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Allosteric small molecule modulators of nuclear receptors

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ABSTRACT
Nuclear Receptors (NRs) are multi-domain proteins, whose natural regulation occurs via ligands for a classical, orthosteric, binding pocket and via intra- and inter-domain allosteric mechanisms. Allosteric modulation of NRs via synthetic small molecules has recently emerged as an interesting entry to address the need for small molecules targeting NRs in pathology, via novel modes of action and with beneficial profiles. In this review the general concept of allosteric modulation in drug discovery is first discussed, serving as a background and inspiration for NRs. Subsequently, the review focuses on examples of small molecules that allosterically modulate NRs, with a strong focus on structural information and the ligand binding domain. Recently discovered nanomolar potent allosteric site NR modulators are catapulting allosteric targeting of NRs to the center of attention. The obtained insights serve as a basis for recommendations for the next steps to take in allosteric small molecular targeting of NRs.

1. Introduction

1.1. Structural organization of nuclear receptors

The nuclear receptor (NR) superfamily consists of 48 members that are structurally related, but have functions in all kinds of physiological processes and, in connection with that, diseases. Their key role in these processes makes them a very attractive drug target. NRs have a conserved domain organization starting at the N-terminus with the highly variable N-terminal domain (NTD). For most NRs this domain contains a ligand-independent activation function (AF1). The NTD is intrinsically disordered (ID), but the interaction with binding partners can induce folding which can enhance transcriptional activity (Kumar and Litwack, 2009; Helsen and Claessens, 2014). The NTD is followed by the DNA binding domain (DBD) which uses its two zinc fingers to recognize specific hormone response elements (HREs). The affinity for specific HREs is dependent on the NR subtype and NR homo- or heterodimerization, or in some cases monomeric binding to extended HREs (Helsen et al., 2012a). The DBD is connected to the ligand binding domain (LBD) via a hinge region that typically contains a nuclear localization signal and undergoes posttranslational modifications (PTMs) (Haelens et al., 2007). The LBD is a highly conserved domain typically consisting of 12 helices which are organized in an antiparallel, globular arrangement (Bourguet et al., 1995). The classical “mouse-trap” model states that when an agonistic ligand binds in the ligand binding pocket (LBP), helix 12 (H12) covers the pocket and creates a surface suitable for coactivator binding (Renaud et al., 1995). Nowadays, this model is under debate and an alternative “dynamic stabilization” model is evoked (Pissios et al., 2000). This concept states that the LBD in its apo form is in a partially molten state and can adopt a wide range of conformations. Agonist binding stabilizes the fold of H12 and the surface elements required for coactivator binding in the active conformation (Rastinejad et al., 2013). Inverse agonists, on the other hand, can increase corepressor recruitment which represses transcription in constitutively active NRs (Kojetin and Burris, 2013). Antagonists do not cause cofactor recruitment, but do prevent agonist binding and therefore passively repress transcription (Kojetin and Burris, 2013). C-terminal to the LBD, certain NRs have an additional F-domain that has a variable length and has functions ranging from interacting with other proteins to stabilizing the ligand bound conformations of the LBD (Patel and Skafar, 2015).

1.2. Inter-domain allosteric regulation of nuclear receptor activity

Up until a few years ago, NR structural information was only available for certain separate domains. This did give a lot of information on ligand- and DNA binding as well as on dimerization of separate LBDs or DBDs, but left a demand for information on the implications of
inter-domain communication (Rastinejad et al., 2013). Since 2008, multi-domain structures obtained via different scattering techniques started appearing (Chandra et al., 2008; Orlov et al., 2012; Rochel et al., 2011). These structures provided more insight into the interplay between the different NR domains. In particular, it became clear that the different domains within a NR act and communicate via modulatory, allosteric mechanisms. Allosterity, in general, is the process where binding of an interaction partner, e.g. ligand or protein, at one site of a protein results in a functional change at another, topographically distinct, site (Motlagh et al., 2014; Nussinov & Tsai, 2013). This means that there is communication over a distance between the binding site, ‘input’, and the site of the biological response, ‘output’, via a conformational change in the protein structure (Changeux and Christopoulos, 2016). Different endogenic types of allosteric regulatory mechanisms have been described for NRs as conceptually summarized in Fig. 1.

First of all, and most obvious, the majority of NR ligands bind to the LBP, which causes a conformational change in the LBD and as a result regulates cofactor recruitment elsewhere on the LBD, outside of the LBP (Fig. 1A). (Hilser and Thompson, 2011) As an illustration, Folkertsma et al. showed that by clustering correlations between the ligand binding mode and peptide recruitment can be delineated (Folkertsma et al., 2007). This can be helpful to identify compounds which cause the anticipated cofactor recruitment (Folkertsma et al., 2007). Besides this prime NR allosteric mechanism, other and frequently more subtle allosteric mechanisms can be found in endogenous NR regulation.

The interplay of the specific HRE with the cofactor binding site of a NR is an allosteric mechanism via inter-domain communication (Fig. 1B). This interplay has been studied, amongst others, in the estrogen receptor (ER). Using a phage ELISA assay, cofactor recruitment in response to various estrogen response elements (EREs) was tested by Hall et al. (2002). They showed that the structure of the coactivator pocket is influenced by the type of ERE, pointing to the ERE as a regulator of biological activity (Hall et al., 2002). A similar example was found for the glucocorticoid receptor (GR). The GR binding sequence (GBS) affinity and transcriptional activity could not be directly correlated and it was found that the only structural difference in the DBD upon binding of different GBSs occurs in the “lever arm” loop that connects the DNA recognition helix and the dimerization domain (Meijising et al., 2009). Helsen et al. proposed that these subtle differences are propagated through the full-length receptor (Helsen et al., 2012b). Molecular dynamics (MD) simulations of the GR DBD in its DNA bound and free state by Frank et al. gave an explanation of the allosteric behavior since conformational sampling of the lever arm is observed to be reduced by DNA binding and dimerization (Frank et al., 2018). The small number of distinct conformations the DNA-bound DBD can adopt, might provide recruitment of different co-regulators (Frank et al., 2018).

Several additional studies have been performed to obtain a better understanding of the allosteric communication between DBD and cofactor binding site, e.g. for the thyroid receptor (TR) (Putcha and Fernandez, 2009), VDR-RXR heterodimer (Zhang et al., 2011) and PPARγ-RXRα complex (de Vera et al., 2017). In these studies, isothermal titration calorimetry (ITC) and hydrogen/deuterium exchange (HDX) techniques were used, showing an enhancement of cofactor recruitment upon binding of the receptor complex to its HRE.

The LBD and DBD can also allosterically influence each-others ligand affinity, as exemplified by Helsen et al. (2012b) (Fig. 1C). By overlaying the androgen receptor (AR) LBD and DBD crystal structures with the full-length heterodimeric PPARγ-RXRα crystal structure, residues in the LBD-DBD interaction surface were identified (Helsen et al., 2012b; Chandra et al., 2008). Residues from this surface that are known to correlate with pathologies were mutated and their effect on ligand and DNA binding was analyzed (Helsen et al., 2012b). Four of these mutations in the LBD led to reduced DNA binding and transactivation while not affecting ligand binding, conversely three DBD mutations reduced ligand binding and transactivation, but not DNA binding (Helsen et al., 2012b).

Another example of allosteric mechanisms in NR regulation is their mediation via post-translational modifications (PTMs) present at one site of the protein that can have various effects, including modifying cofactor interactions, changing cellular localization, regulating protein stability and influencing DNA binding at a different site (Fig. 1D). (Becares Salles et al., 2016) The most intensively studied PTMs on NRs are phosphorylation, acetylation, ubiquitinylation and SUMOylation. Examples of these are known for almost all NRs and their functioning, including allosteric modes, has been reviewed elsewhere (Becares Salles et al., 2016; Faus and Haendler, 2006; Anbalagan et al., 2012; Brunmeir and Xu, 2018).

The NR NTD has also been described to be involved in allosteric communication (Fig. 1E). Khan et al., for example, showed that when the TATA box binding protein (TBP) binds the GR NTD, this induces an increased helical content, as observed in circular dichroism, and the interactions with coactivators and transcriptional activity were, as a result, enhanced (Khan et al., 2011). Similar observations were found for the mineralocorticoid receptor (MR) and progesterone receptor (PR) which supports the notion that there is a common mechanism of regulation via the NTD, despite its poorly conserved sequence (Fischer et al., 2010; Kumar et al., 2013).

Homodimerization or heterodimerization with the retinoid X receptor (RXR) is a common phenomenon for NRs. These processes, similar to the binding of other protein partners, change the structural plasticity, and thereby the behavior of a NR (Jin and Li, 2010). This, in turn, can allosterically affect the recruitment and binding of ligands. As an example, the retinoid acid receptor β (RARβ) is known to be a monomer in solution, but upon binding of cofactors, it forms a homodimeric assembly (Venepally et al., 1997). Similar behavior was found for VDR and TR when bound to coactivators (Takeshita et al., 2000; Velasco et al., 2007). In order to dimerize, the receptor has to undergo a conformational change, which can lead to asymmetry in the dimer complex. For both RARβ and the estrogen receptor α (ERα) it was observed that in the homodimer complex, this asymmetry was altering.
the binding mode of the ligand, as revealed for example via two different poses of the same ligand in each the two monomers (Delfosse et al., 2012; Billas and Moras, 2013). Furthermore, it has been reported that also in the absence of a ligand, the NR homodimer can be asymmetric. The crystal structure of the apo-PPARY LBD contains two monomers in the asymmetric unit forming a homodimer, with only one of the monomers present in its active conformation (Nolte et al., 1998).

The above summary acts to illustrate that NR regulation involves several allosteric mechanisms. For a more detailed review on this subject the reader could consider an extensive review by Fernandez (2018). Despite these various endogenous allosteric mechanisms, the number of actual endogenic molecules known to act via allosteric pockets on the NR, is extremely limited. In this review, allosteric processes in NR modulation using small molecules will be discussed. Allosteric NR modulation by synthetic small molecules is a relatively new area of research, in contrast to the classic targeting of the orthosteric LBP (Moore et al., 2010). While extremely successful, targeting NRs via the orthosteric LBP also brings along certain limitations and challenges (Caboni and Lloyd, 2013). Selectivity issues can arise due to a high degree of similarity in the LBP between certain NRs, which can lead to side effects. Furthermore, mutations can occur in the LBP upon prolonged drug-treatment, which can lead to drug resistance or even agonist/antagonist switching (Caboni and Lloyd, 2013; Lu et al., 2014; Liu et al., 2016). Allosteric modulation of NRs with small molecules, via targeting binding sites beyond the orthosteric pocket, could therefore be a valuable alternative way of targeting NRs that could potentially overcome some of the described shortcomings of orthosteric ligands.

In this review, first the general concept of allosteric modulation in drug discovery is discussed, specifically focused on the different types of receptor classes, as a background setting and inspiration for future NR focused work. The body of the review will then focus on examples of small molecules that allosterically modulate the NR LBD. Finally, the obtained insights will be translated into recommendations for future research.

2. Small molecule allosteric modulation in drug discovery

2.1. Allosteric small molecule modulators and their potential in drug discovery

The vast majority of receptor classes possess endogenous ligands that bind to a defined site, typically termed the orthosteric binding site. Most drugs on the market are similarly targeting these orthosteric pockets and are in essence thus competing with the endogenous ligands (Moore et al., 2010; Caboni and Lloyd, 2013; Tice and Zheng, 2016). Allosteric ligands bind to sites on proteins that do not overlap with the orthosteric site and are therefore potentially resistant to orthosteric competition (van Westen et al., 2014). For multiple receptor classes, such as GPCRs and ligand-gated ion channels, but also for enzymes, a significant number of allosteric modulators have already been identified with considerable success (Foster and Conn, 2017; Taly et al., 2014; Changeux and Christopoulos, 2017; Thal et al., 2018; Gentry et al., 2015). Especially for GPCRs and proteins kinases, multiple allosteric ligands have been identified with some being approved by the FDA, such as Sensipar and Gleevec, and multiple drugs are undergoing clinical trials (Changeux and Christopoulos, 2017; Block et al., 2004; Buchdunger et al., 1996). For some of these proteins, allosteric ligands might be the sole option to selectively target them because of the evolutionarily conserved nature of their orthosteric site, such as the ATP binding site of kinases (Ghoreschi et al., 2009). Within the last two decades, an increasing number of connected publications can be observed, demonstrating the growth in interest and development of allosteric modulators (Fig. 2). During this time, multiple excellent review articles have been published for receptor classes that can be targeted by allosteric modulators (Changeux and Christopoulos, 2016; Foster and Conn, 2017; Taly et al., 2014; Thal et al., 2018; Gentry et al., 2015; Lisi and Loria, 2017). NRs have similarly seen an increase in attention regarding the identification of allosteric ligands, but here the number of publications is still lagging strongly behind that of other receptor classes.

The rapid increase in the attention for allosteric modulators can be explained by several key advantages such ligands have in comparison with orthosteric ligands. First, allosteric sites show greater structural diversity compared to the more conserved orthosteric sites. This could thus lead to a higher degree of selectivity for closely related proteins (Lu et al., 2014; Dixon et al., 2016; Nussinov and Tsai, 2013). Second, as mentioned earlier, allosteric compounds are typically not in competition with the receptor’s endogenous ligand. Therefore, allosteric modulators could eventually be used at lower concentrations resulting in reduced side effects. Furthermore, in the case of drug-resistant mutations in the orthosteric binding site, allosteric ligands can pose a promising alternative entry. In addition, since the binding of the allosteric ligand can potentially modulate the binding affinity and efficacy of the orthosteric ligand, a pathway is provided to fine-tune the physiological response of the both. Finally, the chemical structure properties of allosteric ligands are slightly more beneficial over orthosteric ones, as explored by Westen et al. (van Westen et al. (2014)). Most importantly here, allosteric modulators appear to be more rigid and they adhere slightly better to Lipinski’s rule of 5 which gives a higher potential for good oral drug uptake (van Westen et al., 2014). Despite these favorable characteristics, the challenge of discovering allosteric drugs is a highly relevant current limitation, since allosteric sites are often not identified or lack specific assay formats for target ligand screening (Nussinov and Tsai, 2013). At present, the majority of potential drug molecules are identified using high-throughput screening (HTS), for which the binding affinity of the ligand is often a hallmark to define the quality of the hit. The orthosteric site can, however, be expected to be dominant in this aspect, limiting the identification of ligands with allosteric binding modes. In addition, especially allosteric ligands for GPCRs are notorious to have a flat ‘SAR’, meaning that minor modifications on the modulator scaffold lead to a complete loss of activity, further complicating the discovery and optimization of these ligands (Conn et al., 2012).

The different modes-of-action of allosteric modulators described in literature, as well as the different assay techniques developed for the identification of such allosteric ligands, can serve as inspiration for allosteric targeting of NRs. A compact summary of these concepts,
2.2. Mechanisms of action of allosteric receptor modulators

The mechanisms by which allosteric modulators exert their function have been investigated in molecular detail, especially using crystal- and solution-based structural data. Allosteric modulators can induce an altered physiological response in multiple ways, because their binding changes the preference of populating certain protein conformations, alone or in consort with an endogenous ligand for the orthosteric site (Fig. 3). The section below is geared mostly to membrane proteins and specifically GPCRs as the studies of these proteins serve as important illustrative examples for the much less investigated small molecule NR allosteric modulation.

The majority of the allosteric ligands present in literature bind to pockets in an area surrounding the canonical binding site. This, in turn, influences the binding affinity and efficacy of the orthosteric ligand (Fig. 3A). (Changeux and Christopoulos, 2017; Kenakin, 2014) Benzodiazepines are a group of very successful modulators that bind to the Gamma-aminobutyric acid A (GABA\textsubscript{A}) receptor on the extracellular domain (ECD) (Lu et al., 2014; Smith and Olsen, 2000). Binding induces a significant conformational shift in the protein, leading to a greatly enhanced affinity for the natural ligand GABA (Williams and Akabas, 2000). Nevertheless, an allosteric molecule does not have to be in close proximity of the orthosteric site to alter the binding kinetics of the endogenous ligand. Multiple examples are known in literature where allosteric ligands bind to a distant site while still exerting an influence on the endogenous ligand (Changeux and Christopoulos, 2017; Smerdon et al., 1994; Horn and Shoichet, 2004; Oikonomakos et al., 2000). This arises from the fact that allosterically induced conformational shifts can be at one part of the protein, but can also change the conformation of the entire protein (Oikonomakos et al., 2000; Pargellis et al., 2002).

The dimerization behavior of a protein can also be altered by allosteric ligands (Fig. 3B). Allosteric modulation of receptor tyrosine kinases (RTKs) primarily involves monoclonal antibodies targeting the ECD (Cho et al., 2003; Franklin et al., 2004; Hicklin et al., 2001). The function of the RTK HER2/neu, which is involved in breast cancer, can be inhibited by antibodies using specifically two approaches. Trastuzumab binds to subdomain IV of the HER2/neu RTK thereby inhibiting its activation. Pertuzumab, on the other hand, interacts with subdomain II, which inhibits the activation by blocking dimerization (Cho et al., 2003; Fendly et al., 1990). Also, for the insulin RTK antibodies were identified that influence dimerization behavior (Bhaskar et al., 2012; Corbin et al., 2014). Very recently, the covalent modulator JZ-5209 was identified to bind to the β-Glucocerebrosidase (GCase). This small molecule covalently binds to a lysine residue which is present at the dimerization interface of the GCase, enhancing dimerization and exposing the active site (Zheng et al., 2018).

Intrinsically disordered (ID) protein domains have also come to the forefront for allosteric regulation with small molecules (Fig. 3C). The absence of a defined secondary structure in these domains makes them devoid of a classical binding pocket (Dunker et al., 1998; Wright and Dyson, 2015). Nevertheless, small molecules and macromolecules have been shown to be capable of binding to these regions and of subsequently inducing a defined secondary structure (Motlagh et al., 2012, 2014). The protein tyrosine phosphatase 1B is a therapeutic target for both diabetes and obesity. This protein contains an ID domain at its C-terminus which is located close to the catalytic domain. The small molecule MSI-1436 binds to this ID site with high affinity, thereby locking the protein into an inactive conformation and reducing the phosphatase activity (Smith et al., 2017).

Bitopic ligands, also known as dualsteric ligands, are allosteric modulators in which the orthosteric and allosteric pharmacophore are covalently linked, allowing them to bind to both pockets illustrated with selected highlights, as described for other receptor classes is therefore discussed below.
simultaneously (Fig. 3D). Apart from ensuring a stronger binding of the bitopic ligand to the receptor, the additional allosteric pharmacophore can lead to an improved selectivity and a nuance in receptor activation (Catterall et al., 2007; Lane et al., 2013; Mohr et al., 2010). The bitopic ligand THRX160209 targets the muscarinic M2 acetylcholine receptor, which is a member of the GPCR protein family. For this receptor, the orthosteric site, located in the narrow cavity formed by the seven transmembrane helices, and the allosteric site, located in the ECD, are in close proximity of each other. By combining both pharmacophores, the binding affinity was enhanced with several orders of magnitude. Interestingly, THRX160209 showed to have a superior specificity for the M2 subtype over other four very homologous subtypes (Steinfeld et al., 2007). A similar mode of action is observed for the bitopic ligand SB269652, which binds to both D2 and D3 dopamine receptors with high affinity. In the D2R dimer, SB269652 binds to one monomer which reduces the binding of the orthosteric agonists and inverse agonists in the other monomer (Lane et al., 2014).

Certain receptor proteins contain allosteric pockets featuring a reactive group that can be addressed with a covalent ligand (Fig. 3E). From a therapeutic point-of-view, such reactive groups are especially relevant when they represent mutated residues correlated to disease development. An example of a covalent modulator is the previously discussed compound JZ-5209, which enhances the dimerization of GCase (Zheng et al., 2018). In addition, the GTPase K-Ras can be targeted mutant specifically, exploiting the oncogenic G12C mutation. This mutation diminishes the GTP hydrolysis and is closely located to both the nucleotide and the effector binding pocket. By covalently linking compounds to this cysteine residue, the compounds allosterically control the GTP affinity and the normal function of the enzyme can be restored. Since these compounds make use of a mutated residue, the natural protein is not affected (Ostrem et al., 2013).

A final example of allosteric modulation relates to ion channels, responsible for the ion exchange over the cell membrane and of particular relevance in the central nervous system. In general, ion channels are either activated by ligand binding to the extracellular domain, in the case of ligand-gated ion channels, or through a change in membrane potential, in the case of voltage-gated ion channels (Catterall and Swanson, 2015; Castèlle and Catterall, 2000). In recent years, these venom were used as an inspiration to synthesize synthetic ligands and peptides that target these channels, some of which are used in the treatment of heart diseases and cancer (Catterall and Swanson, 2015; Buchanan and McCloskey, 2016).

2.3. Techniques for the identification of allosteric ligands

The increase in focus on the modulation of proteins by allosteric ligands comes with a concurrent demand for novel screening techniques that both aid in the identification of allosteric pockets and in the discovery of small molecules specifically targeting these pockets. Some of the most promising techniques and examples, including computational approaches, fragment soaking, tethering and phage display, are shortly summarized mentioned here to act as a potential framework to be translated to NRs.

Crystal structures of ligand-bound proteins provide high-resolution information about the binding mode of the ligand. The resulting static picture can be further refined with techniques providing dynamic information (Carvalho et al., 2010). Over the last decades, numerous computational methods have been developed to provide a better understanding of the dynamic behavior of the protein, which is of high relevance for the identification of allosteric sites (Huang et al., 2013, 2015; Lu et al., 2018; Greener and Sternberg, 2018; Lu and Zhang, 2017). The computer-aided drug design allows to help understand how allosteric modulators exert their function (Macalino et al., 2015). Making use of molecular dynamics, ligands could successfully be identified that allosterically bind to the ß2-adrenergic class of GPCRs. Also, a hidden allosteric site in the oncogenic K-Ras4B protein was found using molecular dynamics by sampling a conformation of the protein which was not observed in the crystal structure (Lu et al., 2018).

Fragment-based drug discovery is achieving a strong impact for discovering allosteric binding sites, for example via X-ray crystallography based fragment soaking approaches (Kuo, 2011). Typically, cocktails of 5–10 fragments are soaked into a crystal of the protein of interest (Kuo, 2011; Bauman et al., 2014). When one or more fragments bind to the protein the additional electron density directly reveals the location of the fragment's binding pocket and the structure of the fragment. As an example, for the HIV-1 reverse transcriptase enzyme, three new binding sites were found that also proved to be inhibitory in enzymatic assays (Bauman et al., 2014). Similarly, for tyrosine phosphatase 1B 11 binding sites outside the active site have been elucidated (Keedy et al., 2018).

Tethering is not only a technique applied to discover covalent drug binding or orthosteric drugs, but it could also be considered as a technique to identify ligands for allosteric pockets. Different tethering strategies have been developed, of which for example disulfide tethering is well-known. This technique involves the formation of a reversible disulfide bond via the linking of a thiol-containing fragment to a, potentially genetically introduced, cysteine residue in a protein (Erlanson et al., 2004; Hardy, 2008). At equilibrium, the mixture will consist predominantly of the protein bound to the fragment with the highest binding affinity. By use of the covalent bond formation between the fragment and the protein, the affinity of the fragment for the target protein is amplified, which can be useful for detection at lower concentrations. The direction of the ligands to a specific site makes the approach particularly useful in case of a known allosteric site for which novel allosteric ligands should be identified.

Phage display methods have been used occasionally in the search for allosteric receptor modulators. The technique involves the expression of a random library of peptides on the coat proteins of bacteriophages (Pande et al., 2010). By physically linking the peptide and its genetic material, novel ligands with desired properties can be easily identified via this approach. A recent study by Tipps et al. uses a combination of phage display technology and standard electrophysiological testing for the discovery of novel allosteric modulators for the glycine receptor, a member of the superfamily of ligand-gated ion channels (Tipps et al., 2010). The unbiased nature of the phage display technique provides potential for the identification of allosteric modulators for targets of which specific allosteric pockets have not yet been identified.

3. Nuclear receptor allosteric modulators

NRs, like other receptors, have been targeted on allosteric sites. At the moment, the most studied allosteric modulators include those that target the activation function 2 (AF-2) cofactor binding site, the DBD or DNA response elements, modulate the receptor dimerization, modulate PTMs or target alternative sites in the LBD. These different sites will be discussed briefly, with the main focus on the alternative pockets in the LBD. In the context of this review, allosteric compounds are considered as compounds that bind at a different site than the orthosteric site and lead to stabilization of other NR conformations, and not only inhibit, for example, DNA or coactivator binding.

3.1. Approaches for allosteric modulation of NRs on and beyond the ligand binding domain

NRs are typically activated by the binding of agonistic ligands, resulting in the recruitment of specific cofactors leading to a certain biological response via enhanced gene transcription. One type of
alternative NR modulation is, therefore, via the direct inhibition of cofactor binding at the AF-2 site (Fig. 4A). (Wang et al., 2006) Many types of AF-2 site inhibitors have been described, such as constrained peptides, peptide mimetics and small molecule inhibitors, all typically mimicking the helical LXXLL cofactor binding motif (Moore et al., 2010; Caboni and Lloyd, 2013; Tice and Zheng, 2016). This form of direct inhibition of NR-protein interactions is described by other reviews in this issue and therefore not further elaborated here.

Interactions between the NR DBD and the DNA are crucial for the expression of the NR target genes. Targeting the DBD is, therefore, another potential strategy for NR modulation (Moore et al., 2010; Caboni and Lloyd, 2013). The first option is the blocking of specific HREs at the DNA, inhibiting the binding of the NR DBD (Fig. 4D). The most common examples of this approach are hairpin polyamides that non-covalently bind to the minor groove of DNA and thereby disrupt the interaction with the NRs such as ERx and AR (Caboni and Lloyd, 2013; Gearhart et al., 2005; Nickols and Dervan, 2007). The second strategy is via ligands that target the zinc fingers of the DBD (Fig. 4E). Zinc fingers play an important role in stabilizing the DBD, meaning that disrupting these zinc fingers results in a destabilization of the DBD leading to a reduction of DNA binding (Caboni and Lloyd, 2013; Whittal et al., 2000). Most modulators are oxidizing agents that focus on disrupting the Zn-S interaction; they displace the zinc ion in the zinc finger and cause disulfide bond formation of the cysteines that normally coordinate the zinc ions via electrostatic interactions (Caboni and Lloyd, 2013). The most challenging issue for designing such modulators is to achieve selectivity over other NR members since the DBD is the most highly conserved domain in this class of proteins (Caboni and Lloyd, 2013). Targeting the DBD and HRE interactions will also be discussed in detail elsewhere in this issue.

NR homo- and heterodimerization also provide entries for allosteric modulation via small molecules (Fig. 4B). Through its ability to form heterodimeric assemblies, RXR can modulate and activate multiple NR partners such as PPAR, LXR and Nur1. In general, RXR dimerization partners can be arranged into two classes, being permissive and non-permissive heterodimer partners. For permissive heterodimers, the sole presence of the RXR ligand is enough to make the heterodimer transcriptionally active. If the partner's ligand is also present, this results in a stronger activation. In contrast, for non-permissive heterodimers, the partner's ligand is required for activation, since they are not responsive to rexinoids on their own. However, like permissive heterodimers, the presence of an RXR ligand does enhance the biological response (Forman et al., 1995; Kurokawa et al., 1994). The structural mechanism by which RXR transduces its signal to its dimer partner has been further elucidated recently (Kojetin et al., 2015). As an example of the fine interplay of RXR heterodimer regulation, in a Parkinson’s disease mouse model, treatment with the RXR agonist bexarotene enhanced the clearance of β-amyloids, improving the neural function. This is believed to be caused by the activation of the permissive RXR-PPAR and RXR-LXR heterodimers (Cramer et al., 2012). Furthermore, it has been shown that the type of RXR ligand can have a substantial effect on the dimerization behavior of RXR and therefore on the transactivation to its dimer partner (Scheepstra et al., 2017; McFarland et al., 2013; Giner et al., 2015). The RXR ligand LG101506 was shown to selectively activate RXR-PPAR heterodimers over other heterodimers e.g. RXR-RAR, RXR-LXR and RXR-FXR. Selective activation of RXR-PPAR has been shown to be beneficial in the treatment of type 2 diabetes by improving insulin sensitivity while simultaneously keeping the triglyceride levels consistent (Leibowitz et al., 2006; Michelys et al., 2003). Lastly, in a recent study, the effect of minor modifications on RXR ligand scaffolds on influencing the heterodimerization of RXR with the orphan NR Nur1 was analyzed. Interestingly, ligands that introduced steric bulk towards either H7 or H11 of RXR significantly inhibited the heterodimer formation. It is postulated that this behavior is observed because of the presence of Nur1 H12 making contact with H7 and H11 of RXR at the dimerization interface (Spatis et al., 2017).

Several ligands are known that allosterically modulate NRs by acting at or via PTMs (Fig. 4C). For PPARγ, for example, phosphorylation of S273 was found to decrease insulin sensitivity by upregulating a specific set of target genes without fully activating the receptor (Choi et al., 2010). When compounds with minimal agonistic activity bind the PPARγ LBP, there is inhibition of S273 phosphorylation without causing PPARγ activation associated side-effects (Choi et al., 2011). A similar modulatory effect might be achievable for other NRs since ligand-induced PTMs are common. In the case of the farnesoid X receptor (FXR), agonist-activated SUMOylation at K277, which is necessary for the transrepression of pro-inflammatory genes, is inhibited by acetylation at K217 in obese individuals (Kim et al., 2015). Targeting this acetylation site is postulated as an approach to improve hepatic inflammation and glucose tolerance (Kim et al., 2015). A different approach to target a PTM using small-molecules to alter NR activity was shown for ERα, for which a phosphorylation site in the F-domain was identified which interacts with the hub-protein 14-3-3 (De Vries-van Leeuwen et al., 2013). This interaction can be stabilized by the small
molecule Fusococcin, which reduces ERα dimerization, resulting in inhibition of downstream gene expression (De Vries-van Leeuwen et al., 2013). Such PTMs beyond the LBD might provide an advantage over ligand-induced resistance mutations in the LBD (Zwart et al., 2007).

3.2. Allosteric pockets on and in the NR LBD

The major part of this review is dedicated to compounds that bind to allosteric sites within the LBD, which we have categorized into three sub-groups, depending on their binding location (Fig. 5). The first group, targeting expanded orthosteric sites, binds (part of) the orthosteric LBP and subsequently extends from there into additional pockets that are unique for a certain NR. The compounds in the second group, the dual site binders, bind the LBD in a two to one stoichiometry where typically one of the two ligands binds in the LBP and the second ligand binds a surface exposed site elsewhere on the LBD. The third group of compounds addresses pockets fully outside of the orthosteric LBP, which we will term alternative pockets.

3.2.1. Expanded orthosteric site

The size and plasticity of the orthosteric pocket greatly varies between different NRs, ranging from barely existing to as big as 1600 Å³ (Gallastegui et al., 2015). In the past, there has been structural evidence that ligands, especially fatty acid analogs, can bind to the orthosteric LBP of various NRs with a different binding mode. Therefore, NRs can recognize the same ligand but binding can lead to another biological response (Egea et al., 2002; Itoh et al., 2008). In this section, we explore ligands that take it one step further by binding to the orthosteric LBP and extending out to neighboring pockets connected to the classical LBP. These ligands are often found in NRs that contain a larger orthosteric pocket like PPAR and FXR. Apart from inducing possible alternative protein conformations, the targeting of these ‘novel’ pockets potentially allows for enhancing NR subtype selectivity. Below an overview is provided of examples of ligands acting via the expanded orthosteric site mechanisms, with a focus on those allosteric ligands for which structural information is available.

3.2.1.1. FXR

The farnesoid X receptor (FXR) contains an extra binding site connected to the orthosteric LBP. FXR is a NR that regulates lipid, glucose and amino acid metabolism (Massafran and van Mil, 2018). The extra binding site was initially found by a molecular modeling study which docked E/Z-guggulsterone in a new pocket defined by both the loop between H1 and H2, and H3, H5, and H6 (Fig. 6A). (Meyer et al., 2005) The location of this pocket, defined as the S2 site, was later confirmed via HDX-MS studies, but no crystallographic data is currently available (Yang et al., 2014). When bile acids, the endogenous ligands of FXR, bind to the receptor the carboxylic acid moiety of the side chain points towards the back of the LBD close to the loop between H1 and H2, and H3 and H5 (Fig. 6A). (Mi et al., 2003; Pellicciari et al., 2006) This is of particular interest, since for other NRs that bind cholesterol metabolites this part of the ligand points in the direction of H12. When overlaying the crystal structure of FXR co-crystallized with chenodeoxycholic acid (CDCA), the most potent endogenous ligand, with the docked pose of E/Z-guggulsterone it can be appreciated that the carboxylic acid tail points towards the S2 site (Pellicciari et al., 2006). Pellicciari et al. used this information to design a ligand library with the carboxylic acid position of CDCA exchanged with chemically diverse groups (Pellicciari et al., 2006). Modification of this part of the ligand scaffold yields compounds that expand out of the classical LBP which leads to effects ranging from antagonism to partial and full agonism. This research was continued by further exploring the bile acid scaffold, leading to the discovery of molecules with a high efficacy and affinity (Gioiello et al., 2011; Pellicciari et al., 2016). This approach of gaining selectivity by targeting the unique properties of a single NR exemplifies the utility of targeting allosteric pockets. In addition to the S2 site, it has been shown that the orthosteric LBP of FXR can expand by shifting H2 and H6 outwards, generating additional space in the LBP. Therefore, FXR can accommodate larger sized compounds such as ivermectin that alters the cofactor binding pattern of FXR by allosterically enhancing the flexibility of the cofactor binding site (Fig. 6B) (Jin et al., 2013).

3.2.1.2. LRH-1

The binding of liver receptor homolog-1 (LRH-1) to phospholipids is also an example of NR modulation via addressing an expanded orthosteric site. Tuning the activity of LRH-1 can be beneficial in multiple types of cancer and metabolic diseases (Lazarus et al., 2012). In a study by Musille et al., the crystal structure of LRH-1 with phospholipid diaroylphosphatidylcholine (DLPC) was solved at a resolution of 1.8 Å, revealing the binding site of this phospholipid (Fig. 6C). (Musille et al., 2012) It could be clearly observed that DLPC is located near the β-sheet-H6 region and makes contacts with residues of H3, H5, H6 and H7. In this binding mode the compound occupies not only the LBP, but also extends out of the canonical pocket. In a second study, crystallography and molecular modeling were combined to identify an allosteric network connecting the novel extended binding pocket with the AF-2 site via the orthosteric LBP, elucidating an allosteric mechanism for the activation of LRH-1 by phospholipids such as DLPC (Musille et al., 2016). A highly similar binding mode is observed in the other member of the steroidogenic factor-like subfamily, the steroidogenic factor-1 (SF-1) (Sabin et al., 2009). Blind and coworkers confirmed via biochemical and structural analysis that phospholipid PIP stabilizes the previously determined critical H2-3 and H11-12 loops and thereby enhances coactivator recruitment (Sabin et al., 2009; Blind et al., 2014). Finally, a related example is the glucocorticoid deacylcytovazol (DAC) which binds to an extension of the LBP located at the top half of the LBD of GR, thereby effectively doubling the size of the binding pocket (Suino-Powell et al., 2008).

3.2.1.3. PPARγ

A NR that is well-known for having one of the largest orthosteric binding-pockets is PPARγ. The PPARγ pocket is composed of three sub-pockets which have a combined volume of > 1200 Å³ (Brust
et al., 2017). PPARγ plays an important role in energy homeostasis and inflammation and therefore modulating its activity can be beneficial in e.g. obesity and diabetes (Varga et al., 2011). Hughes et al. discovered that at high concentrations the agonist MRL-20 still increased the interaction between PPARγ and coactivator when the orthosteric site was blocked using a small covalent modifier (Hughes et al., 2014). Using NMR and TR-FRET they confirmed that MRL-20 binds with high affinity to the orthosteric site and with lower affinity in an adjacent pocket, in both cases causing agonistic behavior (Hughes et al., 2014). The agonistic behavior of the first binding site is caused by direct

Fig. 6. Overview of allosteric ligands binding to an expanded orthosteric site and their corresponding crystal structures. A) FXR with CDCA in blue (PDB: 1OSV). GS (shown in teal) is positioned at the predicted binding site. B) FXR with ivermectin (PDB: 4WVD). C) LRH-1 with DPLC (PDB: 1YUC). D) PPARγ with MRL-20 (blue) (PDB: 2Q59). The second MRL-20 (in teal) is positioned at the predicted binding site. E) PPARγ with T2384 (PDB: 3K85). F) PXR with EE2 and TNC (PDB: 4X1G).
stabilization of H12 and the AF-2 surface, whereas the second binding site likely stabilizes H3 which facilitates interactions with the loop between H11 and H12 and with that causes stabilization of the coactivator binding region (Hughes et al., 2014). Covalent modifiers with different properties were used to define the binding site of MRL-20 more precisely (Fig. 6D). (Brust et al., 2017) Another example where one compound is able to bind two connected sites at the same time in the PPARγ LBD is the flexible ligand T2384 (Li et al., 2008). In the crystal structure, T2384 is both observed in a U-shape in the canonical LBP and in a S-shape in the canonical LBP in combination with an extra molecule in the alternate site (Fig. 6E). (Li et al., 2008) By making mutant proteins that lack one of the two binding sites, it was elucidated that the combined antagonist/agonist behavior that is observed for this compound in the native protein is caused by an agonistic effect of binding in the orthosteric pocket and an antagonistic effect of binding in the connected allosteric pocket (Li et al., 2008). These binding-modes were confirmed with NMR where it was concluded that both the U and S compound-conformation in the orthosteric pocket exist when adding one equivalent of the compound and the alternate binding site gets populated when adding more than one equivalent (Hughes et al., 2016). In addition to the instances where one compound binds to both the orthosteric and a connected alternate site, there are also a couple of examples where a synthetic ligand and a naturally occurring lipid together occupy the two pockets of PPARγ (Jang et al., 2017; Shang et al., 2018).

3.2.1.4. PXR. Finally, the pregnane X receptor (PXR) also has a unique way in which its orthosteric binding pocket can be expanded (Delfosse et al., 2015). PXR is known for binding structurally diverse ligands and with that serving as a hydrophobic toxin sensor (Kliewer et al., 2002). Agonists for PXR. However, when these compounds are combined they significantly prevent coactivators from binding. For the scope of this review, one equivalent (Hughes et al., 2016). In addition to the instances where one compound binds to both the orthosteric and a connected alternate site, there are also a couple of examples where a synthetic ligand and a naturally occurring lipid together occupy the two pockets of PPARγ (Jang et al., 2017; Shang et al., 2018).

3.2.2. Dual site binding

3.2.2.1. VDR. The vitamin D receptor (VDR), in addition to its binding to the endogenous ligand vitamin D, also acts as a sensor for the toxic secondary bile acid lithocholic acid (LCA) (Makishima et al., 2002). LCA acts as an agonist for VDR which, in turn, facilitates the clearance of LCA metabolites. A recent crystal structure of zebrafish VDR LBD co-crystalized with LCA showed LCA to bind both in the orthosteric pocket as well as to a surface exposed site near the cofactor binding site (between L1-3, H2' and H3) (Fig. 7). A combination of biological, computational and structural analysis showed that both sites have to be occupied by LCA for it to act as a VDR agonist. The LCA molecule at the secondary site does not interact with H12 directly but is in close proximity of the coactivator. Comparison of the B-factors of the crystal structures of VDR bound to either one or two LCA molecules showed that the presence of both LCA molecules leads to a stabilization of H12, the loop between H11 and H12 and the cofactor. Therefore, binding of a ligand to this site might reveal a mechanism of ligands to induce coactivator selectivity (Belorusova et al., 2014).

3.2.2.2. PPARα. The potent and PPARα-selective agonist WY-14643 (also known as pirinixic acid) was shown to inhibit NF-kB activity and decrease anti-inflammatory responses. In addition to binding to the orthosteric pocket, WY-14643 binds to an additional binding site in a pocket between H2', H3 and the loop between H11-H12 (Fig. 7). Using mutational studies, it was shown that binding of the second ligand significantly enhances the activation of PPARα. Because the second ligand is not in contact with H12 directly, the increased activation was further investigated using MD. In this simulation, WY-1463 stabilized the otherwise flexible Ω-loop (between H2 and H3) which, in turn, indirectly stabilized H12 (Bernardes et al., 2013). This indirect modulation of H12 via another part of the protein has already been reported earlier in literature for this receptor class. Orthosteric ligands of PPARγ have been shown to stabilize the Ω-loop and β-sheet region in PPARγ, both leading to the stabilization of H12 (Puhl et al., 2012; Waku et al., 2009a, 2009b; Bruning et al., 2007).

3.2.2.3. TRα. In 2011, HDX studies were used to show that the natural ligand of the thyroid receptor (TR), tri-iodothyronine (T3), was likely to also bind in a region of the protein between H9, H10 and H11, located at the opposite site of the AF-2 (Figueira et al., 2011). Three years later, the crystal structure was solved of TRα with T3 bound to the orthosteric pocket and either T3 or T4 located at the earlier predicted position (Fig. 7). (Souza et al., 2014) The same group also showed that in an earlier solved crystal structure of the TRβ-selective ligand GC-24 in complex with human TRβ, an additional GC-24 molecule was similarly present in this region. The undefined electron density was initially presumed to be an artifact (Souza et al., 2014; Borngraeber et al., 2003). Interestingly, the position of this binding site is the same as the predicted binding site of the F-domain of TRα. It is, therefore, hypothesized that the secondary binding site suppresses the activation of TRα when there is a high concentration of ligand present by preventing recruitment of certain cofactors (Souza et al., 2014). In addition, T3 also binds to other NRs such as AR and ER (Bona et al., 1979). Interestingly, when AR was co-crystalized with both 5-alpha-dihydrotestosterone and T3, another allosteric pocket was found for T3 at the other side of H9 compared to the TRα structure (Tanenbaum et al., 1998).

3.2.3. Alternative pockets in the LBD

The two previously mentioned classes of LBD allosteric sites are either an expansion of the orthosteric site or a secondary binding site for an orthosteric ligand (dual site binding). The third class, discussed below, deals with allosteric pockets on the LBD fully independent from the orthosteric pocket in terms of the type of ligand or spatial overlap. Such allosteric sites are known and structurally described for RORγt, Nur1, Nur77, RXR, and AR (Fig. 8). Of course, it should be noted that nevertheless, the actual ligand binding to the orthosteric and allosteric pockets could potentially still influence each other, because of allosteric effects.

3.2.3.1. RORγt. Retinoic acid related orphan receptor γ t (RORγt) is a
key regulator protein for the development of T cells into Th17 cells, which results in tissue inflammation by the excretion of pro-inflammatory cytokines such as IL-17a (Ivanov et al., 2006; Korn et al., 2009; Yang et al., 2008). Inhibition of RORγt has, therefore, been found to be a promising strategy in the treatment of autoimmune diseases by diminishing the inflammatory response (Isomoto et al., 2014). Many synthetic small molecule inverse agonists for RORγt have been identified in literature, most of them targeting the orthosteric LBP (Fauber and Magnuson, 2014; Kamenecka et al., 2013). However, a few years ago a novel acyl-indazole series of RORγt modulators was disclosed by the company Merck/MSD containing a different chemotype in comparison with the already known modulators (Karstens et al., 2012). Scheepstra et al. identified that these ligands were binding at an alternative binding pocket in the LBD of RORγt (Scheepstra et al., 2015). For four of these acyl-indazole ligands (MRL-871, MRL-299, MRL-367, MRL-673), a high resolution crystal structure has been elucidated, in which it can be clearly observed that these modulators bind in an allosteric binding site, distal to the orthosteric binding pocket, at the position where H12 is normally located in its agonistic conformation. This allosteric binding site is shown illustratively for MRL-871 in Fig. 8A. The allosteric pocket is formed by H4, H5, H11 and the repositioned H12, which has a unique conformation by folding back over the ligand. In this conformation, the recruitment of coactivators to the AF-2 is prevented. Via this binding mode, the allosteric ligands are functionally acting as inverse agonists with a similar effect as regular orthosteric inhibitors, but in contrast, not in competition with the orthosteric ligands. The acyl-indazole ligands interact with the allosteric pocket mainly via hydrophobic interactions and additionally via H-bonding between the carboxylic acid moiety of the ligand and the Q329 side chain (H3) as well as the main chain amide of A497 and F498 (H12) of the protein. In addition to structural studies, the allosteric modulators were also tested in time-resolved fluorescence resonance energy transfer (TR-FRET) coactivator recruitment assays and cellular assays, which showed IC50 values in the nanomolar range. This means that these compounds can be considered as NR allosteric compounds with a potency that reaches clinical demands.

With the discovery of these RORγt allosteric ligands, follow-up research on these compounds has been performed by many companies and research groups, most extensively by Genentech, Glenmark Pharmaceuticals and Ouvry et al.. Genentech performed an extensive SAR study around the MRL series of compounds, varying the indazole core and also making changes to the carboxylic acid and 3-acylarene moiety (Fauber et al., 2015). They specifically attempted to improve the selectivity for RORγt over other NRs, since a remarkable cross-reactivity with PPARγ was observed for the MRL derivatives (Scheepstra et al., 2015). From this study, GNE-0946 and GNE-6468 (Fig. 8) were identified as highly potent compounds in both biochemical and cellular assays, with improved selectivity and physicochemical properties. Glenmark Pharmaceuticals performed a scaffold hopping approach with MRL-871 as a starting point, resulting in a compound with a thiophenopyrazole core (Fig. 8) as one of the best hits (Gege, 2015; Chaudari et al., 2015). The compounds were tested in biochemical TR-FRET assays, where one of the compounds showed an IC50 value of less than 50 nM. Finally, Ouvry et al. mainly focused on improving the metabolic stability of MRL by replacing the heterocyclic amide linkage, since related compounds have shown in vivo instability due to hydrolytic cleavage (Ouvry et al., 2016). For this purpose, different linkers were incorporated on a reverse indazole scaffold, which was derived from one of the MRL analogs, and the library was screened in a RORγt/Gal4 cell-based assay. Incorporation of an ether linker, compound 27 (Fig. 8), resulted in the most potent ligand. Furthermore, the ligand was also tested in phototoxicity assays where it turned out to have a phototoxic irritancy factor above the toxic level, which could not be improved by some further structural changes. Interestingly, the crystal structure of RORγt with phenoxindazole 27 was elucidated which revealed binding to the same allosteric pocket as MRL-871 in a similar orientation (Ouvry et al., 2016).
the LBD as discovered for RORγt. Two crystal structures were solved showing two different cyclopentenone prostaglandins (cyPGs) covalently bound to C566 of Nurr1. Binding of the molecule resulted in a significant shift of H12 compared to the apo structure. In an in vitro luciferase reporter assay, cyPGs enhanced the transcriptional activity of Nurr1 in HEK293T cells (Yoon et al., 2018).

Recent crystallography studies indicate that also Nur77 can bind ligands at alternate sites. Wu et al. identified the cytosporone B analogue TMPA to bind Nur77 at two surface exposed sites compromised by either H4, H11 and H12 or H1, H5, H7 and H8 (Zhan et al., 2012). Via mutational and functional studies it was discovered that the first site, which has similarities to the allosteric pocket in RORγt, is important for mutually exclusive TMPA binding but also liver kinase B1 (LKB1) interaction (Zhan et al., 2012). Nur77 also plays a suppressing role in lipopolysaccharide (LPS)-induced sepsis by associating with p65 and blocking its binding to κB (Li et al., 2015). This interaction is, however, diminished by p38α phosphorylation of T27 and T143 in the N-terminal domain of Nur77 (Li et al., 2015). Binding of PDNPA, which resembles cytosporone B even closer, to the same site where TMPA and LKB1 can bind, blocks binding of p38α to Nur77 resulting in reduced LPS-induced inflammation (Li et al., 2015). Via similar methods, the same group identified the trihydroxyphenyl derivative THPN that, instead of disrupting a protein-protein interaction, creates a binding interface for NIP3-like protein X (Nix) (Wang et al., 2014). THPN binds in a surface exposed cavity between H5, H7, H8, H9 and H10 of Nur77 (Wang et al., 2014). After THPN binding, Nur77 translocates to the mitochondria through binding to the mitochondrial outer membrane protein Nix which, on its turn, starts a cascade leading to autophagy (Wang et al., 2014). By combining THPN with Akt2 inhibitor administration, this approach can be expanded to other cancer types where Nur77 phosphorylation inhibits THPN-induced mitochondrial targeting (Wang et al., 2015).

3.2.3.3. RXR. A secondary binding site on the retinoid X receptor alpha

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Fig. 8. Overview of NRs targeted via alternative allosteric pockets. A) RORγt with MRL-871 (overlay with the protein with T0901317 (PDB: 4YPQ & 4NB6)). B) RXRα with K-8008 (overlay with the protein with 9-cis retinoic acid (PDB: 4N8R & 1FBY)). C) AR with T3 and DHT (PDB: 2PIT). The orthosteric ligands were shown in red (the licorice representation was used if the ligand was used as a reference compound in the orthosteric site, a spherical representation is used if the ligand is actually present in the crystal structure) and the allosteric ligands in blue. Also, a selection of reported RORγt, RXRα, and AR allosteric ligands is shown.
(RXRα) was discovered a few years ago by Chen et al. In their study, two Sulindac-derived analogs, K-8008 and K-8012 (Fig. 8), were identified as novel RXRα allosteric antagonists, inhibiting coactivator recruitment with IC₅₀ values around 10 µM (Chen et al., 2014). These ligands were identified as inhibitors, which interact with the interaction between RXRα and the p85α subunit of the phosphatidylinositol-3-OH kinase (PI3K), resulting in a decrease in the activation of the TNFα-activated PI3K/Akt pathway and therefore led to induction of apoptosis (Chen et al., 2014; Zhang et al., 2015). The X-ray crystal structures of the compounds in complex with the RXRα LBD revealed a homo-tetrameric structure of RXRα LBDs, with one ligand binding to each dimer partner (Fig. 8B).

In this structure, the ligands bind at a novel site, distinct from the orthosteric LBP. This hydrophobic binding site is located at the surface of the LBD, near the dimer-dimer interface of the tetramer. Specifically, it is formed by H3, H5 and H10/H11, nearby the entry of the canonical LBP, but not overlapping with it. Ligands binding to the tetrameric structure may help to stabilize the tetramer, which might explain the diminished interaction of RXRα with p85α. In additional experiments, the therapeutic relevance of targeting RXRα with the ligands was shown, since they were able to induce apoptosis in vitro and also inhibit tumor growth in animals without apparent toxicity. Thus, despite their relatively weak affinity for RXRα, the compounds could be clinically relevant for developing RXRα modulators in cancer therapies.

3.2.3.4. AR. The androgen receptor (AR) is an important regulator for the growth and function of the prostate gland, meaning that overstimulation of the receptor can lead to prostate cancer (Biron and Bédard, 2016). Therefore, AR inhibition is a relevant strategy for the treatment of this type of cancer. Currently, most AR antagonists act by binding to the canonical LBP, but often resistance to these antiandrogen drugs is developed over time in cancer patients (Biron and Bédard, 2016). Therefore, there is a high demand for alternative strategies to target AR.

Around a decade ago, an allosteric binding site in the AR was discovered, termed binding function-3 (BF-3). This hydrophobic binding site has a size that is comparable to the AF-2 site and is located at the junction of H1, the loop of H3-5 and H9, adjacent to AF-2 (Fig. 8C). (Biron and Bédard, 2016) Estebanez-Perpina et al. used a fluorescence polarization-based assay in combination with a crystallographic screening approach, in which a large library screening was performed and three inhibitors were identified; flufenamic acid (FLUF), T₈₉, which was already discussed as a TRα dual site binder, and triodothyraoectic acid (TRIAC) (Fig. 8). (Biron and Bédard, 2016; Estebanez-Perpina et al., 2007) These compounds were shown to preferentially interact with the BF-3 site (IC₅₀ values ~50 µM in FP assays) and to have some minor cross-talk to the AF-2 site. A number of follow-up studies were performed by Cherkasov et al. (Biron and Bédard, 2016; Lack et al., 2013; Munuganti et al., 2013, 2014; Ban et al., 2014), specifically focused on improving the affinity and selectivity for the BF-3 site, while also inhibiting prostate cancer cell growth. Recently, VPC-13566 (Fig. 8) was found as a highly potent (IC₅₀ = 50 nM) and selective compound, also inducing a significant inhibition of cancer cell growth (Lallous et al., 2016). Although the BF-3 site is an allosteric one, the conformational change induced in the AR protein appears to be limited. Recently it was found that the way in which the BF-3 site targeted compounds regulate AR activity is via the inhibition of the binding of co-chaperones (Bag-1L (Jehle et al., 2014), FKBP52 (Stope and Burchardt, 2012) and SGTA (Lallous et al., 2016)), which normally interact with the AR LBD via the BF-3 site.

4. Conclusion & perspective

NRs are a promising target in pathology because of their regulating role within the human genome. The primary focus of NR targeting has classically been via the orthosteric LBP. Small-molecule modulators binding to this part of the protein have yielded great successes, nevertheless issues such as cross-reactivity, selectivity, and drug resistance remain major challenges. In recent years, allosteric modulation of protein receptors has gained attention, with drugs with allosteric modes of action on the market for GPCRs and kinases, and multiple drugs undergoing clinical trials. The first allosteric ligands for NRs were shown to have low binding affinities towards their target. But, recently, nanomolar potent allosteric site modulators have been discovered for several NRs, bringing allosteric targeting of NRs also to the center of attention.

The LBD is presently the most promising NR domain for allosteric targeting due to the high affinities and the functional effects of the allosteric compounds that have been found. In addition, there is considerable structural information available for this domain, making de novo drug synthesis, computational guided studies, and medicinal chemistry more straightforward. Nevertheless, the scarcity of full-length structures and the absence thereof in the presence of allosteric ligands, still limits a concise delineation of the allosteric effects on the multi-domain protein level for these new kind of compounds.

Allosteric modulation via nuclear receptor dimerization, mainly via the LBD, has delivered interesting entries for drug development that could be further investigated. To date, the mechanism by which the activation of specific dimers is controlled is still rather poorly understood. The structural diversity of the amino acid residues present at the dimerization interface between NRs is significant. Therefore, the possibility arises to generate synthetic ligands that can selectively bind to the dimerization interface to either stabilize or inhibit specific homo- and heterodimer formation. These synthetic ligands can serve as interesting approaches to target specific pathways, but also to fundamentally understand the importance of the protein dimers.

Although most success in NR allosteric targeting has been on the LBD, other strategies such as allosteric modulation via PTMs also show high potential and have already yielded some successes. The intrinsically disordered nature of the NTD of NRs together with the fact that this domain contains multiple sites for PTMs makes this domain of NRs of particular interest for allosteric modulation via addressing the PTMs (Simons et al., 2014; Hill et al., 2012). Allosteric modulators which either enhance or abolish these PTMs selectively, can lead to an improved understanding on the modulatory role of these PTMs and can potentially be interesting for drug discovery.

Reflecting on the allosteric mechanisms found for other receptor classes, it becomes clear that NRs possibly also harbor analogous sites with the potential to be similarly allosterically modulated. To date, the vast majority of these approaches, such as bitopic ligands and covalent attaching, have remained unexplored for NRs while the potential for drug discovery can be substantial. Allosteric sites that are in close proximity of the canonical LBP can be targeted by bitopic ligands to more accurately force a desired biological response. Additionally, for NRs with an expanded orthosteric site, the orthosteric ligand can be combined with pharmacophores that occupy directly neighboring pockets. This will ultimately potentially result in a modulation of efficacy, enhanced affinity, and selectivity of the ligand for the specific NR. These type of ligands can, therefore, reduce the cross-reactivity of ligands to other NRs, which remains a serious problem in NR research (Moras and Gronemeyer, 1998). Also covalent attachment of molecules is an interesting approach to target NRs. Covalent modulators have the potential to more selectively target NRs using a lower dose, and perhaps also specifically target NR mutants.

A major challenge for the field is the actual discovery of such allosteric ligands, which is in part due to the high potency of orthosteric ligands, biasing screening campaigns. Several entries could be explored in order to identify allosteric modulators more easily. (1) By blocking the orthosteric site with a transcriptionally active or silent covalent modulator, NRs can subsequently only be modulated using an allosteric ligand, which can then be detected by conventional screening assays. (2) By (temporary) covalent tethering of compounds at a specific,
allosteric site of the protein. (3) By exploiting the orthosteric ligand as entry for generation of bitopic ligands for both expanded LBP s and pockets that are in close proximity. (4) Besides the above mentioned specific techniques and targets, also more diverse and directed screening methods, such as those based on computational approaches, fragment-based screening, and phage display could be further optimized or developed towards allosteric site targeting.

NRs have been shown to harbor multiple allosteric sites that can potentially be used for drug discovery. A number of promising examples of small molecule allosteric NR targeting concepts have recently been reported, illustrating the high potential of this concept. Inspiration for novel modes of allosteric NR modulation can be found in other receptor classes or, alternatively, via novel chemical concepts such as tethering, bitopic ligands and fragment based screening. The future for allosteric small molecule modulators of Nuclear Receptors is highly promising.

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References

