Orphan nuclear receptors in drug discovery

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Orphan nuclear receptors provide a unique resource for uncovering novel regulatory systems that impact human health and also provide drug targets for a variety of human diseases. Ligands of nuclear receptors have been used in several important therapeutic areas, such as breast cancers, skin disorders and diabetes. Orphan nuclear receptors, therefore, represent a tremendous opportunity in understanding and treating human diseases. Here, I highlight recent advances in the use of orphan nuclear receptors and their potential as targets for drug discovery in diabetes, obesity, neurodegenerative diseases and other related disorders.

Introduction

Nuclear receptors are ligand-dependent transcription factors that have important roles in several biological processes, including cell proliferation, differentiation and cellular homeostasis [1]. The primary function of nuclear receptors is to mediate transcriptional responses to hormones and other metabolic ligands through the recruitment of a range of positive and negative regulatory proteins, referred to as co-activators or co-repressors. The recruitment of co-activator complexes is a crucial step in ligand-induced transcription, whereas the recruitment of co-repressor complexes mediates active repression of unliganded nuclear receptors. The target genes of nuclear receptors comprise a complex genetic network, in which their coordinated activity defines the physiological hormonal responses.

Nuclear receptors contain several functional domains that are defining structural features for members of the nuclear receptor superfamily. In general, the receptor structure comprises an amino-terminal activation domain AF-1, a DNA-binding domain (DBD), a hinge region, a conserved ligand-binding domain (LBD) and a second activation domain, AF-2, which is located at the carboxy-terminal end of the LBD and mediates ligand-dependent transactivation by nuclear receptors (Figure 1a). The LBD mediates nuclear localization and contains sites for co-activator and co-repressor interactions. Members of the nuclear receptor superfamily include the well-known endocrine receptors, the adopted orphan receptors, for which ligands have been identified in recent years, and the orphan receptors, ligands of which have not yet been identified (Figure 1b). The identification of selective small molecule ligands is one of the major goals of orphan nuclear receptor research, which will make new therapeutic interventions available for a variety of human diseases. Here, I discuss recent findings on drug discovery using orphan nuclear receptors peroxisome proliferator-activated receptors (PPARs) and Nur-related protein 1 (NURR1) and the potential application of retinoid acid receptor-related orphan receptors (RORs) and tailless homolog (TLX) as targets for drug discovery.

Ligand identification for orphan nuclear receptors

Identification of novel ligands for orphan nuclear receptors is likely to lead to the discovery of new drugs. Because nuclear receptors are important regulators of human physiology and pathology, ligands that interact with nuclear receptors to modulate the activity of these receptors have direct implications in drug discovery. Ligands of nuclear receptors have been used in many important clinical areas; for example, the estrogen receptor (ER) antagonist, tamoxifen, is used in the treatment of breast cancers; and retinoic acid receptor (RAR) agonists, retinoids, are used in the treatment of skin disorders [2]. PPARγ is another excellent example of a drug target as thiazolidinediones (TZDs), which are ligands of PPARγ, are widely used in the treatment of type II diabetes [3]. In addition to ligands that bind directly to the conserved ligand binding pocket, compounds that target regions

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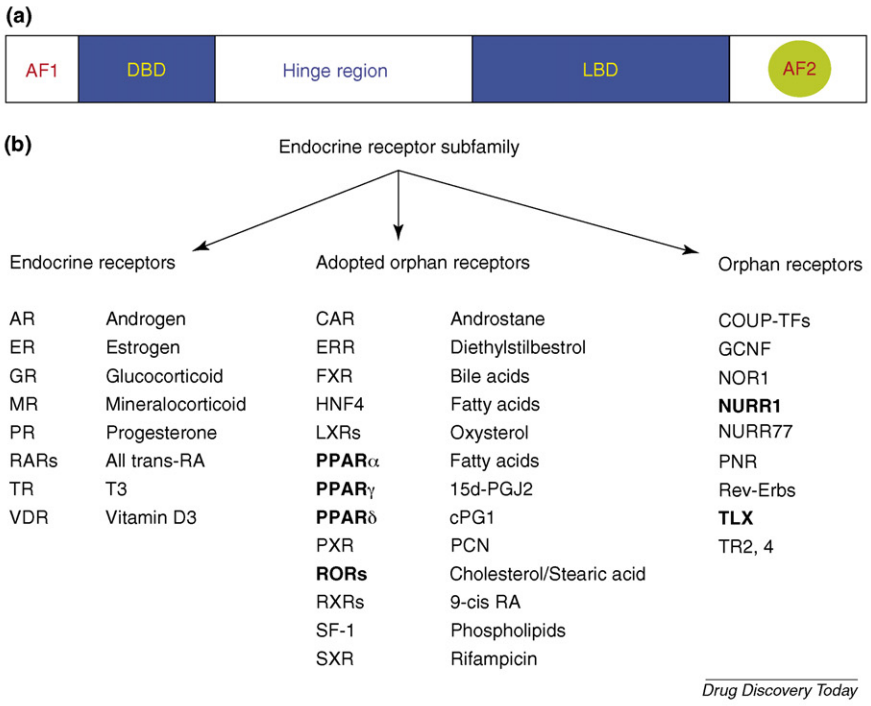

FIGURE 1

Structure–function domains and the superfamily members of nuclear receptors. (a) Nuclear receptor domain structure. In general, the receptor structure comprises an amino-terminal activation domain AF-1, DBD, a hinge region, a conserved LBD and a variable C-terminal region with a second activation domain AF-2. **(b)** The nuclear receptor superfamily includes the endocrine receptors, the adopted orphan receptors and the orphan receptors. The orphan nuclear receptors that are discussed here are in bold.

outside of the LBD in the receptor can also regulate receptor–co-factor interactions and receptor functions, and thus could also be potential drugs for related diseases. One such example is NURR1 agonist, 6-mercaptopurine (6-MP), which activates NURR1 through its AF-1 domain [4].

Attempts to identify ligands for orphan receptors have been conducted using a variety of methods. The most frequently used approach is cell-based assays using cultured mammalian cells transfected with a receptor construct and a reporter gene. Nuclear receptor LBD fused to Gal4 (a positive regulator of galactose-induced genes in *Saccharomyces cerevisiae*) DBD is often used as the receptor. The transfected cells are treated with candidate ligands and assayed for the activity of the reporter gene product. Using this strategy, ligands have been identified for RARs, retinoid X receptors (RXRs), PPARs, liver X receptors (LXRs), farnesoid X receptor (FXR), pregnane X receptor (PXR), steroid and xenobiotic receptor (SXR) and constitutive androstane receptor (CAR) [5,6].

The most straightforward methods for ligand identification are those based on direct binding, in which the target protein is immobilized on a solid support. Cell lysates or mixtures of compounds containing possible ligands are passed over the immobilized target protein and, after extensive washes, the putative ligand is eluted and characterized by analytical methods such as mass spectrometry. The ligand-dependent nuclear receptor–co-activator interactions have also been used for ligand screening. Examples of this strategy include fluorescence resonance energy transfer (FRET) assay [7], in which ligand-induced receptor–co-activator interactions lead to energy transfer between their tags, fluorescent proteins such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). The resulting fluorescent intensity change can be detected by using fluorescence microscopy. The AlphaScreen assay (Amplified Luminescent Proximity Homogenous Assay) is also based on ligand-dependent interactions between receptors and co-activators [8]. In this assay, the receptor is conjugated to a donor bead and the co-activator to a receptor bead. When a ligand-dependent interaction between a receptor and its co-activator brings their conjugated beads into proximity, singlet oxygen generated by the donor beads initiates a luminescence or fluorescence cascade in the nearby acceptor beads, leading to a highly amplified signal in the 520–620 nm range. This assay has been used to screen for phospholipid ligands that regulate steroidogenic factor-1 (SF-1)–co-activator interactions [9].

Structures of orphan nuclear receptor LBDs have also helped to identify ligands of these receptors. To date, crystal structures of LBDs have been reported for most of the nuclear receptors [10] and provide a detailed picture of their ligand binding pockets, which greatly facilitates designing pharmacologically active ligands for the receptors. Structure ligands, small molecules that are found in the ligand binding pocket of receptors in crystal structures, have been identified for orphan receptors, including RORs, hepatocyte nuclear factor α (HNFα), SF-1 and liver receptor homolog-1 (LRH-1). For example, cholesterol was identified as a ligand for RORα in a structural analysis [11]. One aim for the near future is to elucidate the structures of LBD for the remaining orphan nuclear receptors. These studies will provide new insights into ligand-mediated regulation of nuclear receptors and will help to identify ligands

for the remaining orphan receptors. The identification of ligands is, in turn, likely to lead to the discovery and design of new drugs.

Virtual screening of molecular compound libraries has recently emerged as a powerful method for drug discovery [12,13]. Based on the crystal structure of the target protein and high-throughput molecular docking using compound database, virtual screening enables a large number of compounds to be scanned with reasonable accuracy and speed. It has been used to identify RAR and thyroid hormone receptor (TR) antagonists [14,15] and to screen for selective ER modulators (SERMs) [16]. Computer-aided high throughput docking provides a rapid and economic approach for orphan nuclear receptor ligand screening and is a valuable tool for drug discovery.

PPARs: drug targets for diabetes and obesity

Since their discovery, PPARs have received attention as potential pharmacological targets for combating diabetes and obesity because of their important roles in cell metabolism regulation. There are three members of the PPAR family: PPARα, PPARγ and PPARδ. PPARα is expressed most prominently in the liver, kidney, heart, skeletal muscle and brown adipose tissue. In addition to its activation in response to peroxisome proliferators, PPARα is also activated by a variety of medium- and long-chain fatty acids and stimulates lipid metabolism by the induction of peroxisomal β-oxidation and fatty acid ω-hydroxylation [17]. Mice lacking functional Pparα are incapable of responding to peroxisome proliferators and fail to induce expression of a variety of genes required for the metabolism of fatty acids [18]. For a long time, the PPARα-activating fibrates, a class of amphipathic carboxylic acids, have been used in the treatment of dyslipidemia. In dyslipidemic patients, these drugs improve the plasma lipid profile by lowering triglyceride and, to a lesser extent, low-density lipoprotein (LDL) cholesterol levels and by increasing high-density-lipoprotein (HDL) cholesterol levels [19]. These effects are achieved by a variety of mechanisms, such as an increase in lipoprotein lipase expression, reduction of apolipoprotein CIII expression, inhibition of triglyceride synthesis and very low-density lipoprotein production.

PPARγ is abundantly expressed in adipose tissue and has a central role in adipogenesis [20,21]. Pparγ-null mice are embryonic lethal owing, in part, to disrupted placental function [21]. Rescue of the placental defect results in lipid dystrophy and neonatal death [22,23]. PPARγ is activated by 15-deoxy-(12,14)-prostaglandin J2 (15d-PGJ2) or its synthetic analog TZDs [24,25]. TZDs represent the best studied class of PPARγ agonists and are used clinically as insulin-sensitizing drugs for the treatment of type II diabetes [3]. Activation of PPARγ by TZDs induces genes involved in adipocyte differentiation and lipogenesis, which are thought to be responsible for the insulin-sensitizing actions of these drugs. However, an unwanted side effect of TZD is weight gain. Partial PPARγ agonists, compounds that selectively activate PPARγ glycemic control function with weaker adipogenic potential, are now being developed to avoid this adverse side effect [26]. PPARγ antagonists that promote glycemic control and decrease adiposity will also become valuable tools for drug discovery in diabetes and obesity.

PPARδ is widely expressed at relatively high levels in brain, macrophages, lung, adipose tissue and skeletal muscle [27]. Recent data from transgenic mouse models have implicated it in the regulation of energy expenditure as well as in glucose and lipid metabolism, highlighting the potential use of PPARδ modulators as therapeutic agents for type II diabetes and obesity [28]. Muscle-specific expression of an activated form of Pparδ (VP-Pparδ) in mice resulted in resistance to diet-induced obesity, increased metabolic rate and lipid utilization, and decreased intramyocellular triglyceride levels [29]. In addition, there was an increase in the type I muscle fibers, which correlated with increased exercise endurance in transgenic mice. Mice expressing VP-Pparδ in adipose tissue had reduced white adipose tissue mass along with increased energy expenditure and lipid utilization [30]. These mice were resistant to diet-induced obesity and hyperlipidemia. PPARδ agonists have validated this receptor as a therapeutic target for the treatment of obesity and diabetes. Treatment of obese, insulin-resistant monkeys and diabetic rodent models with GW501516, a potent and selective synthetic PPARδ agonist, decreased fasting insulin and triglyceride levels and increased HDL cholesterol levels [31], supporting the idea that PPARδ is an important drug target for the treatment of diabetes and obesity.

Recently, the concept of the selective PPAR modulators (SPPARM) was introduced [19]. SPPARM is a new pharmacological approach that is based on selective receptor–co-factor interactions and differential target gene regulation. It encompasses the principle of chemical alteration of PPAR-specific ligands to create compounds that selectively activate specific PPAR functions. For example, selective PPARγ modulation could lead to potent insulin sensitization without adverse effects such as weight gain [32]. Several compounds have now been identified as SPPARMs, some of which are already in clinical testing [32–35]. In addition, pan-agonists combining PPARα, γ and δ agonism have the potential to improve insulin resistance and dyslipidemia without causing weight gain. These compounds have recently entered clinical trials [28]. The development of SPPARMs and pan-PPAR agonists will contribute significantly to drug discovery in diabetes and obesity.

Nurr1: a drug target for Parkinson's disease

The nuclear receptor subfamily 4A (NR4A) comprises three members, NURR1 (NR4A2), NUR77 (NR4A1) and NOR1 (NR4A3) [36–38] (symbols in parentheses reflect current nomenclature). They largely function as immediate–early genes, the expression and activation of which is regulated in a cell-type specific manner in response to a range of signals, such as mitogenic and apoptotic stimuli [39]. NURR1 is expressed predominantly in the central nervous system (CNS), especially in substantia nigra, the ventral tegmental area, the midbrain and limbic areas [40]. Several lines of evidence have indicated that it is essential for the development, migration and survival of dopaminergic neurons [41]. As Parkinson's disease (PD) results from the loss of dopaminergic neurons, the prospect of using NURR1 as a drug target for PD is promising.

Experimental studies in *Nurr1*-knockout mice indicated that Nurr1 deficiency resulted in impaired dopaminergic function and increased vulnerability to apoptosis in midbrain dopaminergic neurons, which degenerate in PD. Mutations in the gene encoding Nurr1 have also been associated with PD [42]. Decreased Nurr1 expression was found in PD midbrains, particularly in neurons containing Lewy bodies, abnormal aggregates of proteins that are associated with neuronal degeneration in such brains [43].

Moreover, Nurr1 overexpression in embryonic stem cells is sufficient to generate differentiated dopaminergic neurons [44]. These studies suggest that Nurr1 is not only essential in the development and survival of mensencephalic dopaminergic neurons, but also has a role in the pathogenesis of PD.

For many years, much effort was put into finding ligands that bind and activate the NURR1 receptor. Although the NURR1 LBD is folded in much the same way as in other nuclear receptors, the space that in other nuclear receptors is normally occupied by ligands is entirely filled by hydrophobic amino acid side chains in NURR1, hence preventing ligand binding in this pocket [45]. However, a novel hydrophobic interaction surface outside of the classic ligand binding domain has been identified that could bind co-activators and could be used as a molecular target for NURR1-activating compounds [46]. The identification of potent and selective agonists of NURR1 will enable the development of new therapeutic interventions for CNS disorders. Recently, 6-MP was reported as a modest agonist of NURR1; it activates NURR1 through its amino-terminal AF-1 instead of the classic ligand binding domain [4]. More NURR1 agonists with high potency (EC50: 8–70 nM) have been identified, which can activate NURR1 transcription activity with excellent bioavailability and can easily cross the blood–brain barrier [47]. These compounds are currently being tested in both *in vitro* and *in vivo* PD models [43]. They enhance tyrosine hydroxylase and dopamine transporter expression in primary mensencephalic cultures and exert a beneficial effect on dopaminergic neurons in animal models of PD [43]. These new NURR1 agonists could have the potential to be developed into therapeutic tools for PD.

RORs: drug targets for CNS and cholesterol-related diseases

The ROR proteins RORα, β and γ are encoded by three different genes, *RORA*, *RORB* and *RORC*, respectively [48]. RORα is expressed in specific areas of the brain, including the Purkinje cells in the cerebellum and the suprachiasmatic nucleus of the hypothalamus. It is also expressed in the spleen, thymus and macrophages. The initial studies on the *in vivo* function of RORα came from an animal model known as *staggerer* mice, which have a deletion in *Rora* [49]. These mice exhibit ataxic phenotype resulting from a massive neurodegeneration in the cerebellum, which is caused by a developmental defect in Purkinje cells [50]. Additional phenotypes in the *staggerer* mice include abnormal circadian behaviors, osteoporosis, muscular atrophy, dyslipidemia and enhanced susceptibility to atherosclerosis. RORβ is expressed specifically in areas of the CNS that are involved in the processing of sensory information and in primary components of the mammalian circadian system, including the suprachiasmatic nuclei, the pineal gland and the retina. The expression profile suggests a role for RORβ in the processing of sensory information and in the circadian rhythm [51]. *Rorb*-knockout mice display a duck-like gait, transient male infertility, abnormal circadian behavior and retinal degeneration [52]. RORγ is found at high levels in skeletal muscle and thymocytes [53,54]. Analysis of *Rorc*-knockout mice revealed significant roles of Rorγ in both thymocyte development and lymphoid organogenesis [55].

Ligands have been identified for both RORα and RORα but no ligands have been described for RORα. Studies of the RORα LBD structure revealed the presence of a relatively large ligand binding pocket [56]. Stearic acid was found in the ligand pocket of RORβ in a crystal structure but was not able to activate RORβ in a reporter assay, arguing against it to be a real RORβ ligand [56]. All-trans retinoic acids (ATRA) have been proposed as RORβ ligand after a co-crystal complex was reported [57]. RORβ transactivation was strongly inhibited by retinoids, suggesting that ATRA acts as an antagonist for the constitutively active RORβ. This finding suggests that retinoids can become valuable tools for drug discovery in RORβ-related CNS diseases.

The pineal gland hormone melatonin, a drug that has been used to treat sleep disorders, was reported as a natural ligand for RORα [58]. The gene encoding human 5-lipoxygenase was shown to be the first RORα/melatonin-responding gene [59]. More recently, cholesterol was identified as being a ligand for RORα [11]. Cholesterol is an important membrane component of mammalian cells and a biosynthetic precursor of the corticosteroid and sex steroid hormones [60]. Depleting cellular cholesterol led to a reduction in RORα activity, whereas addition of cholesterol or its analogs reactivated its activity. Interestingly, RORα has been shown to regulate directly transcription of apolipopoprotein A-I, a key component of HDL particles that transport cholesterol. Together these data suggest that RORα senses cholesterol levels and regulates cholesterol flux [60]. The fact that changes in cholesterol levels can modulate the transcriptional activity of RORα suggests that cholesterol is a 'real' ligand rather than just a structural cofactor of RORα [11]. RORα could have a key role in the regulation of cholesterol homeostasis and, thus, represents an important drug target in cholesterol-related diseases. The identification of RORα as a cholesterol 'receptor' will significantly aid the search for pharmacologically active synthetic RORα ligands for the discovery and design of new drugs in RORα-related diseases.

TLX: potential drug target for neurodegenerative diseases

Orphan receptor TLX (tailless homolog, NR2E1) is specifically expressed in the brain and has an important role in vertebrate brain functions [61,62]. The human and mouse TLX are highly conserved and are homologous to the *Drosophila* tailless (Figure 2a). *Tlx*-knockout mice are viable and appear normal at birth, although the *Tlx* gene is required for the formation of superficial cortical layers in embryonic brains [63], to regulate the timing of neurogenesis in the cortex [64] and to control patterning of lateral telencephalic progenitor domains during development [65]. Mature *Tlx*-knockout mice have significantly reduced cerebral hemispheres [61] and severe retinopathies [66–69]. Behaviorally, adult *Tlx* mutants exhibit increased aggressiveness, decreased copulation, progressively violent behavior, late onset epilepsy and reduced learning abilities [61].

TLX is an essential regulator of neural stem cell maintenance and self-renewal in the adult brain [62] and it maintains adult neural stem cells in the undifferentiated and self-renewable state (Figure 2b). The TLX-expressing cells isolated from adult TLX-heterozygote brains can proliferate, self-renew and differentiate into all neural cell types *in vitro*. By contrast, *Tlx*-null cells isolated from the brains of adult *Tlx*-knockout mice fail to proliferate. Reintroducing Tlx into *Tlx*-null cells rescues their ability to proliferate and self-renew [62]. *In vivo*, *Tlx* mutant mice show a loss of

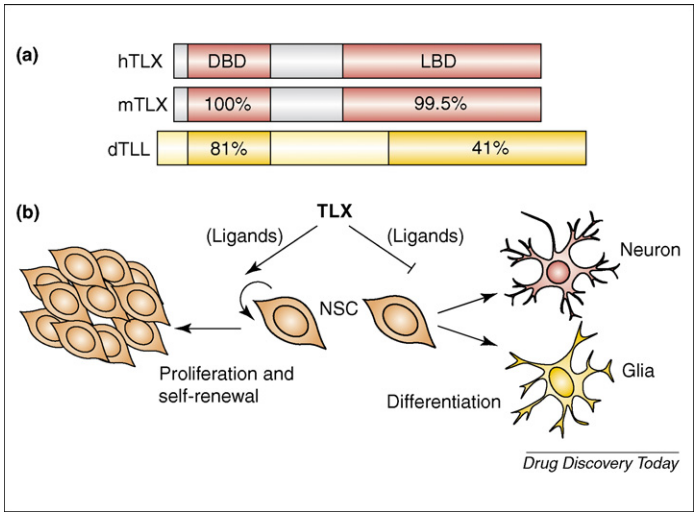

FIGURE 2

Orphan nuclear receptor TLX. (a) Structure–function domains and sequence homology of human (h), mouse (m) TLX, and *Drosophila* Tailless (dTLL). **(b)** A model of TLX-mediated neural stem cell (NSC) maintenance and self-renewal. TLX stimulates NSC proliferation and self-renewal and inhibits NSC differentiation to maintain neural stem cells (NSCs) in the undifferentiated and self-renewable state. Ligands of TLX can modulate these events through regulating TLX activity.

cell proliferation and reduced neural precursors in the neurogenic areas of adult brains. TLX represses the expression of astrocyte markers, such as GFAP, in neural stem cells, suggesting that transcriptional repression is crucial in maintaining the undifferentiated state of neural stem cells. Similarly, the *Drosophila* tailless acts as a dedicated repressor in the early *Drosophila* embryo to support normal embryonic development and establish accurate patterns of gene expression [70].

In addition to its function in neural stem cells in the brain, TLX is a key component of retinal development and is essential for vision [66]. It is expressed in retinal progenitor cells in the neuroblastic layer during the period of retinal layer formation and is crucial for controlling the generation of appropriate numbers of retinal progenies [67]. The *Tlx*-knockout neural retinas were significantly thinner than those of wild-type littermates during development [68]. In the postnatal mouse retina, *Tlx* is strongly expressed in the proangiogenic astrocytes and acts as a proangiogenic switch in response to hypoxia [69].

Given the essential role of TLX in regulating the maintenance and self-renewal of adult neural stem cells, ligands of TLX are likely to be important modulators of neurogenesis and neuronal regeneration. Identification of TLX ligands will provide potential pharmacological tools for neurodegeneration. Ligands of TLX can lead to enhanced neurogenesis in normal brains and increased neuroregeneration in neurodegenerative brains. The role of TLX in retinal progenitor cells suggests that TLX is also an important drug target for retinodegeneration. In summary, ligand screening for TLX provides a future direction for drug discovery in neurodegenerative diseases.

Conclusions

The search for ligands of orphan nuclear receptors has led to the discovery of many signaling pathways and has revealed a direct link of nuclear receptors to human conditions such as diabetes, obesity and neurodegenerative diseases. Ligand identification of orphan receptors will lead to the discovery of novel hormone response systems and will open many new therapeutic avenues for a variety of human diseases. Identification of compounds with selective activities for specific orphan receptors is of clinical and pharmacological importance and promises a bountiful harvest in the near future.

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