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REVIEW

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Natural products as modulators of retinoic acid receptor-related orphan receptors (RORs)

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Retinoic acid receptor-related orphan receptors (RORs) belong to a subfamily of the nuclear receptor superfamily and possess prominent roles in circadian rhythm, metabolism, inflammation, and cancer. They have been subject of research for over two decades and represent attractive but challenging drug targets. Natural products were among the first identified ligands of RORs and continue to be of interest to this day. This review focuses on ligands and indirect modulators of RORs from natural sources and explores their roles in a therapeutic context.

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1 Introduction

The retinoic acid receptor-related orphan receptors (ROR) α, β, and γ are a subfamily of nuclear receptors, encoded by the *RORA-C* (or *NR1F1-3*) genes. In general, nuclear receptors are ligand-dependent transcription factors that translate endocrine and dietary signals into differential gene expression patterns. An endogenous ligand for RORs has not been unequivocally confirmed, however, intermediates and metabolites of cholesterol metabolism have been suggested.[1–3]

All members of the nuclear receptor superfamily feature a significant sequence homology and conserved structure. The ligand-independent activating function 1 (AF1) is located at the N-terminus, followed by a DNA-binding domain (DBD), hinge region, and ligand-binding domain (LBD). The most conserved region is the DBD, which contains two zinc-finger motifs that mediate binding to response elements located in the promoter region of target genes and are involved in receptor dimerization. ROR response elements (ROREs) consist of the AGGTCA consensus sequence proceeded by an A/T-rich region. Nuclear receptors can either bind DNA as monomers like RORs, homodimers, or heterodimers with a member of the retinoid X receptor subfamily as partner. The LBD consists of twelve α-helices that create a hydrophobic cavity to which ligands can bind. The AF2 domain, also referred to as helix 12 and included

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in the LBD, provides the structural surface for the interaction with co-activator and co-repressor proteins.[4,5]

In their unliganded basal state, nuclear receptors are bound to co-repressor proteins and act as transcriptional repressors. Binding of agonistic ligands leads to conformational changes, primarily stabilizing helix 12, which entails the displacement of co-repressor and recruitment of co-activator proteins, ultimately leading to the modulation of target gene expression. Interestingly, inverse ROR agonists like digoxin have been reported to destabilize helix 12, resulting in loss of co-activator interaction, however, without increased recruitment of co-repressors (for more details on digoxin's mechanism of action, see chapter 3.4.1).[6] Co-activator proteins are bound to the LBD *via* their LXXLL interaction domain by a charge clamp (reviewed in ref. 4 and 7). X-ray studies revealed that, in addition, stabilization of the active conformation of RORs is established by formation of a hydrogen bond between a histidine residue in helix 11 and a tyrosine residue in helix 12 (His–Tyr lock). Both, histidine and tyrosine further form π–π interactions with a phenylalanine residue on helix 12, which overall stabilizes the active conformation. If the His–Tyr lock is broken, the aromatic interactions are also terminated and helix 12 is destabilized as a consequence.[8–10] Inverse agonistic ligands are characterized by their ability to repress the transcriptional activity of its nuclear receptor below basal level *via* recruitment of additional co-repressors and have been shown to disrupt the active conformation of helix 12. Co-repressors contain a different interaction motif than co-activators with a similar amphipathic core (φXXφφ; φ is a hydrophobic amino acid) but additional flanking sequences and increased length (reviewed in ref. 11). Other mechanisms include NCoR or SMRT tethering *via* other transcription factors[12] (reviewed in ref. 13) or "water trapping", which was proposed for two synthetic compounds by Kallen *et al.* in 2017.[9] This mechanism involves a water molecule becoming "trapped" in a partially hydrophobic environment when the inverse agonists are bound to the RORγt-LBD. Subsequent release of the water molecule into bulk solvent leads to destabilization of helix 12.[9] However, many more mechanisms are involved in transcriptional regulation by nuclear receptors (reviewed in ref. 14).

RORs have been reported to influence various physiological processes such as circadian rhythm, neuronal cell development, and immune cell differentiation. At the same time, they are implicated in several pathologies like autoimmune, inflammatory, and metabolic diseases. RORα is expressed in many peripheral tissues like the liver, skeletal muscle, skin, lung, and adipose tissue. RORβ expression is restricted to brain, retina, bone, and pineal gland. The *RORC* gene encodes two isoforms *via* the use of alternative promoters. RORγ (RORγ1) differs from RORγt (RORγ2) only at the first 100 nucleotides at the N-terminus.[15] RORγ1 is expressed in muscle tissue, prostate, pancreas, heart, liver, and testicles, whereas RORγt is exclusively expressed in lymphatic tissues.[5,14,16] With the recent discovery of several ligands interacting with ROR receptors, interest for such ligands in drug development has increased.[14,17–19]

Due to their diversity and still often undiscovered biological potential, natural products are an important source for lead structures in the development of novel drugs.[20–23] Even though natural products have been an important source for medicinal preparations, the focus on them in the pharmaceutical industry

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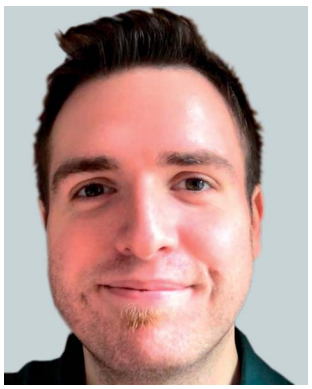

Patrik Schwarz studied Pharmacy at the University of Vienna (Austria) where he graduated in 2019. Since March 2020, he works as a doctoral student at the Department of Pharmacognosy, University of Vienna, under the supervision of Verena Dirsch. He is interested in finding novel (natural) ligands of the nuclear receptor RORγ.

Verena Dirsch received her PhD from the University of Munich (1993) and then joined as postdoctoral fellow of the German Research Council the group of Koji Nakanishi at the Columbia University, New York. From 1995 to 2004 she held several academic positions in the group of Prof. Angelika Vollmar at the University of Munich. Since 2004, she is full professor at the University of Vienna and since 2006 head of the Department of Pharmacognosy. She also served as Vice-Dean at the Faculty of Life Sciences (2008–2014). Her main interest is to study the molecular mechanisms of natural products.

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has diminished over the last decades. With high-throughput screening (HTS) and combinatorial chemistry on the rise, natural products were believed not to fit the requirements of these systems.[20,21] While many chemical probes have been discovered by screening, it is not a magic bullet.[21,22] Natural products offer a wide range of pharmacophores and a high number of stereocenters, which provides libraries containing such compounds a higher hit rate (reviewed in ref. 22 and 23). As a guide to obtain sufficient oral bioavailability, Lipinski's rule of five is used. As many natural products are substrates for active cellular transporters, they often do not have to fit these rules. This is a big advantage, as it makes such compounds more likely to succeed.[23] This is apparent by the fact that between 1981 and 2014, approximately 50% of newly approved drugs are inspired by natural products, be it natural products, natural product analogues, or synthetic mimetics.[23]

2 Biological roles of RORs and their potential as drug targets

2.1 Biological roles of RORs

As the biological roles of RORs have already been explored in depth earlier (examples: ref. 16, 17 and 24–26), preference will be given to the aspects necessary to understand the studies covered in the present review.

The first encounter with the at that time still unknown RORs was made, when a naturally occurring mutant strain of mice was discovered in the 1960s.[27] These mice were called "staggerer" because of their staggering gait. Severe cerebellar underdevelopment with a lack of up to 90% of the Purkinje cells compared to wild type (WT)[28] and a shorter life span were noticed, among others.[27] Over 30 years later, it was discovered that staggerer mice possess a deletion in the *Rora* gene (thus also referred to as $Rora^{sg/sg}$ mice) that leads to the elimination of the LBD, leaving the nuclear receptor inactive as interactions with co-activators are not possible anymore.[29] The phenotype of $Rora^{-/-}$ mice is very similar compared to that of staggerers.[30] Interestingly, $Rora^{sg/sg}$ mice experience a variety of metabolic benefits (see chapter 2.2) but also deficiencies like an impaired immune system, increased inflammation, osteopenia, muscle atrophy, atherosclerosis (all reviewed in ref. 31), and irregularities in circadian rhythm.[32] Since RORβ is found mainly in the CNS, especially in areas connected to processing of sensory information (retina) or involved in circadian rhythm (suprachiasmatic nucleus),[33] it is not surprising that $Rorb^{-/-}$ mice show several related issues including retinal degeneration and blindness in adulthood as well as abnormalities in circadian rhythm and male sexual behavior.[34] Lastly, RORγ was proven to be key for the development of lymphatic tissues and reduces apoptosis of $CD4^+CD8^+$ cells by up-regulating the anti-apoptotic gene Bcl-XL.[35,36]

Interestingly, circadian rhythm and RORs have been shown to be directly connected. RORs upregulate the expression of brain and muscle ARNT-like 1 (*Bmal1*),[32,37] a subunit of a key transcription factor in circadian regulation called CLOCK-BMAL1 (CLOCK = circadian locomotor output cycles kaput) (reviewed in ref. 38). Of note, neuronal PAS domain protein 2 (NPAS2), a paralog of CLOCK, can substitute for it[39] and was also shown to be under the control of RORs.[40] Briefly, CLOCK-BMAL1 controls the expression of cryptochrome (*Cry*) and period (*Per*) as well as various other clock-regulated genes. After being expressed in a sufficient amount, the CRY-PER dimer can inhibit CLOCK-BMAL1 and is later subjected to ubiquitination, after which the cycle can start anew.[38] On the other hand, RORs themselves experience a rhythmic expression.[41] For instance, RORγ was shown to be under the control of CLOCK-BMAL1 in certain tissues (*e.g.* the liver) while its isoform RORγt exhibits constitutive expression.[42] It was proposed that RORs act as "intermediaries" between the circadian clock and the cyclic expression of certain genes, affecting the extent of gene expression rather than rhythmicity itself.[43,44] This was proven for metabolic genes like insulin induced gene 2a (*Insig2a*), elongation of very long chain fatty acids 3 (*Elovl3*), *Cyp8b1*, glucose-6-phosphatase (*G6pc*) and phosphoenolpyruvate carboxykinase (*Pepck*), among others.[43,44] Furthermore, the expression of numerous phase I and II enzymes was shown to be controlled by RORα and RORγ and thus a connection to bile acid synthesis, drug and fatty acid metabolism as well as glutathione conjugation was established, to name a few.[45]

Several types of cancer are linked to an increase or decrease in the activity of all three RORs (reviewed in ref. 46) as well. In short, RORα showed tumor suppressive activities that were amongst others mediated by p53 (ref. 47–50) and the value of RORγt as a target in tumor therapy is currently under investigation (see chapter 2.2).

RORγ and especially its isoform RORγt are the most researched RORs due to their connection to various inflammatory and autoimmune diseases such as multiple sclerosis,[51] rheumatoid arthritis (RA),[52] systemic lupus erythematosus (SLE),[53] psoriasis,[54,55] asthma,[56,57] and, again, cancer[46,58,59] (also reviewed in ref. 60). This is due to the role of RORγt as a critical regulator of Th17 cell differentiation.[61] The underlying mechanisms of Th17 cell differentiation are complex (Fig. 1). The expression of RORγt requires IL-6 and TGF-β.[61] Upon activation, both cytokines are secreted by dendritic cells, which promotes differentiation of $CD4^+$ cells into Th17 cells. Then, these cells up-regulate the IL-23 receptor and increase the expression of key cytokines like IL-17A/F.[61] Subsequently, IL-17 is able to promote IL-6 production in various cell types (reviewed in ref. 62). While IL-23 is not necessary for Th17 cell differentiation, it is required to maintain their differentiated state.[63] *Via* signal transducer and activator of transcription 3 (STAT3), IL-6 also increases the expression of *Il21*, which henceforth acts in an autocrine manner, promoting Th17 differentiation.[64] Of note, both cytokines can increase RORγt protein levels STAT3-dependently (reviewed in ref. 65). However, not only RORγt, but also RORα expression is necessary for Th17 differentiation through the aforementioned cytokines and STAT3.[66] It is known that by upregulating forkhead box P3 (*Foxp3*), TGF-β promotes an immunosuppressive response *via* Treg differentiation,[67] while FOXP3 also inhibits RORγt function and thus Th17 differentiation.[68] However, TGF-β is also required for Th17 differentiation *via* up-regulation of *Il23r*.[69] It was elucidated that

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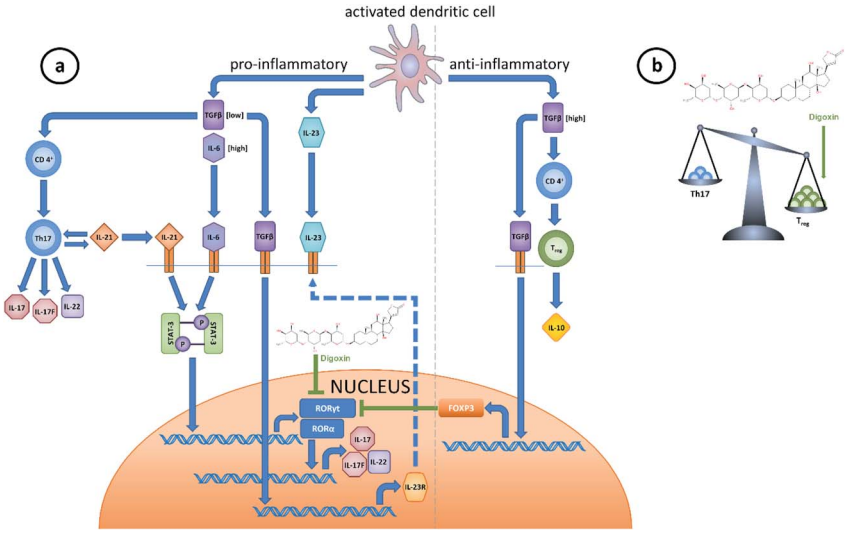

Fig. 1 (a) Differentiation of CD4$^+$ cells into Th17 or Treg cells. Activated dendritic cells secrete a variety of cytokines like IL-6, IL-23 and TGF-β. These cytokines at varying concentrations are responsible for either Th17 or Treg differentiation. High concentrations of IL-6 and low concentrations of TGF-β favor Th17 differentiation. STAT3 downstream of IL-6 signaling is responsible for the expression of IL-21, which henceforth acts in an autocrine manner. *Via* STAT3, IL-6 and IL-21 promote RORα and RORγt expression, the latter being an important regulator of Th17 cell differentiation. Both RORs then drive IL-17A/F and IL-22 expression. Inverse agonists of RORγt like digoxin were shown to inhibit Th17 differentiation. IL-23 is necessary for the maintenance of the Th17 phenotype and TGF-β seems to promote the expression of its receptor. Tregs are created in the presence of higher TGF-β concentrations and in the absence of pro-inflammatory cytokines like IL-6. *Via* up-regulation of FOXP3, TGF-β inhibits RORγt function, thus favoring Treg differentiation. (b) Inverse RORγ(t) agonists like digoxin can lead to a re-balance of the Th17/Treg ratio in favor of anti-inflammatory Tregs.

these phenomena were dependent on the TGF-β concentration and the presence or absence of certain cytokines during differentiation: high TGF-β concentrations led to an increase in *Foxp3* expression and a decrease in *Il23r* expression, thus favoring Treg differentiation. On the other hand, low concentrations of TGF-β were found to enhance *Il23r* and inhibit *Foxp3* expression in concert with IL-6 and IL-21,[64,70,71] thus favoring Th17 differentiation.[68,72]

2.2 Consequences of ROR (inverse) agonism and RORs as drug targets

Inverse agonists of RORα will face difficulties to succeed as drug targets, mainly due to the aforementioned tumor suppressive capabilities of this nuclear receptor (see chapter 2.1). From a metabolic perspective, however, there are some interesting implications of RORα inverse agonism that were first discovered in *Rora*$^{sg/sg}$ mice, including drastically reduced triglyceride and apo-CIII levels,[73] enhanced breakdown of fatty acids, reduction in lipogenesis, prevention of weight gain[74] and elevated glucose uptake in skeletal muscle cells.[75] Furthermore, the synthetic RORα inverse agonist SR3335 was shown to decrease the expression of two major gluconeogenic enzymes in mice, *G6pc* and *Pepck*, thus lowering blood glucose levels and potentially being useful in type 2 diabetes therapy.[76] Of note, the inhibition of G6PC as a therapy strategy for type 2 diabetes has been proposed before,[77] although not in the context of RORα inverse agonism. Conversely, RORα agonists could play a role in the therapy of inflammatory diseases (*e.g.*, RORα promotes *Ikba* expression[78]), atherosclerosis (*e.g.*, RORα promotes *Abca1*/*Abca8*/*g1* and *Apoa1* expression, thus increasing cholesterol efflux and HDL formation[74,79]), cancer (*e.g.* the synthetic RORα agonist SR1078 increased p53 stability[49]) or possibly even disorders linked to circadian rhythm. Furthermore, RORα was shown to promote *Ibsp* expression, with the according protein being involved in bone mineralization.[80] While the therapeutic potential of RORβ has not been explored much hitherto, in a more recent study, a connection between this nuclear receptor and bone loss was reported *via* RORβ-dependent inhibition of RUNX2.[81] Thus, RORα and RORβ possibly could be targets in osteoporosis therapy. Moreover, in the last few years an aminothiazole compound has been identified as a dual inverse agonists of RORβ and RORγ[82] and derivatives thereof were reported to be neutral antagonists of RORβ.[83] These findings could benefit further research on RORβ. Both, RORγt agonists and inverse agonists were shown to have the potential to be used as therapeutics. RORγt agonism using the synthetic compound SR0987 showed an increase in IL-17 and a decrease in programmed cell death protein 1 (PD-1) mRNA levels *in vitro*, which indicates a possible beneficial combination in cancer therapy. More importantly, they found a decline in T cells expressing PD-1 on their surface following SR0987 treatment, although it is unclear how exactly RORγt and PD-1 are connected.[84] Still, the mechanisms involved in an antitumor activity of RORγt agonism seem to be far more complex than that, with a wide range of co-stimulatory receptors (*e.g.* CD137) up-regulated and co-inhibitory receptors (*e.g.* TIGIT) down-regulated in T17 cells in response to a RORγt agonist.[85] Interestingly, the synthetic RORγt agonist cintirorgon (= LYC-55716) was deemed safe for use in various types of cancer in a recent

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phase I clinical trial.[86] Myeloid-derived suppressor cells (MDSC) are known suppressors of the immune system – especially T cells – (reviewed in ref. 87) and can directly exert various pro-tumor effects (reviewed in ref. 88). In a paper published in 2015 by Strauss *et al.*,[89] a connection between MDSC-expansion and RORγ was described. RORγ acts by promoting positive (C/EBPβ) and suppressing negative (Socs3 and Bcl3) transcriptional regulators of myelopoiesis.[89] When transplanting bone marrow of *Rorc*-deficient mice into lethally irradiated WT mice, they saw a significant decrease in tumor growth, metastasis and splenic MDSC in the recipients. Conversely, treating tumor-bearing WT-mice with the RORγ agonist SR1078 increased lung metastatic burden and splenic MDSC.[89] RORγ(t) inverse agonists are interesting due to their anti-inflammatory potential. Most of the RORγ(t) ligands currently in clinical development have psoriasis as their target indication (reviewed in ref. 19). This is probably due to the promising results gathered from compounds like A213.[90] A213 was successfully used for oral treatment of psoriasis in two different mouse models of this disease.[90] Although one of the most promising candidates in this field, the RORγt inverse agonist VTP-43742, failed in phase II, the development of novel compounds is on the rise.[19] In 2016, Wang *et al.* reported that RORγ is overexpressed in tumors of patients suffering from metastatic castration-resistant prostate cancer (mCRPC) and able to increase the expression of the androgen receptor. Consequently, RORγ inverse agonists (*e.g.* SR2211) were found to inhibit androgen receptor signaling and could therefore represent novel therapy options in mCRPC.[91] Recently it was discovered that RORγ is a pivotal regulator of cholesterol biosynthesis in triple-negative breast cancer cells and that its inhibition exhibits antitumor effects, for instance in patient-derived xenografts.[92] From a metabolic perspective, *Rorc*$^{-/-}$ mice displayed a time-dependent decrease in gluconeogenesis and an improvement in glucose tolerance and insulin sensitivity, suggesting a therapeutic potential for RORγ inverse agonism in diabetes type II as well.[43] Noteworthy, RORα often exerts its effects in synergy with RORγ. For instance, staggerer-*Rorc*$^{-/-}$ double knockout mice showed significantly lowered blood glucose levels compared to WT littermates, though these effects could not be observed in either, staggerer or *Rorc*$^{-/-}$ mice alone.[45] Regarding anti-inflammatory capabilities, it was shown that Th17 differentiation is not completely abolished in the absence of RORγt alone,[61] but rather by a RORα–RORγ-double deficiency.[66] Importantly, mice that lacked both nuclear receptors (*Rora*$^{sg/sg}$/*c*$^{-/-}$) experienced complete protection against experimental autoimmune encephalomyelitis (EAE),[66] an animal model for multiple sclerosis.[93] Both examples indicate that in some instances, inhibition of more than one ROR at once is desirable.

3 Natural ligands directly binding to RORs

An overview of natural ligands directly binding to RORs is depicted in Fig. 2 and Table 1.

3.1 Steroids

3.1.1 Cholesterol and cholesterol sulfate. The first hint in the search for endogenous ROR ligands was provided by the finding that cells stimulated with fetal calf serum (FCS) show an increased transactivation of RORs in different cell systems.[94,95]

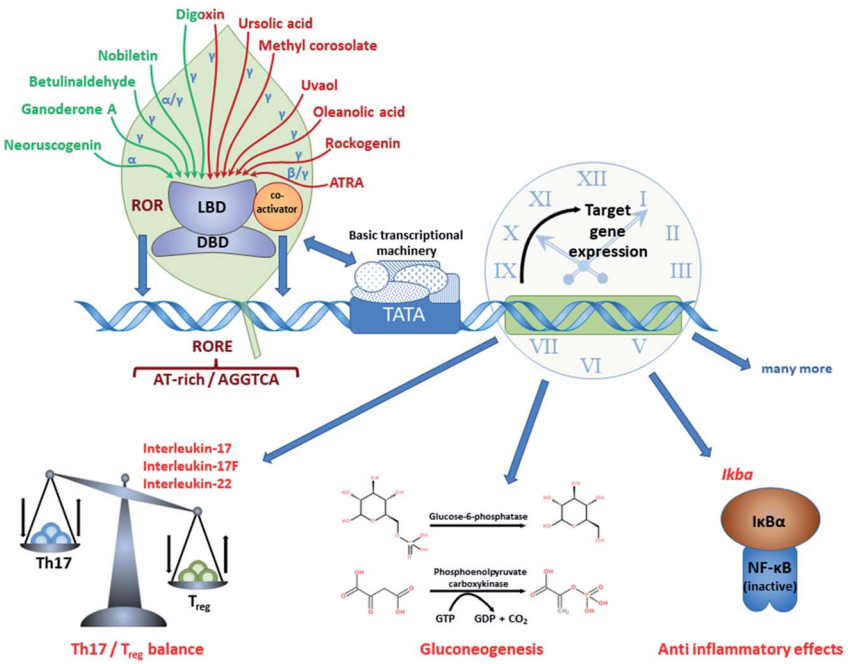

Fig. 2 Natural products as ligands of RORs. Various natural products were shown to act as ROR (inverse) agonists, thus affecting ROR target gene expression.

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Table 1 Selected direct ROR ligands

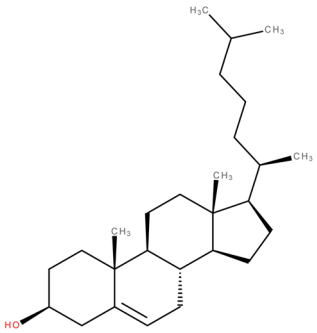
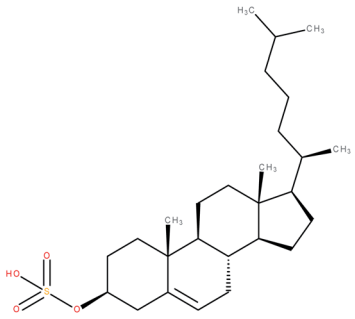
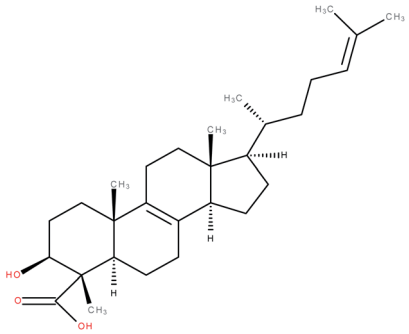
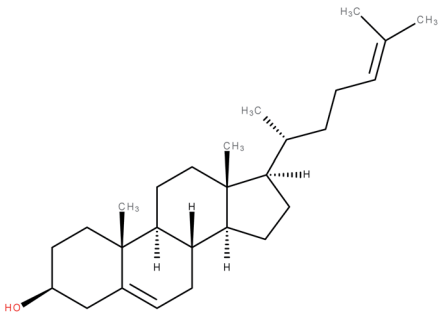
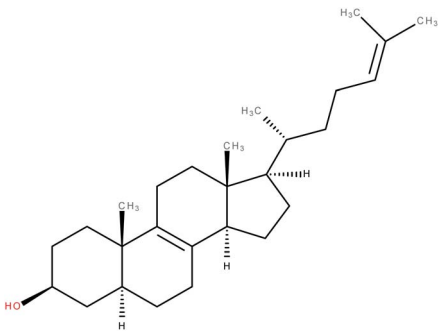
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Cholesterol		RORα agonist	EC_{50} = 200 nM (co-activator binding to RORγ LBD in AlphaScreen® assay)[100] No activity in RORα/β/γ-LBD:Gal4-DBD luciferase assay,[1,100–103] RORγ Pcp2 promoter luciferase co-transfection assay in Cos-7 cells,[100] co-activator binding to RORα LBD in AlphaScreen® assay,[100] 25-[^{3}H]OHC competition assay[101]	1, 8, 96 and 100–103
Cholesterol sulfate		RORα agonist	EC_{50} = 7.1 nM Activity = 88% (FRET assay)[9]	2, 8, 9, 96 and 103–105
4α-Carboxy, 4β-methyl-zymosterol		RORγ agonist		1
Desmosterol		RORγ agonist	EC_{50} = 0.08 μM (co-activator binding to RORγt LBD using a TR-FRET assay)	2
Zymosterol		RORγ agonist	EC_{50} = 0.11 μM (co-activator binding to RORγt LBD using a TR-FRET assay)	2

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Table 1 (*Contd.*)

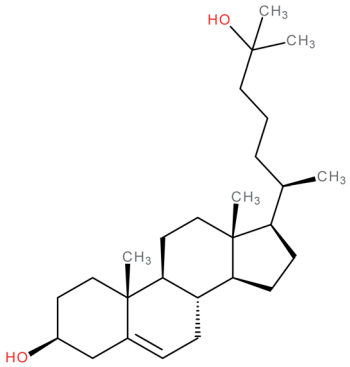
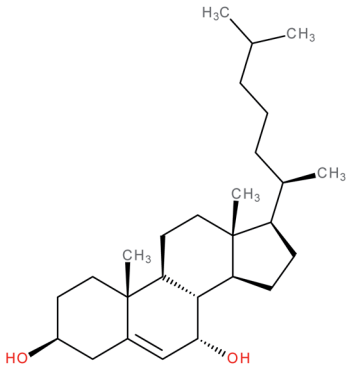
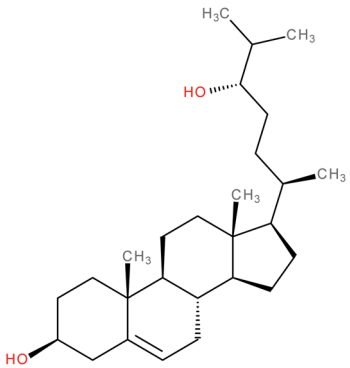
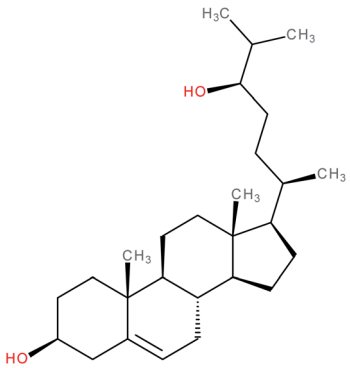
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
25-Hydroxycholesterol		RORγ agonist	EC_{50} = 20–40 nM (co-activator binding to RORγ LBD in AlphaScreen® assay)[100] K_d of [^{3}H]-25-OHC for RORα LBD = 3.3 ± 0.89 nM (ref. 103) K_d of fluorescein labeled 25-OHC for RORγ LBD = 109 nM (ref. 155)	1, 96, 100, 103, 108, 155 and 172
7α-Hydroxycholesterol		RORα/γ inverse agonist	IC_{50} = 1.3 μM (RORα-LBD:Gal4-DBD luciferase assay) IC_{50} = 1.6 μM (RORγ-LBD:Gal4-DBD luciferase assay) K_i (RORα LBD) = 12–18 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC) K_i (RORγ LBD) = 17–31 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC) IC_{50} = 1.3 μM (RORα G6PC promoter luciferase co-transfection assay in HEK-293 cells) IC_{50} = 1.7 μM (RORγ G6PC promoter luciferase co-transfection assay in HEK-293 cells)	103
Cerebrosterol (24*S*-hydroxycholesterol)		RORα/γ inverse agonist	IC_{50} = 620 nM (RORα-LBD:Gal4-DBD luciferase assay) IC_{50} = 1300 nM (RORγ-LBD:Gal4-DBD luciferase assay) K_i (RORα LBD) = 27 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC) K_i (RORγ LBD) = 25 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC)	112
24*R*-Hydroxycholesterol		RORγ inverse agonist	IC_{50} = 90 nM (RORγ-LBD:Gal4-DBD luciferase assay) K_i (RORγ LBD) = 102 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC)	112

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Table 1 (*Contd.*)

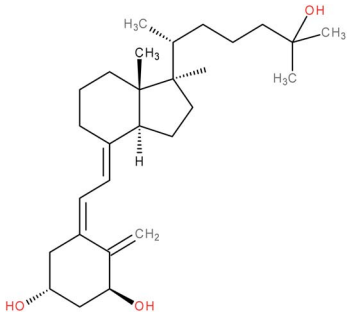
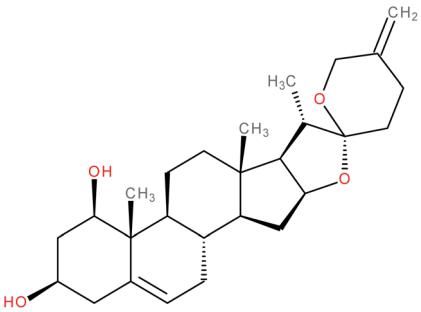
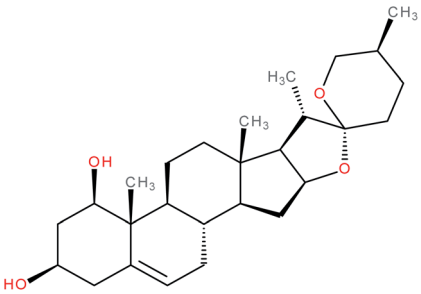
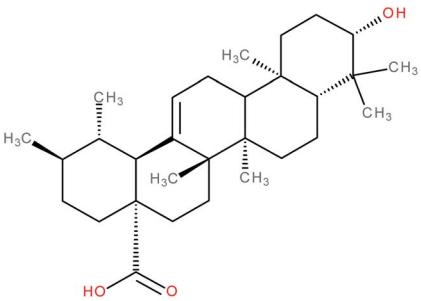
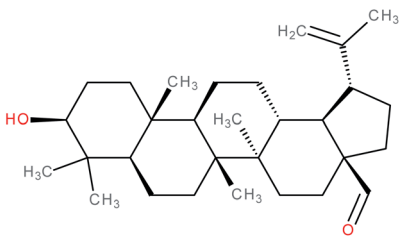
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Secosteroids *e.g.* $1,25(OH)_2D_3$		RORα/γ inverse agonist	IC_{50} = 0.1–0.01 nM (luciferase reporter assay in RORα or RORγ stable transfected CHO Tet-on cells co-transfected with 5 × RORE)	119
Neoruscogenin		RORα agonist	EC_{50} = 0.11 μM (pull-down assay)	121
25*S*-Ruscogenin		RORα agonist	EC_{50} = 0.78 μM (pull-down assay)	121
Ursolic acid		RORγ(t) inverse agonist	IC_{50} = 0.68 ± 0.1 μM (co-activator binding to RORγt LBD using a TR-FRET assay)[123] IC_{50} = 0.56 ± 0.1 μM (Th17 cell differentiation)[123] EC_{50} = 0.25 μM (co-activator release from RORγ LBD in FRET assay)[138] IC_{50} = 1.3 μM (inhibition of co-activator binding to RORγt LBD in AlphaScreen® assay)[139] IC_{50} = 6.5 μM (inhibition of co-repressor binding to RORγt LBD in HTRF assay)[139] K_d = 3.20 μM (SPR binding assay with RORγt protein)[139]	123, 124, 138 and 139
Betulinaldehyde		RORγt agonist	EC_{50} = 11.4 μM (co-activator binding to RORγt LBD in AlphaScreen® assay) IC_{50} = 15.6 μM (inhibition of co-repressor binding to RORγt LBD in HTRF assay) K_d = 2.99 μM (SPR binding assay with RORγt protein)	139

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Table 1 (*Contd.*)

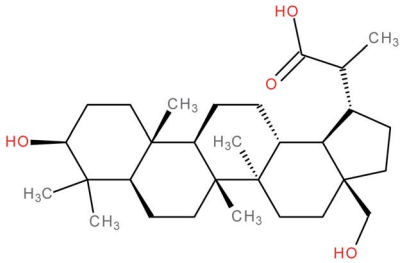
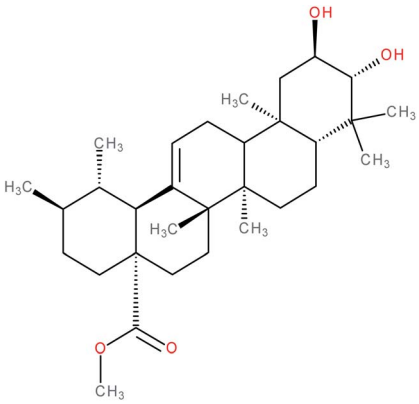
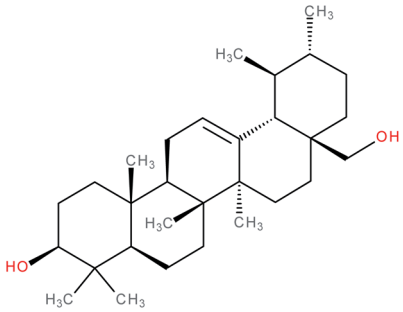
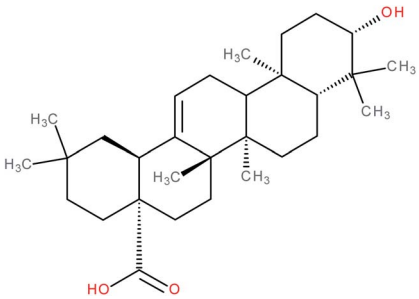
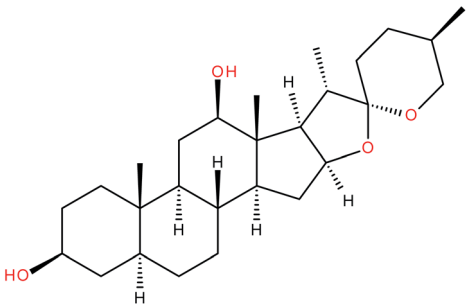
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
3β,28-Dihydroxy-lupan-29-oic acid		RORγt inverse agonist	IC_{50} = 6.8 μM (inhibition of co-activator binding to RORγt LBD in AlphaScreen® assay) IC_{50} = 19.8 μM (inhibition of co-repressor binding to RORγt LBD in HTRF assay) K_d = 1.47 μM (SPR binding assay with RORγt protein)	139
Methyl corosolate		RORγt inverse agonist	IC_{50} = 6.512 μM (RORγ-LBD:Gal4-DBD luciferase reporter assay in Jurkat cells)	141
Uvaol		RORγt inverse agonist	IC_{50} = 4.254 μM (RORγ-LBD:Gal4-DBD luciferase reporter assay in Jurkat cells)	141
Oleanolic acid		RORγt inverse agonist	IC_{50} = 8.589 μM (RORγ-LBD:Gal4-DBD luciferase reporter assay in Jurkat cells)	141
Rockogenin			IC_{50} = 0.2 μM (IL-17 production in Th17 cells)[143] IC_{50} = 5.1 μM (RORγ-LBD:Gal4-DBD luciferase reporter assay)[143] EC_{50} = 2.5 μM (co-activator displacement FRET assay)[138,143]	138 and 143

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Table 1 (*Contd.*)

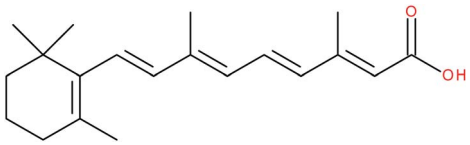
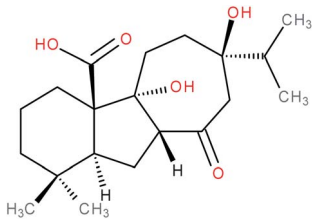
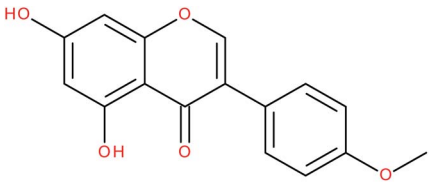
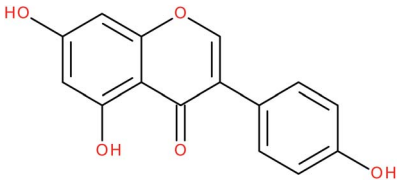
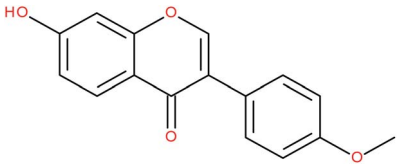
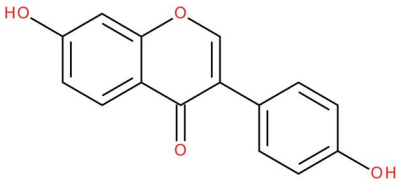
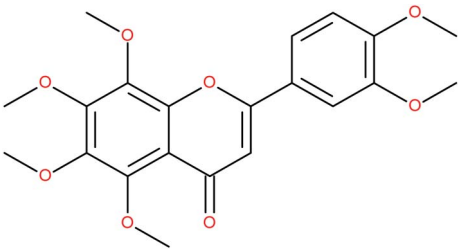
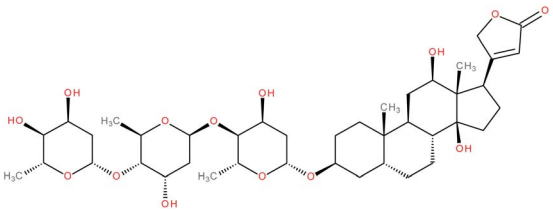
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
All-trans retinoic acid		RORβ inverse agonist	K_d = 280 nM (radioligand competition assay)[102] IC_{50} = 0.15 nM (RORβ-LBD:Gal4-DBD luciferase reporter assay)[102]	102 and 144
Amethinol A		RORγ(t) inverse agonist		145
Biochanin A		RORα/γ-agonist		147 and 148
Genistein		RORα/γ-agonist		147 and 149
Formonenetin		RORα/γ-agonist		147
Daidzein		RORα/γ-agonist		147
Nobiletin		RORα/γ-agonist	K_i (RORα-LBD) = 53.4 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC)[152] K_i (RORγ-LBD) = 8.0 nM (radioligand binding assay *vs.* [^{3}H]-25-OHC)[152]	152–154
Digoxin		RORγ(t) inverse agonist[155] and RORγ(t) agonist[171]	IC_{50} = 1.98 μM (RORγ-LBD:Gal4-DBD luciferase reporter assay in S2 cells)[155] IC_{50} = 4.1 μM (*in vitro* competition assay with fluorescein-labelled 25-OHC)[155] IC_{50} = 1.8 μM (displacement of NCOA3-1b co-activator peptide)[155] IC_{50} = 3.9 μM (promotion of NCOR2 co-repressor peptide binding)[155]	6, 155, 157, 161, 162, 165–167 and 171

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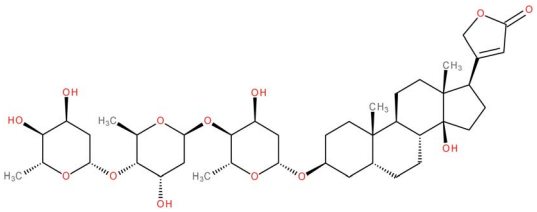
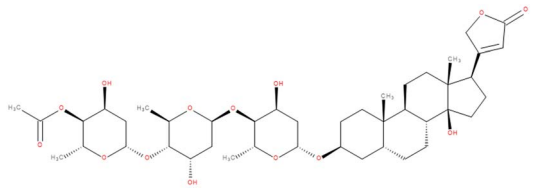
Table 1 (*Contd.*)

Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Digitoxin		RORγ(t) inverse agonist	IC_{50} value "similar" to digoxin according to the authors (luciferase reporter assay in S2 cells)	155
β-Acetyldigoxin		RORγ(t) inverse agonist	IC_{50} value "similar" to digoxin according to the authors (luciferase reporter assay in S2 cells)	155

When the crystal structure of the human RORα LBD was first elucidated the authors found a ligand within its ligand binding pocket that turned out to be cholesterol.[8] This was confirmed using mass spectrometry (MS).[8,96] Mutations in the LBD impairing cholesterol binding as well as inhibition of cholesterol synthesis using a statin resulted in a decrease in transcriptional activity in a full-length RORα luciferase reporter assay. Depleting cells of cholesterol using hydroxypropyl-β-cyclodextrin (HPCD) and a statin also led to a decrease in transcriptional activity, which could be reversed by the addition of *e.g.* cholesterol and, even more effectively, 7-dehydrocholesterol (= provitamin-D_3) at 10 μM.[8] Although helix 12 in the LBD is in an active conformation when bound to cholesterol, cholesterol does not directly interact with this helix.[4,8,97] However, it is known that agonists can function without this ability, for instance by stabilizing the hydrogen bond of the His–Tyr lock[10] after binding, which in turn stabilizes helix 12.[9,98,99] Hence, after mutating this tyrosine residue to phenylalanine, effectively eliminating the His–Tyr lock, a decrease – but no obliteration – in transcriptional activity was observed by the authors,[8] indicating that other interactions must be able to stabilize the active conformation as well. In later studies, cholesterol could increase RORγ co-activator recruitment, while having no effect on co-repressor interaction and on RORα co-activator recruitment, indicating RORγ-specific agonistic properties. The affinity of cholesterol was, however, much lower when compared to other cholesterol metabolites.[100] Notably, cholesterol was not able to upregulate transcriptional activity in a RORγ-Gal4, RORα-Gal4, and RORβ-Gal4 reporter assay as well as a full-length RORγ transactivation assay and was not able to displace 25-[^{3}H]OHC in a competition assay.[1,100–103]

Interestingly, cholesterol sulfate was able to replace cholesterol in the ligand binding pocket of RORα and was predicted to bind the RORα LBD with even higher affinity by docking, which was proven experimentally *via* electrospray ionization (ESI)-MS and differential scanning calorimetry.[8,96,104] The crystal structure of cholesterol sulfate in complex with the RORα LBD[104] was structurally very similar to that of cholesterol and showed an agonistic conformation.[8] A luciferase reporter assay in cholesterol-depleted and statin-treated cells (as described before[8]) showed that treatment with 10 μM cholesterol sulfate led to a higher transcriptional activity relative to cholesterol. On the other hand, a critical mutation (RORα Cys 288 → Gln) within the LBD selectively decreased the affinity of cholesterol sulfate while not affecting cholesterol binding and thus resulted in a reduced transcriptional activity relative to cholesterol.[104] Moreover, cholesterol sulfate was active in a RORγ co-activator recruitment assay, RORγ-Gal4 and RORα-Gal4 reporter assays in the presence of either the inverse agonist ursolic acid or an azole (CYP51 inhibitor).[2] However, another study could not observe an activity of cholesterol sulfate at 500 μM in RORγ-Gal4 or RORα-Gal4 reporter assays where cells were cultivated in lipid depleted and statin and mevalonate supplemented medium, although cholesterol sulfate was active in a 25-[^{3}H]OHC competition assay.[103] In a cell-based study, 40 μM cholesterol sulfate increased the mRNA expression levels of RORα and the RORα-regulated epidermal barrier precursor protein profilaggrin in a RORα-dependent manner in normal human epidermal keratinocytes.[105]

Taken together, due to the low affinity for the LBD of RORs, cholesterol seems unlikely to be a physiological ligand. However, cholesterol sulfate has been shown to be present in Th17 cells and although functional assays have not been performed, data with desmosterol sulfate (see chapter 3.1.2) suggests similar properties for cholesterol sulfate.[2]

3.1.2 Cholesterol biosynthetic intermediates. In 2015, Santori *et al.*[1] were able to show for the first time that sterols from the cholesterol biosynthetic pathway are a sufficient requirement for RORγ transcriptional activity. RORγ activity in an insect cell-based reporter system was dependent on sterol lipids, with a broad specificity for a wide range of sterols. In different mammalian cells lines, such as HEK293T, Hela, or HepG2 cells, cultivated in cholesterol-free medium, RORγ is

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ubiquitously active supporting the notion that one or more common metabolites act as endogenous RORγ ligands. RORγ activity was altered only by enzymes of the cholesterol biosynthetic pathway and correspondingly loss of sterol biosynthesis abolished RORγ activity. To identify the enzymes and products responsible for RORγ, shRNA knockdown, overexpression, and co-immunoprecipitation experiments were carried out and narrowed the possibilities down to non-canonical lanosterol products and canonical cholesterol biosynthetic intermediates. One canonical cholesterol biosynthetic intermediate studied in more detail was 4α-carboxy, 4β-methyl-zymosterol, which acted as an agonist on RORγ in transactivation assays, bound the RORγ-LBD with high affinity in a competition assay, enhanced co-activator recruitment, restored RORγ reporter activity when competing with an inhibitor and increased thermal stability of the RORγ-LBD. Co-crystals of the RORα-LBD or RORγ-LBD and 4α-carboxy, 4β-methyl-zymosterol showed that helix 12 was in an active conformation. Taken together, they pinned down their search for a physiological endogenous ligand of RORγ to a cholesterol biosynthetic intermediate downstream of lanosterol and upstream of zymosterol. Intracellular concentrations of canonical cholesterol biosynthetic intermediates range between 50 to 5000 nM and estimates in this study assume that an endogenous ligand at 500 nM can occupy 80% of available RORγ.[1]

In the same year Hu *et al.*[2] found that endogenous sterol metabolites control Th17 differentiation *via* RORγ agonism. Notably, the inhibition of the mevalonate-cholesterol synthetic pathway *via* statins reduced Th17 differentiation and IL-17A production.[106,107] Desmosterol as well as zymosterol potently increased co-activator recruitment in the presence of the inverse agonists ursolic acid or digoxin and RORγ-Gal4 transcriptional activity, thus suggesting that these compounds occupy the same binding site. They also both increased IL-17A production and Th17 differentiation in the presence of ursolic acid. Moreover, in Th17 cells desmosterol increased RORγ target gene expression but not RORγt itself. The RORγ-dependence of the IL-17A increase elicited by desmosterol was confirmed *via* knockdown of RORγ with siRNA during differentiation and the use of T cells from RORγ knockout mice. Quantification of selected sterols in Th17 cells revealed that only cholesterol and desmosterol were detectable. Furthermore, sulfated sterols, especially desmosterol sulfate, were basally or in the presence of ursolic acid more potent agonists of RORγ than the corresponding 3-OH sterols. Higher production of sulfated sterols together with the fact that desmosterol sulfate as well as cholesterol sulfate could be quantified in Th17 cells, suggests that sterol sulfates might act as endogenous RORγ agonists in Th17 cells.

Taken together, upregulation of cholesterol biosynthesis and uptake and simultaneous downregulation of cholesterol metabolism and efflux during Th17 differentiation leads to the accumulation of the cholesterol precursor desmosterol and its sulfate conjugates, which then act as endogenous RORγ agonists in Th17 cells.[2]

3.1.3 Oxysterols. Several oxysterols have been investigated regarding their effect on different RORs.

20α-Hydroxycholesterol (20α-OHC), 22(*R*)-hydroxycholesterol (22*R*-OHC), and 25-hydroxycholesterol (25-OHC) were all active with similar affinity in RORγ co-activator recruitment assays with EC_{50} values between 20 and 40 nM, while being inactive in RORγ co-repressor recruitment and RORα co-activator recruitment assays.[100] Notably, in another study 22*R*-OHC and 22*S*-OHC (10 μM) were both not able to elicit an effect in RORγ- or RORα-Gal4 assays.[103] Co-crystal structures with the RORγ LBD, the co-activator peptide NCOA2-2 and the putative ligands 20α-OHC, 22*R*-OHC, and 25-OHC revealed very similar LBD structure for all of them with the C-terminal AF-2 in the active conformation, suggesting they act as RORγ agonists. Although they take up the same position in the LBD, binding is dependent on unique pocket residues relevant for size and polarity, as could be shown with a mutagenesis approach. Transcriptional activation of RORγ-Gal4 and full-length RORγ confirmed the agonistic properties of these oxysterols and mutation of the unique binding residues in the RORγ LBD abolished the activity of the respective oxysterols. Interestingly, sulfation of cholesterol and oxysterols carried out *via* SULT2B1 overexpression led to a decrease in transcriptional activity and re-supplementation of these oxysterols partly reversed this effect. It was further revealed that 25-OHC interacts with the two amino acid residues involved in the His–Tyr lock, indirectly with tyrosine on helix 12 and directly with histidine on helix 11.[9,10] Although 25-OHC was not active in a RORα co-activator recruitment assay,[100] it was shown to directly bind to the LBD of RORα in a MS approach[96] and slightly but non-significantly to reduce activity in a RORα-Gal4 assay,[103] suggesting inverse agonism. Interestingly, the enzyme CH25H , which is responsible for 25-OHC production, is downregulated in bone marrow-derived macrophages from sg/sg mice.[108] Lipid storage is disturbed in these cells, which could be restored by treatment with physiological concentrations of 25-OHC,[109] pointing to 25-OHC as endogenous ligand.

Next to the already mentioned compounds, other oxysterols, like 22*S*-OHC and 27-OHC did not influence RORα or RORγ activity.[100,103]

Given the role of RORs in bile acid metabolism,[45] 7α-hydroxycholesterol produced by CYP7A1, the key enzyme in bile acid synthesis,[110] and other related 7-oxysterols (7-OS, 7β-hydroxycholesterol and 7-ketocholesterol) were investigated on RORα/γ.[103] A competitive radioligand binding assay performed against tightly bound 25-[^{3}H]OHC indeed showed that all these 7-OS bind with high affinity to the RORα/γ LBD. Furthermore, 7-OS decreased transcriptional activity in a RORα/γ-Gal4 and full-length RORα/γ assay with the RORE-containing G6PC promoter[43,111] in HEK-293 cells, which suggests an inverse agonistic mechanism of action. When mutating this RORE, the effects of the sterols vanished.[103] Furthermore, 7-OS inhibited the mRNA expression of G6PC in HepG2 cells and ChIP experiments revealed a 7-OS-dependent decrease in NCOA-2 recruitment to the G6PC promoter. A reChIP experiment using 7α-OHC confirmed the RORα dependence of the decrease in NCOA-2 recruitment to the promoter. Together with the data obtained by hydrogen deuterium exchange MS, the authors proposed a model of RORα/γ being in a constitutively active (and thus co-

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activator-bound) state, where inverse agonists such as 7-OS can interfere with this process. Finally, in a more functional setting using RORα/γ siRNA in murine hepatocytes, the metabolic effects of 7-αOS (decrease in *G6pc*/*Pepck* gene expression and glucose output) was obliterated, as expected from direct ligands like 7-OS. Additionally, the tested 7-OS showed no affinity towards LXR in a Gal4 luciferase assay. Possible effects on other nuclear receptors were not examined.[103] In addition to these three 7-OS, GC-MS revealed that 7-dehydrocholesterol acts as a ligand of RORα.[96]

Later additional oxysterols were investigated.[101] In RORγ-Gal4, full-length RORγ and RORγt reporter assays several oxysterols showed significant agonistic activity in the presence of the inverse agonist ursolic acid, with the highest potency and efficacy observed for 27-OHC and 7β, 27-OHC. Notably, in this assay 7α, 25-OHC was inactive, while 7-keto, 27-OHC was only active in the Gal4 but not in the full-length transcriptional assays. Several oxysterols that were previously reported as RORγ or RORα inverse agonists or agonists[101,103,112] including 25-epoxycholesterol and 7-ketocholesterol were only weakly to moderately active. Cholestenoic acid derivatives of 27-hydroxylated sterols were only barely active, emphasizing the importance of the hydroxyl group at carbon 27 for RORγt agonism. Many of the tested oxysterols had activities on other nuclear receptors, however, 7β, 27-OHC and 7α, 27-OHC seemed to be the most selective RORγt agonists. Direct binding of oxysterols was investigated in thermal shift assays with RORα/β/γ LBD and the co-activator peptide NCOA1, where 27-OHC oxysterols bound most potently to the RORγ LBD (7β, 27-OHC > 7-keto, 27-OHC > 27-OHC > 7α, 27-OHC). Notably, 27-OHC binding to the RORγ LBD was significantly lower in the presence of NCOA1, suggesting that this oxysterol is no endogenous RORγ agonist.[101] In a functional approach 7β, 27-OHC and 7α, 27-OHC, but not 7α, 25-OHC increased the number of IL-17A producing cells from total or naïve mouse and human $CD4^+$ T cells under Th17 differentiating conditions in the absence or presence of ursolic acid. RORγt dependency was confirmed with RORγt-deficient mouse $CD4^+$ T cells. In addition, it was confirmed that 7β, 27-OHC and 7α, 27-OHC do not activate RORα, as functional RORα is expressed in RORγt knockout cells. The production of 27-OHCs are dependent on the enzyme CYP27A1. Mouse *Cyp27a1* knockout-naïve $CD4^+$ cells showed significantly reduced Th17 differentiation and exogenous addition of 7β, 27-OHC restored this effect, suggesting a physiological role of 27-OHC oxysterols in this process. The importance of CYP27A1 and 7β/α, 27-OHCs for Th17 differentiation was confirmed *in vivo* in mice. *Cyp27a1* knockout mice had elevated 25-OHC levels, which led the authors to the conclusion that this oxysterol is unlikely to function as endogenous RORγt agonist.[101]

Another oxysterol studied in more detail is 24*S*-OHC (cerebrosterol), mostly found in the brain.[112,113] It showed similar effects on RORα and RORγ transcriptional activity in Gal4 assays as 7-oxysterols. Using ChIP-reChIP assays with the coactivator NCOA2, 24*S*-OHC was demonstrated to reduce recruitment of this peptide to RORα. Notably, also 24(*S*),25 epoxycholesterol, found in micromolar concentrations in the liver and brain,[114,115] and 24*R*-OHC act as specific partial inverse agonists for RORγ with an IC_{50} of 280 nM and 90 nM in a Gal4 assay and a K_i value of 20 nM and 102 nM in a competition assay against 25-[^{3}H]OHC, respectively, with no activity on RORα.[112] The high abundance of cerebrosterol in young children[113] and its RORα agonistic properties led the authors to propose a role for it in the developing brain.[112] It is interesting to see that only small structural changes in the molecules can evidently lead to a specificity for a certain ROR protein, which can be explained by the differences in the LBD amongst RORs (reviewed in ref. 24). Noteworthy, all compounds tested in this study were previously identified to be agonists of LXR.[116]

In differentiated Th17 cells, oxysterols could not be detected[2] but in naïve T-cells, 27-OHCs were quantifiable.[101] Moreover, the level of 27-OHC has been reported to be 5 times lower than that of desmosterol in human plasma.[117,118] The physiological relevance of different sterols thus still seems to be not completely clear and needs further evaluation.

3.1.4 Secosteroids. The secosteroids $20(OH)D_3$, $20,23(OH)_2D_3$, and $1,25(OH)_2D_3$ have been shown to inhibit RORE-driven activation of a reporter in human epidermal keratinocytes and melanoma cells. In a RORE-driven reporter assay in CHO cells, with RORα or RORγ overexpressed, $20(OH)D_3$ and $20,23(OH)_2D_3$ inhibited doxycycline-induced transactivation of both receptors, while $20(OH)D_2$, $1,25(OH)_2D_3$, and vitamin D_2 were considerably less potent. In a mammalian two-hybrid system, used to evaluate the interaction of the RORα or RORγ LBD with the coactivator peptide EBIP96, $20(OH)D_3$ dose-dependently decreased this interaction. In full-length transactivation assays with the *Bmal1* or *G6pase* promoter, $20(OH)D_3$, $20,23(OH)_2D_3$ and $20(OH)D_2$ acted inhibitory, while $1,25(OH)_2D_3$ and vitamin D_2 had no or much weaker activity. Moreover, $20(OH)D_3$ and $20,23(OH)_2D_3$ were both able to dose-dependently repress the transactivation of the *IL17* promoter in Jurkat cells and to inhibit the production of IL-17A in mouse splenocytes. *In silico* docking studies for $20(OH)D_3$ and $20,23(OH)_2D_3$ produced scores for binding to RORα and RORγ and binding pose similar to 25OHC.[119] In a follow up study IC_{50} values of several CYP11A1-derived secosteroids were determined in the RORE-driven RORα and RORγ reporter assay. Notably, all tested compounds ($20(OH)D_3$, $1,20(OH)_2D_3$, $20,23(OH)_2D_3$, $1,20,23(OH)_3D_3$, $20,24(OH)_2D_3$, $1,20,24(OH)_3D_3$, $20,25(OH)_2D_3$, $1,20,25(OH)_3D_3$, $20,26(OH)_2D_3$, $1,20,26(OH)_3D_3$, $17,20,23(OH)_3D_3$, $1,25(OH)_2D_3$), elicited similar IC_{50} values in the range of 0.1 to 0.01 nM. Additionally, molecular docking studies predicted binding of these vitamin D analogues to RORα and RORγ.[120] Taken together, the studied secosteroids acted as inverse agonists on RORα and RORγ, which might explain the multitude of effects elicited by vitamin D.

3.1.5 Neoruscogenin. Neoruscogenin, a steroidal sapogenin, which can be found in *Ruscus aculeatus* (Asparagaceae), was identified as an agonist of RORα.[121] A novel HTS method utilizing a variant of a pull-down assay, in which the ligand-dependent recruitment of a co-activator peptide to RORα LBD is quantified using luminescence, was able to identify RORα ligands within fractionated plant extracts. Using this method combined with subsequent isolation and chromatographic

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purification steps led to the identification of 25*S*-ruscogenin (from *Dalbergia cambodiana*, Fabaceae) as a potent RORα agonist. Due to its better availability, neoruscogenin, which was found to be even more potent in the HTS method than 25*S*-ruscogenin, was subjected to further studies, which proved the agonistic properties of neoruscogenin in a RORα-Gal4 luciferase assay and by a significant increase in gene expression of RORα target genes in HepG2 cells. The latter was later confirmed *in vivo* after treating mice with the compound and harvesting their livers (*e.g. Bmal1* and *G6pc* were up-regulated).[121] Regarding selectivity, it must be noted that although being specific for RORα and not activating other RORs, neoruscogenin was found to increase the transcriptional activity of PXR in a Gal4 luciferase assay, whereas other nuclear receptors (such as LXR and FXR) were not affected.[121]

3.2 Terpenoids

3.2.1 Ursolic acid. Ursolic acid, a pentacyclic triterpenoid carboxylic acid common in most plant species,[122] was found to selectively inhibit RORγt.[123] It was able to inhibit Th17 differentiation of naïve $CD4^+$ T cells (murine, human) and down-regulated IL-17 but not RORα/γt gene expression. Other cell types (*e.g.* Th1, Treg) were not affected as much, although a slight rise in IFN-γ^+ cells upon ursolic acid treatment was detected. Measurements of (i) *Il17a/f* expression after transduction of RORs into neutrally differentiated T cells, (ii) transcriptional activity in a luciferase assay when RORE reporter and RORα/γt were transfected in HEK293T cells and (iii) co-activator peptide binding to the LBDs (TR-FRET assay), revealed selective inhibition of RORγt by ursolic acid.[123] In a mouse EAE model, ursolic acid delayed the onset of the disease by a few days, but after seven days, 80% of the mice were sick in both, the control and the ursolic acid group. Clinical scores, CNS invading helper T cells (Th17 and Th1 cells) and splenic IL-17 production, on the other hand, were decreased significantly in the treatment group.[123]

In another study[124] ursolic acid lessened the incidence and severity of collagen-induced arthritis in mice while decreasing the expression of the proinflammatory cytokines TNF-α, IL-1β, IL-6, IL-21, and IL-17 and the oxidative stress markers iNOS and nitrotyrosine. Ursolic acid moved the balance between Treg and Th17 cells in the spleen of these mice to the Treg side, consistent with a reduced expression of IL-17, IL-21, phosphorylated (p)-STAT3, and RORγt. Moreover, the inhibitory effect of ursolic acid on Th17 cell differentiation was confirmed in an *in vitro* model. However, in this model the amount of Treg cells was not influenced, as shown *via* Foxp3 expression.[124] Some of these results are in contrast to the findings from Xu *et al.* as outlined before.[123] In their study ursolic acid did not influence STAT3 phosphorylation and RORγt mRNA levels in Th17 cells. The authors argued that these discrepancies might result from different Th17 differentiation cocktails or cell types used.[123,124] Moreover, ursolic acid has been reported to inhibit STAT3 activation in many other model systems.[125–131] STAT3 has been previously shown to be required for Th17 differentiation *in vivo* and to act upstream of RORγt.[3,61,132] Furthermore, ursolic acid is a known inhibitor of the NF-κB pathway.[133] Inhibition of NF-κB leads to decreased expression of IL-6, which in turn acts as activator of STAT3 signaling.[134–137] This suggests that ursolic acid modulates the transcriptional activity of RORγ also indirectly *via* NF-κB inhibition. More mechanistically, ursolic acid was able to displace a co-activator and co-repressor peptide *in vitro*.[138,139] Direct interaction of ursolic acid with the LBD was suggested *via* an increase in melting temperature (T_m) in a thermal shift assay and *via* surface plasmon resonance (SPR).[139] A co-crystal structure of ursolic acid and the RORγ-LBD suggested a unique mode of action. Ursolic acid was shown to form a hydrogen bond with a histidine residue leading to a flip of helix 11, moving it closer towards helix 12, thereby causing a disordered C-terminus. They hypothesize that this flip leads to the displacement of the co-activator peptide and prevents the recruitment of a co-repressor.[138] Lastly, RORγt selectivity (over RORα and RORβ) was evaluated by an AlphaScreen® assay.[139]

Since ursolic acid has many other targets besides RORγt (for a comprehensive overview, see ref. 140) it must be carefully evaluated to what extent this could become an issue when using it for therapeutic purposes in humans.

3.2.2 Betulinaldehyde. In a recent study,[139] betulinaldehyde and 3β,28-dihydroxy-lupan-29-oic acid were discovered as agonist and inverse agonist of RORγt, respectively. Betulinaldehyde was able to enhance co-activator and suppress co-repressor binding to the RORγt LBD in an AlphaScreen® and HTRF® assay, respectively, while 3β,28-dihydroxy-lupan-29-oic acid inhibited both, co-activator and co-repressor binding. Thermal shift assays saw an increase in T_m for both substances (3β,28-dihydroxy-lupan-29-oic was the more effective one, even at lower concentrations), suggesting a direct interaction between the substances and the RORγt LBD. K_d values were determined by SPR and the selectivity of the substances for the RORγt LBD was demonstrated by yet another AlphaScreen® assay.[139] Binding modes were proposed by employing molecular docking, suggesting that the His–Tyr lock was indirectly stabilized (similar to cholesterol sulfate[9]) through hydrophobic interactions in the case of betulinaldehyde but was disrupted when 3β,28-dihydroxy-lupan-29-oic acid was docked, confirming the experimental results.[139]

3.2.3 Ganoderone A. Ganoderone A, a triterpenoid from *Ganoderma pfeifferi* (Ganodermataceae), was identified as a potent agonist of RORγt.[9] X-ray analysis of RORγt LBD in complex with ganoderone A revealed stabilization of the receptor *via* the disruption of the His–Tyr lock in favor of two new direct hydrogen bonds established between ganoderone A and the two amino acid residues in helix 11 and 12. Probably due to these direct interactions, ganoderone A was found to be slightly more potent in a HTS-FRET assay compared to the indirect stabilizer of the active conformation cholesterol sulfate.[9]

3.2.4 Methyl corosolate, uvaol and oleanolic acid. Three triterpenoids isolated from *Eriobotrya japonica* (Rosaceae), methyl corosolate, uvaol and oleanolic acid, were found to possess RORγt inhibitory effects in a Gal4 luciferase assay performed in Jurkat cells.[141] Murine Th17 differentiation and *Il17a/f* gene expression were significantly and dose-dependently

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inhibited.[141] Subsequent *in vivo* studies were performed in lupus nephritis (LN) mice using only oleanolic acid but not the other two compounds due to poor extraction yields. Particularly uvaol would have been interesting to study since it possessed the lowest EC_{50} value and effectively inhibited Th17 differentiation even in the nanomolar concentration range. Anti-dsDNA antibodies (markers for SLE, but their role as such have been questioned in the past[142]) were reduced significantly in the blood of LN mice treated with oleanolic acid.[141] Lastly, kidney damage and renal IgG/IgM depositions were found to be reduced in mice treated with oleanolic acid.[141] Interestingly, treatment with oleanolic acid had a greater effect compared to the positive control prednisolone acetate *in vivo*. However, different doses were used (50 mg kg^{-1} oleanolic acid *vs.* 15 mg kg^{-1} prednisolone acetate), making a direct comparison difficult.

3.2.5 Rockogenin. The plant sterol rockogenin, isolated from *Agave gracilipes* (Asparagaceae), inhibits IL-17 production in Th17 cells, decreases RORγ transactivation, and displaces a co-activator peptide.[138,143] Co-crystal structures with rockogenin, the RORγ-LBD and the co-repressor peptide SMRT22 could determine that rockogenin interacts with the RORγ-LBD *via* two direct hydrogen bonds and van der Waals contacts. The suggested mechanism involves the disruption of the His–Tyr lock. This then leads to the release of the co-activator and the recruitment of co-repressor peptides.[138]

3.2.6 Retinoids. The vitamin A metabolite and RAR agonist all-trans retinoic acid has been reported to specifically bind the RORβ LBD and completely replace the fortuitous pseudoligand stearate, which copurifies with the RORβ and acts as a filler molecule from the expression host *E. coli* without influencing transcriptional activity,[144] in an ESI-MS assay. The formation of co-crystals with the RORβ LBD, all-trans retinoic acid, and the co-activator peptide NCOA1 revealed that the binding pose of all-trans retinoic acid did not include interactions with helix 12. A scintillation proximity assay showed that [^{3}H] all-trans retinoic acid specifically binds to the LBD of RORβ. Moreover, in a Gal4 reporter assay in HT22 cells, all-trans retinoic acid inhibited RORβ and RORγ transactivation, while being inactive on RORα. Using the same assay, different cell types were tested and it was revealed that all-trans retinoic acid inhibited transcriptional activity in the neuronal cells HT22 and Neuro2A but not in NIH3T3, HEK293 or P19 cells, suggesting some kind of cell-type specificity. The authors suggest that all-trans retinoic acid might be important for crosstalk between RAR and ROR pathways.[102]

3.2.7 Amethinol A. Amethinol A, a diterpene isolated from *Isodon amethystoides* (Lamiaceae), was shown to possess inhibitory effects on RORγt at 10 μg ml^{-1} in a Gal4-based luciferase assay performed in Jurkat cells.[145] No further pharmacological information was provided by the authors.

3.3 Polyketides

3.3.1 Isoflavones. Isoflavones are common plant constituents from the family of Fabaceae and the multitude of their beneficial properties, for example in the prevention or treatment of cancer, metabolic syndrome and cardiovascular disease, have been reviewed recently.[146] The isoflavones biochanin A, genistein, formononetin, and daidzein (0.1–10 μM) have been reported to dose-dependently enhance RORα- and RORγ-mediated transcriptional activity in a transactivation assay with a RORE-responsive reporter in CHO and the *Il17a* promoter in Jurkat cells. Furthermore, these four isoflavones dose-dependently increased the interaction between the RORα- or RORγ LBD and the co-activator LXXLL-peptide EBIP96 and the mRNA expression of IL-17A in mouse T lymphoma EL4 cells. Biochanin A, in particular, was shown to increase IL-17A mRNA levels RORα/γ- and STAT3-dependently and to enhance the interaction between RORγt and the co-activator NCOA1 as shown with immunoprecipitation and immunoblotting assays.[147,148] Furthermore, biochanin A increased STAT3 phosphorylation in a Src kinase-dependent manner.[148] In another study, genistein treatment delayed the onset and reduced the severity of EAE. Interestingly, genistein-treated mice had a lower expression level of RORγt and reduced production of IL-6 in the spinal cord, however, IL-17 levels were not changed.[149] The isoflavones formononetin and isoformononetin, reduced IL-17a production and Th17 differentiation in a mouse model of osteoporosis.[150] The isoflavone puerarin, found in the herbal medicine Puerariae radix (*Pueraria lobata*, Fabaceae), decreased the amount of Th17 cells found in blood in a rat model of acute lung injury.[151]

These contradicting results regarding IL-17 for isoflavones might stem from the different model systems used.

3.3.2 Nobiletin. Nobiletin is a natural polymethoxylated flavone found in citrus peels. In a competitive radio-ligand binding assay for RORs using 25-[^{3}H]OHC nobiletin showed robust competitive binding to the LBDs of RORα and RORγ, but with higher affinity to RORγ. In addition, nobiletin was active in RORα- and RORγ-Gal4 mammalian one-hybrid assays, indicating direct binding of nobiletin to the RORα and RORγ LBD. Moreover, nobiletin dose-dependently and RORα/γ-dependently increased RORα and RORγ transactivation of the *Bmal1* promoter in Hepa1-6 cells and the expression of ROR target genes such as *Cyp7b1*, *IkBa*, and *Gck* in mice livers with diet-induced obesity.[152] Follow up studies showed a beneficial effect of nobiletin on metabolic fitness in naturally aged mice fed a regular diet or a high-fat diet *via* increased ROR-dependent mitochondrial respiration. In skeletal muscle of high-fat diet fed mice, expression of ROR target genes (*Bmal1*, *Npas2*) were increased upon nobiletin treatment and RORα and RORγ protein levels were induced at zeitgeber time 18.[153] Based on these metabolic effects,[153] the impact of nobiletin on cholesterol homeostasis in metabolically challenged aged mice was investigated by the same group. Overall, the cholesterol profile of nobiletin treated high-fat diet fed aged mice improved and the reduced hepatic expression of ROR target genes involved in bile acid synthesis in these mice was abolished.[154]

3.4 Cardiac glycosides

3.4.1 Digoxin and derivatives. In 2011, digoxin, a cardenolide from *Digitalis lanata* (Plantaginaceae) was identified among nearly 5000 substances as a ligand of RORγ using a RORγ-Gal4

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mammalian one hybrid assay in insect cells.[155] Digoxin acted as an inverse agonist, decreasing the transcriptional activity of RORγ (but not RORα or other nuclear receptors, *e.g.* hAR or LXR) when pretreated with 22-OHC at 10 μM by binding to its LBD.[6,155] Binding to the LBD was proven by an *in vitro* competition assay using fluorescein-conjugated 25-OHC as well as circular dichroism analysis. Importantly, the related cardenolides digitoxin and β-acetyldigoxin were also shown to possess RORγ(t) inhibitory activity while the aglycone of digoxin, digoxigenin, was inactive. Interestingly, according to the authors,[155] digoxigenin does not even bind to the RORγt LBD, a notion challenged by later observations.[156] Treatment of murine $CD4^+$ cells with digoxin during polarization resulted in a selectively reduced expression of RORγt-controlled genes (*e.g. Il17a*/*f*, *Il23r*). Reduction of Th17 differentiation upon digoxin treatment was specific, since other cells lines (*e.g.* Th1, Treg) were not affected.[6,155] Moreover, only naïve murine $CD4^+$ cells transduced with RORγt (and not RORα[66]) experienced a decrease in *Il17* expression upon digoxin treatment, as shown using flow cytometry. Gene expression profiling (GEP) revealed that digoxin treatment and RORγt deficiency mostly (>90%) impacted the same genes. This led to the suggestion that RORγt is the dominant target of this cardenolide.[155] Moreover, RORγt gene expression itself was left unaltered by digoxin, indicating a direct inhibitory effect. Using ChIP analysis, digoxin was shown to inhibit RORγt-binding to key gene loci (*Il17a*/*f*, *Il23r*) of Th17 cells. Furthermore, co-activator binding to the LBD of RORγt was decreased while co-repressor binding was promoted using digoxin *in vitro*.[6,155] Importantly, this mechanism of action diverges from the ones observed using certain sterol ligands, where only co-activator binding to ROR, but not binding of the nuclear receptor itself to the promoter was inhibited, indicating the existence of different modes of action of ROR inverse agonists.[103,112] The selective influence of digoxin on already differentiated Th17 cells was proven using *in vitro*, *ex vivo* and *in vivo* experiments, implying that digoxin treatment promoted dedifferentiation of Th17 cells (*e.g.* absence of *Il17* expression) due to RORγt inhibition.[155] Interestingly, digoxin was effective only in relatively high concentrations (≥1 μM).[6] Moreover, the co-crystal of the human RORγt LBD bound to digoxin was solved and revealed that digoxin occupies the same site within the ligand binding pocket as the agonist 25-OHC (shown in ref. 100). Furthermore, helix 12 is destabilized through hydrogen bonding between digitoxose and the histidine on helix 11 involved in the His–Tyr lock.[9,10] Also, digoxin impedes the proper agonistic positioning of helix 12 by sticking out between helices 3 and 11 with its sugar moieties, thus hindering co-activator recruitment.[6] Importantly and in accordance with previously collected data,[155] the lack of sugars would explain why digoxigenin, the aglycone of digoxin, did not act in an inverse agonistic fashion in contrast to the glycoside.

In contrast to the studies mentioned above, digoxin showed no effect on murine Th17 cell differentiation at 10 μM in a later study.[157]

After digoxin was established as an inhibitor of RORγ(t),[6,155] its value for treating various, mostly inflammatory diseases was examined in several preclinical studies.

3.4.1.1 Preclinical studies performed with digoxin. In a study by Huh *et al.*, digoxin but not its aglycone was able to ameliorate EAE in mice when compared to the vehicle control DMSO.[155] The onset of the disease was pushed back by a few days and the clinical scores were consistently (and significantly) lower in the treatment group. A significantly decreased amount of Th17 cells were found in the spinal cords of mice in the treatment group compared to control while Th1 cells were left mostly unaffected,[155] which contrasts with other observations.[123] Previously, it has been shown that transplant rejection is connected to Th17 cells and IL-17,[158,159] and direct antagonism of IL-17 (ref. 160) could suppress this process in rats. Accordingly, in a study investigating digoxin's effect on heart transplant rejection in mice, treatment with the cardenolide doubled the survival time compared to control. Moreover, inflammation and necrosis of allografts were decreased and re-balancing of the Th17/Treg ratio in favor of Tregs was accomplished.[161] Another study suggested a benefit when treating abdominal aortic aneurysm (AAA) using digoxin in mice,[162] which is in line with the important role of IL-17 in AAA pathophysiology.[163] The authors found a reduction of aortic diameter (key for the diagnosis and risk assessment of AAA[164]), a reduced incidence of AAA and a re-balancing of the Th17/Treg ratio in favor of Tregs. The survival ratio did not change, indicating a rather prophylactic value of digoxin for this indication.[162] Furthermore, digoxin was shown to possess prophylactic and therapeutic capabilities *in vivo* as it was able to suppress and ameliorate collagen-induced arthritis in mice.[165] Inflammation and cartilage loss were markedly reduced in the ankles of digoxin treated mice, as were arthritis scores and disease incidence in general. Lower expression levels of certain proinflammatory cytokines (*e.g.* IL-6, -17 and -21) in arthritic joints were accompanied by a significant decrease in Th17 and a rise in Treg cells in murine spleens.[165] Another study examined the therapeutic potential of digoxin regarding atherosclerosis in $ApoE^{-/-}$ mice on a western-type diet.[166] Histologically, a reduction of atherosclerosis could be quantified. After 12 weeks, a statistically significant reduction in total cholesterol, triglyceride and LDL levels was found, while HDL levels were unchanged. mRNA levels of three RORγ target genes involved in metabolism (*Insig2a*, *Elovl3*, *Cyp8b1* (ref. 44)) were examined and found to be decreased significantly in the digoxin groups. In the spleens, flowcytometric measurements saw a significant decrease in Th17 cells and a significant increase in Tregs following digoxin treatment and the same was true for Tregs in atherosclerotic plaques. Also, Th17 cell invasiveness of the plaques was decreased upon digoxin treatment.[166] A further study used a murine model in order to examine the possible benefits of digoxin treatment in inflammatory bowel diseases (IBD).[167] Weight loss due to colitis and colon colitis scores were reduced significantly in the treatment group and fewer proinflammatory $CD3^+$ T cells were found in the colonic mucosa. Th17 cells (together with IL-17A and IL-23R mRNA levels) were decreased and Tregs (together with IL-10 mRNA levels) were increased significantly in the colon due to digoxin treatment. When colitis was induced in mice using $CD4^+$ cells of *Il10* knockout mice, digoxin treatment led to a significant decrease

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in weight loss, but histological scores were not significantly different between the groups. Therefore, they suggested a beneficial effect of digoxin treatment in disorders like Crohn's disease through direct inhibition of RORγt in a partly IL-10 dependent fashion.[167]

Importantly, these studies were all performed in mice. Cardiac glycosides are known to bind to the Na^+/K^+-ATPase α1 subunit of rodents with a significantly decreased affinity. Therefore, concentrations that are toxic in humans can be expected to be tolerated well in mice.[168–170] For this reason, the results of such studies cannot simply be applied to humans but give an impression of the clinical values of RORγ(t) inhibition in general.

3.4.1.2 Digoxin as RORγ(t)-agonist? Surprisingly, another group reported agonism of digoxin on RORγ(t) in a RORγ transactivation assay.[171] This contrasts with the results published in 2011 by Huh *et al.*[155] and Fujita-Sato *et al.*[6] who proposed the exact opposite. While Huh *et al.* and Fujita-Sato *et al.* worked in the micromolar concentration range using mostly insect or murine cells,[6,155] Karaś *et al.* employed 100 nM, since cytotoxic effects on HepG2 and human Th17 cells were observed at concentrations as low as 200 nM. Interestingly, when using 10 μM of digoxin (as in ref. 66 and 155) the authors observed cell viabilities of approx. 40% and 5% for HepG2 and human Th17 cells, respectively. They also reported increased expression of the RORγ-regulated genes *G6PC* and *NPAS2* in HepG2 cells following digoxin treatment. In agreement with previous studies, a mammalian one-hybrid assays with RORγ-LBD:Gal4-DBD and RORα-LBD:Gal4-DBD constructs showed a RORγ specific activity of digoxin.[6,155,171] Interestingly, when overexpressing human and mouse RORγ(t) in (RORE)6-tk-Luc transfected HepG2 reporter cells, digoxin was shown to have a greater effect on the murine compared to the human variants.[171] However, this finding was not explored further. Moreover, the authors performed an electrophoretic mobility shift assay, which showed that digoxin treatment increased nuclear protein binding to a RORE-containing DNA probe. A ChIP assay showed increased RORγt and NCOA-1 (but decreased NCOA-2) binding in the promoter region of RORγ-regulated genes (*G6PC*, *NPAS2*, *IL17*).[171] It would be interesting to see whether the increase in co-activator occupancy is dependent on RORγ *via* a sequential ChIP experiment, as conducted in previous studies.[103,112] Application of 100 nM digoxin during polarization of $CD4^+$ cells into Th17 cells yielded higher IL-17 mRNA and protein levels and a transcriptome analysis of digoxin-treated Th17 cells derived from human donors showed the induction of certain Th17-related genes like *IL17A*/*F*. Additional docking experiments with digoxin gave the best results when the active conformation of the RORγ LBD was used and NCOA-2 was absent. Of note, docking of digoxin to the RORγt LBD domain in the inverse agonistic conformation resulted in the formation of a hydrogen bond to the histidine residue of the His–Tyr lock. Importantly, the hydrogen bond was not established *via* digitoxose (as seen in the co-crystal structure of Fujita-Sato *et al.*[6]) but the carbonyl residue of the butenolide ring instead.[171] Why these results drastically differed compared to the earlier studies[6,155] is not conclusively clarified. The authors speculate that differences in the protein biosynthesis of the insect cells (see ref. 155) – *e.g.* with respect to co-activators – could be responsible. However, this does not explain why Huh *et al.*[155] found a decreased co-activator and an increased co-repressor binding to the RORγt LBD *in vitro* when applying digoxin, although different co-activators were used in both studies. The conversion of digoxin into its aglycone digoxigenin under experimental conditions could be another explanation for the different results, since the agonistic effect of digoxigenin was suggested in an earlier study.[156] Assuming that no conversion took place, questions remain to be answered, *e.g.* why the X-ray analysis by Fujita-Sato *et al.*[6] suggested an inverse agonistic rather than an agonistic mechanism of action of digoxin. Further X-ray or NMR analyses of digoxin in complex with RORγ(t) LBD could potentially provide clarification on this subject. Also, it would be interesting to repeat some key experiments of Huh *et al.*[155] and Fujita-Sato *et al.*[6] at lower digoxin concentrations to see if the results of this study can be reproduced. Lastly, it could indeed be possible that digoxin may function as both, agonist and inverse agonist at different concentrations, as the authors suggested.[171]

4 Natural products indirectly affecting RORs

4.1 Melatonin

The first proposed endogenous ligand for RORs was the amino acid hormone melatonin (*N*-acetyl-5-methoxytryptamine).[95,173] Melatonin is produced in the pineal gland and regulates circadian rhythm, sleep–wake cycles, and seasonal reproduction in mammals, among others. Although direct binding of melatonin to RORβ has been shown initially, these results were not reproducible and the respective report has been retracted.[95] However, a study from the same group showing a direct interaction of melatonin with RORα has not been withdrawn.[173] In a more recent study,[94] melatonin was shown to decrease RORα transactivation in the human breast cancer cell line MCF-7 in the presence of 10% FCS to 34% of control at a concentration of 10 μM and to decrease the ability of RORα to bind to its response elements, as shown in a transfection assay and a gel mobility shift assay, respectively, without affecting RORα protein levels. It has been shown before that increased $[Ca^{2+}]_i$ levels and enhanced Ca^{2+}/calmodulin (CaM)-dependent protein kinase IV activity stimulates RORα transcriptional activity.[174] CaM kinase IV may influence the phosphorylation status of ROR co-factors, thereby modulating its activity. Accordingly, a calmodulin antagonist, calmidazole, decreased RORα transactivation similarly than melatonin.[94] Melatonin is known to modulate $[Ca^{2+}]_i$ levels *via* G-protein coupled membrane receptors and act as CaM antagonist.[175–177] In MCF-7 cells, melatonin had no direct effect on $[Ca^{2+}]_i$ levels, suggesting that melatonin influences RORα activity *via* CaM antagonism.[94] In *in vitro* cultured goat spermatids, 0.1 μM melatonin increased RORα

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Table 2 Selected indirect modulators of RORs

Substance class	Examples	Effect on RORs	Study results
Steroids and terpenoids	Dioscin, pristimerin, 3β-acetyloxy-oleanolic acid, saikosaponin A	• Decrease of RORγt mRNA[189,190] and protein[180,183] levels • Inhibition of RORγt transcriptional activity[190]	• Amelioration of inflammatory diseases in rodents[180,183,189,190] • Decrease of proinflammatory cytokines (*e.g.* IL-17)[180,183,189,190] • Decrease in Th17 cell differentiation[183,189,190]
Polyketides	Bavachalcone, poncirin/ponciretin, quercetin, baicalein	• Increase of RORα transcriptional activity[191] and *Rora*/*RORA* expression[191,192] • Decreased expression of RORγt[193,194]	• Increased expression of RORα target genes (*e.g. BMAL1*, *Fgf21*)[191,192] • Decrease of proinflammatory cytokines (*e.g.* IL-17)[193,194] • Decrease in Th17 cell differentiation[193,194] • Protection from liver damage in rodents[194]
Cardiac glycosides	Uscharin, calcein, calotropin, digoxigenin, dihydroouabain, strophanthidine	• Decrease of RORγt transcriptional activity[157] • Decrease of RORγt protein levels[157] • Increase of RORγ transcriptional activity[156] • Direct interaction with RORγt suggested by docking[156,157]	• Decrease in Th17 cell differentiation[157] • Increased expression of RORγ(t) target genes (*e.g. G6PC*, *IL-17*)[156] • Favorable scores when docked into RORγ-LBD[156,157]
Other substance classes	Arctigenin (lignan), epigallocatechin-3-gallate, astragalus polysaccharide & astragaloside IV (saponin), oxymatrine (quinolizidine alkaloid), rapamycin (macrolide), α-mangostin (xanthone)	• Decrease of RORγt mRNA[184,186,195–198] and protein[185,198] levels • Decrease of RORγt transcriptional activity[199]	• Amelioration of inflammatory diseases[184,185,195,196,198] and emphysema[186] in rodents • Decrease of proinflammatory cytokines (*e.g.* IL-17)[184,185,195,198,199] • Decrease in Th17 (ref. 184 and 199) and Tc17(ref. 186) cell differentiation
Extracts	Grape seed proanthocyanidin extract, ginger extract, compound sophorae decoction	• Increase of cyclic *RORA* expression[200] • Increase of RORα transcriptional activity in a Gal4 system, thus direct interaction suggested[200] • Decrease of RORγt mRNA levels[181,182]	• Amelioration of inflammatory diseases in rodents[181] • Decrease of proinflammatory cytokines (*e.g.* IL-17)[181] • Decrease in Th17 cell differentiation[181]

mRNA and protein levels. However, in CHO cells and human keratinocytes, melatonin was not able to inhibit RORα or RORγ transactivation. In addition, relatively low docking scores in *in silico* modeling have been obtained for RORα or RORγ, suggesting low affinity of melatonin for these receptors.[119] In summary, the ability of melatonin to influence ROR transcriptional activity seems to be cell-type specific and at least in human breast cancer cells a link to the calmodulin antagonism of melatonin is suggested. However, a causal relation could not be established. Moreover, it is likely that melatonin modulates ROR expression *via* its influence on circadian rhythm.[178,179]

4.2 Selected indirect modulators of RORs

A summary of studies on selected indirect ROR modulators is available in Table 2. The mechanism of action of most indirect ROR-modulators has not been elucidated, though hypotheses have been proposed. For instance, saikosaponin A has been suggested to influence RORγt protein expression *via* inhibition of NF-κB activation and hence IL-6 expression, a known activator of STAT3 signaling.[134–137,180] Similarly, anti-inflammatory mechanisms might explain the effects of compound sophorae decoction[181] and ginger extract[182] on ROR. Other proposed mechanisms include the STAT5-dependent modulation of IFN-γ, or PPARγ expression, ultimately decreasing RORγt levels (*e.g.* primisterin,[183] arctigenin,[184] astragalus polysaccharide[185]) and the downregulation of the transcription factor HIF-1α *via* mTOR, leading to a decrease in *RORC* transactivation as suggested for rapamycin[186,187] (also reviewed in ref. 188).

It must be stated that the extent of RORs' influence on the outcomes of these studies was not explicitly investigated in most cases. It can be assumed (and is often pointed out by the authors themselves) that other mechanisms are involved as well.

5 Conclusion and outlook

Many studies discussed the question as to whether the transcriptional activity of ROR is ligand dependent. Interestingly, one study showed that apo-RORα (expressed in *E.*

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coli and therefore deemed ligand free) is active,[103] but in most reports, RORs were active only in the presence of sterol ligands (examples: ref. 1, 8, 104 and 155). To explain this discrepancy, the concept of "silent ligands" was brought up,[18] which, however, also excludes RORs in an apo-state. Additionally, expression of RORs in *E. coli* do not necessarily yield empty LBDs.[144] It is likely that intermediates and metabolites of the cholesterol metabolism act as endogenous ligands for RORs.[1–3] Furthermore, it was shown that 500 nM of an endogenous ligand are sufficient to occupy 80% of available RORγ. Calculations revealed that a further increase in occupancy would require very high concentration of ligands, which might explain the moderate effect of exogenous ligands in reporter assays containing serum.[1] When discussing natural products interacting with RORs, one of the most prominent and heavily investigated ligands is the cardiac glycoside digoxin. Despite its relatively high IC_{50} value,[155] its benefits were examined in numerous preclinical studies in rodents. The cardenolide was deemed both, inverse agonist[6,155] and agonist[171] of RORγ(t) in different studies. Variations in experimental settings and the possibility of observing concentration-dependent effects were proposed as explanations,[171] but further validation is needed. As inhibitors of the Na^+/K^+-ATPase, cardiac glycosides like digoxin are used to treat heart failure, among other conditions, due to their positive inotropic effects. However, the cardenolide is used with caution and only when strictly indicated due to its narrow therapeutic window.[201] Digoxin dose-dependently decreased cell viability in 10 human tumor cell lines with a mean IC_{50} of 80 nM (ref. 168) and it is recommended to aim for serum concentrations not exceeding 0.8 ng ml^{-1} (approx. 1.0 nM) when treating patients.[202] However, digoxin's inhibition of RORγ occurs at the micromolar concentration range[6,155] and even its possible agonistic effect occurs at much higher concentrations.[171] In a study on digoxin's use in atherosclerosis therapy in mice, the authors measured plasma levels after the last injection and ascertained that they were "at or below the therapeutic range for humans",[166] at least in the low dose group. But even if digoxin could be used for its effects on RORγ(t) in humans, pharmacokinetic issues arising for instance from kidney impairments[203] would then have to be taken into account to prevent poisoning.

In general, RORs are challenging targets, since their apparent therapeutic potential is accompanied by various difficulties that still need to be overcome. RORs were first discovered in the mid-90s, the crystal structures were solved in the early 2000s and yet, to this day, there are no drugs on the market that have RORs as their target. As RORs are connected to several prominent biological systems, from circadian rhythm to metabolism and cancer, caution and a targeted approach is vital. The question to be answered is how natural products can be of use in this regard. Most of the compounds described in this study are either (i) toxic in the concentrations needed (*e.g.* digoxin), (ii) too ineffective (*e.g.* betulinaldehyde), (iii) not bioavailable enough (*e.g.* uvaol), and/or (iv) lack selectivity (*e.g.* ursolic acid). On the other hand, natural products have provided important mechanistical insights and may serve as templates for improved synthetic substances, as it was often the case in the past. Such substances would ideally be selective for one ROR protein, with certain exceptions (see chapter 2.2), and active in lower nanomolar concentrations while having no to little off-target activity and good safety. A good example for such an approach is the chemical conversion of digoxin to 20,22-dihydrodigoxin-21,23-diol which did not exhibit cytotoxic effects on human cells even at 40 μM while still inhibiting RORγ with an IC_{50} of 12 μM (*in vitro* competition assay).[155] In the last couple of years, progress has been made in this regard[204,205] (some further examples reviewed in ref. 206).

6 Conflicts of interest

There are no conflicts of interest to declare.

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