

*Minireview*

# **Small Molecule Modulation of Nuclear Receptor Conformational Dynamics: Implications for Function and Drug Discovery**

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Abbreviations: NR, nuclear receptor; ER, estrogen receptor; GR, glucocorticoid receptor; PPAR, peroxisome proliferator-activated receptor; AF-1, activation function-1; AF-2, activation function-2; DBD, DNA-binding domain; LBD, ligand-binding domain; VDR, vitamin D receptor; RXR, retinoic acid X receptor; HDX, hydrogen/deuterium exchange; SERM, selective estrogen receptor modulator; SAR, structure-activity relationship; rosiglitazone, 2,4-THIAZOLIDINEDIONE, 5-[[4-[2-(METHYL-2-PYRIDINYLAMINO)ETHOXY]PHENYL]METHYL]-(9CL); MRL20, (2S)-2-(2-[[1-(4-METHOXYBENZOYL)-2-METHYL-5-(TRIFLUOROMETHOXY)-1H-INDOL-3-YL]METHYL}PHENOXY)PROPANOIC ACID; MRL24, (2S)-2-(3-[[1-(4-METHOXYBENZOYL)-2-METHYL-5-(TRIFLUOROMETHOXY)-1H-INDOL-3-YL]METHYL}PHENOXY)PROPANOIC ACID; nTZDpa, 5-CHLORO-1-(4-CHLOROBENZYL)-3-(PHENYLTHIO)-1H-INDOLE-2-CARBOXYLIC ACID; GW1929, N-(2-BENZOYLPHENYL)-O-[2-(METHYL-2-PYRIDINYLAMINO)ETHYL]-L-TYROSINE HYDROCHLORIDE.

## Abstract

Nuclear receptors are targets for a wide range of ligands, both natural and synthetic, which regulate their activity and provide a means to pharmacologically modulate the receptor. Recent emphasis in the nuclear receptor field has focused on selective nuclear receptor modulators, which can display graded transcriptional responses and tissue selective pharmacological responses that deviate from the prototypical agonist or antagonist. Understanding the molecular mechanism of action of these selective modulators will provide significant insight towards the development of the next generation of modulators. Although most nuclear receptor structural studies have primarily focused on obtaining ligand-receptor co-crystal structures, recent studies implicate an important role for protein dynamics in the mechanism of action of nuclear receptor ligands. Here we review nuclear receptor studies reporting how ligands modulate the conformational dynamics of the nuclear receptor ligand-binding domain (LBD). A particular emphasis is placed on protein NMR and hydrogen/deuterium exchange (HDX) techniques, and how they provide complementary information that, when combined with crystallography, provides detailed insight into the function of nuclear receptors.

## Introduction

Nuclear receptors are modular domain transcription factors that regulate the expression of genes controlling a wide range of physiological processes. Nuclear receptors are generally considered ligand-regulated transcription factors, although only about half of the 48 members in the human nuclear receptor superfamily have identified physiological ligands. These ligand-regulated receptors have been successful targets for drugs treating a variety of human diseases. Primary examples include estrogen receptor (ER), the target for tamoxifen in breast cancer therapy; glucocorticoid receptor (GR), the target for dexamethasone and prednisolone as anti-inflammatory therapies; and peroxisome proliferator-activated receptors (PPARs) such as PPAR $\gamma$ , which is the target for rosiglitazone in type 2 diabetes therapy. Nuclear receptors share a conserved modular domain structure (Figure 1A), including a N-terminal activation function-1 (AF-1) region and central DNA-binding domain (DBD). However, the primary target for drug discovery is the C-terminal ligand-binding domain (LBD), which contains the activation function-2 (AF-2) surface that serves as a binding site for coregulator proteins. The nuclear receptor LBD is the physiological binding site for natural ligands such as 17 $\beta$ -estradiol (ER), cortisol (GR) and 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> (vitamin D receptor; VDR). In the absence of ligand, parts of the receptor LBD are conformationally mobile, or dynamic. Ligand binding stabilizes the receptor LBD conformation, which facilitates interactions with coregulator proteins that remodel chromatin, which controls polymerase binding and the expression of target genes (Figure 1B).

Nuclear receptors can be generally divided into two classes, transcriptional activators and repressors. The accepted mechanism of action for nuclear receptor transcriptional activators (Figure 1C) dictates that an agonist ligand binds to the LBD and increases the recruitment of coactivator proteins, which in turn increases the transcription of target genes. In the classical sense, an antagonist would block the binding of the agonist to the LBD and prevent the agonist from inducing a conformational change in the receptor. However, many antagonists described for nuclear receptors display inverse agonist activity for receptors with significant basal or constitutive transcriptional activity, where binding of the ligand increases recruitment of corepressor proteins and actively represses transcription. The mechanism of action of nuclear receptor ligands is complex, as the same ligand can have different tissue-, cell- and promoter-specific action, often depending on the expression levels of coregulator proteins, and also display graded receptor activity (Kojetin et al., 2008; Shang and Brown, 2002; Shang et al., 2000)—also referred to as selective nuclear receptor modulation. Agonists can also induce corepressor recruitment to nuclear receptor transcriptional activators (Fernandes and White, 2003), whereas some ligands act as agonists in certain tissues and antagonists in others in part depending on the level of coregulator expression in the tissues (Shang and Brown, 2002). Other ligands can modulate posttranslational modification of the receptor impacting function independent of transcriptional agonism (Choi et al., 2010). Transcriptional repressors, such as the Rev-erbs, are oppositely regulated, whereby agonist binding—in this case, the natural porphyrin heme or other

synthetic Rev-erb agonists—induces corepressor recruitment and repression (Raghuram et al., 2007; Solt et al., 2012; Yin et al., 2007).

### **Ligand-receptor crystal structures and the helix 12 structure-function model**

Many advances in our understanding of nuclear receptor function have come from structural biology efforts focused on the receptor LBD. The most common method of choice for these endeavors has been X-ray crystallography. Crystal structures of ligand-receptor complexes provide an atomic “snapshot” into the molecular mechanism of action of the receptor. Hundreds of crystal structures of nuclear receptor LBDs have been reported culminating in a helix 12 structure-function model (Figure 2) describing the molecular basis of ligand-modulated agonism (the “on” or transcriptionally active conformation) and antagonism (the “off” or transcriptionally repressed conformation). The LBD adopts a three-layered  $\alpha$ -helical sandwich fold, consisting of twelve  $\alpha$ -helices with a ligand-binding pocket (Pike, 2006). The hydrophobic AF-2 surface (helix 3/4/5/12 interface) provides a binding site for coactivator proteins with a LXXLL recognition motif (Savkur and Burris, 2004). In the apo or unliganded form, helix 12 is generally thought to be extended away from the LBD, as is the case observed in the apo RXR $\alpha$  LBD crystal structure (Gampe et al., 2000). However, in the case of apo PPAR $\gamma$  (as described below), helix 12 does not adopt a single conformation but rather adopts multiple conformations in solution (Hughes et al., 2012; Johnson et al., 2000). Furthermore, as described below for ERs, helix 12

appears to be stabilized to the same degree in apo or liganded forms (Dai et al., 2009; Dai et al., 2008). It has been generally observed that agonist ligands position helix 12 to cap the ligand-binding site, leaving the AF-2 surface exposed for coregulator binding (Brzozowski et al., 1997). Antagonist ligands induce an unfavorable conformation for coregulator binding, some with bulky portions that perturb the AF-2 surface via directly contact (Pike et al., 2001). Other antagonists function in a passive manner through a lack of appropriate contacts in the ligand-binding cavity, including perturbation of helix 11 (Shiau et al., 2002), which alters helix 12 positioning indirectly to occupy the AF-2 surface (Shiau et al., 1998). Partial agonists are thought to dynamically switch helix 12 between active and inactive structural conformations, perhaps through the ligand binding in different binding orientations (Bruning et al., 2010; Hughes et al., 2012), or through perturbation of helix 12 positioning (Pike et al., 2000a; Pike et al., 1999). These ligand-bound structural observations have been used as a primary to understand the mechanism of action of ligands and are generally used as a guide for nuclear receptor virtual ligand screening and structure-based drug design efforts.

### **Ligand stabilization of LBD conformational dynamics**

Although the helix 12 structure-function model (Figure 2) derived from nuclear receptor LBD crystal structures provides a convenient model to correlate ligand activity via structure-function relationships, this model has not satisfactorily explained the mechanism of action for some types of ligands. The switching

between “on” (agonist) and “off” (antagonist) conformations in crystal structures is generally referred to as structural plasticity of helix 12, though in some cases helix 12 “dynamics” is used to describe this model. Although crystal structures do not readily report on protein dynamics, there is a growing consensus that protein dynamics does indeed impact the function of nuclear receptors. A primary example of this has been illustrated with PPAR $\gamma$ . Unlike the structures of apo RXR $\alpha$  LBD, for example, which displays helix 12 in an extended conformation away from the LBD (Bourguet et al., 1995), crystal structures of the PPAR $\gamma$  LBD in the apo form show the “active” conformation (Figure 3A) (Nolte et al., 1998; Uppenberg et al., 1998). Namely, in the apo PPAR $\gamma$  LBD structures, helix 12 caps the ligand-binding pocket in the same manner that seen when PPAR $\gamma$  LBD was co-crystalized with the PPAR $\gamma$  full agonist rosiglitazone (Nolte et al., 1998). Although the full PPAR $\gamma$  agonist ligand rosiglitazone makes a number of contacts in the ligand-binding pocket, which were postulated to stabilize a conformation suitable to bind coactivator proteins, the apo vs. rosiglitazone-bound structures have not provided a conclusive mechanism for ligand-dependent PPAR $\gamma$  function. It is noteworthy to mention that most crystallization conditions are in fact non-physiological and often use of extreme measures to facilitate protein crystal formation, including extreme buffer pH, salt concentrations, and large amounts of dehydrating or precipitating agents. Thus the crystalized structural conformation could be expected to differ from a structure obtained under physiological or more native-like conditions. On the other hand, below we describe data from other structural techniques suitable to probe the structure and dynamics of the LBD in



solution, under what could be considered more native-like conditions, which have provided detailed insight on the ability of nuclear receptor ligands to stabilize the conformation of the receptor LBD and affect function.

Protein nuclear magnetic resonance (NMR) studies on the PPAR $\gamma$  LBD were among the first to provide insight into the dynamic mechanism of nuclear receptor activation by ligands. In protein NMR experiments, each nucleus (or atom) in the protein has a unique chemical environment that results in a unique NMR resonance with a distinct chemical shift. When collected for different samples or conditions, NMR experiments can detect structural differences between various molecular states (e.g. apo LBD vs. different ligand-bound receptor LBD). In addition, the appearance and shape of the NMR resonances are affected by the local conformational dynamics of the specific atom or nucleus. Protein NMR studies of the PPAR $\gamma$  LBD (Figure 3B) have demonstrated that in the absence of ligand, only about half of the expected NMR resonances are observed (Hughes et al., 2012; Johnson et al., 2000). The missing resonances localize to the ligand-binding pocket and activation function-2 (AF-2) coregulator interaction surface, which includes helix 12. Missing NMR resonances manifest due a motion that occurs on a specific time scale, on the order of microseconds-to-milliseconds ( $10^{-3}$ - $10^{-6}$ /sec), which is generally called “intermediate chemical exchange”. In contrast to apo LBD, the binding of a full or strong agonist, such as rosiglitazone, fully stabilizes the PPAR $\gamma$  LBD conformation allowing the observation of nearly all NMR resonances in the PPAR $\gamma$  LBD (Hughes et al., 2012; Johnson et al., 2000). This binding event

stabilizes the receptor NMR resonances observed to be in intermediate chemical exchange in the apo LBD. Before to these NMR experiments, the nuclear receptor field suggested that the LBD exists in discrete conformational states depending on the specific ligand bound to the receptor and the ligand-binding event shifts the conformation from one state to another. However, the NMR data support a different model, one by which the apo LBD samples an ensemble of multiple conformations, and ligand binding stabilizes a subset of these conformations (Johnson et al., 2000). Similar observations have been made for PPAR $\alpha$  (Cronet et al., 2001) RXR $\alpha$  (Lu et al., 2006).

Hydrogen/deuterium exchange (HDX) studies coupled to mass spectrometry have revealed a similar dynamic phenotype for the LBD upon binding ligands. The HDX experiment involves subjecting protein to D<sub>2</sub>O for different times allowing solvent accessible amide hydrogens to exchange for deuterium. When coupled to mass spectrometry, the protein sample is quenched at specific time points and digested, and the degree of HDX is quantitatively assayed in specific peptides. The number of amide deuteriums is a direct measure of solvent accessibility and conformational dynamics via changes in hydrogen bonding patterns (Hoofnagle et al., 2003) making this experiment sensitive to the conformation of the protein. This experiment is often performed as a differential analysis, where the exchange kinetics of the apo LBD is compared to the ligand-bound LBD. Apo PPAR $\gamma$  LBD exhibits considerable HDX in similar regions observed in intermediate conformational exchange by NMR, namely regions making up the ligand-binding pocket and the AF-2/helix 12 surface (Bruning et

al., 2007; Hamuro et al., 2006; Hughes et al., 2012). Full PPAR $\gamma$  agonists, such as rosiglitazone or GW1929, display robust protection from HDX in these regions suggesting these ligands change the conformation of the protein relative to apo LBD (Figure 3B). The protection from HDX observed in helix 12 in particular appears to correlate with the formation of hydrogen bond between the ligand and a residue in helix 12 (e.g. PPAR $\gamma$  Tyr473). HDX on other receptor LBDs have shown a similar stabilization upon ligand binding, though the specific regions can differ, including data on CAR/RXR $\alpha$  (Wright et al., 2011), ERs (Dai et al., 2009; Dai et al., 2008), GR (Frego and Davidson, 2006), PPAR $\gamma$  (Bruning et al., 2007; Choi et al., 2010; Choi et al., 2011b; Hamuro et al., 2006; Hughes et al., 2012; Malapaka et al., 2012), PPAR $\gamma$ /RXR $\alpha$  (Chalmers et al., 2006; Chandra et al., 2008), ROR (Kumar et al., 2012; Solt et al., 2011), RXR $\alpha$  (Yan et al., 2004; Yan et al., 2006; Yan et al., 2007) and VDR/RXR $\alpha$  (Chalmers et al., 2011; Zhang et al., 2010; Zhang et al., 2011).

Fluorescence anisotropy has also been used to study the effect of ligand binding on the dynamics of helix 12. This technique involves labeling the LBD with a fluorophore at site-specific locations, allowing for the measurement of the anisotropy of the fluorophore. The anisotropy signal is inversely proportional to mobility (dynamics). Thus, this technique is useful to determine how ligand binding affects the dynamics of different sites on the LBD. When a fluorophore was coupled to the C-terminus of helix 12 on the PPAR $\gamma$  LBD, the PPAR $\gamma$  full agonist rosiglitazone caused a dose-dependent increase in helix 12 anisotropy (Kallenberger et al., 2003). Due to the inverse relationship between anisotropy

and mobility, this revealed that rosiglitazone reduces the motion of helix 12 relative to apo LBD. Time-resolved fluorescence anisotropy decay measurements also revealed that rosiglitazone stabilizes helix 12 on a fast motion time scale, revealing that ligand binding resulted in reduced helix 12 mobility. In addition, a significant increase in intermediate motion was observed upon binding rosiglitazone, a motion that approximates the overall motion of the LBD. This suggests that in the apo LBD, helix 12 possesses independent motion compared to the core of the LBD. Rosiglitazone binding immobilizes helix 12, tethering helix 12 to the core of the LBD via the hydrogen bond between the ligand and Tyr473, and thus helix 12 tumbles with core LBD.

In summary, data from these studies are consistent with structural interpretations suggesting that the apo nuclear receptor LBD is a dynamic, molten globule-like domain (Nagy and Schwabe, 2004). In the absence of ligand, there is native-like helical secondary structure but the LBD possesses a dynamic ligand-binding pocket, which perturbs a conformational disorder to nearby functional surfaces, such as the PPAR $\gamma$  AF-2, and negatively affects coactivator protein interaction. In the case of PPAR $\gamma$ , binding of an agonist ligand stabilizes the molten globule-like nature of the ligand-binding pocket and also stabilizes the AF-2 surface to adopt a conformation favoring coactivator protein interaction. To some degree, the dynamic molten globule-like nature of apo LBD is a characteristic of an intrinsically disordered protein domain, however in this case it is one that possess the ability to be fully stabilized by ligands—or, as described below, partially stabilized resulting in graded receptor activity.

## Dynamic features of graded receptor agonism

Although the helix 12 structure-function model derived from nuclear receptor LBD crystal structures details the relationship between the two major and opposing functional states, where the active state is associated with transcriptional agonism and inactive state associated with antagonism, it does not explain very well several features of nuclear receptor function. This includes graded transactivation (also called intermediate or partial agonism) as well as non-classical helix 12-independent activation; selective nuclear receptor modulators fall within this class of ligands. Based on ligand-receptor co-crystal structures, several mechanisms have been suggested for graded/partial agonists.

This includes an intermediate/quasi-antagonist conformation where coregulator interaction can switch helix 12 between different helix 12 positions, either by inducing a conformation that is like the agonist conformation but different enough that it does not induce a robust coregulator interaction therefore providing a reduced transactivation response (Pike et al., 1999; Pike et al., 2000b). Or by the ligand binding in different binding modes, perhaps allowing the receptor to sample active and inactive conformations (Bruning et al., 2010; Hughes et al., 2012). However, partial agonist bound nuclear receptor LBDs have in general been difficult to crystalize (Bruning et al., 2010), and therefore the low sampling of partial agonist bound LBD crystal structures has limited these interpretations. Furthermore, these structural models describing possible mechanisms for

providing a partial agonist response were primarily derived from ER crystallography studies and may not universally apply to other nuclear receptors. As discussed above for PPAR $\gamma$ , nearly all crystal structures of its LBD—whether in the apo form or bound to any ligand of graded agonist activity—shows the same three-dimensional fold with helix 12 in the “active” conformation (Figure 3A) (Bruning et al., 2007; Nolte et al., 1998; Uppenberg et al., 1998). This includes a study on an intact nuclear receptor complex, where full-length PPAR $\gamma$ /RXR $\alpha$  co-crystalized with a PPAR $\gamma$  full agonist, partial agonist or antagonist all show helix 12 in the active conformation (Chandra et al., 2008).

Protein NMR studies were among the first to provide a glimpse into the mechanism of partial agonism. The PPAR $\gamma$  partial agonist nTZDpa, a potent ( $EC_{50}$  = 57 nM) yet weak partial agonist (25% efficacy vs. rosiglitazone), only partially stabilized the PPAR $\gamma$  LBD affording a partial increase in NMR resonances when compared to NMR data for apo PPAR $\gamma$  LBD (Berger et al., 2003). Subsequent HDX mass spectrometry studies revealed additional insight for a variety of PPAR $\gamma$  ligands with graded response profiles (Bruning et al., 2007; Hamuro et al., 2006). Unlike full agonists, PPAR $\gamma$  partial agonists do not stabilize helix 12 and differentially stabilize other portions of receptor ligand-binding pocket, including the  $\beta$ -sheet surface and helix 3 (Figure 3B). Protein NMR studies have also revealed that stabilization of the receptor from intermediate conformational exchange is correlated with the graded response of ligand (Figure 3B) (Hughes et al., 2012). Full PPAR $\gamma$  agonists provide robust

stabilization compared to apo LBD, and weak partial PPAR $\gamma$  agonists provide less stabilization. Interestingly, the PPAR $\gamma$  NMR studies also revealed that PPAR $\gamma$  ligands can bind to the LBD in more than one conformation, suggesting added complexity to the structure-function relationship of PPAR $\gamma$  ligands.

When the PPAR $\gamma$  NMR and HDX studies are considered with co-crystal structures of the PPAR $\gamma$  LBD complexed to these ligands, a fuller picture of the mechanism of action is realized for ligands with graded response profiles. Although the crystal structures for the PPAR $\gamma$  LBD complexed to MRL20 and MRL24 show helix 12 in “active” conformation, only MRL20 is observed to make a hydrogen bond with Tyr473. This explains the protection from HDX on helix 12 observed for MRL20, as well as the slight increase in stabilization resulting in the appearance of data in the protein NMR experiments for residues in helix 12. On the other hand, MRL24 does hydrogen bond to Tyr473, does not afford much protection from HDX in helix 12, and does not stabilize the receptor as much as MRL20 resulting in no assigned NMR resonances for residues in helix 12. When discussing the use of HDX to study ligands with graded response profiles, it is noteworthy to mention that the gradations in HDX observed for these compounds are not a reflection of ligand affinity. This is most apparent when comparing rosiglitazone, a PPAR $\gamma$  full agonist, which has a weaker binding affinity, on the order of 1-2 orders of magnitude lower, than that of the partial agonist MRL24 or the near full agonist MRL20. HDX has also been used to profile VDR full and partial agonists, which also revealed that VDR partial agonists differentially stabilize the AF-2/helix 12 region (Chalmers et al., 2011; Zhang et al., 2010).

Fluorescence anisotropy studies on ER $\alpha$  have also suggested that selective estrogen receptor modulators (SERMs), which display weaker transactivation profiles compared to full ER agonists, increase the local dynamics at the end of helix 11, which precedes the loop going into helix 12 (Tamrazi et al., 2003).

These techniques that report on the conformational dynamics of nuclear receptor LBDs have provided unique insight into the mechanism of action of nuclear receptor ligands, in particular those with graded function. The protein disorder imparted, or rather not stabilized, by graded or partial agonists may allow the receptor to search for different binding partners. This is supported by the studies on PPAR $\gamma$  showing that, unlike full agonists which facilitate binding of coactivators and disfavor binding of corepressors, partial agonists allow coactivator binding while retaining the ability to interact with corepressors (Lee et al., 2002; Leesnitzer et al., 2002; Motani et al., 2009).

### **Conformational dynamics as a guide for nuclear receptor drug discovery**

Several recent studies support the incorporation of dynamic information to help explain the mechanism of action of subclasses of ligands, including the use of dynamic information during structure-activity relationship (SAR) analysis in the drug discovery pipeline. HDX analyses for a variety of SERMs revealed dynamic profiles that correlated with the pharmacological profiles of the ligands with respect to ER $\alpha$  activity (Dai et al., 2008). Similar to the analysis of graded PPAR $\gamma$  agonists (Bruning et al., 2007), SERMs displaying various graded response



profiles also display gradations in the degree of protection from HDX in several structural regions. Interestingly, clustering analysis of the ligand-induced HDX profiles allowed for the prediction of the tissue specificity of the ER compounds, suggesting this relatively high-throughput HDX structural assay, compared to a low-throughput method such as crystallography, may be useful to generate new SERMs with specific tissue specificity and thus reduce side effects. This study was extended to compare HDX profiles between the two ER subtypes, ER $\alpha$  and ER $\beta$  (Dai et al., 2009). These receptors bind many of the same ligands, but have different tissue expression profiles, different affinities for the same ligand, and they are thought to oppositely regulate one another (i.e. ER $\alpha$  is thought to oppose the functions of ER $\beta$ , and vice versa) (Matthews and Gustafsson, 2003; Zhao et al., 2008). Interestingly, the HDX studies revealed that ER $\beta$  undergoes different structural changes when compared to ER $\alpha$  for the same ligand, giving support to the notion that the same ligand can affect the function of ER $\alpha$  and ER $\beta$  differently. What was further interesting in both of these studies is that the dynamics of helix 12 in the ER LBD is not altered, when compared to apo receptor, upon binding agonist or antagonist ligands. Crystal structures of ER LBDs show a clear repositioning of helix 12 when bound to agonists or antagonists. However, unlike PPAR $\gamma$  agonists such as rosiglitazone and MRL20, ER ligands do not stabilize helix 12 through hydrogen bond formation with a residue on helix 12 but rather through interactions with other residues lining the ligand-binding pocket, including His524 on helix 11. Furthermore, apo ER LBD has been difficult to crystalize and was only possibly by making mutations that

introduce a hydrogen bond observed in the agonist- and antagonist-bound helix 12 conformations to stabilize the receptor conformation (Nettles et al., 2008). This is in contrast to studies on PPAR $\gamma$ . Crystal structures of the PPAR $\gamma$  LBD in the apo form or when bound to agonist all show helix 12 in the active conformation with helix 12. In addition, unlike ER, HDX and NMR studies show a clear stabilization of helix 12 upon binding PPAR $\gamma$  agonist ligands (Bruning et al., 2007; Hughes et al., 2012). These studies suggests that although ER ligands may not directly stabilize helix 12 through hydrogen bond formation with a residue on helix 12, they may stabilize helix 12 through other interactions within the ligand-binding pocket and in concert with coregulator proteins that bind to the AF-2 surface, which includes helix 12. Related to ER, a similar and larger profiling study on 87 VDR modulators have revealed statistically significant trends in HDX profiles that correlate with the selective activation properties of the ligands (Chalmers et al., 2011) (P. Griffin, personal communication).

HDX studies have also been used as a guide for SAR analysis in PPAR $\gamma$  drug discovery. It was recently revealed that the anti-diabetic effect of synthetic PPAR $\gamma$  ligands involves a novel mechanism—stabilization of dynamics around LBD residue Ser273, which inhibits Ser273 phosphorylation by the protein kinase Cdk5 (Choi et al., 2010). Notably, blocking of Ser273 phosphorylation by PPAR $\gamma$  ligands was found to be independent of the transcriptional activation properties of the ligand afforded by helix 12 stabilization. In this study, two synthetic PPAR $\gamma$  compounds were studied: the full agonist, rosiglitazone, and the partial agonist, MRL24. While both ligands displayed a similar level in their ability to block

Ser273 phosphorylation, the lower transcriptional activity profile of MRL24 correlated with reduced helix 12 stabilization as well as a better gene expression profile when compared to rosiglitazone. Microarray analysis revealed an overlapping set of genes affected by rosiglitazone and MRL24, which included genes dysregulated in type 2 diabetes and obesity, including adiponectin and adipisin. However, the expression of a large set of genes corresponding to the classic adipogenic genes was increased by rosiglitazone but not by MRL24. These principles provided a pathway whereby classical transcriptional activation could be dissociated from anti-diabetic efficacy on the molecular level via HDX profiles. In a subsequent study, HDX was among several SAR assays used in the design of a proof-of-concept PPAR $\gamma$  non-agonist ligand, SR1664 (Choi et al., 2011a). SR1664 binds directly to the PPAR $\gamma$  LBD and, like MRL24 but unlike rosiglitazone, SR1664 does not stabilize helix 12. Although SR1664 is devoid of transcriptional activation, it displays potent anti-diabetic activity with a reduced side effect profile compared to rosiglitazone in terms of reduced fluid retention, weight gain and interference with bone formation.

## Summary

The studies discussed above reveal the powerful synergy in combining different approaches focused on structure and conformational dynamics to provide a fuller understanding of the mechanism of action of nuclear receptor ligands. Although x-ray crystallography studies have played an important role in understanding

nuclear receptor structure-function relationships, and will continue to do so in the future, they do not readily report on solution-state conformational dynamics. This is quite important, as most biomacromolecules are dynamic, and the inherent dynamics of the protein plays an important role in function. As can be appreciated from the studies reviewed above, modulation of conformational dynamics represents a potentially exploitable feature for drug discovery. Thus, techniques to study biomacromolecular structure and dynamics are critical in understanding the molecular basis of biological function and mechanism of action of ligands.

### **Authorship Contributions**

Participated in research design: n/a

Conducted experiments: n/a

Contributed new reagents or analytic tools: n/a

Performed data analysis: n/a

Wrote or contributed to the writing of the manuscript: Kojetin and Burris

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## Footnotes

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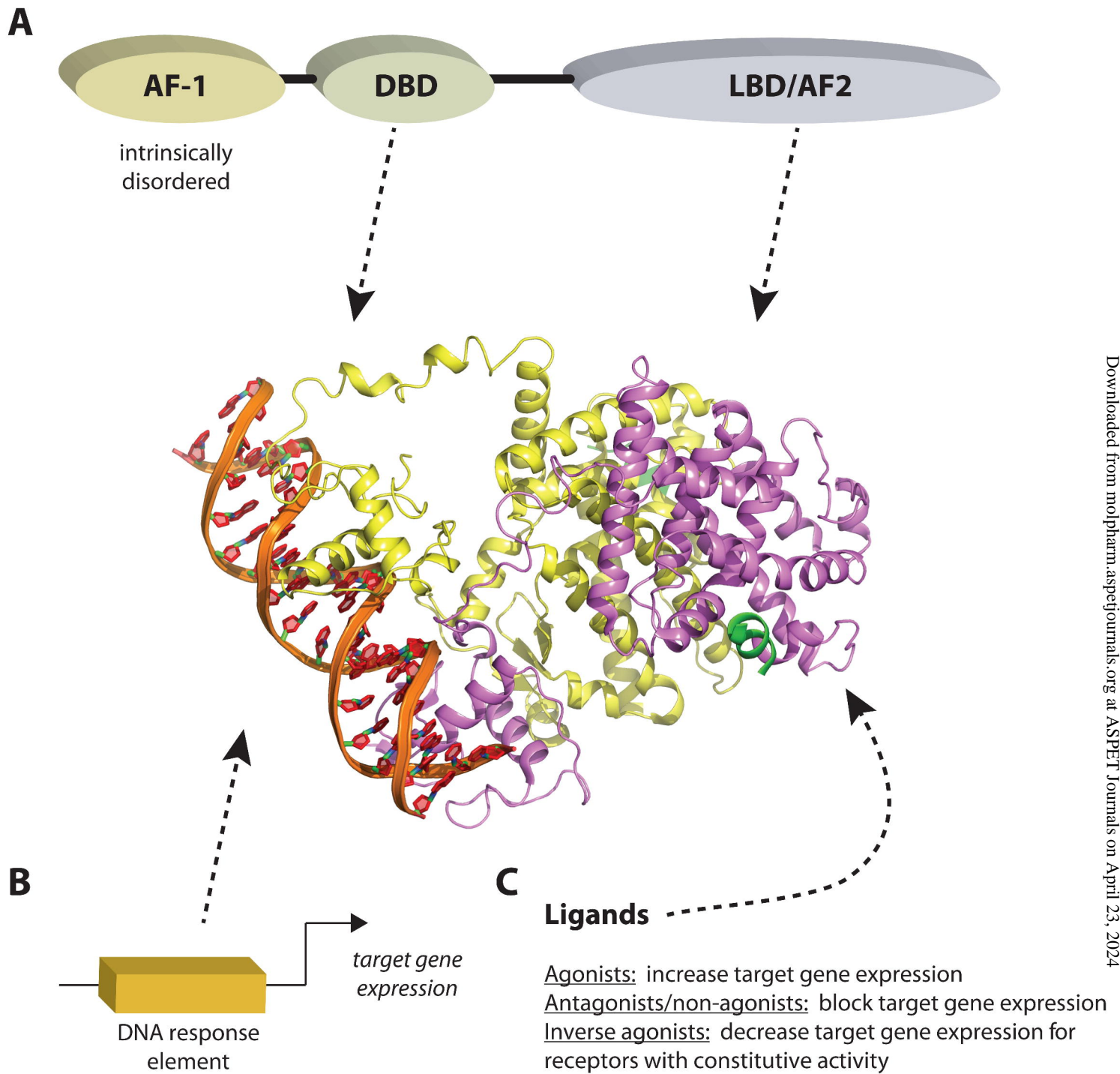
**Figure 1.** Overall structure of nuclear receptors and mechanism of action. A, the conserved domain architecture of nuclear receptors consists of a N-terminal intrinsically disordered region called the activation function-1 (AF-1) domain, followed by a central DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) that contains the activation function-2 (AF-2) region. To illustrate the quaternary structure, the intact PPAR $\gamma$ /RXR $\alpha$  (yellow and pink, respectively) complex is shown bound to DNA, ligands and coregulator peptides (green); PDB: 3DZY. B, nuclear receptors bind to specific DNA response elements, recruit coregulator proteins, which remodels chromatin and controls polymerase binding, all of which controls the expression of specific target genes. C, ligands that bind to the nuclear receptor LBDs elicit a variety of pharmacological responses, including activation (agonists), inactivation (antagonists or non-agonists) and, for receptors that are constitutively active, ligands can down-regulate the constitutive response (inverse agonists).

**Figure 2.** The helix 12 structure-function model is a popular structure-function model in the nuclear receptor field. This model has been derived from ligand-receptor LBD co-crystal structures and correlates the position of helix 12 in the LBD to the functional response of the bound ligand. In the unliganded form, helix 12 is dissociated from the core of the LBD. For some proteins, such as the RXRs, this outward-facing position facilitates tetramer formation. For others, such as PPAR $\gamma$ , helix 12 is conformationally mobile and likely adopts multiple

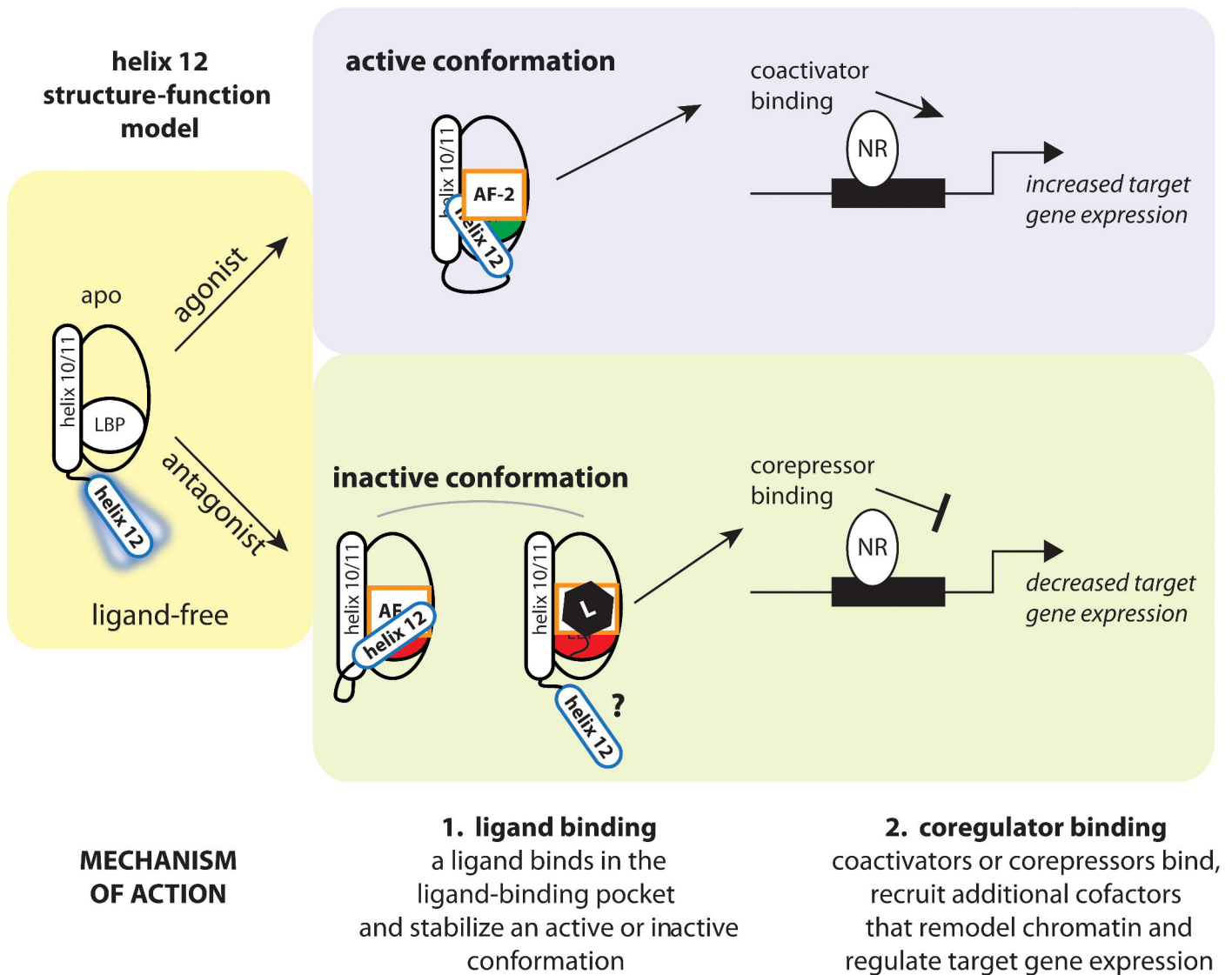
conformations. Upon binding an agonist ligand, helix 12 docks to the core of the LBD and covers the bound agonists in the ligand-binding pocket. This conformation forms the AF-2 coregulator surface that allows for binding of coactivator proteins containing a LXXLL motif—an “active conformation”. However, upon binding an antagonist ligand, the AF-2 surface is blocked, which inhibits coactivator binding and facilitates corepressor binding—an “inactive conformation”. There are two general classes of antagonists: passive and active. Passive antagonists induce a helix 12 conformation that blocks the AF-2 surface. Active antagonists contain structural moieties that physically blocks the AF-2 surface, and for these ligands helix 12 can be found in a variety of positions or is not observed in the crystal structure because of structural disorder. Although this model generally explains the activity of agonist and antagonist ligands, it has not provided detailed insight into the mechanism of action of other classes of ligands, including graded/partial agonists, or the observation that the same ligand can have agonist activity in one tissue or cell type and antagonist activity in another.

**Figure 3.** Ligand-induced differences in conformational dynamics provide a better model for understanding the mechanism of action of nuclear receptor ligands. The helix 12 structure-function model has not adequately explained the mechanism of action of PPAR $\gamma$  ligands, in particular those displaying graded activity. A, crystal structures of the PPAR $\gamma$  LBD all show the same conformation with helix 12 capping the ligand-binding pocket, whether crystallized in the apo form (yellow) or bound to ligand—such as the a full agonist (rosiglitazone; green),

near full agonist (MRL20; pink) or weak partial agonist (MRL24; blue). B, techniques useful in the study of protein conformational dynamics provides mechanistic insight into the activity of PPAR $\gamma$  with graded activity. In the apo form, the PPAR $\gamma$  LBD is conformationally dynamic. In this state, backbone NMR resonances are not observed for residues comprising the ligand-binding pocket, helix 11 and the AF-2 surface, which contains helix 12. Furthermore, these regions show dramatic and rapid exchange in HDX experiments. Full agonists, such as rosiglitazone, provide robust stabilization of the LBD in both NMR (more assigned NMR resonances) and HDX data (robust protection from HDX). Agonists with graded activity, such as MRL20 and MRL24, display gradations in their ability to stabilize the LBD, in particular on helix 3, 11 and 12—all in a manner that correlates with the graded response of the ligand. Namely, stronger agonists afford more stabilization compared to weaker agonists.



**Figure 1**



**Figure 2**

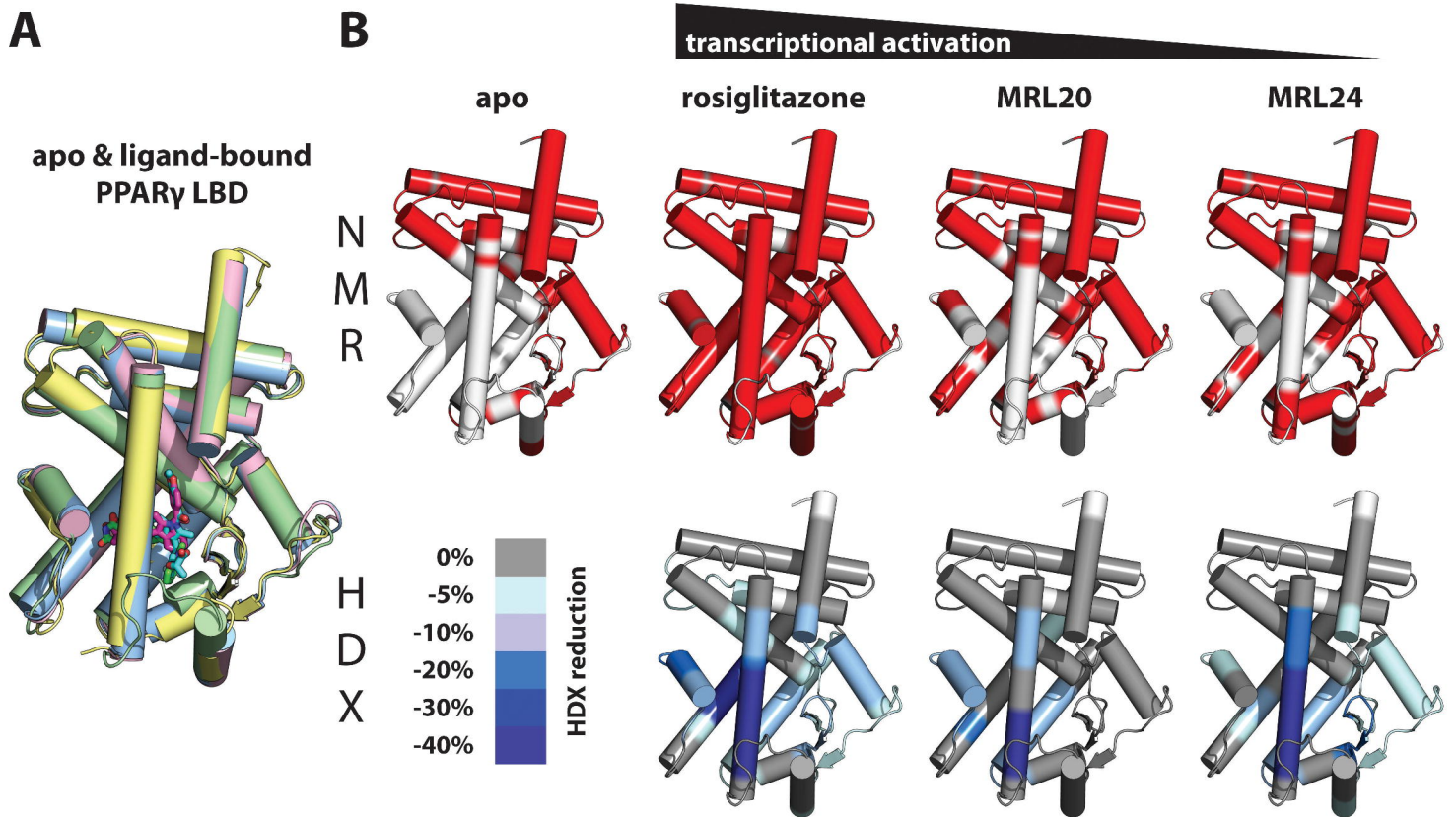


Figure 3