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ALTERNATIVE RETINOID X RECEPTOR (RXR) LIGANDS

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ABSTRACT

Retinoid X receptors (RXRs) control a wide variety of functions by virtue of their dimerization with other nuclear hormone receptors (NRs), contributing thereby to activities of different signaling pathways. We review known RXR ligands as transcriptional modulators of specific RXR-dimers and the associated biological processes. We also discuss the physiological relevance of such ligands, which remains frequently a matter of debate and which at present is best met by member(s) of a novel family of retinoids, postulated as Vitamin A5. Through comparison with other natural, but also with synthetic ligands, we discuss high diversity in the modes of ligand binding to RXRs resulting in agonistic or antagonistic profiles and selectivity towards specific subtypes of permissive heterodimers. Despite such diversity, direct ligand binding to the ligand binding pocket resulting in agonistic activity was preferentially preserved in the course of animal evolution pointing to its functional relevance, and potential for existence of other, species-specific endogenous RXR ligands sharing the same mode of function.

INTRODUCTION

Retinoid X receptors discovered in the early 1990s (Leid et al., 1992; Mangelsdorf et al., 1992) are nuclear hormone receptors (NRs) acting as transcription factors, which in mammals are represented by three isoforms, RXR α , β , and γ (named also NR2B1, 2 and 3 (Germain et al., 2006b). These are encoded by distinct genes (9q34.3, 6p21.1-3, 1q22-23, respectively) displaying different expression patterns (Dolle et al., 1994; Krezel et al., 1999; Mori et al., 2001). RXRs are exceptional in the sense that they can act as homodimers, but also as obligatory heterodimerization partners for different types of NRs including: (i) classical hormone receptors like thyroid receptors (TR α , β ; NR1A1, 2), vitamin D receptor (VDR; NR1I1) and retinoic acid receptors (RAR α , β , γ ; NR1B1, 2, 3), (ii) metabolite or drug sensor receptors such as peroxisome proliferator activated receptors (PPAR α , β/δ , γ ; NR1C1, 2, 3), liver X receptor (LXR α , β ; NR1H2, 3), farnesoid X receptor (FXR; NR1H4), pregnane X receptor (PXR; NR1I2) and (iii) orphan receptors like Nur77 (NR4A1), Nurr1 (NR4A2) and constitutive androgen receptor (CAR; NR1I3). Hence RXRs bridge different signaling pathways whereby they can: (i) act as “silent” partners of “non-permissive” NRs (TRs, VDR), being insensitive to their own ligand in different biological settings (Forman et al., 1995; Thompson et al., 1998), (ii) enhance activity in a ligand-dependent manner of ligand-activated “conditional” partners (RARs and in case of colon cancer or endothelial cells also VDR), here not being able to induce NR/RXR activity on their own (Germain et al., 2006a; Lin et al., 2016; Sanchez-Martinez et al., 2006), and (iii) induce activity of “permissive” heterodimerization partners (PPARs, LXRs, FXR, Nurr1, Nur77) upon binding of RXR ligand (Forman et al., 1995; Germain et al., 2006a; Giner et al., 2015). Importantly, RXR heterodimers with PPARs, LXRs, FXR can also be activated by respective ligands of the RXR partner. On the other hand, RXR ligand remains the only mean to activate heterodimers with orphan receptors or RXR homodimers (see Figure 1). Such promiscuity of RXRs can be critical for harmonized tuning of physiologic programs necessary for adaptive process, but also re-adjustment of the transcriptional programs under pathological conditions, as reflected by clinical or experimental data for some RXR ligands discussed below. Knowledge on RXR architecture and its dynamics is essential for understanding of ligand binding mechanisms and their functional outcome. Here, we will first review RXR structural data focusing much on those that are relevant for binding of different ligands. In contrast to previous reviews on RXRs and their ligands (named rexinoids) which focused much on structural biology, molecular functions and post-transcriptional modulation of RXR functions (Dawson and Xia, 2012), or modulation of metabolic receptors (Hiebl et al., 2018), we will put particular emphasis on known endogenous ligands and their metabolites. The physiological relevance of such ligands will be discussed with its functional and nutritional perspectives. In particular, 9-*cis*-retinoic acid (9CRA), formerly widely accepted as the potential physiological RXR ligand, was found to be essentially undetectable under physiological conditions in vertebrates including human serum and tissue samples. We will review evidence indicating that 9-*cis*-13,14-dihydroretinoic acid (9CDHRA) meets probably best the criterion of a physiological ligand of RXRs. The physiological relevance of 9CDHRA is further supported by its central role in the concept of vitamin A5, identified as a new class of vitamin A, which may depend on a distinct set of nutritionally relevant precursors. This does not exclude the possibility that other endogenous ligands might also be physiologically and nutritionally relevant in specific cell types or in some “challenging” conditions such as nutritional deficits of specific micronutrients, as reviewed below. Characterization of the physiological relevance of any ligand remains both a conceptual and technological challenge, which may require development of new approaches, as discussed below. Additional review of natural phyto-rexinoids, environmental pollutants and synthetic ligands highlights the high diversity of their chemical structures, and thereby of possible mechanisms of ligand binding and transcriptional regulatory activity.

Finally, despite such a diversity, studies of ancestral RXRs point to particular evolutionary pressure to maintain ligand binding for these receptors, and agonistic effect of such interaction. We will thus

discuss the potential physiological and nutritional relevance of endogenous RXR ligand evolution. Characterization of such ligand(s), and their genomic and functional effects, may help to define core, ancient RXR functions and understand how ligand-dependent control of RXR functions could provide evolutionary advantage.

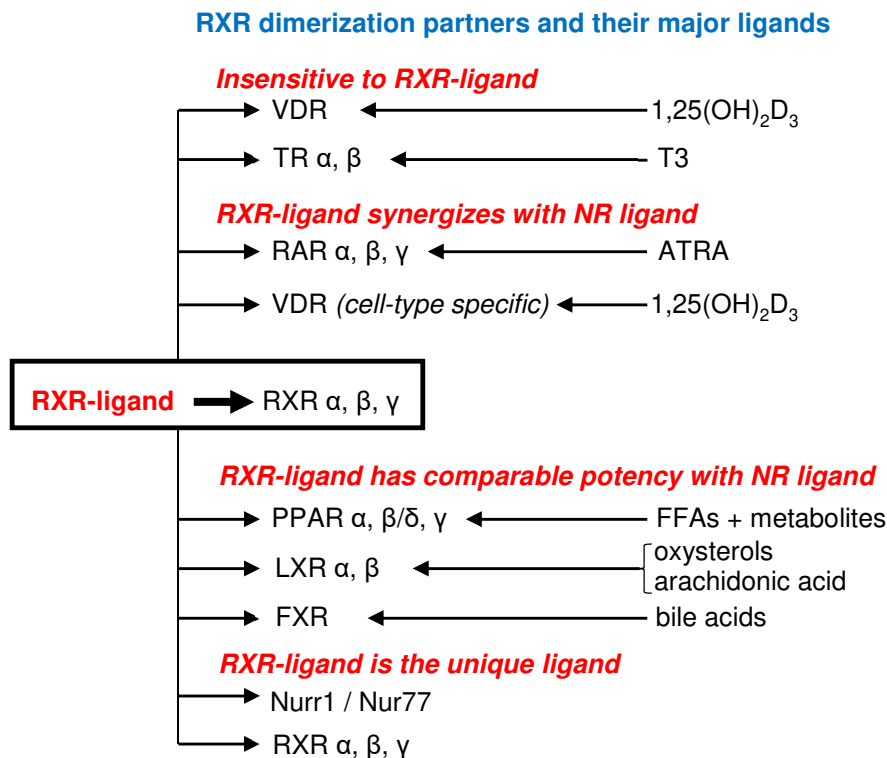


Figure 1: RXR-ligand's ability to mediate transcriptional regulation of RXR-dimers.

THE STRUCTURE OF RETINOID X RECEPTORS

Similarly to other nuclear receptors, RXRs display a domain-like organization with a variable N-terminal domain (NTD) followed by a highly conserved DNA binding domain (DBD) connected by a hinge region with the C-terminal ligand binding (LBD) domain. Although isolated domains display intrinsic activities, such as DNA binding for DBD or ligand binding for LBD, their interactions within the entire receptor are highly dynamic and are under control of multiple allosteric modulators, which underlie pleiotropic activities of RXRs. Such modulators include ligands, which are the focus of this review, but also DNA regulatory elements and post-translational modifications, as reviewed elsewhere (Dawson and Xia, 2012), which determine recruitment of cell-specific heterodimerization partners and co-regulators to control the expression of the corresponding sets of target genes. In the absence of the ligand, RXRs were reported to form tetramers (tRXR) (Kersten et al., 1995). Whereas ligand or DNA binding was shown to dissociate them (Chen et al., 1998), DNA-bound tetramers were also proposed to antagonize transcriptional activation (Kersten et al., 1997). Analyses of DNA binding patterns by RXRs in living cells suggested, however, that in the absence of ligand stimulation, RXRs bind to DNA as homodimers or heterodimers, possibly acting as inhibitors of transcription ready to exchange their interaction partner upon ligand binding (Chatagnon et al., 2015; Nielsen et al., 2008). Ligand binding induces a number of conformational changes, which were first identified in isolated RXR LBD. These initial crystallography studies revealed that the hydrophobic pocket formed by superposition of three helices (H3, H7, H11)

is connected through a hinge region with a hydrophobic channel formed by H3 and H5 helices, forming the L-shaped LBD of RXR α (Egea et al., 2000) (Fig. 2).

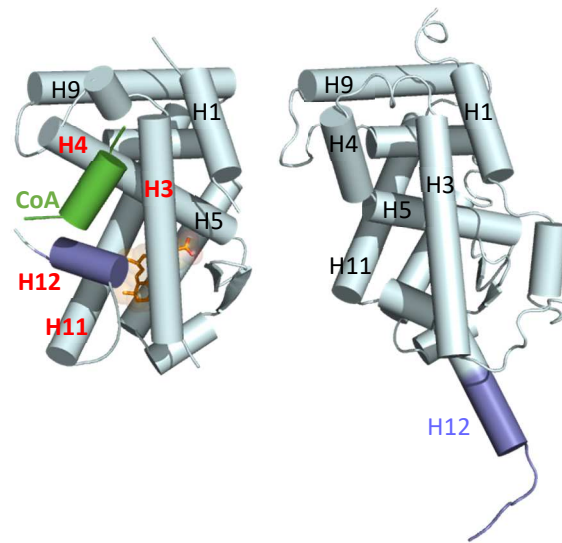


Figure 2. Structure of the RXR α ligand binding domain in holo (ligand-bound) conformation with marked in yellow 9-*cis*-13,14-dihydroretinoic acid (left) and in its apo (without ligand) conformation (right). The AF2 domain interacting with coactivator (CoA) is formed by the helices indicated in red.

This structure of the ligand binding pocket is identical among all three RXR isotypes (Egea et al., 2002). Importantly, ligand binding was shown to induce repositioning of helix 12 next to helices H3/H5 to form the surface called activation function AF2, which by recruiting chromatin modifiers, but also cofactors interacting directly with the transcriptional machinery, may either inhibit or activate transcription of target genes (Rosenfeld et al., 2006). AF2 formed by helices H3, H4, H11 and H12 (Fig. 2) of the ligand binding domain is thus the major ligand-dependent activation domain in RXRs, whereas AF1, an additional activation function domain involved in recruitment of cofactors, is harbored by the N-terminal region which, due to its intrinsically unstructured organization, is less prone to influences of conformational changes induced by ligand or DNA binding (reviewed in (Chambon, 1996; Wurtz et al., 1996). Although AF1 is believed to act in an autonomous manner in RXRs (Belorusova et al., 2016), it may also affect the efficiency and selectivity of gene expression as well as responses to LBD-bound ligands in steroid NRs (Helsen and Claessens, 2014; Hilser and Thompson, 2011; Yi et al., 2015). Thus, analyses of RXR structures in the presence of AF1-interacting cofactors would be particularly interesting as such cofactors could not only locally structure the N-terminal domain, but also make it sensitive to long-range structural rearrangements triggered by ligand or DNA binding. Indeed, more recent studies of RXR LBD and AF2 in the context of the entire receptor alone or with its heterodimerization partners and cofactor peptides, brought into light long-range structural modifications induced by ligand binding and their critical implications for permissiveness of some RXR heterodimers to be activated by ligands of either partner in the RXR heterodimer (Fig. 1). Data reported by Kojetin et al. (Kojetin et al., 2015) indicate that RXR agonist-induced repositioning of RXR H12 into the active conformation can be further stabilized by H12 of PPAR γ when the agonist conformation of the latter is induced by full PPAR γ agonist. In contrast, heterodimerization of RXR with non-permissive TR β destabilizes RXR LBD by rotating helix H11 and twisting H5, deforming the RXR LBD and impeding efficient ligand binding by RXR. Similarly, RXR ligand binding was shown to synergize with FXR ligand binding to induce conformational change of helix H11 in FXR, thus stabilizing FXR coactivator binding surface and coactivator binding (Wang et al., 2018c). Much of our understanding of the ligand-

dependent mechanism of NR activation stems from studies of synthetic RXR ligands, also reviewed in this article. For example, structural analyses of RXR/Nurr1 heterodimers with synthetic derivatives of the natural RXR ligand honokiol (discussed below) revealed that designed biaryl-ligands can compact the LBD, thus allowing the mobility of helix H7 and H11, which may be necessary to accommodate helix H12 of Nurr1, thereby favoring the formation of RXR α -Nurr1 heterodimers instead of RXR α homodimers (Scheepstra et al., 2017). Intriguing also are the opposing transcriptional effects of the natural RXR ligand bigelovin (see below for description) on enhancing transcription of RXR α -PPAR γ heterodimers and inhibiting RXR α -LXR α heterodimers. Crystallography analyses point to alteration of the conformation of helix H10, the core of the dimerization interface, as a potential mechanism (Zhang et al., 2011b).

NATURAL LIGANDS OF RXRS AND THEIR POTENTIAL PHYSIOLOGICAL RELEVANCE

Before discussing different types of ligands, we need first to define concepts of natural vs endogenous and physiological ligands, which are frequently misused. We will thus consider as natural ligands all substances present in Nature that bind with high potency (optimally in the nanomolar range) their receptors leading to conformational changes in the receptor structure, and induce relevant physiological functions. Excluded are all man-made compounds generated *de novo*, which will be referred to as synthetic ligands. Endogenous ligands, although frequently dependent on natural precursors found in nutrition, are unique in a sense that they are produced in the body and do not need to be introduced into the organism from outside to be detected. Such endogenous production is therefore dependent on dedicated metabolic pathway(s) and availability of nutritional precursors, as in the case of small bioactive molecules like retinoids. Although several endogenous ligands were proposed for RXRs, their physiological relevance is under debate, since the presence of the ligand in the right place, at the right time and at sufficient concentration to activate RXRs is not always met and, in most cases, is unknown. Importantly, manipulation of nutritional precursors, relevant metabolic pathways or natural ligands provides a method for regulation of ligand bioavailability ranging from deficiency through maintenance of physiological levels relevant for organismal homeostasis to supraphysiological levels. In the latter cases, referred to as supra-physiological conditions or nutritional intervention, exogenous ligands or endogenous substances not acting as endogenous ligands, may become relevant for the control of biological processes in an RXR-dependent manner.

Fatty acids as potential endogenous and nutritionally regulated RXR ligands

Docosahexaenoic acid (DHA) and other free fatty acids (FFAs). Using bio-guided fractionation of brain-conditioned medium, de Urquiza and colleagues (de Urquiza et al., 2000) identified a series of FFAs capable of inducing RXR transcriptional activation with different efficiencies. Using sensitive reporter assays in JEG-3 or 293T cultured cells, DHA (Fig. 3) displayed the best transactivation activities starting already at 10 μ M (EC_{50} = 50 μ M), whereas docosatetraenoic, oleic and arachidonic acids required higher 100 - 1000 μ M (EC_{50} > 200 μ M) concentrations. Although other studies (Calderon and Kim, 2007; Goldstein et al., 2003) confirmed the requirement of such relatively high concentrations of DHA to induce transcription by RXR, Lengqvist et al. reported an EC_{50} value of 5-10 μ M, by using a different method of DHA application in cultured cells, as well as mass spectrometry evidence for DHA attaching

/ binding to RXR α -LBD (Lengqvist et al., 2004). Direct interaction of DHA with LBD was also documented by crystallography data using RXR α -LBD (Egea et al., 2002).

There is a substantial amount of data supporting the activity of DHA via RXRs under pharmacological conditions, i.e. after DHA treatment or its nutritional supplementations, e.g. in fish oil. In *in vitro* experiments, treatment with DHA (free acid form), similarly to synthetic RXR agonists (LG100268 or BMS649), increased the number of surviving neurons in primary culture cells and such effect was RXR dependent since it was prevented in the presence of the RXR antagonist LG100268 (Wallen-Mackenzie et al., 2003). Importantly, this effect was Nurr1-dependent, pointing to the functional relevance of RXR/Nurr1 heterodimerization and to the possibility of controlling Nurr1-dependent signaling *via* ligand-dependent activation of its RXR heterodimerization partner. DHA-dependent RXR activation, using BSA-complexed free acid form, was also shown to be indispensable for survival of cultured rat photoreceptors during early phases of primary cultures or in response to oxidative stress (German et al., 2013). Accordingly, treatment with 6.7 μ M DHA mimicked the neuroprotective activities of different synthetic RXR agonists (HX630 or PA024), whereas its effect was abolished in the presence of RXR antagonists (HX531 or PA452). The same authors also demonstrated that the availability of DHA in its free acid form was essential for such RXR-mediated neuroprotective activities, since a phospholipase A2 inhibitor preventing DHA release from its phospholipid derivatives (the major form of DHA in cells) prevented the neuroprotective effects of DHA treatment. In addition to neuroprotection, RXR signaling is also critical for DHA cognitive and affective activities *in vivo*. Thus, the beneficial effect of DHA treatment (free acid form) on spatial working memory performance was abolished in mice with a null mutation of RXR γ or in wild-type mice pre-treated with an RXR antagonist (Wietrzych-Schindler et al., 2011). A similar experimental strategy was used to demonstrate the implication of RXR γ in anti-despair effects of DHA in the forced swim task (Wietrzych-Schindler et al., 2011), which may have a direct relevance for antidepressant activities of DHA and other ω -3 polyunsaturated fatty acids, as reviewed elsewhere (Larrieu and Laye, 2018). Finally, there are a number of other functions that are modulated by DHA treatment, but the involvement of RXRs in such functions awaits documentation. For example, DHA enhancement of antibody phagocytosis by microglial cells (Hjorth et al., 2013) could potentially be mediated by RXRs, and Yamanaka et al. have shown that this process could be enhanced by bexarotene (LGD1069), a synthetic RXR ligand (see below), in an RXR α dependent manner, since it was prevented in the presence of relevant siRNAs (Yamanaka et al., 2012).

The role of RXRs in mediating the diverse effects of DHA under supra-physiological conditions (i.e., after treatment or nutritional intervention) and the capability of vertebrates to synthesize DHA from α -linolenic acid as precursor of plant origin (Rajaram, 2014), may suggest that DHA can act as a physiological ligand. However, such a role is questionable due to the likely limited availability of the free acid form. Although the determination of free DHA levels is not evident because of the multiple forms of DHA, its levels were reported to range from 0.1 μ M to 0.01 μ M in animal and human serum or tissue samples (Elabdeen et al., 2013; Szklenar et al., 2013). Such endogenous levels of DHA would not be sufficient for transcriptional activation, which requires at best a concentration range of 5-10 μ M (Lengqvist et al., 2004). Free DHA levels were not examined under conditions of nutritional or pharmacological interventions, and in particular no data are available on its concentration in the nucleus; therefore, it is uncertain whether such interventions really lead to sufficient increase of endogenous free DHA levels to function as a ligand-dependent switch in the nucleus. Moreover, central nervous system activities of DHA (including e.g. mnemonic and antidepressant effects (see (Wietrzych-Schindler et al., 2011) and references therein) might be also difficult to explain by direct DHA-RXR interactions, since transfer of DHA through the blood-brain-barrier is highly selective and of low efficacy for the free acid form, whereas it might be easier for DHA metabolites or synthetic acetyl derivatives (Lo Van et al., 2016; Picq et al., 2010). In this respect, novel DHA metabolites that have been reported during recent years might also be of interest. For example, a number of hydroxylated

transactivation of RXR α activities at 10 μ M. Although phytanic acid competed with [3 H]-9CRA for RXR α binding with a K_i of 4 μ M, it remained a 200-fold less efficient ligand than 9CRA. Activation of RXR α , β and γ isotypes, with EC_{50} of 20 μ M, was also confirmed in a Gal4 reporter assay (Zomer et al., 2000). As an important metabolite of chlorophyll, phytanic acid can be produced in ruminating animals by their rumen bacteria. Humans do not efficiently absorb chlorophyll and cannot metabolize it; thus, levels of phytanic acid in the human body are entirely dependent on meat and milk products ingested in the diet. Consequently, phytanic acid cannot be considered as an endogenous ligand in humans, but may potentially act as such in ruminants. Furthermore, its low levels in human questions the physiological relevance of its activities as a nutritional RXR ligand. Thus, in human plasma phytanic acid concentrations ranged around 1.6 μ M, which was highly dependent on dietary habits of healthy volunteers, with the highest level (5.8 μ M) found in meat eaters and the lowest in vegans (0.9 μ M) (Al-Dirbashi et al., 2008; Allen et al., 2008). Phytanic acid levels ranged around 1 μ M in mouse plasma, but were approximately 10-100 times lower in different tissues (Wanders et al., 2011). Such quantities were increased to 10 μ M levels under supplementation with high amounts of phytol, a phytanic acid precursor (Wanders et al., 2011). Importantly, disruption of phytanol-CoA hydroxylase, the first enzyme in the α -oxidation in Refsum disease, leads to high accumulations of phytanic acid, which may reach up to 1000 times the level of a healthy person (Verhoeven and Jakobs, 2001), with highest levels found in the liver, heart, adipose and peripheral nervous tissues, and much lower in the brain (Cumings, 1971). Despite the wide range of symptoms, including peripheral neuropathy, retinal degeneration and cerebellar ataxia (see (Verhoeven and Jakobs, 2001) and references therein), no abnormalities relevant to general or even exacerbated RXR signaling were reported like, for instance, decreased thyroid hormone T4 levels, elevated triglyceride levels or reduced food intake. This further questions the relevance of phytanic acid and potential phytanic acid metabolites as RXR ligands. Implication of RXR activation in mediating effects of high amounts of phytanic acid on glucose and lipid metabolism or adipocyte differentiation (reviewed in (Roca-Saavedra et al., 2017)) is also not clear as such effects were not tested in competition with an RXR antagonist or in mice/cells carrying null mutations for specific RXR isotypes.

In summary, RXR transactivation by phytanic acid starts at about 10 μ M concentration, whereas physiological levels are on average around 1-6 μ M concentration in plasma, which might be too low to control RXR signaling under physiological conditions. With the exception of plasma, no data are available for human tissue levels. This led us to suggest that with the current knowledge, phytanic acid remains as a nutritional RXR ligand candidate, but its physiological role in RXR activation is uncertain. Further studies of its cell-type specificity and subcellular availability (including the nucleus), as well as potential involvement of its metabolites in RXR-dependent signaling, should be encouraged. Finally, although its physiological role in humans is questionable (or can be relevant under special conditions), it may play an important role in ruminants where, in contrast to humans, phytanic acid may potentially act as an endogenous RXR ligand. This hypothesis remains to be evaluated but, being the case, it could illustrate the variability in the type of endogenous RXR ligand as a function of nutritional habitat, a determinant of available ligand precursors, which may differ between species (see also evolutionary considerations in this review).

Retinoids and alternative carotenoid metabolites

Carotenoids, C40 isoprenoid pigments produced by plants or microorganisms, serve in animals as precursors for a number of bioactive metabolites globally called apo-carotenoids (cleavage products of carotenoids). One of the best examples is apo-15'-carotenoic acid, better known as all-*trans*-retinoic acid (ATRA) acting as "the" physiological ligand of the RARs. In humans and other vertebrates it is

formed by central enzymatic cleavage of all-*trans*- β -carotene (ATBC, also called pro-vitamin A1) originating from plants. ATRA, as well all-*trans*-retinol (ATROL, known as vitamin A1), can be stored in the animal organism as esters (Blaner et al., 2016), which in turn can be a source of ready-to-use ATROL / ATRA once ingested in animals as food products. Recent published and unpublished data indicate the existence of other bioactive retinoids, including dihydroretinoids, some of which act as RXR endogenous ligands, which are derived from different carotenoid precursors. In addition, alternative natural apo-carotenoids also display activity in modulating the transcription of retinoid receptors (Harrison and Quadro, 2018).

Retinoic acids

All-*trans*-retinoic acid (ATRA) is an undisputed endogenous and physiological *bona fide* ligand of RARs (Allenby et al., 1993; Giguere et al., 1987). Indeed, it was shown to be produced in vertebrates from nutritional precursors and can be found in human and other vertebrates at concentrations allowing efficient RAR binding and transactivation under physiological conditions (Allenby et al., 1993). Nutritional deprivation of ATRA precursors, which can be determined mainly as reduced serum levels of ATRA / ATROL, lead to various abnormalities, which were observed in humans and extensively studied in rodents (Rhinn and Dolle, 2012; Wilson et al., 1953). Such abnormalities were recapitulated by genetic inactivation of the two classes of retinoid receptors, the RARs and the RXRs (Mark et al., 2009). In summary, ATRA meets a global criterion of endogenous and physiological ligand of RARs in vertebrates.

The identification of the endogenous retinoid X receptor (RXR) ligand was a bit more fragile, partly due to technical challenges in retinoid research (see below). Initially suggested as an endogenous ligand for RXR α (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992), 9-*cis*-retinoic acid (9CRA, Fig. 4A) became quickly and widely accepted as “the” natural RXR ligand (Desvergne, 2007; Evans and Mangelsdorf, 2014; Kane, 2012; Kane et al., 2010). However, many groups with expertise in the retinoid analysis area failed in detecting endogenous 9CRA in humans and other mammals (Blomhoff and Blomhoff, 2006; Gundersen, 2006; Gundersen et al., 2007; Kane et al., 2005; Kane et al., 2010; Kane et al., 2008; Rühl, 2006; Ruhl et al., 2015; Schmidt et al., 2003; Wongsiriroj et al., 2014) and partly questioned its endogenous existence as well as its physiological and nutritional significance (Kane et al., 2005; Kane et al., 2010; Rühl, 2006; Ruhl et al., 2015). Just like in the original study (Heyman et al., 1992), 9CRA identification, secured only *via* co-elution with a standard compound, was reported in animal models only under supra-physiological conditions after administration of a high dosage of naturally-occurring retinoids (retinol or retinoic acids) or after ingestion of food with high content of vitamin A derivatives (Arnhold et al., 1996; Ulven et al., 2001). As a consequence, this led us to suggest that 9CRA is present in sufficient amounts for binding and activating the RXRs only after pharmacological or nutritional retinoid supplementation, but not under physiological conditions. Technological advances and use of highly sensitive MS-detectors made it possible to detect low levels of 9CRA in the range of 0.03 to 0.003 nM (de Lera et al., 2016; Kane et al., 2008), however such levels are not sufficient to modulate RXR signaling. Indeed, various transactivation assays in diverse reporter cell lines showed that the minimal concentration of 9CRA required for RXR binding and initiation of transcriptional activation is in the range of 10 – 100 nM (Heyman et al., 1992; Levin et al., 1992; Ruhl et al., 2015), thus excluding the possibility of 9CRA acting as endogenous and physiological ligand (de Lera et al., 2016; Ruhl et al., 2015).

The difficulties in studying bioactive molecules, including retinoids, may result not only from their low levels, but also from the type of equipment and the wide variety of additional molecules with similar characteristics, which elute in the same area during chromatographic fractionation. Some of those may not even be detected by UV detectors, often used in retinoid studies, due to the lack of conjugated double-bond substructures. Use of highly sensitive MS detectors allows not only to detect weakly represented retinoids, but also to see those that cannot be sensed with UV detectors. This is especially true for 9CRA, as described by one of our groups, with examples from an HPLC-MS-MS methodology already in 2005 (Kane et al., 2010; Rühl, 2006; Ruhl et al., 2015). In particular, MS-MS detection allowed a better identification of other retinoids with similar structures, including 9-*cis*-13,14-dihydroretinoic acid (Fig. 4).

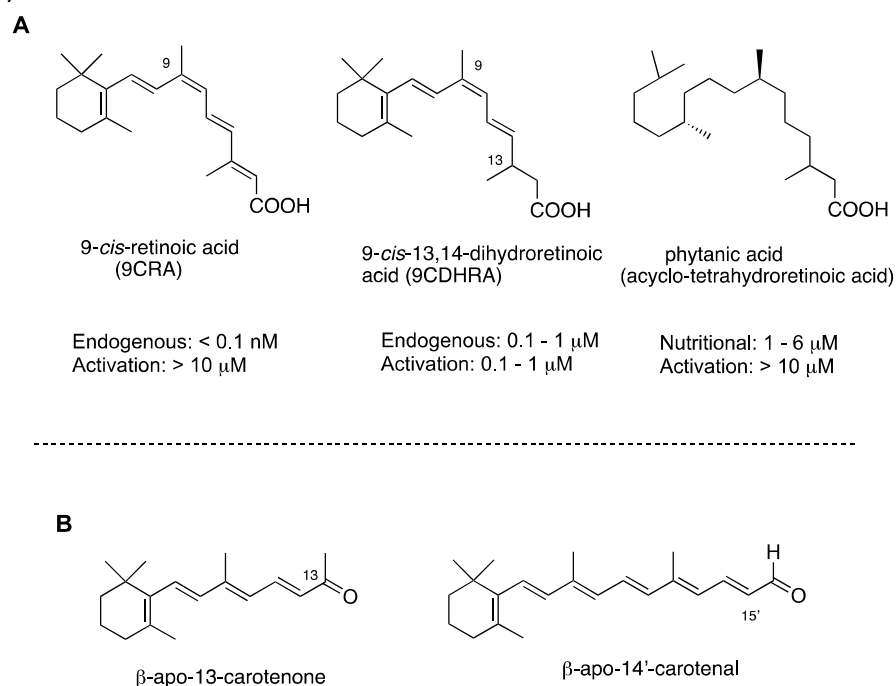


Figure 4 9-*cis*-retinoic acid and 9-*cis*-13,14 dihydroretinoic acid are shown for comparison with exogenous, nutritionally-relevant phytanic acid, which has been drawn in a conformation that mimics a 9-*cis*-configuration, to underline its structural potential for RXR activation (A). Two apo-carotenoids acting as RXR antagonists are found mainly in processed food and can be detected in humans (B).

The new hope - dihydroretinoids

Recently our groups have identified the endogenous presence of 9-*cis*-13,14-dihydroretinoic acid (9CDHRA, Fig. 4A) together with its all-*trans* isomer, in several organs (liver, serum, brain) from mice through a combined liquid chromatography–tandem mass spectrometry using UV analytical set-up and comparison with synthetic standard samples (Ruhl et al., 2015). The quantities measured in mouse serum (~400 nM), liver (~450 nM) and brain (~130 nM) were considered as sufficient for direct interaction with RXRs and to maintain RXR-dependent activities. Accordingly, crystallography analyses revealed that 9CDHRA employs a similar mode of binding to RXRα-LBD as 9CRA (Fig. 2), whereas the affinity of such binding evaluated by fluorescence quenching assay was 90 ± 20 nM, which is very close to the K_d value of 20 ± 10 nM established for 9CRA (Chen et al., 1994). RXR transactivation activity starting at about 100 nM was confirmed with a Gal4 reporter system in Cos1 cells. Importantly, similarly to 9CRA or LG100268, 9CDHRA activated transcription of several target genes of LXR/RXR, PPAR/RXR and RAR/RXR heterodimers in differentiating monocyte-derived human dendritic cells. The physiological relevance of 9CDHRA was documented by deficits of spatial working memory associated

with reduced levels of 9CDHRA in mice with a null mutation of Rbp1, a protein potentially involved in the biogenesis of 9CDHRA. Importantly, such deficits were rescued by 9CDHRA supplementation (Ruhl et al., 2015). Additional functions of 9CDHRA related to RXR signaling are currently under investigation. One of the key questions in such ongoing studies is the nature and origin of metabolic precursors of 9CDHRA (Ruhl et al., 2018). It is not clear whether 9CDHRA is a new bioactive metabolite of ATROL (vitamin A1) and ATBC (pro-vitamin A1), or whether other dihydro- form(s) of retinol or carotenoids exist *in vivo* and could play the role of such a precursor. Thus, if ATROL or ATBC act as precursors of 9CDHRA, the latter could be considered as a new metabolite of the vitamin A1 family. However, if 9CDHRA cannot be efficiently generated from those precursors, being produced from other endogenous retinoid(s) like 9-cis-13,14-dihydroretinol and nutritionally relevant carotenoids like 9-cis-13,14-dihydro- β , β -carotene, it could be considered as a founding member of a new class of vitamin A. In addition to vitamin A1 (ATROL) and vitamin A2 (3,4-didehydroretinol; for recent review see La Frano et al. (La Frano et al., 2018)) it would define a new class of vitamin A present in vertebrates meeting a classical food-to-ligand concept of vitamins. Since the name of vitamin A3 was attributed to 3-hydroxyretinal present in arthropods (Babino et al., 2016) and vitamin A4 to 4-hydroxyretinal found in crustaceans (Goldsmith, 2013), we refer to this potential new class of vitamin A as vitamin A5.

These studies highlight that documentation of the physiological relevance of a ligand necessitates both the knowledge of RXR-related functional markers and the availability of animal model(s) in which not the receptor, but ligand availability, is compromised. Particularly promising for such studies are murine models with compromised activities of enzymes or transport/chaperone proteins involved in ligand biogenesis or uptake. Thus, in order to further identify relevant metabolic pathways and to understand whether 9CDHRA may act as ligand fulfilling the food-to-ligand concept, we hypothesized (Ruhl et al., 2018) and are currently exploring the possibility of its endogenous synthesis from nutritional precursors (Krezel et al., manuscript in preparation).

Alternative carotenoid metabolites

A distinct ATBC derivative, β -apo-13-carotenone (Fig. 4B), has been recently demonstrated in model systems to compromise availability of RXR α to form transcriptionally active dimers by stabilization of its tetrameric, inactive state (Sun et al., 2014b), leading as an end point to the inhibition of RXR α homodimer transcriptional activity on the DR1 ("direct repeat 1" RA-response element)-containing RBP2 promoter (Eroglu et al., 2010). Its binding to RXR α was documented with a K_i of 8 nM in a 9CRA competitive displacement assay, although *in silico* ligand docking analyses with RXR α -LBD suggested different modes of binding (Sun et al., 2014b). Importantly, the same apo-carotenoid also acts as a competitive inhibitor of RARs with a K_i of 4 nM (Eroglu et al., 2012). At equimolar concentration it reduces ATRA-RAR-mediated activity by about 70%. Thus, the presence of β -apo-13-carotenone in human plasma at 3-5 nM concentrations could lead to active synergistic suppression of RAR/RXR signaling by concomitant direct transcriptional inhibition of RARs and tetrameric sequestration of RXR α . Such activity of β -apo-13-carotenone could be potentially deleterious; however, it is not relevant for endogenous carotenoid signaling under physiological conditions, since β -apo-13-carotenone cannot be produced in mammals, and is instead generated by thermal degradation and oxidation of β -carotene during food processing, and otherwise is found only in few fresh fruits (Harrison and Quadro, 2018). Another study from the same group has also reported much lower (1 nM) levels of β -apo-13-carotenone following chronic consumption of β -carotene-enriched diets (Cooperstone et al., 2017). A similar scenario applies to β -apo-14'-carotenal (Fig. 4B), another apo-carotenoid reported to act as a potential inhibitor of RXR α as well as of selected heterodimeric partners (PPAR α , PPAR β/δ , LXRA and LXR β , but not RARs). This apo-carotenoid can be found in several melons,

but its detection in human plasma was not consistent (Harrison and Quadro, 2018). It is therefore unlikely that those apo-carotenoids are physiologically meaningful, although in *in vitro* systems their inhibitory activities could explain the repression of Ap2 / FABP4 and adiponectin transcription in 3T3-L1 cultured adipocytes (Ziouzenkova et al., 2007).

There is a high potential to identify additional bioactive apo-carotenoids acting as RXR ligands, or maybe even as RAR ligands with agonistic and/or antagonistic activity, considering the large diversity of the family of nutritionally-relevant carotenoids and the complex pattern of metabolic conversion in humans and animals (Bohn et al., 2017; Bohn et al., 2019; Rodriguez-Concepcion et al., 2018). In this context, and based on the current knowledge, apo-carotenoic acids and apo-carotenals might be of particular interest. Unfortunately, many pieces of the big picture are still missing to even draw limited conclusions. In addition, there are several differences in carotenoid uptake and metabolism between mouse and human (Lee et al., 1999). Thus, whereas many studies have focused on mice as an experimental model, just a limited number of apo-carotenoids have been identified under physiological conditions and after nutritional intervention in humans, whereas a limited number of apo-carotenoids have been identified in the human food chain (Eroglu et al., 2012; Harrison and Quadro, 2018; von Lintig, 2010). A clear picture of the functional cascade starting from food as source of carotenoids, the relevant concentrations of such carotenoids and their metabolites in human serum and tissues falling in the range for transcriptional RXR activation, is thus missing.

RXR ligands from traditional medical plants/products (phyto-pharmacology).

Quite interestingly, a number of bioactive compounds isolated from traditional medicine sources were found to act as RXR ligands displaying different molecular and biological profiles.

Honokiol (Fig. 5) was one of the first to be reported and probably the most extensively studied (Atanasov et al., 2013; Jung et al., 2010; Kotani et al., 2012; Kotani et al., 2010). Using functional screens based on ABCA1 promoter-driven luciferase reporter, Jung et al. identified this natural product from an extract of *Magnolia officinalis*, as activator of ABCA1 in glioma cells and of ABCA1, ABCG1 and apolipoprotein E (apoE) in THP-1 macrophages, and also of ABCA1 and ApoE in rat neuronal and astrocyte primary cultures with $EC_{50} \sim 10 \mu\text{M}$ (Jung et al., 2010). Direct activation of RXR β -LXR through RXR β was proposed to mediate such transcriptional effects, based on the evidence of honokiol binding to RXR β and the synergistic transcriptional activity of honokiol with pan-LXR agonist T0901317, but was independent from PPAR-signaling. Such activities, confirmed by a parallel study, were functionally relevant as honokiol reduced cholesterol efflux from macrophages (Kotani et al., 2010). These effects may be highly cell-type and/or dose specific, since in 3T3-L1 cells honokiol displayed only little or no transcriptional effect on similar transcriptional targets alone, but efficiently synergized with LXR selective ligands (Kotani et al., 2012). In contrast, it activated in the same cells RXR/PPAR γ signaling, with evidence of direct binding and transcriptional activation of PPAR γ as well as induction of glucose uptake at low concentrations (1-10 μM) (Atanasov et al., 2013). Whereas the effects of honokiol on ApoE / ABCA1 expression and cholesterol metabolism and trafficking, and its anti-inflammatory activities (Rickert et al., 2018) might be of interest for the prevention of atherosclerosis and Alzheimer's disease, the effects on glucose metabolism were shown to be relevant for reduction of glucose levels and body weight in a genetic mouse model of diabetes (Atanasov et al., 2013). It remains to be investigated whether anti-inflammatory, neuroprotective or antitumor activities of honokiol demonstrated more recently in animal models of Alzheimer's or Parkinson's disease may also involve direct RXR activation in addition to the proposed stimulation of PPAR γ signaling (Chen et al., 2018; Wang et al., 2018b). Similar attention was paid to magnolol (Fig. 5), isolated from another extract from *Magnolia officinalis*, which through crystallization and binding studies was shown to bind RXR α and

PPAR γ with EC₅₀ ~40 μ M and EC₅₀ ~2 μ M, respectively. The data might explain its preference for activation of RXR/PPAR γ transcription at EC₅₀ ~10 μ M (Zhang et al., 2011c).

Drupanin (Fig. 5) was isolated from Brazilian green propolis (bee glue), a resinous mixture that honey bees produce by mixing saliva and beeswax with additional exudates from *Baccharis dracunculifolia* (as aster family plant), here produced by Africanized honey-bees (*Apis mellifera*). It is a natural compound with selective, dual RXR/PPAR γ agonistic profile in transactivation and binding assays (no binding to RARs, LXRs, VDR was observed) (Nakashima et al., 2014). This profile was similar to that reported for magnolol, with the exception that drupanin can bind with higher efficacy to all RXR isotypes (EC₅₀ = 2-7 μ M) than to PPAR γ (EC₅₀ ~15 μ M). Consistent with the activation of the RXR/PPAR γ pathway, drupanin increased Ap2 / FABP4 expression and accelerated lipid accumulation in differentiated 3T3-L1 cells.

Prenylated flavonones. RXR agonistic activity was also identified for two prenylated flavonones (Fig. 5) isolated from *Sophora tonkinensis* (Inoue et al., 2014), a herb used in traditional Chinese medicine. They could both bind similarly to all three RXR isotypes, at the nanomolar range, and did not bind or activate RAR α , LXR α or PPAR α , β/δ or γ . Transcriptional profiling for selected target transcripts revealed partially overlapping to synthetic RXR-agonist bexarotene (LDG1069), but also the necessity of at least one range higher concentration to induce such activities. Importantly, either one or the other, or both flavonones, induced expression of several transcripts known to be direct targets of RXR heterodimers with LXR, PPAR β/δ , PPAR γ , but differentially or not at all induced by bexarotene. Involvement of such genes in lipogenesis (e.g. lipoprotein lipase), counteracting inflammation and being antioxidant (heme oxygenase-1) points to a different transcriptional specificity of prenylated flavonones and bexarotene, which highlights the potential biomedical interest of those flavonones.

Danthron and rhein. RXR antagonistic activity was observed for several naturally-occurring compounds. The anthraquinones danthron and rhein (Fig. 5), isolated from Chinese rhubarb *Rheum palmatum*, were reported to antagonize 9CRA in RXR α -LBD, Gal4-luciferase transactivation assays at relatively low concentrations, with IC₅₀ values of 10 and 70 μ M, respectively (Zhang et al., 2011a; Zhang et al., 2011d). Crystallography studies revealed that through direct interactions with LBD, leading to exacerbated displacement of H12 helix, anthraquinones induce the recruitment of SMRT (silencing mediator for retinoid and thyroid hormone receptors) corepressor and stabilize an inactive, tetrameric form of RXR making it inaccessible for formation of transcriptionally functional homo- or heterodimers. As a consequence, danthron and rhein inhibit RXR-mediated transactivation through RXR-homodimers, but also through heterodimers with FXR, LXR or PPAR γ . Although those ligands were suggested as potentially useful for the treatment of diabetes due to improvement of insulin tolerance in mouse models (Wang et al., 2018d; Zhang et al., 2011a; Zhang et al., 2011d), the direct involvement of RXRs in those activities is unlikely. In support of this assumption, respective treatments induced activation of some RXR target genes like ABCA1 and ABCG1, which is opposite to what could be expected from an RXR antagonist. An alternative mechanism, proposed to involve the action on AMPK signaling for danthron (Zhou et al., 2013) or the modulation of microbiota for rhein (Wang et al., 2018d), might be thus more relevant.

Bigelovin (Fig. 5) is a sesquiterpene lactone isolated from the flowers of *Inula helianthus-aquatica* or *Inula hupehensis*, which are used in folk medicine for their anti-cancer virtues as confirmed also by some experimental studies (see for example anti-colon cancer (Li et al., 2018) and anti-liver cancer (Wang et al., 2018a)). Although bigelovin binds to RXR α -LBD (no binding was identified for LXR α , FXR or PPAR γ), it displays very peculiar transcriptional activities since it transactivates RXR α -PPAR γ heterodimers, but fails to transactivate RXR α -FXR heterodimers and suppresses RXR-homodimers or RXR α -LXR heterodimers on their respective response elements in the 1-10 μ M concentration range

(Zhang et al., 2011b). Although the relevance of such specific transcriptional activities is not clear, crystallographic studies indicated a specific mode of action of bigelovin binding to RXR-LBD, revealing that by ligand-mediated remodeling of LBD structure it may discriminate not only between RXR-homodimer and heterodimer interactions, but also induce opposing activities of specific RXR heterodimers.

Valerenic acid. Recently, the screen of the Dictionary of Natural Products based on isofunctional mimetics of structures of natural RXR modulators allowed to identify valerenic acid (Fig. 5) as a novel RXR agonist (Merk et al., 2018b). This sesquiterpenoid, isolated from *Valeriana officinalis* (a flowering plant mainly present in Europe and Asia), showed agonistic activity in the micromolar range, which was reflected in the efficient induction of the RXR transcription target genes ABCA1 and ApoE. The Gal4-based reporter assays performed in HEK293T cells revealed intriguing partial selectivity for RXR β , since valerenic acid showed better EC₅₀ (5 μ M) and about 10-times higher transactivation of RXR β as compared to RXR α (EC₅₀ = 27 μ M), and RXR γ (EC₅₀ = 43 μ M). In addition, no activation of RARs, PPARs, LXRs, FXR, CAR, VDR and PXR was observed. Molecular modeling studies suggested similar binding modes to RXR α and RXR β as classical rexinoids (Merk et al., 2018b). This first-in-class ligand with RXR-isotype selectivity has stimulated further development of synthetic ligands (see below).

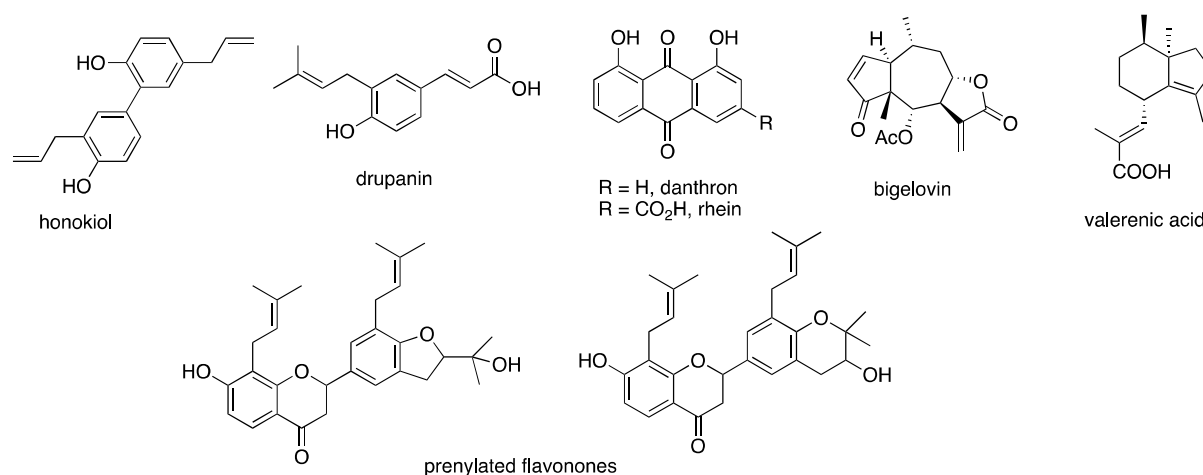


Figure 5: Naturally-occurring RXR ligands from plants with phyto-pharmacological relevance.

SYNTHETIC LIGANDS

The design of synthetic ligands was much instructed by the chemical structures of endogenous ligands, the structure of RXR LBD and data on ligand – LBD interactions. Inversely, challenging RXR LBD with such ligands and millions of molecules *in silico* and in functional screens followed by determination of their structure-activity relationship (SAR), and sometimes even crystal structure, was highly informative about: (i) flexibility of LBD in binding different compounds, (ii) how such flexibility affects specificity of RXR dimerization with other nuclear receptors, and (iii) alternative modes of binding to RXRs. Importantly, whereas some compounds like organotinins can bind efficiently RXR LBD despite their small size and thanks to covalent bonds, other compounds can bind in the proximity of LBD or at

cofactor binding hydrophobic surface of AF2. Collectively, such diverse modes of interactions suggest a possibility of existence of alternative natural or endogenous ligands with such novel or yet unknown modes of interaction.

“Classical” ligands generated by mimetics of endogenous ligands

Crystal structures of RXR-ligand complexes (Huang et al., 2014) have shown that RXR ligands hold or adopt frequently a bent structure including a twisted polyene side-chain in order to bind to the L-shaped ligand-binding pocket of RXR (Egea et al., 2000). Thus, conformational flexibility and modular structure of 9CRA and 9CDHRA, and also certain unsaturated fatty acids, allows them to bind both RAR and RXR by optimal adaptation to (and filling of) the LBP. Rationally-designed rexinoid scaffolds making reference to these natural ligands, and called therefore “classical”, usually contain three structural modules: the carboxylic acid, a linker fragment allowing to achieve permanent or induced skeletal twist and the hydrophobic carbo- or heterocyclic terminal ring (see respectively red, blue and green domain in Fig. 6).

The linking unit is quite amenable to modifications, which according to their chemical nature and functional groups can be divided (Fig. 6) into 5 groups: A) polyenoic acids including 9-*cis*-locked compounds, B) aryl rings connected by trigonal (Csp^2) or tetrahedral (Csp^3) carbon, C) biphenyl rings with *ortho* substituents, D) diarylamines, and E) fully condensed rings, such as dibenzodiazepines (Dominguez et al., 2017). Comparison of these structures points to the high adaptability exhibited by the RXR LBD, which is also supported by the ligand-bound crystal structure complexes (Huang et al., 2014). On line with the above structural considerations, RXR antagonists usually contain certain substituents attached to the central linker region (see arrows in Fig. 6) which, depending upon their overall size, fully or partially disrupt the hydrophobic interactions that stabilize the agonist conformation of H12 (Egea et al., 2000). The functional transition from agonists to partial agonists/antagonists within a family of rexinoids by changes on the length or bulk of these substituents is usually observed.

Recent revisions and arbitrary classifications of the most important rexinoid scaffolds, for which comprehensive structural and functional information has been provided, can be consulted (Dominguez et al., 2017; Mendoza-Parra et al., 2015; Su et al., 2017; Wagner et al., 2017). Only an overview of the most potent and selective rexinoids will be described, with a particular emphasis on their structural diversity, mode of binding to RXR, and activity on specific RXR homodimers and RXR/NR permissive heterodimers.

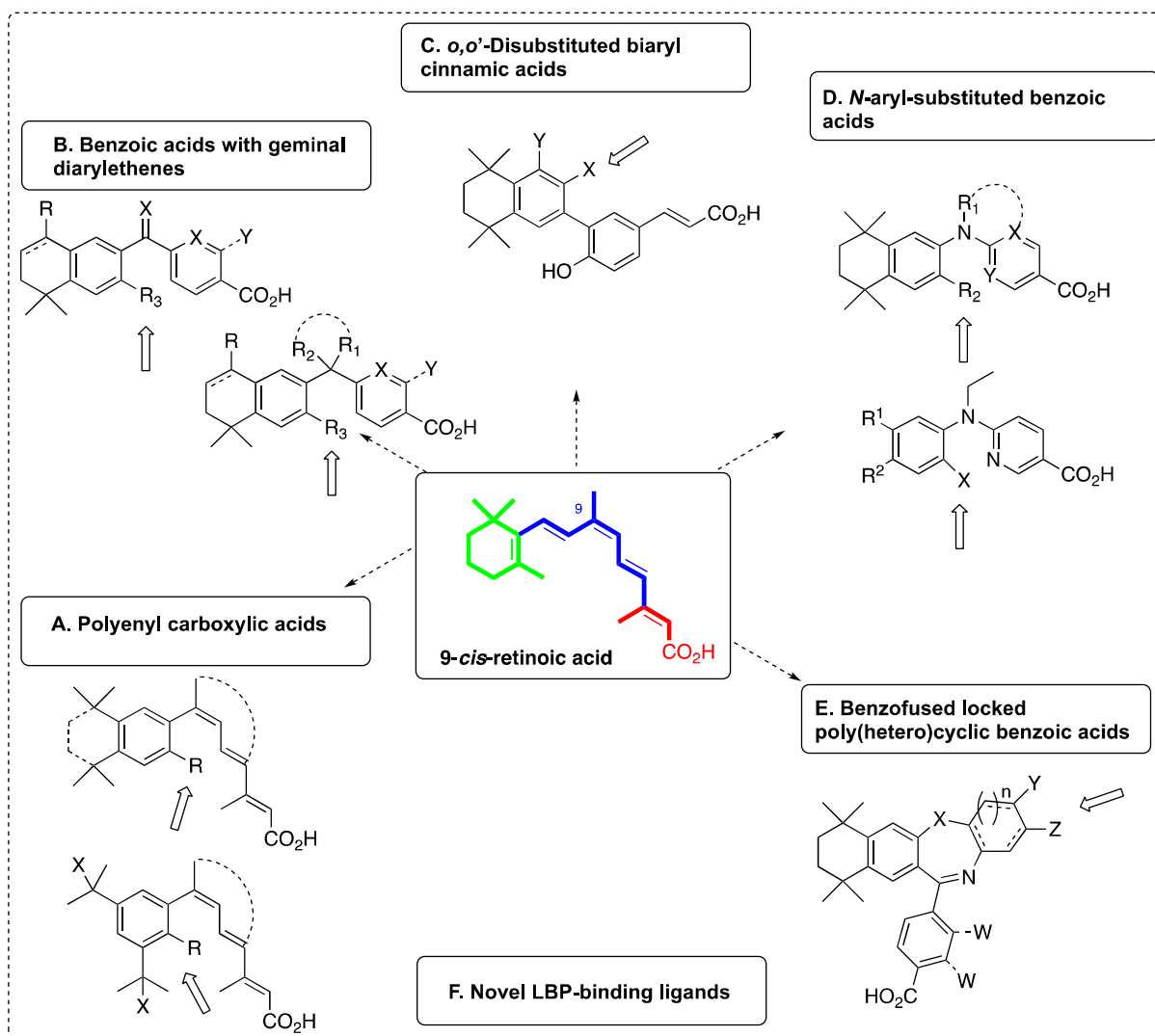


Figure 6. Structures of the rationally-designed rexinoid classes organized in series collecting their structural diversity as the combination of building blocks with hydrophobic features (green), linkers (blue) and polar motifs (red). Antagonist feature groups attached to the arrowed positions are shown.

Group A. Polyenyl carboxylic acids (Fig. 7A)

This group of rexinoids maintains part of the acyclic polyene chain of 9-*cis*-retinoic acid. Compound LG100754 (Canan Koch et al., 1996) is transcriptionally neutral by itself, and functions as a competitive RXR antagonist in RXR homodimers, and as an agonist for PPAR α /RXR heterodimers, but does not activate LXR/RXR, FXR/RXR, or Nurr77/RXR heterodimers (Canan Koch et al., 1996). It inhibited transactivation in a concentration-dependent manner at low concentrations (IC₅₀ 20 nM) and also antagonized transactivation of RXR homodimers by rexinoid agonists LG100268 (Fig. 7A) or 9-*cis*-retinoic acid. Moreover, LG100754 binding to RXR α did not affect the binding of the SRC-1 NR2 peptide to the RAR α apo form (Sato et al., 2010b). Crystallography studies confirmed that LG100754 binding leads to stabilization of H12 in an unfolded position typical for inactive RXR (Sato et al., 2010a; Sato et al., 2010b).

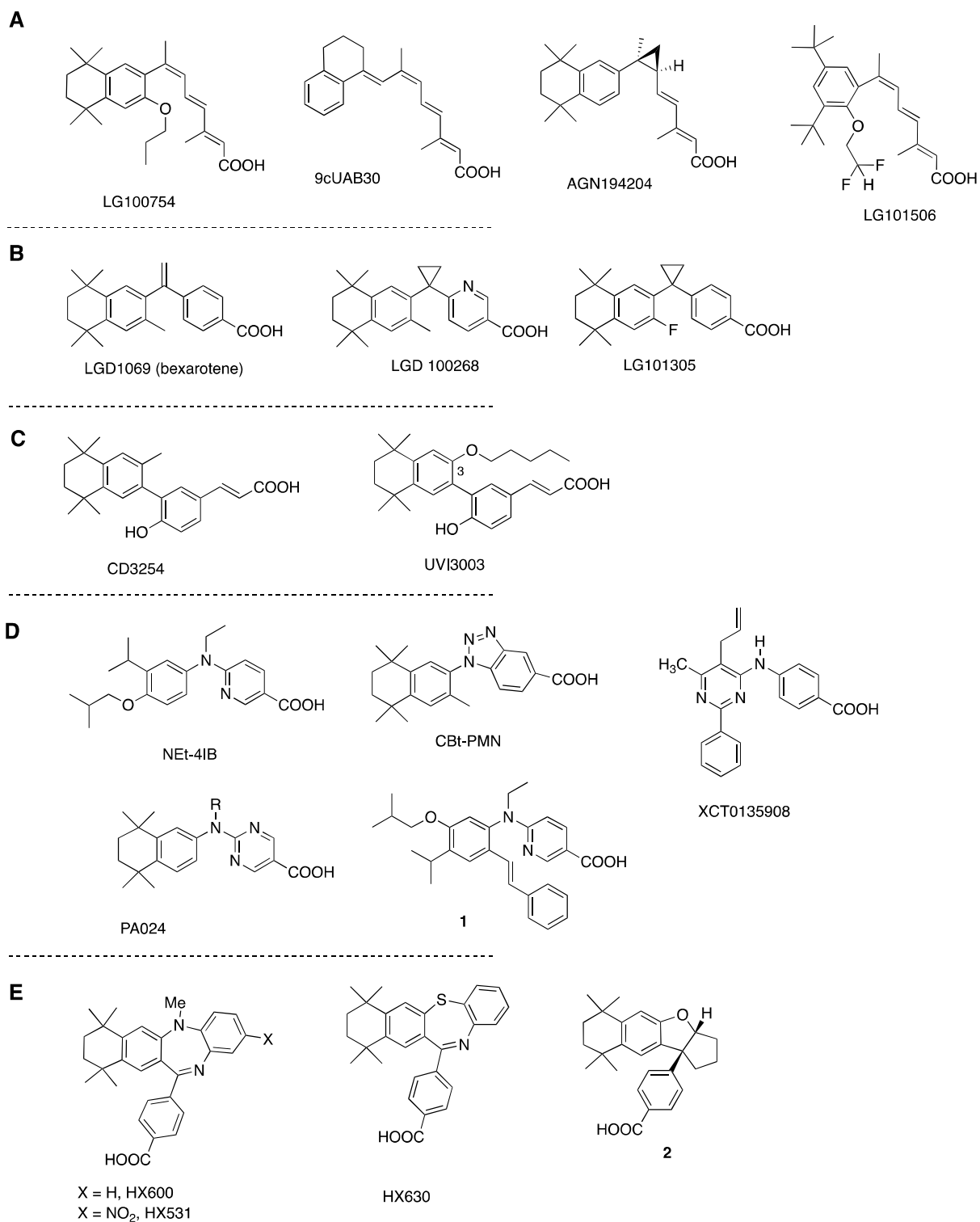


Figure 7. Structure of polyenyl retinoids (A), retinoids with geminal diarylethene units (B), retinoids with biaryl cinnamic acid substructures (C), retinoids with *p*-N-aryl benzoic acids (D), and benzofused poly(hetero)cyclic retinoids (E).

9cUAB30 (Muccio et al., 1998) showed induction of apoptosis, reduction of proliferation and prevention of mammary cancer in rodent models, similarly to 9-*cis*-retinoic acid (Atigadda et al., 2003;

Grubbs et al., 2006). National Cancer Institute clinical trials of 9cUAB30 as cancer chemopreventive agent revealed no significant toxicity (Kolesar et al., 2010), although hepatomegaly was observed in animal studies at high doses (Kapetanovic et al., 2010).

AGN194204 appears to be the most potent and specific agonist reported (Vuligonda et al., 1996) with EC₅₀ values ranging from 0.08 nM (RXR γ) to 0.8 nM (RXR β). It also showed a potent hypoglycemic effect in the *db/db* mouse model (genetically defective in leptin signaling), perhaps due to the activation of the PPAR γ /RXR or other heterodimeric-dependent pathways.

LG101506 was identified as a heterodimer-selective RXR modulator (Leibowitz et al., 2006) that binds RXR with high affinity (K_i values of 3.0 \pm 0.8; 9.0 \pm 1.7; 11.0 \pm 3.6 nM for RXR α , β , and γ , respectively) and was also characterized as a RXR homodimer partial agonist. LG101506 selectively activated PPAR γ /RXR and PPAR α /RXR (Haffner et al., 2004; Leibowitz et al., 2006), but not RAR α /RXR, LXR α /RXR, LXR β /RXR or FXR α /RXR heterodimers (Michellys et al., 2003). When administered daily at 30 mg/Kg body weight to *db/db* mice, a model of type 2 diabetes, this compound induced lowering of glucose levels. Preclinical studies have shown that LG101506 caused beneficial metabolic effects without inducing undesired hypertriglyceridemia or thyroid suppression and did not induce weight gain in rodents (Leibowitz et al., 2006).

Group B. Benzoic acids with geminal diarylethene groups and analogues (Fig. 7B)

LGD1069 (bexarotene, Targretin[®]) was found to bind RXR with high affinity (K_d values of 14 \pm 3; 21 \pm 4; 29 \pm 7 nM) and showed poor binding affinity for the RAR isotypes (K_d >1000 nM)(Boehm et al., 1994; Boehm et al., 1995; Lehmann et al., 1992). It has been approved for the treatment of cutaneous T-cell lymphoma (CTCL) (Tanaka and De Luca, 2009), although as observed with other rexinoids, the drawbacks associated to the use of LGD1069 are the induction of hypothyroidism due to antagonism of the TR receptor with ligand-activated RXR (Sherman et al., 1999), and hyperlipidemia and cutaneous toxicity as a result of residual RAR agonism. Bexarotene was found to activate Nurr1/RXR heterodimers, rescue dopamine neurons and restore behavioral functions in a rat model of Parkinson's disease (McFarland et al., 2013).

The structural analogue LG100268 (Fig. 7B) was found to be even more RXR-specific (K_d of 3 nM for RXR subtypes; K_d > 1000 nM for RARs), with a binding affinity somewhat higher than LGD1069. This compound gave 50% maximal activation at a concentration at least 10-fold lower than that of 9-*cis*-retinoic acid (Boehm et al., 1995). By activation of RXR homodimers (Szeles et al., 2010) as indicated by transcriptome profiling, LG100268 was able to induce a number of target genes during differentiation of monocytes to dendritic cells or during osteoclast differentiation (Menendez-Gutierrez et al., 2015). LG100268 strongly activates the LXR/RXR (Faul et al., 2001) and the PPAR γ /RXR heterodimers (Cesario et al., 2001; Forman, 2002).

Rexinoid LG101305 (Fig. 7B) was shown to suppress the transactivation of androgen receptor (AR) in a reporter gene assay (Chuang et al., 2005).

Group C. *o,o'*-Disubstituted biaryl cinnamic acids (Fig. 7C)

Sterically hindered compounds such as CD3254 were characterized as selective RXR agonists (Pérez-Santín et al., 2009). The incorporation of *O*-alkyl substituents led to antagonist UVI3003 (Nahoum et al., 2007), which showed no effect on the tetrameric state (Sun et al., 2014a). Analogs with shorter alkoxy side chains were found to act as partial agonists, and their crystal structures when bound to RXR α LBD and co-activator peptide TIF2 NR2 revealed the structural reasons for their activity (Nahoum et al., 2007; Pérez-Santín et al., 2009).

Group D. Benzoic acids substituted with N-aryl groups at C4 (Fig. 7D)

These analogues (Fig. 7D) were found to display a full range of rexinoid ligand functions, including the differential modulation of RXR heterodimers (Takamatsu et al., 2008a; Takamatsu et al., 2008b). NET-41B ($EC_{50} = 169$ nM), which showed a 50-60% efficacy towards all RXR subtypes in a luciferase reporter gene assay (Kawata et al., 2015), was characterized as a RXR partial agonist (Ohsawa et al., 2013) since it recruited both coactivators (CoA) and corepressors (CoR). Oral administration of NET-41B to mice resulted in antidiabetic effects without the undesired side effects of full agonists.

CBt-PMN (Kakuta et al., 2012; Ohsawa et al., 2013) is a representative member of a class of ligands having reduced conformational flexibility. In vitro, CBt-PMN activated selectively the RXR α -PPAR γ and RXR α -LXR α heterodimers as a partial agonist ($EC_{50} = 143$ nM, $E_{max} = 75\%$), whereas in vivo ($EC_{50} = 15$ nM, $E_{max} = 67\%$) it showed beneficial effects (potent glucose-lowering, improvement in insulin secretion and glucose tolerance) in type 2 diabetes mice models (Kakuta et al., 2012). Interestingly, X-Ray structures revealed that CBt-PMN could bind hRXR α ligand-binding pocket (LBP) in two different conformations leading to the formation of hRXR α tetramers that consist of activated (H12 in folded conformation) and repressed (H12 in opened conformation) forms of hRXR α , thus explaining potentially partial agonistic activity (Miyashita et al., 2019).

PA024 was found to activate PPAR γ /RXR and LXR/RXR heterodimers, whereas alkoxy derivatives modified at the lipophilic region as in analogue **1** (Fig. 7D) led to selective activation of PPAR γ /RXR α vs LXR α /RXR α heterodimers (Ohsawa et al., 2010).

XCT0135908 compound (discovered after screening of a chemical library) selectively activated the Nurr1-RXR heterodimers in African green monkey CV-1 cells (Wallen-Mackenzie et al., 2003). As indicated above, Nurr1/RXR specific rexinoids might be of interest for the treatment of neurodegenerative diseases (Parkinson's disease) (Wallen-Mackenzie et al., 2003).

Group E. Benzofused locked poly(hetero)cyclic benzoic acids (Fig. 7E)

HX600 was shown to play a dual function in the regulation of retinoid receptor activities, acting as a RXR synergist (rexinoid) or as a RAR antagonist at low and high concentrations, respectively (Umemiya et al., 1997). Modeling studies indicated that HX600 occupied the RXR α LBP and maximized the cavity occupancy when anchored similarly to 9-*cis*-retinoic acid (Egea et al., 2000). Albeit a weak RXR agonist, HX600 was able to selectively activate NGFI-B/RXR (via an allosteric effect) and Nurr1-RXR heterodimers (Morita et al., 2005).

Modifications of the skeleton generated RXR antagonists (Kagechika and Shudo, 2005), such as the nitroderivative HX531, which inhibited both RXR transactivation and HL-60 cell differentiation mediated by RAR/RXR heterodimers, and transactivation of RARs induced by an RAR agonist (Ebisawa et al., 1999). It also antagonized PPAR γ /RXR, but not the PPAR α /RXR heterodimer. Thus, even in the case of permissive RXR heterodimers, some rexinoids appear to adopt formal agonistic and antagonistic structures, depending upon the partner and/or related cofactors (Shulman et al., 2004).

Other rexinoids have been developed to also function as Nurr1/RXR heterodimer activators, with sterically constrained enantiomer **2** being the most potent of the series (Sundén et al., 2016).

Group F. Novel LBP-binding ligands (Fig. 8)

Structurally novel ligands have emerged from computer-aided screens (Katsila et al., 2016; Sliwoski et al., 2014), increasing the structural diversity and revealing novel modes of ligand-LBP interactions. In

this regard, computational search of a collection of commercially available compounds (3.38 million) aimed to discover new RXR ligands revealed the agonistic activity of structurally novel RXR ligands, such as **3**, which showed micromolar potency and some moderate RXR β and RXR γ selectivity (Merk et al., 2018a).

In addition, screening the Dictionary of Natural Products based on isomimetics of natural RXR ligands led to identification of valerenic acid as new natural RXR ligand (Merk et al., 2018b) (see section on natural ligands for detailed description). *De novo* design based on this natural product afforded analogue **4**, which showed 3-times enhanced activation of RXR as compared with valerenic acid and retaining some selectivity to RXR β activation (Merk et al., 2018a).

Structure-based virtual screening of RXR α LBP using a collection of 1280 drugs approved by the FDA in different conformations into the LBP of the agonist- and antagonist-bound structures of RXR α afforded several potential ligands. Statin drugs (which are inhibitors of sterol biosynthesis) were predicted to be RXR α antagonists, including pitavastatin (and its bis-dihydrated derivative), fluvastatin and analogues (Xu et al., 2017). Rosiglitazone (as maleate) and domperidone behaved as agonists only in the presence of 9-*cis*-retinoic acid, and therefore enhanced the effect of the latter. Surface plasmon resonance experiments indicated binding of these compounds to the purified RXR α -LBP protein although Molecular Dynamics simulations suggested that pitavastatin and fluvastatin bind to the LBP, with very fast rates of association and dissociation, and the hydroxyl groups do not make any contact with residues of the LBD (Xu et al., 2017).

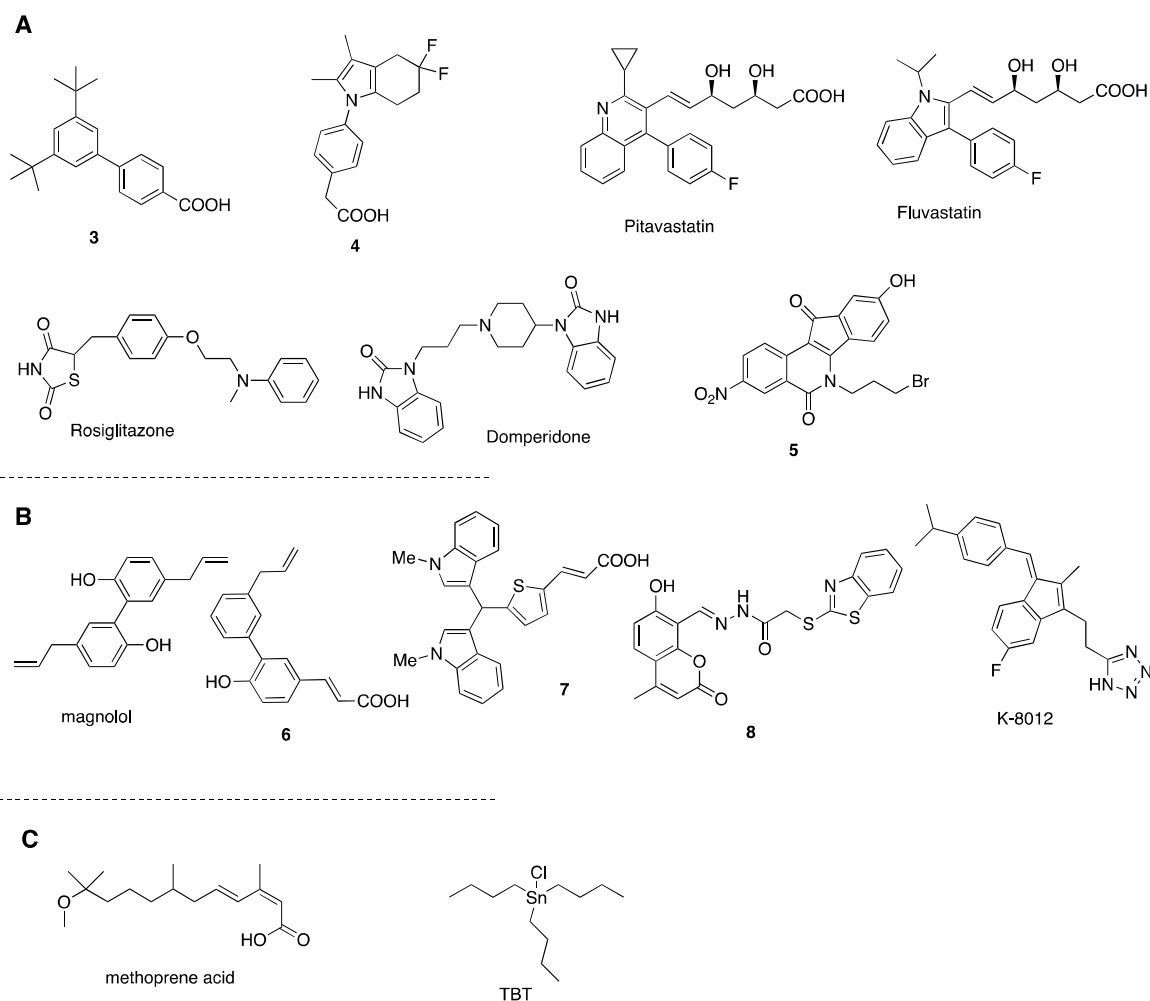


Figure 8. RXR modulators from virtual ligand screening and computational-based drug discovery (A), non-canonical RXR modulators (B), and ligands of environmental relevance (C).

Compound **5** (Fig. 8A) was discovered as a selective RXR α agonist after a screening campaign of a 5000 compound library. It was found to induce apoptosis of MCF-7 breast cancer cells and inhibit proliferation of HL-60 cells (Park et al., 2011). Although subsequent comprehensive SAR studies carried out after docking of the lead structure suggested structural modification to improve binding efficiency (among them, the presence of a carboxylic acid side chain), they did not reveal the structural basis of the interaction with RXR (Conda-Sheridan et al., 2013).

Although the discovery of some of these new RXR ligands resulted from the evaluation of structurally diverse collections of natural products and their metabolites, as well as commercial libraries, their relatively small structural diversity, easy adaptation to the LBP and, in most of the cases, their smaller size result in suboptimal occupancy of the ligand binding cavity of RXR. However, some of the existing structural differences (see Fig. 8) are just an indication of the conformational adaptability of RXR LBP to bind compounds with a variety of skeletons (Dominguez et al., 2017). Synthetic and medicinal chemists are now capable of further developing these scaffolds in order to improve potency and selectivity.

Non-canonical modulators of RXR

Other RXR modulators lack the carboxylic acid, such as the natural product honokiol (Fig. 5), and the positional isomer magnolol (Fig. 8B), which induced the transactivation of the PPAR γ /RXR α heterodimer, but not that of the RXR α /RXR α homodimer (Park et al., 2011). The crystal structure of the ligand-bound RXR α LBD revealed that the *p*-allyl phenol moiety of honokiol occupies the L-shaped arms of the LBD and one of the phenol hydroxyl groups forms a hydrogen bond with Asn306 on N-terminal helix H5. More recent Molecular Modeling and NMR spectroscopy studies concluded that honokiol targets RXR at both sides of the interface, acting on the coactivator side of the dynamic activation function (AF2) or alternatively switches from one to the other side of the interface. Rational design allowed to split the dual-binding properties of the natural product honokiol, and led to compound **6** (Fig. 8B), a first-in-class modulator reported to inhibit co-activator binding at the solvent-exposed side of AF2 (as confirmed by the X-Ray structure) (Scheepstra et al., 2014).

Compound **7** is representative of a new RXR scaffold (Dawson et al., 2009) that did not activate RARs or PPAR γ . Docking experiments proposed the stabilization of the complex by formation of the salt bridge of the carboxylic acid of **7** with Arg316, and interactions of the indole rings with Cys269 and Cys432, and the *N*-methyl group with hydrophobic residues (Dawson et al., 2009).

A VLS campaign using the coactivator binding site of the crystal structure of RXR α -CD3254-CoA peptide led to the discovery of the new antagonist **8** (Chen et al., 2014a), which offered some selectivity for RXR α over other NRs, as well as for the RXR heterodimers (Chen et al., 2014a). Competition experiments with tritiated 9-*cis*-retinoic acid allowed to discard compound binding to the LBP and supported binding of **8** to the coregulator binding site. Compound **8** inhibited AKT-dependent activation in several cancer cell lines in the absence or presence of TNF α , and this effect might be related to its ability to induce apoptosis through inhibition of the interaction of tRXR α with p85 α (Chen et al., 2014a). More recent studies have proven that the compound sits in a coactivator-binding groove (Xu et al., 2016).

Sulindac analogs with increased affinity for tRXR α have been designed (Zhou et al., 2010), in particular K-8012 (Fig. 8B). K-8012 showed improved activity in the inhibition of the tRXR α -mediated PI3K/AKT signaling pathway. It was characterized as antagonist of RXR α in co-transfection assays, and in cell-based assays it was more potent than Sulindac in the induction of apoptosis and inhibition of the binding of tRXR α to the p85 α regulatory subunit of PI3K (Chen et al., 2014b). Strikingly, it failed to displace tritiated 9-*cis*-retinoic acid from its binding site, thus discarding the possibility of binding to the canonical LBP. A time-resolved FRET RXR α coactivator peptide competition assay showed that unlike 9-*cis*-retinoic acid, and similarly to Sulindac, K-8012 targets the hydrophobic receptor surface region near the entry and the edge of the cognate LBP (Chen et al., 2014a), resulting in stabilization of the tetrameric conformation of RXR α with two homodimers packed in a bottom-to-bottom manner. This might explain why K-8012 fails to compete with the binding of 9-*cis*-retinoic acid but can still inhibit cancer cell growth (Zhang et al., 2011d).

Environmentally relevant synthetic ligands

Discussing RXR ligands to which human and multiple animal species are exposed in daily life, it is necessary to mention environmental pollutants. The group of compounds which may modulate RXR activities include phthalates, plasticizers, certain herbicides, organotins, biocides, and pharmaceuticals. These have been extensively reviewed elsewhere (Rogers et al. 2013; Delfosse et al. 2014, 2015). In this review we will focus only on those that have been well documented to bind RXRs and modulate their activities (Fig. 8C).

Methoprene acid (MPA) is one of the very widely used pesticides for mosquito control. It is a synthetic analogue of the juvenile hormone, acting as its mimetic. It was originally identified as a transcriptional activator of RXR in a screen of synthetic and natural isoprenoid compounds (Harmon et al., 1995). MPA acts as a RXR-selective agonist, without activation or binding to other nuclear receptors. The mode of MPA action involves direct binding of L-shaped LBD thanks to the flexibility of its polyene chain. It was documented by competition with 9CRA, but also Gal4-RXR α reporter assay and ultimately by crystal structure of apo-RXR α -LBD with MPA (Svensson et al., 2003). It was shown to induce transcription at RXR-specific CRBP II promoter through all three isoatypes of RXRs with EC₅₀ of 2 μ M in insect and 20 μ M in mammalian cell lines. Whereas some preference for RXR γ over RXR α and RXR β was observed in insect Schneider cells, only RXR β responded weakly in CV1 mammalian cell line (Harmon et al., 1995). Methopren, a parent molecule of MPA, was reported to upregulate Abca1 and Abcg1 in cultured rat astrocytes, leading to cholesterol efflux (Chen et al., 2011).

Organotins constitute a large group of tin compounds that are widely present in the environment since the 1960's due to their application in many agricultural and industrial processes like antifouling paints for ships and fishing nets (Appel, 2004; Fent, 1996). Tributyltin chloride (Fig. 8C, TBT) is among the best-documented organotins for its action on RXRs. Crystallography analyses revealed a very particular mode of binding to RXR α LBD. Despite the fact of its much smaller size than any other RXR ligand, but also absence of a carboxylate group with long aliphatic chain, it could bind RXR α -LBP with high, nanomolar affinity through limited number of molecular interactions including covalent S-Sn binding with C432 of helix H11 (Hiromori et al., 2015; le Maire et al., 2009). Accordingly, tributyltin chloride induced transcriptional activity of PPAR γ /RXR as well as LXR/RXR and NURR1/RXR heterodimers (Germain et al., 2006b; Grun and Blumberg, 2006; Hiromori et al., 2015). Such transcriptional activities were shown to be relevant for control of aromatase gene expression (Nakanishi et al., 2005) and were found to promote adipocyte differentiation at nanomolar concentrations (Grun and Blumberg, 2006; Kanayama et al., 2005), and to cause pseudohermaphroditic transformation in some gastropode females known as "imposex" leading to reproduction failure (Urushitani et al., 2018).

Further analyses of environmental pollutants may lead to the identification of additional RXR ligands as suggested by recent *in silico* analyses of molecular interactions, which predict strong binding of easily absorbed bisphenol PH to hPPAR α , β , γ and hRXR α (Sharma et al., 2018). It is worrying that some of the most toxic bisphenol analogues, bisphenol M and bisphenol AF, were also predicted to strongly bind to hRXR β and hRXR γ .

EVOLUTIONARY CONSIDERATIONS

To better understand the origins but also the functional relevance of ligand-dependent regulation of RXRs in vertebrates, we need to travel in time a billion years backwards and look into the evolution of RXRs, their ligands and their functions. Phylogenetic analyses identified an RXR ancestral gene in Placozoans (Baker, 2008; Srivastava et al., 2008) and Cnidarians (Fuchs et al., 2014; Kostrouch et al., 1998), the simplest multicellular organisms and most ancient animal lineages. In particular, *Trichoplax adhaerens* (an ameboid-like multicellular organism, a member of Placozoans present in seawater) is the first organism in the animal lineage to have a well-documented RXR orthologue, the TaRXR (Baker, 2008; Srivastava et al., 2008). This RXR prototype was recently shown to share a number of important structural and functional similarities with mammalian RXRs. First, it displayed high degree of homology with RXRs from several species, which for human hRXR α attains 81% of homology for the DBD and 70% for the LBD (Novotny et al., 2017; Reitzel et al., 2018). This homology is functionally relevant as DNA-binding motif specificity as revealed by protein binding microarrays was conserved up to 85% as compared to hRXR α (Reitzel et al., 2018). Similarly to human RXRs, TaRXR also bound with high affinity 9CRA which modulated its transcriptional activity (Novotny et al., 2017; Reitzel et al., 2018). Importantly, the possibility of ligand-dependent TaRXR-mediated induction of an orthologue of vertebrate L-malate-NADP⁺ oxidoreductase places this receptor and its ligand(s) as key modulators of an ancestral Krebs cycle, a cornerstone of the evolution of cellular metabolism and central regulator of different metabolic pathways. Such an important function necessitates a high level of precision in the control of TaRXR activities and might have exercised an evolutionary pressure to limit the number of endogenous and/or physiological RXR ligands. Thus, ancestral TaRXR could have acted as the first high-affinity receptor for a limited number of ligand(s), with the main function to enhance global metabolic activity in environmentally favorable conditions to produce energy (e.g. to ensure reproduction), or reduce energy production in unfavorable conditions (e.g. to avoid exhaustion). Several arguments favor this hypothesis. Firstly, in contrast to several other NRs acting as metabolic sensors and capable of binding different types of ligands (individually or even simultaneously) in a large (>1000 Å³) ligand binding pocket (Holzer et al., 2017), the RXR across different species has relatively well conserved small ligand binding pocket of about 400 Å³ in volume (Egea et al., 2000; Tocchini-Valentini et al., 2009). Such small volume favors interactions with only limited number of ligands, like 9CRA, which fills about 60% of this space assuring thereby a tight fit and specificity. Secondly, in addition to conservation of the structure of the RXR LBD across a vast evolutionary distance (Tocchini-Valentini et al., 2009), its capacity to bind 9CRA was conserved from the earliest metazoans (Reitzel et al., 2018), through mollusks (de Groot et al., 2005) or chordate-like amphioxus (Tocchini-Valentini et al., 2009), to *Homo sapiens*, documenting the strong evolutionary pressure to conserve high-precision in binding the same type of ligand(s). The exception would mostly be insect RXR orthologues like ultraspiracle (USP) from *Mecopterida* (winged insects) and non-*Mecopterida*, which do not bind and are not activated by RXR ligands (Iwema et al., 2007) and can act as silent, non-permissive partners of the ecdysone receptor. The loss of ligand binding capacity emerged, however, relatively late during insect evolution as RXR orthologues from some primitive insects like *Tribolium* (beetles) (Iwema et al., 2007) or *Locusta migratoria* (a grasshopper species) (Nowickyj et al., 2008; Palli et al., 2005) preserved potential to bind 9CRA. The fact that TaRXR shows DNA-binding specificity mostly to a single half site rather than to repeated sequences (Reitzel et al., 2018) also indicates that in *Trichoplax adhaerens* RXR was most probably acting as a monomer, avoiding thus the influence of other signaling pathways in the control of global metabolic activity.

The remaining question is, what is this mysterious ancestral ligand? Was it produced in *Trichoplax adhaerens* from nutritional precursor(s) to act as endogenous ligand, or was it originally a nutritional ligand and organisms adopted it as “endogenous” through evolution of dedicated metabolic

pathway(s)? Whereas 9CRA was certainly a very valuable experimental ligand to document conservation and high-affinity binding properties of RXR ligand binding pocket across evolution, it does not seem to be a physiologically relevant ligand as it was detected at moderate levels only in arthropod *Locusta migratoria* (Nowickyj et al., 2008; Palli et al., 2005), but not in other phyla or species. Thus, it is tempting to hypothesize that 9CDHRA, a physiological RXR ligand in vertebrates (Ruhl et al., 2015), could be an ancestral retinoid and prototypic ligand for RXRs. This possibility is supported by the identification of dihydroretinoids or their precursors in non-vertebrates (Foster et al., 1993) and vertebrates (Aydemir et al., 2013; Moise et al., 2007; Moise et al., 2004). Understanding the metabolic origin of 9CDHRA and its nutritional precursors will be the focus of future research, as they could be directly relevant to the diet of *Trichoplax adhaerens*. To address this theory, studies of representatives of ancestral organisms in the context of their endogenous habitats and food chain are necessary. The obtained data should provide insights into the interplay between genetic and environmental constraints shaping ligand-receptor interactions with RXR as model NR. Of particular interest for such investigations, but also for the identification of new nutritionally or pharmacologic valuable RXR ligands, might be the studies of micronutrient diversity in the oceans, the cradle of animal life (Markov et al., 2018).

CONCLUSIONS AND FUTURE DIRECTIONS

Since the initial identification of 9CRA as an agonist of RXR and the demonstration that RXR homo- and heterodimers can be modulated by RXR ligands, much research has been performed to dissect their physiological and biological relevance, and the molecular mechanisms of ligand-dependent RXR control. Investigation of natural and synthetic RXR ligands have been very fruitful in revealing the high diversity of ligand binding modes and the possibility of specific/selective activation of certain NR/RXR heterodimers to control biological functions. In particular, recent identification of ligands with novel modes of binding outside the conventional ligand binding pocket should revive synthetic ligand-design studies. Although such data will have biomedical implications, the question of the endogenous RXR ligand remains open as 9CRA, widely accepted as endogenous and physiological ligand, is detected in vertebrates only after pharmacological treatment or after nutritional intervention with high doses of retinoids, whereas in physiological conditions 9CRA is either not detected or is found at very low levels, 10-100 fold below its range for RXR transactivation.

Free fatty acids, and in particular DHA, were proposed as endogenous ligands of RXRs. However, here again an involvement of DHA in control of RXR signaling under physiological conditions is still not clear, since the endogenous concentration of DHA as free acid available for transcriptional activation of RXRs in the nucleus is unknown, and in few reported cases of plasma levels in human or tissue levels in rodents, such concentration is below the transactivation range. Data on tissue levels in humans under basal conditions and after dietary stimuli are not available. In turn, a larger array of fatty acid metabolites, including those from DHA, were reported to be present endogenously but again, not much knowledge is available on the potential overlap between RXR transactivation properties and endogenous/nutritionally relevant ranges.

In this context, identification of 9CDHRA as an endogenous and physiologically relevant ligand of RXRs was an important step in understanding RXR signaling *in vivo*. Accordingly, this retinoid is present in mice and human at sufficient concentrations to activate RXR transcription and its absence in mice induces cognitive deficits associated with deficient RXR signaling. Although at present 9CDHRA fulfills the best criteria for an endogenous ligand, there are still many questions to be addressed, relating to its nutritional precursor(s), metabolic pathway(s), the biological functions it may control, and more generally its bioavailability in health and disease. Importantly, determination of whether ATROL and ATBC are precursors of 9CDHRA or whether there are other precursors, will clarify the link with the

known “canonical” vitamin A1 pathway. Addressing such questions may be useful for better understanding the physiopathology of RXR-related diseases, and to identify novel biomarkers and/or design pharmacological or nutritional treatments and disease-prevention schemes.

Among the unresolved challenges in RXR-ligand research is the physiological relevance for co-existence of alternative RXR ligands, like 9CDHRA, DHA and its metabolites or the nutritionally acquired phytanic acid. As discussed above, the relevance of one ligand may potentially become evident in the absence of the other, as in case of raising levels of DHA in vitamin A-deficient conditions. What is the physiological meaning of it? Are the alternative ligands functionally redundant (at least as far as RXR signaling is concerned) or will they activate different physiological programs that are best adapted to homeostatic conditions or adaptation to environmental challenges? Furthermore, such mutual regulations would imply a crosstalk between metabolic pathways involved in the biogenesis of such ligands. Although such mechanisms may potentially involve transcriptional regulation and sharing of some transport proteins, this area of research remains essentially unexplored and will require more holistic nutritional and analytical studies.

Finally, the difficulty in understanding the physiological relevance of individual RXR endogenous ligands, but also their crosstalk, comes from the lack of cellular resolution in RXR-ligand studies. Similarly to a high degree of cell-type specificity of transcriptomic profiles revealed by single-cell genomic analyses, we could expect to dissect intra- vs intercellular diversity in ligand specificity and functions. This calls for development of new approaches that will allow to combine single-cell metabolomics or nuclear identification of the ligand with single-cell transcriptomic profiles.

The biomedical relevance of RXR ligands was emphasized in many studies on metabolic diseases, as several of the permissive RXR heterodimerization partners act as metabolic sensors. However, in recent years the nervous system appears to be a promising but little explored area for RXR research. Report of efficient clearance of amyloid plaques in mouse brain (Cramer et al., 2012), although not completely reproduced (see (Landreth et al., 2013) and reference therein), stimulated interest in RXR signaling and its ligands in the context of Alzheimer’s disease and microglia research. RXR signaling is also of direct relevance to other pathological conditions including multiple sclerosis, where RXR γ is the only known factor positively stimulating remyelination (Huang et al., 2011), in Huntington’s disease, where decreased expression of RXR γ may potentially contribute to some aspects of its physiopathology, or in depression, where RXR cistrome was strongly associated with major forms of the disease (Wray et al., 2018). Addressing those various questions will certainly have clinical and nutritional relevance and may contribute not only to diagnosis and treatment of such diseases, but also to preventing or retarding their onset.

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