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SÍTIOS DE INTERAÇÃO ALTERNATIVOS EM RECEPTORES NUCLEARES E SUA VIABILIDADE COMO ALVOS TERAPÊUTICOS USANDO TRIAGEM COMPUTACIONAL E EXPERIMENTAL

Dissertação apresentada ao Programa de Pós-Graduação em Biologia da Relação Patógeno-Hospedeiro do Instituto de Ciências Biomédicas da Universidade de São Paulo, para obtenção do título de Doutor em Ciências.

Área de concentração: Biologia da Relação Patógeno-Hospedeiro

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Versão original

São Paulo
2017

RESUMO

Kronenberger T. Sítios de interação alternativos em receptores nucleares e sua viabilidade como alvos terapêuticos usando triagem computacional e experimental. [tese (Doutorado em Biologia da Relação Patógeno-Hospedeiro)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2017.

Receptores nucleares controlam a transcrição em células eucarióticas quando ativados por ligantes e, além do sítio de interação com ligantes, há outros sítios alternativos em sua superfície que podem ser alvo de compostos capazes de interferir com as interações proteína-proteína desativando o RN. A ativação do Receptor X de Pregnano (RXP) e do Receptor Constitutivo de Androstano (RCA) resulta na indução do metabolismo e efluxo de fármacos. Portanto, RXP/RCA sao responsáveis por causar reações adversas ou falhar terapias. Uma abordagem combinando a triagem experimental à nível cellular, em uma biblioteca de fármacos, e validação com ensaios *in vitro* e *in silico*, conseguimos identificar três novos antagonistas de RXP e cinco novos contra RCA, cada um com um perfil único de interação.

Palavras-chave: Receptor Constitutivo de Androstano, Receptor X de Pregnano, High-throughput screening, Agonistas inversos, Antagonistas, Descoberta de fármacos

ABSTRACT

Kronenberger T. Targeting Alternative Ligand-Binding Sites in Nuclear Receptors Using Computational and Experimental Screening. [Ph. D. thesis (Host-Pathogen Relationship Biology)]. São Paulo: Instituto de Ciências Biomédicas, Universidade de São Paulo; 2017.

Nuclear receptors can control transcription in eukaryotic cells in a ligand-dependent manner and, besides the ligand-binding pocket there is evidence of the existence of alternative ligand-binding sites on the surface, which can be addressed by small organic molecules that disrupt specific protein-protein interactions and thereby may antagonise NR function. Activation of pregnane X receptor (PXR) and constitutive androstane receptor (CAR) results in the induction of first-pass metabolism and drug efflux. Hereby PXR/CAR may cause adverse drug reactions or therapeutic failure of drugs. Therefore, PXR and/or CAR antagonists can minimise adverse effects or improve therapeutic efficiencies. Combination of cellular high-throughput screen identified CAR and PXR potent antagonists in a library of approved and investigational drugs. Further validated by cellular and *in vitro* assays, as well as molecular docking, suggesting additional or exclusive binding outside the classical ligand binding pocket. In conclusion, we here have identified three approved drugs as novel potent PXR antagonists and five potential CAR inverse agonists with differential receptor interaction profiles.

Keywords: Constitutive Andronstane Receptor, Pregnane X Receptor, Antagonist, High-throughput screening, Inverse Agonist, Drug-Discovery

1 INTRODUCTION

1.1 Nuclear Receptors

Biological systems are often characterised by a great complexity accomplished by a multitude of diverse interactions between its integral parts. This applies not only to the macroscopic level, but is also valid for the smallest biological building blocks, the cells, whether of protozoan or metazoan nature. Intracellular communication is required for retention of the organisation and the physiological properties of the cell as well as its adaption capability to varying conditions. In multi-cellular organisms the intercellular interactions play also a significant role exemplified by diverse endocrine signals. Referring to signal transduction, intracellular communication processes often comprise successive biochemical reactions triggering changes in the gene expression profile, energy status or cell locomotion, respectively. Chemical signals comprise small molecules such as steroidal hormones as well as cyclic nucleotides and phosphoinositide derivatives.

During the last decades, nuclear receptors (NRs) have emerged as key elements in the intracellular signal transduction of metazoans [1]. By responding to a large variety of hormonal and metabolic signals, NRs act as ligand-activated transcription factors, thus playing a crucial role in the regulation of gene expression. Moreover, NRs are targeted by other signalling cascades and integrate diverse signal transduction pathways involving them in numerous physiological processes comprising development, differentiation, homoeostasis and reproduction [2]. Although the signal molecules such as steroid and thyroid hormones have been isolated in the early 20th century, the targets of those compounds remained unknown for several decades. In 1974, the correlation between hormone action and alterations in the gene expression status was reported [3]. Later studies revealed the classic model of the NR signalling pathway described in detail in the next section [4]. The first NRs were cloned in 1985 and represented the starting point of the modern NR research [5–7]. Additional NRs were subsequently identified suggesting the existence of a large NR superfamily, which evolved from one ancestral orphan receptor up to the current status, composed of altogether six sub-families (NR1-NR6) [8–10]. The numerous and often delusive denotations of NRs finally led to an unified nomenclature system that relies on the homology to other NRs in the most conserved regions [11]. The number of NR genes between species is quite diverse. As an example, 21 NR genes have been revealed in *Drosophila melanogaster*, whereas in *Caenorhabitis elegans* more than

270 genes have been identified [12]. In humans altogether 48 NRs have been discovered so far, but the number of functionally different NR proteins is by far larger due to alternative splicing processes [13].

1.1.1 Signal-transduction and NR activity

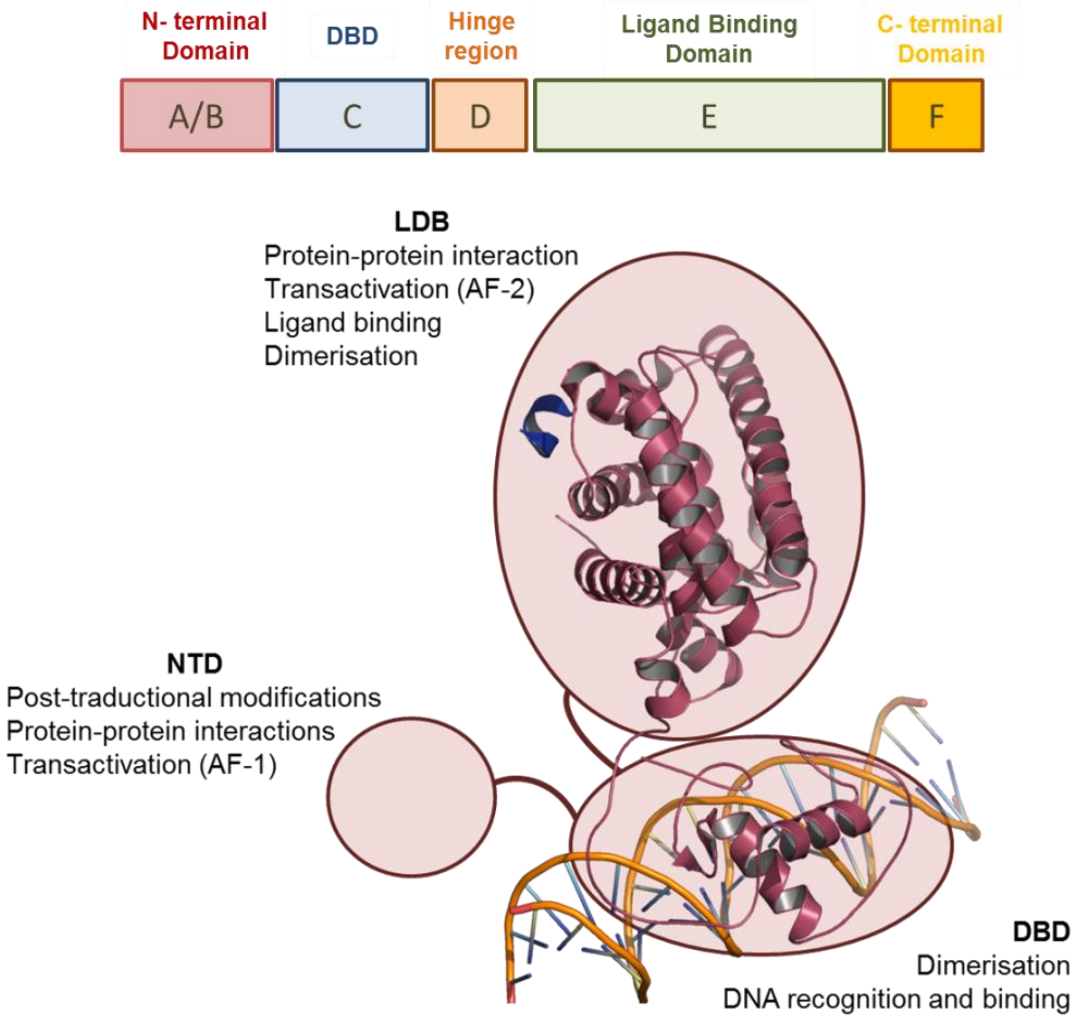
Gene transcription and protein translation processes are strictly regulated. Usually, transcription is prevented by the chromatin into which the DNA is assembled. Chromatin is the structural building block of a chromosome composed of nucleosomes [14]. Each nucleosome is composed of a core that is constituted by histone proteins around which the DNA is wrapped. Besides providing the lowest level of DNA compaction, nucleosomes are also important for gene regulation. Depending on the acetylation state of histone proteins, the chromatin adopts a more condensed or a more open form that prevents or allows the access of the basal transcription machinery, thus repressing or initiating protein biosynthesis. NRs regulate the gene expression by modulating the histone acetylation status of chromatin at their target gene, thus initiating or silencing the first step of the protein biosynthesis, the transcription process. NRs recognise and bind to specific binding sites in the promoter region of the gene referred to as response element (RE) [15]. Depending on the type of RE, NRs not only stimulate gene expression (positive RE), but also may have silencing effects via negative elements that are located in close vicinity of the transcription initiation site or even downstream of the TATA box [16–18]. The canonical core recognition motif of REs consists of a central hexameric element having the consensus sequence 5’-AGGTCA-3’ [19]. Number and configuration of the core motif, as well as the 5'-flanking region, determines the specificity and affinity of the binding NR [20,21]. The length of the spacer region between the core motifs influences the NR specificity as well [22,23]. Usually, NRs bind as homo- (Type I) or heterodimers (Type II) to their respective REs whose core motifs can be configured as direct repeats (DR), everted repeats (ER) or palindromes. Steroid hormone receptors (*e.g.* ER, AR, GR) almost exclusively recognise REs organised as palindromes whereas non-steroidal receptors (*e.g.* VDR, PPAR, RXR) recognise response elements of different configurations [24]. Activation of gene expression requires co-activators and other protein factors to be recruited to

the promoter-bound NR that serves as a nucleation site for a large multi-protein complex containing histone modifying and chromatin remodelling activities [25]. Usually, un-liganded NRs are complexed to co-repressors such as the silencing mediator of retinoid and thyroid receptors (SMRT) or the nuclear receptor co-repressor (NCoR) [26,27], which are both recruiting histone deacetylases and chromatin and, remodelling proteins thus rendering the promoter transcriptionally silent [28]. Distinct groups of co-activators with different properties are necessary for NR-dependent transcription: Bridging co-activators act as connectors between NRs and proteins carrying histone modifying or chromatin remodelling activities [29]. These type of co-activators, formed by members of the steroid receptor co-activator (SRC) [30] family, bind to NRs via their nuclear receptor interaction domain (NRID), an amphipathic helix containing a conserved LxxLL motif (L is leucine whereas x is any amino acid) [31,32]. Other types of co-activators are constituted by histone-modifying proteins (histone acetyltransferases, histone methyltransferases and histone kinases) and chromatin remodelling complexes both opening the promoter by unpacking the DNA from histones [28,33,34]. Furthermore, mediator complexes facilitate binding of RNA polymerase II to the promoter and thus help to establish the pre-initiation complex consisting of general transcription factors (*e.g.* TFIID) and the RNA polymerase II [35]. In contrast to NR activation, antagonist binding can stabilise the NR-co-repressor interactions or even prevent the NR from adopting an active conformation as shown for raloxifene or tamoxifen in the oestrogen receptor [36–38].

1.1.2 Structural Organisation

Nuclear receptors share a conserved structural and functional organisation (Figure 1). Altogether four distinct regions have been characterised which comprise the N-terminal region A/B, a conserved DNA-binding domain (DBD, region C), a linker region D and a ligand-binding domain (region E). Some nuclear receptors also contain a C-terminal extension (region F) of yet unknown function.

Figure 1 – Schematic illustration of a nuclear receptor.



Top – Schematic 1D of a nuclear receptor. Bottom – 3D structural illustration of the DBD (bound to DNA) and LBD (bound to hormone) regions of the nuclear receptor. N-terminal domain (A/B), hinge region (D), and C-terminal domain (F) are given in red, purple, and orange dashed lines, respectively. X-ray crystal structure (PDB code 3DZY of the PPARγ (violet) and RXRα (orange) DBD and LBD (cartoon representation) bound to DNA (CPK representation). The structure was solved in complex with NR agonists rosiglitazone (PPARγ) and 9-cis retinoic acid (RXRα) as well as coactivator peptides (blue ribbons).

1.1.3 DNA-Binding Domain

Nuclear receptors recognise their specific target gene via the DNA-binding domain (DBD). The DBD represents the most conserved domain in NRs that is composed of two zinc-finger motifs and a C-terminal extension (CTE) domain encoded by roughly 60-70 amino acids [39]. Each zinc atom is coordinated in a tetrahedral arrangement by four highly conserved cysteine residues [40]. The three-dimensional structure of the DBD has been revealed by NMR and X-ray studies (Figure 1) [41–43].

Recognition of the target DNA is achieved by the so-called P-box located in the first zinc-finger whereas the second one harbours the D-box which provides the dimerisation determinants [23]. DBD post-translational modifications have been reported to modulate the receptor activation, such as CAR (Thr38) and TR [44]. Unlike the core DBD sequence, the CTE is not conserved among NRs and may adopt diverse structural motifs with diverse functions comprising sequence recognition or receptor dimerisation, respectively

1.1.4 Ligand Binding Domain

The LBD is a multifunctional domain that, besides the ligand binding site, also carries the ligand-dependent transcription activation function 2 (AF-2) [45,46] as well as a dimerisation motif for RXRα [47,48] and a nuclear localisation signal [49]. Additionally, the LBD harbours binding sites for heat shock and co-regulatory proteins [29,50]. Despite a considerable variability in sequence, the LBDs of all nuclear receptors possess a canonical structure in which 12 to 14 α-helices, together with a (2-5) stranded-sheet, which are arranged in an anti-parallel, three-layered helix sandwich (Figure 1) [51–53]. Located between the outer sandwich layers, the ligand-binding pocket (LBP) is mainly constituted by hydrophobic aminoacids [54]. The size of the LBP can diverge considerably among different NRs ranging from 100 $Å^3$ (ERR) up to 1300 $Å^3$ (PPAR) [55–57], with exceptions such as NURR1 with an uncommonly small ligand binding cavity [58].

Nuclear receptors are associated with many diseases and therefore represent promising drug targets. Androgen receptor (AR) and oestrogen receptor α (ERα) are targeted for the treatment of various forms of cancer (AR: prostate cancer, ERα: breast cancer) while PPARγ plays a role in cancer and diabetes and, depending on the disease is addressed by agonists and antagonists. Hepatocyte nuclear factor 4 α (HNF4α) is involved in maturity-onset diabetes of the young, type 1. Pregnane X receptor (PXR) in turn is responsible for drug metabolism and is also involved in metabolic diseases.

1.2 Drugs Modulating Nuclear Receptors

Besides other drug targets such as G protein-coupled receptors, ion channels, receptor tyrosine kinases and immunoglobulin-like receptors, nuclear receptors represent another major receptor target class. Of the 48 nuclear receptors in humans, about a third is already targeted by approved drugs. Most of these receptors belong to the subfamilies NR1 and NR3 and the main indication areas are cancer and hormone replacement. As of 2011, 76 approved drugs targeting 17 nuclear receptors were available [59].

A classical NR-drug is tamoxifen, the first approved agent for breast cancer prevention and treatment. The compound is a potent antagonist of the oestrogen receptor (ER), belonging to the steroid hormone receptors. Being a prodrug, tamoxifen requires conversion by cytochromes CYP2D6 and CYP3A4 in the liver into the bioactive forms 4-hydroxytamoxifen and N-desmethyl-4-hydroxytamoxifen, respectively. The metabolites have improved affinity for the ER by the fold of up to 100 times [60]. The tamoxifen metabolites can be taken up to the breast tissue, acting then as competitive inhibitors of oestrogen hormones in the ligand pocket of the ER. Curiously, in other tissues such as the endometrial, tamoxifen acts as potent ER agonist [61]. ER three-dimensional structures, determined in co-crystallisation studies with both the drug and peptide of the protein partners, show that the ligand induces an inactive conformation of the receptor, by sterically displacing H12 from the active conformation [57].

Although significant anti-breast cancer effects of tamoxifen justify its application, the drug reveals several serious side effects. Tamoxifen can act either as an agonist or antagonist on different tissues, depending on the estradiol concentration and hence the menopausal status of the patient. The effects of the use of tamoxifen use during the pregnancy are also adverse, foremost resulting in foetus malformation [62].

Another selective oestrogen receptor modulator is the benzothiophene raloxifene. The compound is not a prodrug as tamoxifen. Also, its effect is different as raloxifene does not show any antitumor activity. Instead, the compound is used for prevention of osteoporosis and may reduce the incidence of breast cancer in postmenopausal women.

Both SERMs are T-shaped molecules. The core structure of both compounds is planar and binds in a similar orientation into the ligand-binding pocket as the natural

ligand 17β-estradiol. The side chain protruding from the core structure is responsible for the sterical displacement of AF-2 from the active conformation, thereby preventing co-activator binding and eventually inactivating the receptor.

The ligand-binding pocket of several nuclear receptors has already been exploited for pharmaceutical purposes. Several approved drugs either activate or inhibit nuclear receptor function by competing with the natural ligand. The first drug targeting a nuclear receptor was cortisone that GR, using the purified cortisone from gland extracts, in order to reduce the inflammatory effect.

The cortisone synthesis opened the door for more potent glucocorticoid receptor agonist currently in use for therapy of inflammatory diseases, such as the dexamethasone and the prednisolone. The crystal structure of the GR with dexamethasone is an example of how the ligand binding can influence the LBD conformation since the AF-2 helix is present in an active position allowing the co-activator protein to interact [63]. On the other hand, GR structures with the antagonist (RU486) showed a dimethylaniline group preventing the AF-2 to assume the active position [64]. Those two studies together suggest that AF-2 helix, as well the region nearby, can be disposed to high flexibility leading to ligand-induced conformational changes.

This structural knowledge allowed the chemistry to synthesise compounds which are able to activate only a subset of the GR functions, modulating the expression of specific genes, thus retaining the anti-inflammatory effect, altogether reducing the side-effects on the bone metabolism and glucose control [65,66].The entire steroid hormone ligand binding pocket linger common structures that allow them to bind natural hormones, for instance, 75% of their residues are hydrophobic accommodating the steroid hormone common scaffold. It is also known that the few conserved polar residues are responsible by orientating the hormone towards the proper fitting within the pocket between helices 3 and 5 [67]. Besides the GR and the ER mentioned before, other steroid receptors which have been exploited as drug-target, are the androgen receptor (AR). Unlike the other receptor mechanism, testosterone (or its reduced form 5-dihydrotestosterone) binds to the NR and induces the FxxLF motif of the N-terminal domain to interact with the AF-2 region [68]. 5-dihydrotestosterone dissociates more slowly than testosterone from AR and thereby better stabilises the AR complex.

AR is known to be involved in several diseases, among those the prostate cancer is worthy to be highlighted since the androgen can stimulate growth, survival and also inhibit apoptosis. One common therapy includes the androgen deprivation, however more recent approaches indicate the interference with androgen binding [69,70]. There are two major pathways targeted in prostate cancer: i) the inhibition of CYP17A1, an enzyme required for the androsterone biosynthesis by drugs such as abiraterone and ii) the direct interference with the androgen receptor by antagonists (*e.g.* galeterone and enzalutamide).

The enzalutamide (or MDV3100) is an androgen receptor antagonist, designed to bind to the ligand binding pocket and thereby preventing AR translocation to the nucleus [71]. Enzalutamide was able to prolong the life of cancer patients, who did not receive chemotherapy, with only a few registered side-effects [72].

Mutations in LBD render the competitive inhibitors, therefore, several groups have focused on the DBD. The use of virtual screening combined with the chemical improvement of compounds resulted in molecules able to inhibit enzalutamide-resistant cells [73,74].

Another receptor targeted by FDA-approved drugs belong to the group of peroxisome proliferator-activated receptors (PPAR) [75]. Three PPAR subtypes have been identified: alpha, delta (also called beta) and gamma. All receptors have different roles in the regulation of the lipid and glucose metabolism. Drugs targeting PPARs are mainly used for the treatment of metabolic syndrome, in particular for lowering triglycerides and blood glucose level. Endogenous ligands of PPARs comprise free fatty acids and eicosanoids. Some compounds are specifically binding to PPAR subtypes such as prostaglandins for PPARγ.

Fundamentally, the full agonist is able to interact with polar residues within the ligand binding pocket stabilizing the interaction with the H12, more exactly the Tyr473 within the AF2 helix, while the partial agonist does not have this polar interaction or interact on someplace else [76,77].

The ligand pocket of PPARs is normally large in order to accommodate different fatty acids, the PPAR pockets can be divided into three arms: one rich in polar residues and other two hydrophobic. The first one with polar residues includes the activation function 2 (AF-2 helix), able to bind to the thiazolidinedione group. The hydrogen bond network between the ligand and the amino-acid residues Tyr464, Tyr314 and

Ser280 on PPARα and Tyr473, His449, His323 and Ser289 on the other two PPAR [78].

Compound selectivity for the different PPARs can be achieved by the exploitation of just a few residues by contacting of the compounds with the lateral residue of those mentioned amino-acids, as following: PPAR alpha has the major affinity towards saturated fatty acids; PPAR gamma and delta presents two histidine residues allowing the interaction with unsaturated fatty acids and PPAR delta has a couple of specific bulky residues Val334, Leu339 and Ile364, important for the selectivity of the ligands [76,79].

Contrary to PPAR alpha and gamma, which has been widely exploited as drug-targets, the PPAR delta has no clinically relevant ligands. Besides several computational efforts towards selecting specific agonists [80–82], just one molecule has reached phase II clinical trial for the treatment of dyslipidaemic syndrome or diabetes [83]. In the last years, the focus has been drawn on PPAR delta which established the receptor as new drug-target. This has been corroborated by the function of PPAR-delta in the fatty acid metabolism, insulin resistance, reverse cholesterol transport and inflammation as discovered by knockout animals and specific drugs [84–86]

PPAR-delta was initially exploited by the use of nonsteroidal anti-inflammatory drugs [87]. The use of PPAR-delta antagonists and agonists have been tested on the cancer therapy, due to the high expression levels of this receptor in prostate cancer cells and colorectal tumours respectively [88].

At last, the development of non-specificity drugs leads to the appearance of pan-drugs (either agonists or antagonists), which are able to bind and modulate common interfaces or pockets among the different PPARs. Due to the significant similarity among the different PPAR isoforms, several synthetic dual- or even pan-agonists were developed.

The use of pan-agonists was first proposed with the glitazars, aiming specifically the alpha and gamma subtypes, are looking forward have the benefit of the TZD, such as the reduction of the sugar levels without the cardiovascular risks [89]. The most well-known fibrate with a pan-agonism profile of activity is the bezafibrate, proven to delay the diabetes progression in patients with sugar imbalance, increase of HDL cholesterol and a reduction of triglycerides [90].

1.3 Xenobiotic metabolism and the role of xenosensors

Most nuclear receptors are addressed because of their direct involvement in a disease. However, some members of the NR superfamily are interesting because of their involvement in drug metabolism. This process comprises three phases and involves modification processes (*e.g.* oxidation, reduction, hydroxylation), coupling reactions with hydrophilic compounds (*e.g.* glucuronic acid, glycine) and finally excretion of the metabolised molecules from the cell. Both pregnane X receptor (PXR) and constitutive androstane receptor (CAR) are responsible for ligand-dependent induction of gene expression of proteins involved in phase I-III. Due to their ability to bind structurally and chemically diverse molecules, both receptors are also termed xenosensors.

It is suggested that CYP3A4 (Phase I enzyme) is responsible for the turnover of about 50% of the known drugs and, which correspond to the multi-resistance drug-gene (MDR1) spectrum of drugs [91]. Several authors have pointed out the leadership of CAR and PXR on the xenobiotic-sensing and detoxification (as reviewed by [92,93]) and regulation of either the cytochrome protein expression (highlighting the CYP3A4) or drug efflux mediated by the MDR1 [94].

1.3.1 Constitutive Androstane Receptor (CAR)

The constitutive androstane receptor (CAR, NR1l3) is a transcription factor from the nuclear receptor family, whose activity is regulated by endogenous and exogenous ligands [95]. Originally designated as orphan receptor due to the absence of any known endogenous ligand [96], a large set of compounds modulating CAR activity has been identified up to now [93]. CAR, together with the pregnane X receptor (PXR, NR1I2), is involved in the metabolism of drugs and environmental chemicals by regulating the expression of important phase I and phase II metabolising enzymes as well as transporter proteins in a ligand-dependent manner [97,98].

Another nuclear receptor involved regulation of drug metabolism is the constitutive androgen receptor (CAR). Known CAR ligands comprise 5-androstan-3-ol and 5-androst-16-en-3-ol, but also pregnanedione [96,99]. The main expression of CAR is in the liver tissue and the intestine and reveals a pronounced basal activity *in vitro*

[100,101]. Alternatively spliced variants of CAR have been also detected in adrenals, brain, spleen, prostate and testis that do not possess basal activity [102,103].

In a similar way, as PXR does, CAR can dimerise with RXR and bind an ER6 (everted repeat of the response elements separated by 6 base pairs) in order to induce the expression of CYP3A4 genes [104]. Furthermore, when bound to phenobarbital, CAR can bind to DR4 motifs (*i.e.* two direct repeat response elements separated by 4 bp) within the enhancer PBREM (phenobarbital-responsive enhancer module) region of CYP2B genes [105]. Phenobarbital-like molecules such as pesticides or contaminants can act as CAR agonists, that would include chlorpromazine, phenytoin, dichlorodiphenyltrichloroethane (DDT), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) as well as polychlorinated biphenyls [105,106].

Despite the interplay and overlap of ligands and target genes between CAR and PXR, specific CAR activation can contribute to drug resistance [107] and drug-induced hepatotoxicity [108]. Additionally, it is well known that CAR can remain constitutively active even in the absence of ligands, depending on the cellular context [109]. Besides exogenous compounds, CAR activity can also be modulated by endogenous ligands, *e.g.* bile acids, dehydroepiandrosterone, androstenol and androstanol [96,110]. In this sense, CAR modulation participates in physiological conditions like obesity, diabetes and tumour development; as well it regulates pathways related to hepatic energy homeostasis [111], insulin signalling [112] and cell proliferation [113,114].

Several different CAR splice variants have been reported, of which most are inactive. [115,116]. Only three of these have been described to be physiologically relevance: the canonical CAR1 (also termed CAR-SV1, 348 amino acids), CAR2 (CAR-SV3, containing a four amino acid insertion – VSPT – between helix 6 and 7) and CAR3 (CAR-SV2, containing a 5 amino acid insertion – APYLT – between helix 8 and 9) (Auerbach *et al.*, 2003). Different isoforms possess distinct functional profiles. While both CAR1 and CAR2 are constitutively active, CAR3 does not possess a basal activity. The ligand spectrum of CAR1 and CAR2 is different, CAR1 is inducible by CITCO or pregnanedione and the basal activity can be reduced by androstanol or meclizine [118,119] whereas only a few agonists have been identified for CAR2 (*e.g.* the plasticizer di(2-ethylhexyl) phthalate), which are specific for that isoform [106]. In contrast to CAR1 and CAR2, CAR3 is not constitutively active and is more sensitive

to ligand activation, such as CITCO [120]. The weak CAR1 antagonist clotrimazole was, surprisingly, an agonist for CAR3 and for the chimerical construct CAR1+A, which mutates the five amino acids insert for a single alanine [118,121]. On the other hand, strong CAR1 antagonist PK11195, a benzodiazepine receptor ligand, cannot further reduce the low CAR3 activity, regardless of CITCO treatment [122]. Recently, a qHTS assay has been used to identify CAR1+A non-specific modulators, resulting in the discovery of 115 CAR1+A activators and 154 antagonists [123].

Regardless of the new molecular study tools for CAR and the emergence value of this receptor as a drug target for several syndromes, so far only few inverse agonists are known: clotrimazole, meclizine, androstenol, PK11195 [96,118,122,124] and more recently CINPA1 and its analogues [125,126]. All compounds share at least some degree of cross-reactivity with other receptors, mainly PXR (Chai *et al.*, 2016).

1.3.2 Pregnane X Receptor

The pregnane X receptor (PXR, NR1I2) together with the closely related constitutive androstane receptor (CAR, NR1I3) constitutes the xenobiotic sensing system in liver and intestine [127,128].

PXR is one of the most promiscuous nuclear receptors as it binds several clinically used drugs, among them calcium channel blockers, statins, antidiabetic drugs, HIV protease inhibitors and also artemisinin and its derivatives [127,129]. PXR X-ray crystal structures have unravelled the basis for the pronounced ligand promiscuity of the receptor. Due to a broken and kinked helix 6 as well as a long loop connecting H1 and H3 that is part of the ligand-binding pocket, the receptor can adapt to structurally and chemically diverse molecules. The molecular weight of compounds binding to PXR varies from 200 to more than 800 Da with rifampicin as largest known PXR agonist. PXR is constitutively expressed in the liver and intestine [130,131]. Upon activation, the receptor forms heterodimers with RXR. In particular activation of PXR may result in drug-drug interactions, adverse drug reactions or therapeutic failure of drugs [132,133]. PXR is activated by various small molecules, including clinically used drugs, environmental pollutants and natural products, but also endogenous compounds such as bile acids and pregnane metabolites [93,134].

In contrast to a large number of known PXR agonists, only few PXR antagonists have been reported so far. These comprise natural products such as ecteinascidin 743 [135], sulforaphane [136], coumestrol (Ekins *et al.*, 2008; Wang *et al.*, 2008), camptothecin [139], fucoxanthin [140] and sesamin [141], as well as synthetic compounds PCB197 [142], A-792611 [143], SPB00574 and SPB03255 [138] as well as the synthetic ketoconazole derivative FLB-12 [144,145]. In addition, several approved drugs, including ketoconazole, fluconazole, enilconazole, itraconazole [138,146], metformin [147] and leflunomide [138] have been identified to inhibit PXR.

Besides the conventional model of NR inhibition, which requires antagonist binding to the ligand-binding pocket (LBP), another mode of action is based on inhibitors addressing non-conventional sites on the ligand-binding domain (LBD) surface [148]. In addition, coumestrol has been shown to effectively inhibit constitutively active PXR mutants containing bulky LBP-filling amino acids (Wang *et al.*, 2008), which suggests an additional binding site. FLB-12 has been suggested to exclusively bind outside of the LBP, as these compounds did not compete for binding to PXR with an established agonist *in vitro* but still inhibited the constitutive activity of the LBP-filled mutant [145]. In addition, molecular *In silico* pharmacophore modelling and molecular docking studies suggested binding of several known antagonists to the surface of the AF-2 region [138,149].

PXR antagonists could provide the opportunity to prevent or minimise drug-drug interactions or could be applied in order to reduce hepatic clearance, thus allowing a lower drug dosage [150]. By screening a compound library of approved and investigational drugs using a cell-based assay, followed by a set of confirmatory cellular assays, as well as *in vitro* binding and molecular docking investigations, we here have identified and characterised three drugs as novel PXR antagonists, which differentially interact with the receptor.

1.4 Methods to assess ligand binding and/or activation of nuclear receptors

In the last decades, a couple of molecular and cell biology methods have been applied to investigate nuclear receptor function and regulation [151]. In the following, a selection of most relevant methods is presented that are frequently used in NR research in academia and pharmaceutical industry.

Standard molecular biology techniques like cDNA cloning have been used to identify the genes for orphan nuclear receptors. For the identification of hormone-response elements, electrophoretic mobility shift assays (EMSA) and chromatin-immunoprecipitation (ChIP) have been used as well as GST-pull down assays [152,153]. For the identification of natural ligands standard transactivation assays, GAL4-LBD fusion transactivation systems and also image based receptor translocation assays were used [154,155].

In order to demonstrate the direct and high-affinity binding of a ligand, a variety of compound binding assays have been developed. These can be divided into several classes. The fundamental ligand binding assay measures the competition for binding to the receptor with a known ligand [156]. For investigation of receptor conformational changes after ligand binding protease sensitivity and non-denaturating gel electrophoresis assay formats have been used. Another assay type that reports molecular consequences of ligand binding is called Co-activator-dependent receptor ligand assay (CARLA), which refers to a modified GST-pull-down assay [157]. More complex approaches include the protein nuclear magnetic resonance (NMR), which allows the analysis of conformational dynamics due to ligand binding in solution [158,159]. Recent studies associate an important role for protein dynamics in the mechanism of action of nuclear receptor ligands [160]. Detailed analyses of the macromolecular interaction of ligand binding including affinity and binding kinetics are performed by the surface plasmon resonance (SPR) technology [161]. This sensitive technology overcomes the limitations of old-fashioned filter binding assays, which were limited to low affinities or indirect non-equilibrium methods. In the standard SPA BIAcore approach small amounts of receptor protein are immobilised onto a solid phase, while the ligand is introduced to flow over the surface. Binding of the ligand leads to an increase in the refractive index. A linear relationship exists between the mass (concentration) of molecules bound the surface of the ship. SPR allow analysis of high-affinity protein-protein, protein-DNA interaction and the binding of small molecules [161].

Due to their relevance as therapeutic targets, the pharmaceutical industry prioritised the development of novel assay systems that allowed speeding up the throughput and the screening of large compound collections. Therefore in the last decade, a couple of academic laboratories, as well as big pharmaceuticals and biotech companies, spent much effort in the development of HTS compatible screening

assays which assess ligand binding and/or activation of nuclear receptors (Reviewed by [162,163]). These efforts led to modified methodologies with higher throughput. Despite the fact that these assays have been designed for certain targets the principles could be expanded to any NR, making these assay formats accessible to screening purposes.

Most common screening systems for nuclear receptor activation are cell-based„ transactivation assays“. These assays rely on the potential of nuclear receptors to activate transcription in response to binding of a ligand [164,165]. This is achieved by transfection of a cell line with an expression vector for the receptor and a reporter vector that has the binding site for the receptor and also encodes for a protein that, when incubated with the appropriate substrate, yields a product that can be measured. The most common method for evaluation NR activation involves transient transfection of a receptor and a response element-reporter gene construct [97]. Binding of a ligand to the receptor leads to a binding of the activated receptor to the binding site on the reporter construct and an activation of transcription of the reporter gene. The main advantage of these cell-based assay is that they allow screening of large compound libraries in reproducible fashion [163].

By far, the most common method involves transient transfection and until now many cells lines have been described as possible recipients of these vectors, including CHO, HuH7, MCF-7, HEK293, HepG2 and Caco-2 cells [98]. Using transient transfection systems a couple of investigators identified activators for human PXR, CAR and AhR (extensively reviewed by [93]). Testing known drugs within these systems were able to identify therapeutic agents which activate NR and cause Drug-Drug Interactions (DDI) with high correlation [162,164,166]. Comparing those results with the ones from cell-based systems using primary cultures of hepatocytes, it was found that primary hepatocytes do not allow this identification due to the co-regulation of the receptors and the crosstalk for target genes found among human CAR, PXR and AhR [167].

To reduce the variability associated with transient transfection stable cell lines have been generated that contain NR and the NR response element-reporter genes. In the past, a couple of cell lines have been generated to express hPXR and were used to predict the potential for DDI of new drugs in a rapid and simple approach [168]. The system allows multiplexing, which means that a single well can be used to determine a parameter for receptor activation, cell viability and induced enzyme activity [168–

170]. Results from stable transfected cells can vary from standard transiently transfected assays and even primary cell-lines, which have to be taken into consideration [171,172].

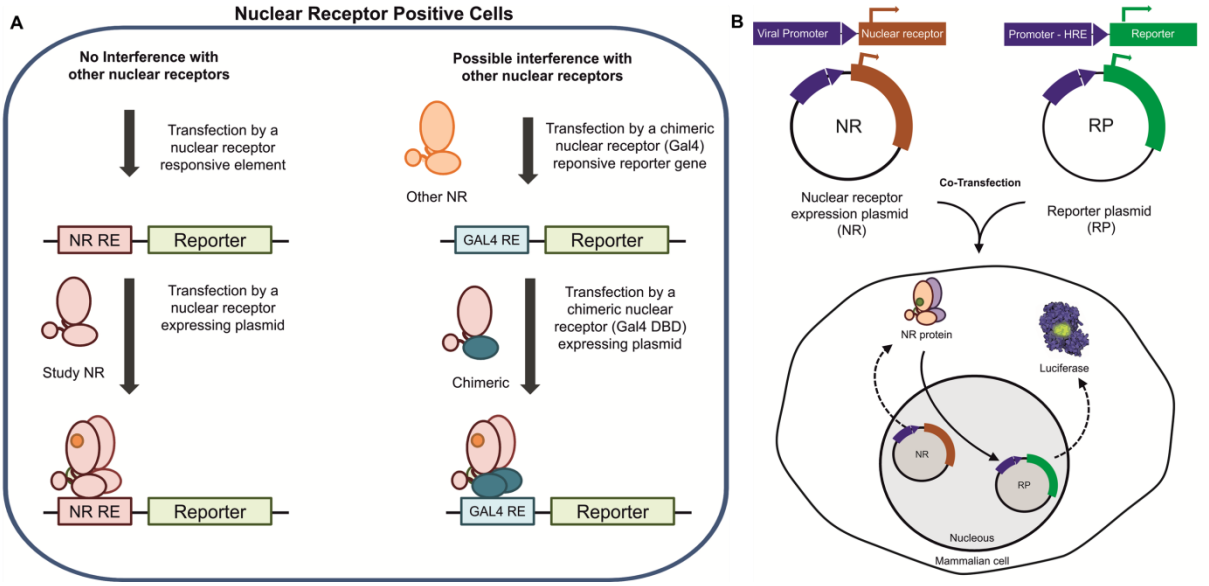
An alternative or even complementary to transactivation assay system is the mammalian two-hybrid system. This assay system represents a powerful approach for detecting protein-protein interactions in cells, which has evolved from the original two-hybrid system into a method for identifying NR ligands. The system is based on the finding that co-activators and co-repressors are involved in the regulation of NR function. Recruitment of co-activators upon ligand binding is a critical step in NR-mediated transcriptional activation. Following ligand binding, many NR undergoes a conformational change (*e.g.* PXR) and form a specific co-activator binding pocket, which permits the interaction with the receptor. In the mammalian two-hybrid approach, chimerical receptors containing the LBD of interest are fused to the DBD of the yeast transcription factor GAL4. The DBD binds to specific DNA response elements. The interaction between NR and its co-activator is detected using a reporter gene containing multiple copies of the GAL4 upstream activating system (UAS). Agonist binding to the LBD induces the formation of a complex which promotes the assembly of RNA polymerase II complex and subsequently enhances the transcription of the reporter gene [173].

Examples are mammalian two-hybrid assays consisting of the LBD of hCAR and co-activator SRC-1 fused to GAL4 (DBD). In that assay, the ligand binding enhances the interaction between LBD and SRC-1, which is detected by the reporter gene activity [120]. With similar assay setup, a couple of agonists and inverse agonist were identified for human CAR even if some results were contradictory [174]. It was speculated that the use truncated of chimerical receptors or resulted in subtle conformation changes and unspecific protein-protein interactions [98], leading to the conclusion that full-length receptors would be more sensitive and closely to the *in vivo* situation [163].

The study of PPAR inhibitors greatly benefited from the use of the double transfection system, using an expression plasmid of the NR and reporter gene construction. Despite the variety of different constructs, they all follow the same pattern; the NR construct is normally a plasmid that facilitates constitutive expression of the entire open reading frame of the NR in mammalian cells. The reporter construction resides commonly the open reading frame of luciferase which is cloned

downstream of a promoter region fragment coupled to the hormone responsive element (HRE) or an enhancer [175]. Both plasmids can have resistance cassettes for stable transfection or be used on the basis of a transient expression system Figure 2B.

Figure 2 – Cell based assays for nuclear receptor studies



A) Transactivation assay rationale, the plasmid with constant expression B) Flow chart for decision between the different transactivation strategies, classical and one-hybrid assays, and the situations requiring each technique

Within this framework, the normal NR action follows: A mammal expression vector is used for the expression of the NR, which binds to the hormone, dimerises and interacts with the DNA sequence of the HRE at the reporter gene plasmid leading to the liberation of luciferase expression. In the presence of compounds with inhibitory/modulatory activity, the functionality of the NR can be verified by differences in the luciferase expression levels (Figure 2A). Another different methodology uses a similar general strategy however the mammalian expression vector resides the NR LBD fused to a GAL4 yeast DNA binding domain followed by the GAL4 promoter and the luciferase gene [176].

In principle, the two amended transactivation assays are applicable to high-throughput screening (HTS) and valuable for drug discovery. Quality criteria which characterise a certain assay for HTS are robustness, the sensitivity and

reproducibility in identifying compounds that display significant NR binding or activation. A very useful parameter which describes the criteria is the screening window coefficient (termed Z-factor), which is used to define the ratio of the separation band to signal dynamic range of a given assay [59]. This coefficient takes into account the signal dynamic range, the data variation associated with reference and control measurements. The Z-factor is a well-accepted parameter for evaluating HTS assay quality (from 0.5 – 1.0) and a characteristic parameter of hit identification for a given assay under screening conditions [163,177].

With regard to high throughput ambitions, a couple of non-cell based assay formats were developed which passed these criteria (128-130). Rouleau & Bossé (2006) described Assays based on AlphaScreenTM Technology, for oestrogen receptor alpha (ERα) and retinoic acid receptor gamma (RARγ) as models to demonstrate important steps and principles to the development of AlphaScreen assays for NRs.

AlphaScreen^{TM} (Amplified Luminescent Proximity Assay) is a non-radioactive homogeneous proximity assay that relies on the transfer of energy between an acceptor and a donor bead brought into proximity via biological interaction. The donor beads are embedded with a photosensitizer (phthalocyanine), which converts oxygen to an excited state upon illumination. If a biomolecular interaction drags an acceptor bead into close proximity of a donor bead, the excited singlet oxygen will transfer its energy to the acceptor bead leading to the emission of light depending on the fluorophores in the acceptor beads (Tioxene, anthracene and rubrene, emission of light at 520 – 620 nm). Each donor bead is capable of generating up to 60.000 singlet oxygen with a half-life of 0.3 sec, allowing measurements in a time-resolved mode and with substantial signal amplification. The technology can use to rapidly develop high-throughput screening (HTS) assays for NRs [59,178].

The principle of the assay involves two major steps: a) a ligand-activated biomolecular interaction between NR and its co-activator, followed by b) the detection of that interaction using AlphaScreen compatible reader technology. It is well established that following agonist binding, allosteric changes in the LBD of NR will allow the interaction between the AF-2 domain in the LBD and the NR box present in the coactivator structure. A consensus sequence present on all NR co-activators (LxxLL motif) is sufficient for the interaction with the agonist-bound receptor LBD. To establish assay configurations for certain NR, different components can be chosen depending on their cost and availability. Two general configurations are possible: i)

Interaction between agonist, antagonist bound receptors and co-activator/co-repressor; ii) Interaction between agonist bound to the ligand pocket domain and LxxLL motif-containing peptide. Depending on the detection reagents, the binding partners can be wild-type, truncated, or tagged with, for example, biotin, FITC, GST, Flag, c-Myc, HA or His_6 [179].

An assay based on the interaction between receptor and co-activator will generate a signal increase upon agonist binding while a signal decrease is following antagonist binding. The interaction with co-repressors could also be used to find ligands acting at different molecular events. A significant study by Rouleu & Bossé (2006) showed, that AlphaScreen Assays for NR can be performed in 384 well plates with an intraplate variability generated CV 10% and Z values > 0.7 [179].

Another well-validated assay type for studying NR-ligand interactions is based on the Lance^{TM} Technology [179]. In the Lance assay, a signal is generated when a donor molecule labelled with chelated Europium (Eu) gets into proximity of an acceptor molecule by its time labelled with allophycocyanin (APC). When a biological interaction brings the donor and the acceptor into close proximity, excitation of the Eu-chelate at 340 nm allows Fluorescence Resonance Energy Transfer (FRET) to the acceptor APC molecule resulting in fluorescence emission at 665 nm. Long stakes shift and excited-state lifetimes of Europium complex of hundreds of microseconds make this technology perfect for Time-Resolved FRET (TR-FRET). In principle different binding partners can be used: i) Interactions between agonist-bound receptor or receptor LBD and LxxLL motif-containing peptide and/or ii) interaction between an apo- or holo-receptor and the co-repressor interaction domain.

In the literature, a couple of examples are present describing the use of LANCE assays based on the interaction between the receptor and co-activator-derived peptide [180–182] (134-136). Most of these assays involve the interaction between biotinylated LxxLL peptides derived from a co-activator sequence and tagged receptor LBD. The complex formation is detected using Eu-labelled antibody and APC-labelled streptavidin. When both binding partners are biotinylated, the Lance assay can be performed using commercially available Eu-streptavidin and streptavidin-APC [183]. A great advantage of applying the LANCE technology is the long signal stability, which can reach more than 48 h, allowing batch processing in HTS.

Besides experimental approaches, also computational methods are extensively applied in order to study nuclear receptor function. The availability of X-ray crystal data allows employment of virtual screening methods by which 3D structures of a virtual compound library are docked into the ligand-binding pocket of a receptor and the resulting binding modes are evaluated using a scoring function. Top-scored molecules are usually purchased from vendors and tested *in vitro* for example in transactivation assays as described above in order to validate the computational results. Once identified as real NR modulators, further experimental and computational studies can be conducted in order to investigate the mechanism of action or interaction partners in the cell.

A large number of successful virtual screening campaigns suggest that the various docking algorithms are well suited in order to identify NR agonists and antagonists. In case a NR structure is not available homology modelling techniques can be used in order to obtain structural data. For NRs this approach works quite well due to the highly conserved LBD structure and successful virtual screening campaigns against LBD models have been reported as well [184].

1.4.1 Alternative Binding sites

In recent years several alternative ligand-binding sites next to the ligand-binding pocket, located on the surface of the ligand-binding domain, have been discovered. The first identified alternative binding pocket is the co-activator binding site (AF-2 site), that can be targeted by small molecules in order to prevent NR-co-activator interactions, thereby antagonising the receptor. A second site has been identified on the surface of AR (BF-3 site) that is conserved in related receptors (*e.g.* NURR1 and PPARγ) and which allosterically communicates with the AF-2 site. Hence addressing BF-3 also disrupts co-activator binding (Buzon, Carbo et al. 2012). In addition, the LBD-DBD interface in the DNA-bound HNF4α homodimer provides a third alternative binding site for receptor modulators (Chandra, Huang et al. 2013). So far only very few AF-2 and BF-3 targeting compounds have been identified (Moore, Mayne et al. 2010).

5. Concluding Remarks

Nuclear receptors are an important protein family involved in many physiological processes and therefore attractive drug targets. There are many successful stories about nuclear receptor modulation by small molecules and several NRs drugs have been approved. As only a fraction of receptors is addressed by drugs, there is still a tremendous potential for future drug discovery. Despite the classical LBP capacity as interesting a drug-target, alternative approaches using other interaction sites on the protein surface suggest further possibilities for interference with nuclear receptors by small molecules.

This study provides evidence that CAR and PXR receptor inhibition by approved drugs may result in unwanted drug-drug interactions and also corroborates the existence of several ligand-binding sites on the LBD that allow different ligand interaction profiles and modulation.

Our validation protocol by cellular and *in vitro* assays, as well as molecular docking, suggests additional or exclusive binding outside the classical ligand binding pocket for most of the discovered ligands. In conclusion, we here have identified three approved drugs as novel potent PXR antagonists and five potential CAR inverse agonists with differential receptor interaction profiles.

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