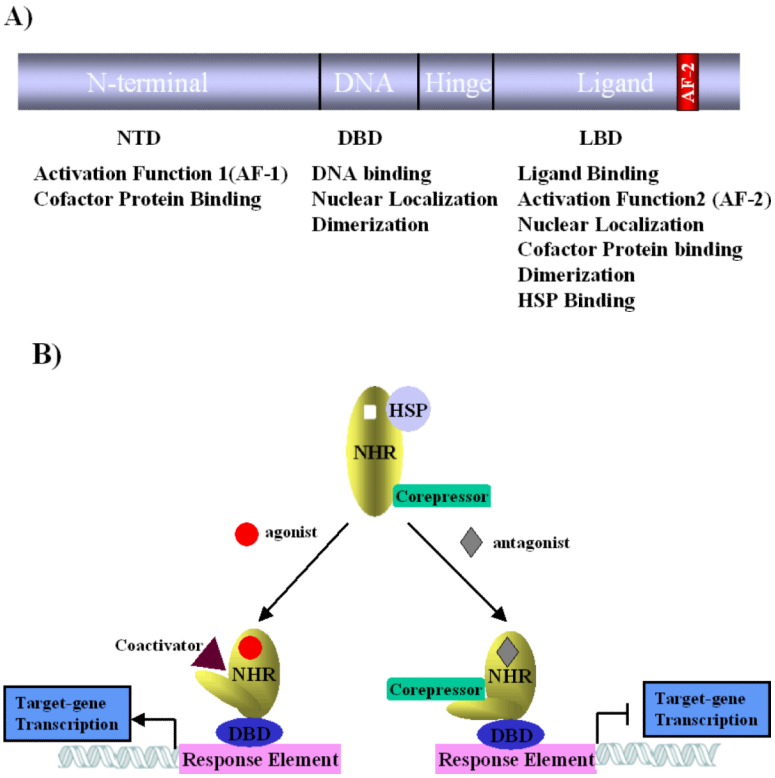

Figure 1.

Domain structures of NRs and mechanism of action upon ligand binding. A) The NR superfamily shares a common domain structure consisting of a NH_2-terminal domain (NTD), a central DNA-binding domain (DBD), a carboxy-terminal ligand-binding domain (LBD), and a hinge domain between DBD and LBD. Functions for each domain are also listed. B) General model for transcriptional activation and repression in presence of agonist and antagonist. Upon agonist binding, heat shock protein (HSP) and corepressor are dissociated from receptor. Conformational changes occur in the LBD during coactivator recruitment, then activation complexes are formed with other cofactor proteins to turn on target gene expression. Antagonism of NRs is complex and not completely understood. Here we present one known silencing mechanisms associated with antagonist binding. Antagonist binding may induce a difference conformation of LBD, therefore prohibiting coactivator binding or promoting the recruitment of corepressors. It is important to note many NRs work as homo- or heterodimers (and possibly higher order multimers). The monomer is displayed here for simplicity.

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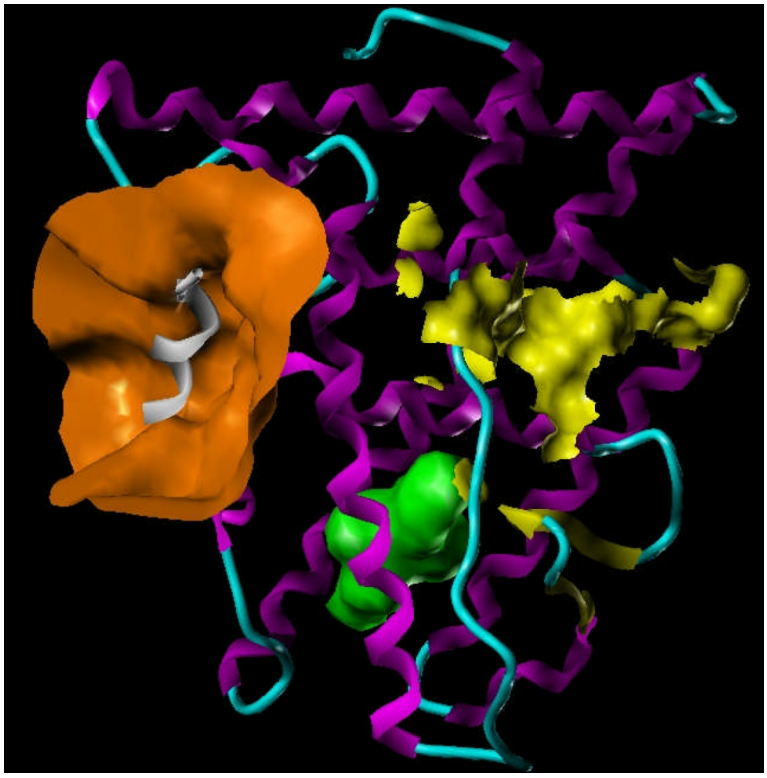

Figure 2.
Functional sites on the LBD of NRs. The ER is used as an example. The ligand binding pocket is shown as a green surface. The cofactor protein binding site is colored in orange. The computationally identified possible steroid receptor-specific functional site is shown with a yellow surface. The helices of ER are rendered by a ribbon representation and colored in magenta, while the coactivator motif is in white.

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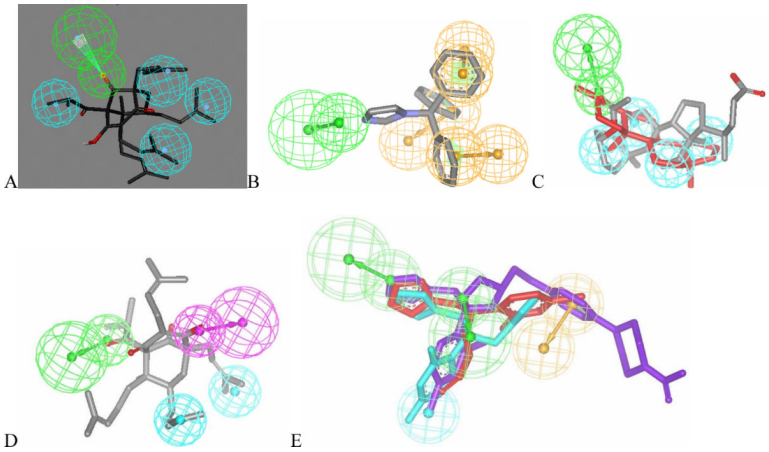

Figure 3.

A–D). 3-D agonist pharmacophores derived from different training sets [51,70] E). Antagonist pharmacophore [70,86]. All pharmacophores were generated with Catalyst (Accelrys, San Diego). Pharmacophore features represent: green = hydrogen bond acceptor; cyan = hydrophobic, Orange = ring hydrophobic and Purple = hydrogen bond donor.

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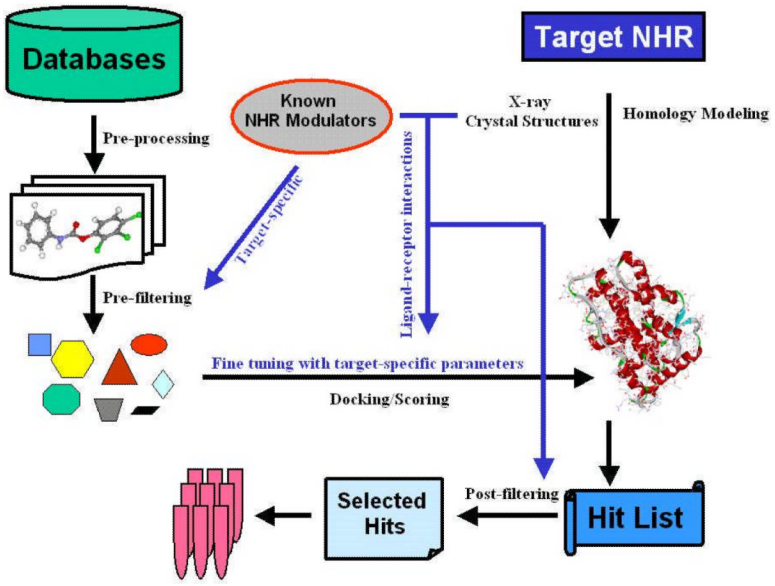

Figure 4.

A Virtual Screening scheme for NRs. First, the corporate or commercial databases need to undergo prescreening processing that incorporates input file formatting, pre-filtering for the drug-like compounds, exploring the conformational space of compounds, as well as their protonation, tautomeric, and stereochemical stages. Studies have identified the “hidden” impact of database preprocessing on VS results [91]. It is common that the number of screening compounds in the database can reach thousands to millions. Since docking/scoring is the most CPU-intensive and rate-limiting step, it is useful to apply certain prescreening filters to speed up the process. Generally these filters can be derived from structural and activities information from known modulators based on pharmacophore modeling and structural similarity. Structural information about the target receptor can be extracted from resolved X-ray crystallography structures or homology modeling. Abundant knowledge about ligand-protein interactions can be utilized to customize or fine tune important parameters for the docking/scoring step of VS. The list of hits will be then post-processed by target-specific filters. This step tries to increase the retrieval rate of the active compounds from the whole database and minimize the occurrence of false positives. Finally selected hits that pass through all requirements are suggested to be evaluated biologically.

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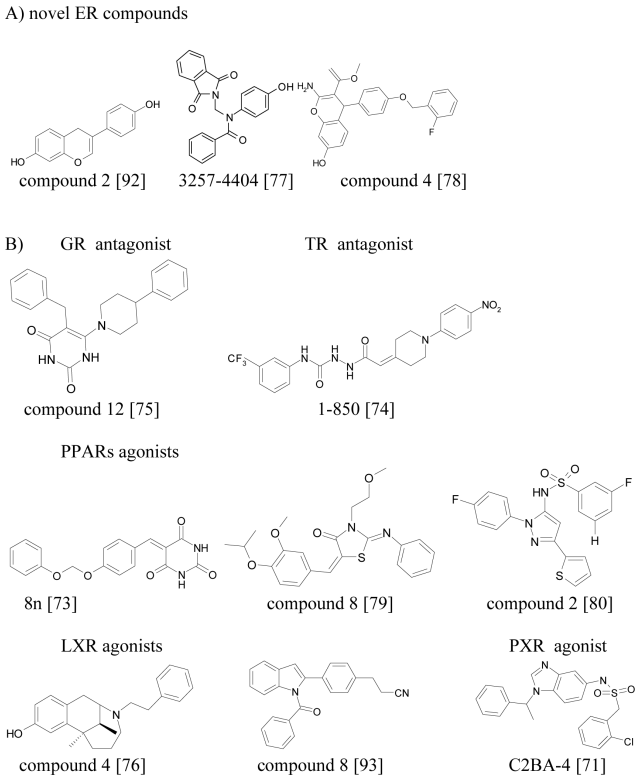

Figure 5.

Structural drawings of some computationally discovered NR modulators using identifiers in the original references. These compounds bind to the LBP of the corresponding NRs.

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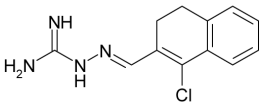
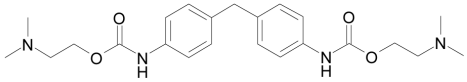
A) Novel SRC-3 inhibitors for ER [83]

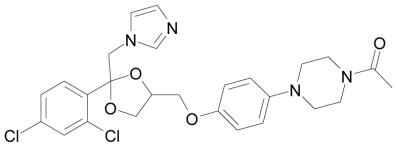


ERI-5

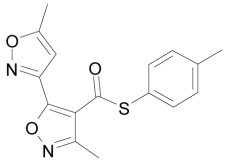


ERI-7

B) Novel PXR antagonists [70,86]



Ketoconazole



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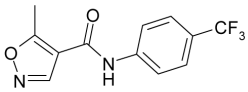

leflunomide

Figure 6.

Structural drawings of some computationally discovered NR antagonists using identifiers in the original references. These compounds bind to the alternative site on the surface of NRs.

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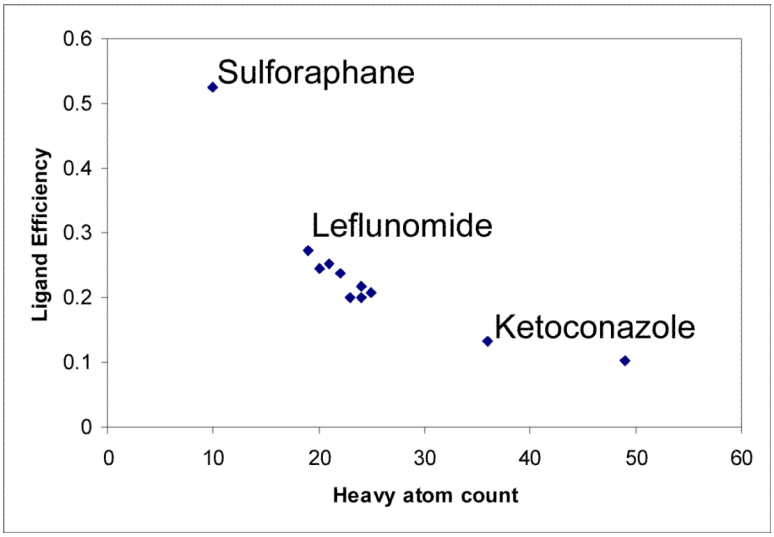

Figure 7.

Ligand efficiency versus heavy atom count for PXR antagonists which highlights the relative positions of the three compounds of interest. When we consider the ligand efficiency ($\log K_i$/heavy atom count (no hydrogens) versus heavy atom count, there is an exponential decrease in efficiency between 10–20 heavy atoms, which is comparable with observations for much larger datasets across different targets [87].