

REVIEW

View Article Online
View Journal | View Issue



Cite this: *Nat. Prod. Rep.*, 2021, 38, 757

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

Angela Ladurner,[†] Patrik F. Schwarz[†] and Verena M. Dirsch[†] ^{*}

Covering: 1994 to 2020

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.

Received 2nd July 2020

DOI: 10.1039/d0np00047g

rsc.li/npr

1	Introduction
2	Biological roles of RORs and their potential as drug targets
2.1	Biological roles of RORs
2.2	Consequences of ROR (inverse) agonism and RORs as drug targets
3	Natural ligands directly binding to RORs
3.1	Steroids
3.1.1	Cholesterol and cholesterol sulfate
3.1.2	Cholesterol biosynthetic intermediates
3.1.3	Oxysterols
3.1.4	Secosteroids
3.1.5	Neurosteroids
3.2	Terpenoids
3.2.1	Ursolic acid
3.2.2	Betulinolaldehyde
3.2.3	Ganoderone A
3.2.4	Methyl corosolate, uvaol and oleanolic acid
3.2.5	Rockogenin
3.2.6	Retinoids
3.2.7	Amethinol A
3.3	Polyketides
3.3.1	Isoflavones
3.3.2	Nobiletin
3.4	Cardiac glycosides
3.4.1	Digoxin and derivatives
3.4.1.1	Preclinical studies performed with digoxin
3.4.1.2	Digoxin as ROR γ (t)-agonist?
4	Natural products indirectly affecting RORs

4.1	Melatonin
4.2	Selected indirect modulators of RORs
5	Conclusion and outlook
6	Conflicts of interest
7	Acknowledgements
8	References

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 preceded 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

Department of Pharmacognosy, University of Vienna, Vienna, Austria. E-mail: Verena.Dirsch@univie.ac.at

[†] These authors contributed equally to this work.



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 (ϕ XX ϕ ; 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 factors¹² (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 ROR γ -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. ROR α is expressed in many peripheral tissues like the liver, skeletal muscle, skin, lung, and adipose tissue. ROR β expression is restricted to brain, retina, bone, and pineal gland. The *RORC* gene encodes two isoforms *via* the use of alternative promoters. ROR γ (ROR γ 1) differs from ROR γ t (ROR γ 2) only at the first 100 nucleotides at the N-terminus.¹⁵ ROR γ 1 is expressed in muscle tissue, prostate, pancreas, heart, liver, and testicles, whereas ROR γ t 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



Angela Ladurner studied Molecular Biology at the University of Vienna (Austria) and completed her doctoral studies on natural product research in the group of Verena Dirsch in 2013. She then worked as a postdoctoral scientist at the Medical University of Vienna and at the University of Vienna. Her research focused on the characterization of the mechanism of action of natural products.



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 ROR γ .



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.



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 “stag-gerer” 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 ROR β 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, ROR γ 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 it³⁹ 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, ROR γ was shown to be under the control of CLOCK-BMAL1 in certain tissues (e.g. the liver) while its isoform ROR γ t 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 ROR α and ROR γ 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, ROR α showed tumor suppressive activities that were amongst others mediated by p53 (ref. 47–50) and the value of ROR γ t as a target in tumor therapy is currently under investigation (see chapter 2.2).

ROR γ and especially its isoform ROR γ t 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 ROR γ t as a critical regulator of Th17 cell differentiation.⁶¹ The underlying mechanisms of Th17 cell differentiation are complex (Fig. 1). The expression of ROR γ t 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 ROR γ t protein levels STAT3-dependently (reviewed in ref. 65). However, not only ROR γ t, but also ROR α 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 ROR γ t 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



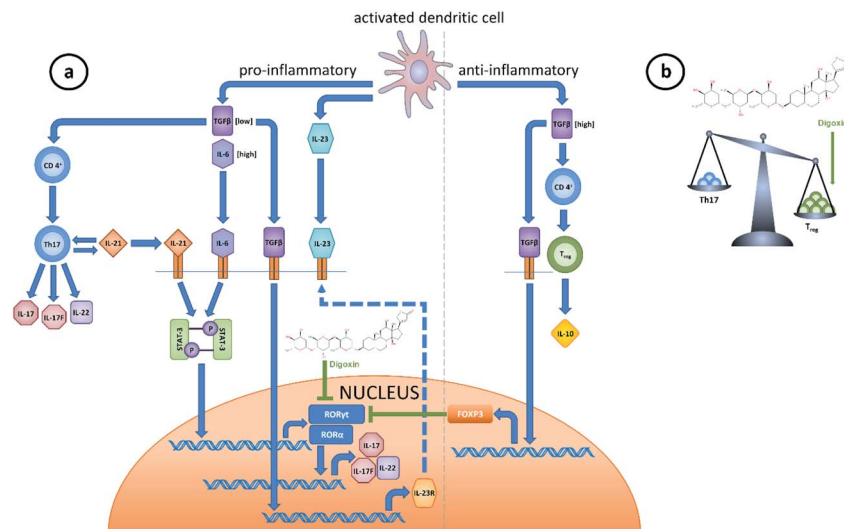


Fig. 1 (a) Differentiation of CD4⁺ cells into Th17 or Treg cells. Activated dendritic cells secrete a variety of cytokines like IL-6, IL-23 and TGF-β. These cytokines at varying concentrations are responsible for either Th17 or Treg differentiation. High concentrations of IL-6 and low concentrations 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 gain⁷⁴ 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* expression⁷⁸), 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 stability⁴⁹) 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 RORγ⁸² 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



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 ROR γ was described. ROR γ acts by promoting positive (C/EBP β) 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 ROR γ agonist SR1078 increased lung metastatic burden and splenic MDSC.⁸⁹ ROR γ (t) inverse agonists are interesting due to their anti-inflammatory potential. Most of the ROR γ (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 ROR γ t 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 ROR γ 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, ROR γ 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 ROR γ 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 ROR γ inverse agonism in diabetes type II as well.⁴³ Noteworthy, ROR α often exerts its effects in synergy with ROR γ . 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 ROR γ t alone,⁶¹ but rather by a ROR α -ROR γ -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}

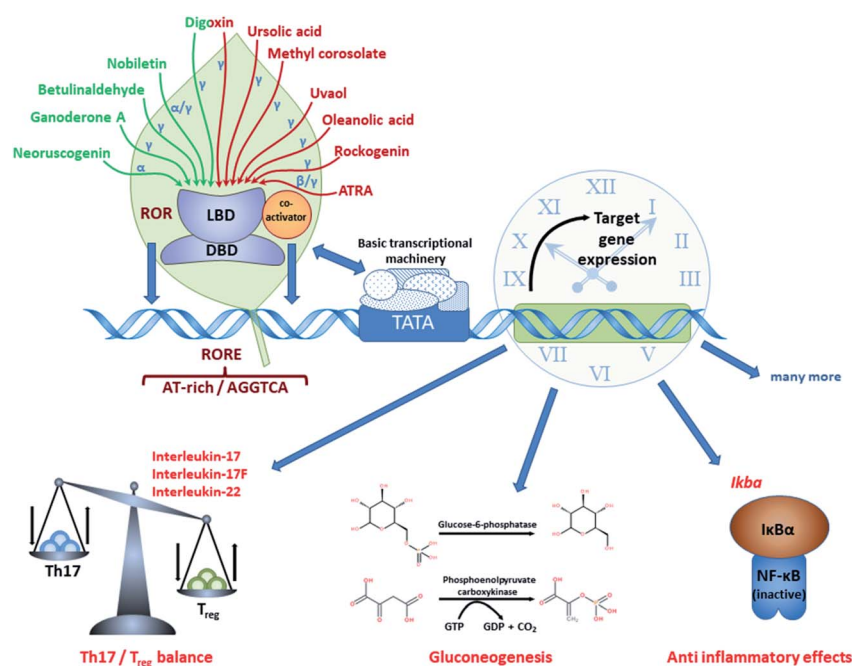


Fig. 2 Natural products as ligands of RORs. Various natural products were shown to act as ROR (inverse) agonists, thus affecting ROR target gene expression.



Table 1 Selected direct ROR ligands

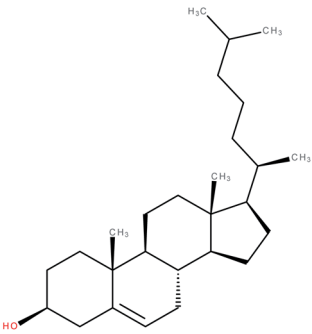
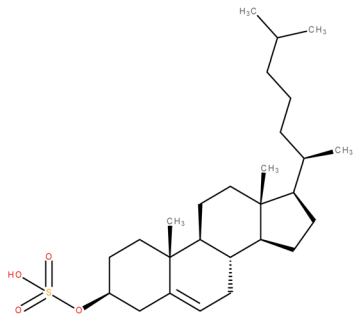
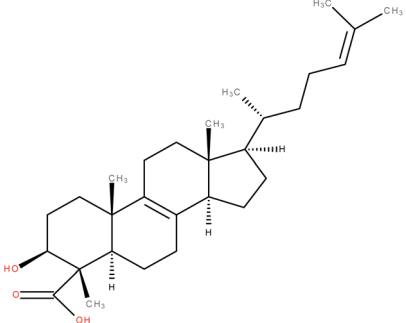
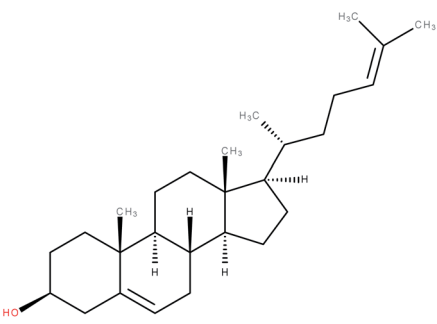
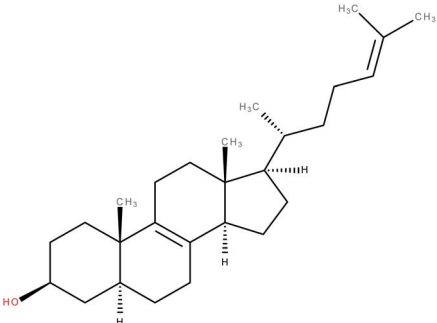
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Cholesterol		ROR α agonist	EC ₅₀ = 200 nM (co-activator binding to ROR γ LBD in AlphaScreen® assay) ¹⁰⁰ No activity in ROR α / β / γ -LBD:Gal4-DBD luciferase assay, ^{1,100–103} ROR γ Pcp2 promoter luciferase co-transfection assay in Cos-7 cells,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



Table 1 (Contd.)

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



Table 1 (Contd.)

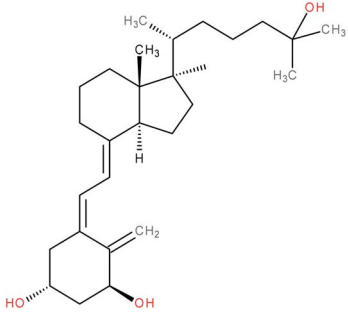
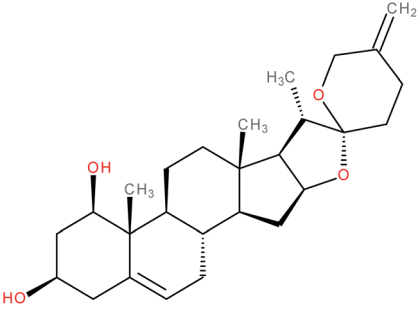
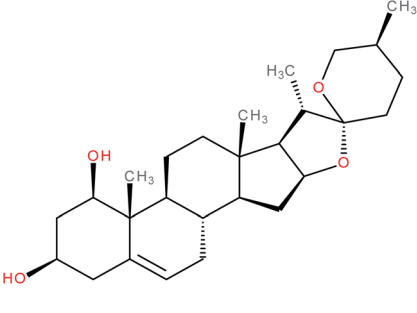
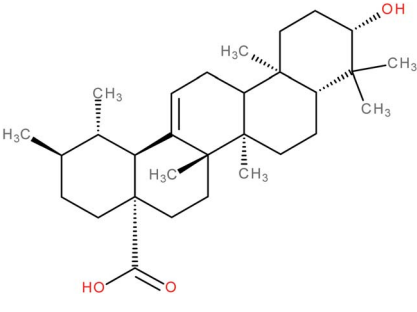
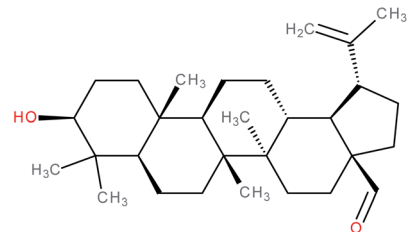
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Secosteroids <i>e.g.</i> 1,25(OH) ₂ D ₃		ROR α / γ inverse agonist	IC ₅₀ = 0.1–0.01 nM (luciferase reporter assay in ROR α or ROR γ stable transfected CHO Tet-on cells co-transfected with 5 × RORE)	119
Neoruscogenin		ROR α agonist	EC ₅₀ = 0.11 μ M (pull-down assay)	121
25S-Ruscogenin		ROR α agonist	EC ₅₀ = 0.78 μ M (pull-down assay)	121
Ursolic acid		ROR γ (t) inverse agonist	IC ₅₀ = 0.68 \pm 0.1 μ M (co-activator binding to ROR γ t LBD using a TR-FRET assay) ¹²³ IC ₅₀ = 0.56 \pm 0.1 μ M (Th17 cell differentiation) ¹²³ EC ₅₀ = 0.25 μ M (co-activator release from ROR γ LBD in FRET assay) ¹³⁸ IC ₅₀ = 1.3 μ M (inhibition of co-activator binding to ROR γ t LBD in AlphaScreen® assay) ¹³⁹ IC ₅₀ = 6.5 μ M (inhibition of co-repressor binding to ROR γ t LBD in HTRF assay) ¹³⁹ K _d = 3.20 μ M (SPR binding assay with ROR γ t protein) ¹³⁹	123, 124, 138 and 139
Betulinaldehyde		ROR γ t agonist	EC ₅₀ = 11.4 μ M (co-activator binding to ROR γ t LBD in AlphaScreen® assay) IC ₅₀ = 15.6 μ M (inhibition of co-repressor binding to ROR γ t LBD in HTRF assay) K _d = 2.99 μ M (SPR binding assay with ROR γ t protein)	139



Table 1 (Contd.)

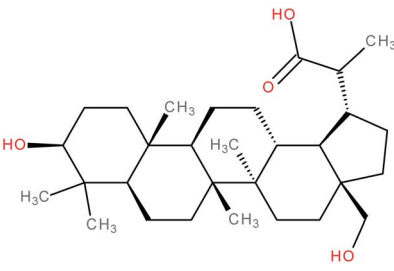
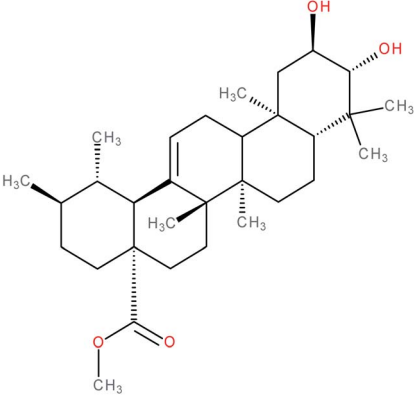
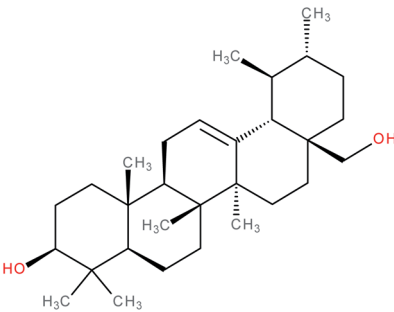
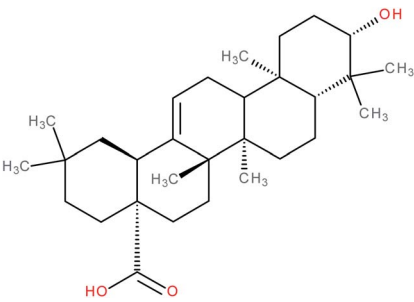
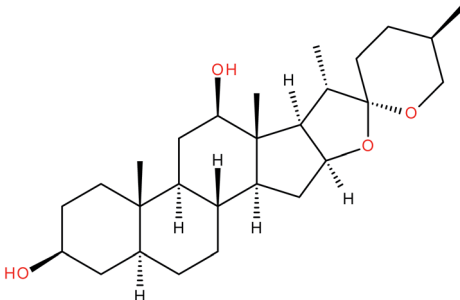
Natural product	Structure	Target(s)	Comment(s)	Reference(s)
3 β ,28-Dihydroxy-lupan-29-oic acid		ROR γ t inverse agonist	IC ₅₀ = 6.8 μ M (inhibition of co-activator binding to ROR γ t LBD in AlphaScreen® assay) IC ₅₀ = 19.8 μ M (inhibition of co-repressor binding to ROR γ t LBD in HTRF assay) K _d =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

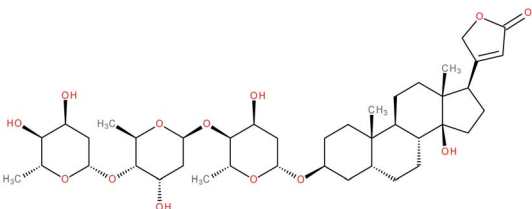
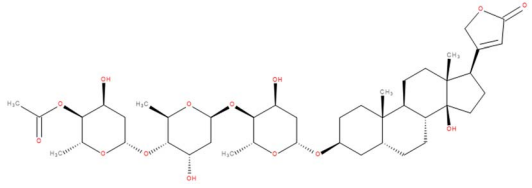


Table 1 (Contd.)

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



Table 1 (Contd.)

Natural product	Structure	Target(s)	Comment(s)	Reference(s)
Digitoxin		ROR γ (t) inverse agonist	IC ₅₀ value "similar" to digoxin according to the authors (luciferase reporter assay in S2 cells)	155
β -Acetyldigoxin		ROR γ (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 ROR α 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 ROR α 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-D₃) at 10 μ M.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 lock¹⁰ 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 ROR γ co-activator recruitment, while having no effect on co-repressor interaction and on ROR α co-activator recruitment, indicating ROR γ -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 ROR γ -Gal4, ROR α -Gal4, and ROR β -Gal4 reporter assay as well as a full-length ROR γ transactivation assay and was not able to displace 25-[³H]OHC in a competition assay.^{1,100–103}

Interestingly, cholesterol sulfate was able to replace cholesterol in the ligand binding pocket of ROR α and was predicted to bind the ROR α 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 ROR α LBD¹⁰⁴ 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 before⁸) showed that treatment with 10 μ M cholesterol sulfate led to a higher transcriptional activity relative to cholesterol. On the other hand, a critical mutation (ROR α Cys 288 \rightarrow 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 ROR γ co-activator recruitment assay, ROR γ -Gal4 and ROR α -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 μ M in ROR γ -Gal4 or ROR α -Gal4 reporter assays where cells were cultivated in lipid depleted and statin and mevalonate supplemented medium, although cholesterol sulfate was active in a 25-[³H]OHC competition assay.¹⁰³ In a cell-based study, 40 μ M cholesterol sulfate increased the mRNA expression levels of ROR α and the ROR α -regulated epidermal barrier precursor protein profilaggrin in a ROR α -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 ROR γ transcriptional activity. ROR γ 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, ROR γ is



ubiquitously active supporting the notion that one or more common metabolites act as endogenous ROR γ ligands. ROR γ activity was altered only by enzymes of the cholesterol biosynthetic pathway and correspondingly loss of sterol biosynthesis abolished ROR γ activity. To identify the enzymes and products responsible for ROR γ , 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 4 α -carboxy, 4 β -methyl-zymosterol, which acted as an agonist on ROR γ in transactivation assays, bound the ROR γ -LBD with high affinity in a competition assay, enhanced co-activator recruitment, restored ROR γ reporter activity when competing with an inhibitor and increased thermal stability of the ROR γ -LBD. Co-crystals of the ROR α -LBD or ROR γ -LBD and 4 α -carboxy, 4 β -methyl-zymosterol showed that helix 12 was in an active conformation. Taken together, they pinned down their search for a physiological endogenous ligand of ROR γ 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 ROR γ .¹

In the same year Hu *et al.*² found that endogenous sterol metabolites control Th17 differentiation *via* ROR γ 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 ROR γ -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 ROR γ target gene expression but not ROR γ itself. The ROR γ -dependence of the IL-17A increase elicited by desmosterol was confirmed *via* knockdown of ROR γ with siRNA during differentiation and the use of T cells from ROR γ 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 ROR γ 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 ROR γ 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 ROR γ agonists in Th17 cells.²

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

20 α -Hydroxycholesterol (20 α -OHC), 22(R)-hydroxycholesterol (22R-OHC), and 25-hydroxycholesterol (25-OHC) were all active with similar affinity in ROR γ co-activator recruitment assays with EC₅₀ values between 20 and 40 nM, while being inactive in ROR γ co-repressor recruitment and ROR α co-activator recruitment assays.¹⁰⁰ Notably, in another study 22R-OHC and 22S-OHC (10 μ M) were both not able to elicit an effect in ROR γ - or ROR α -Gal4 assays.¹⁰³ Co-crystal structures with the ROR γ LBD, the co-activator peptide NCOA2-2 and the putative ligands 20 α -OHC, 22R-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 ROR γ 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 ROR γ -Gal4 and full-length ROR γ confirmed the agonistic properties of these oxysterols and mutation of the unique binding residues in the ROR γ 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 ROR α co-activator recruitment assay,¹⁰⁰ it was shown to directly bind to the LBD of ROR α in a MS approach⁹⁶ and slightly but non-significantly to reduce activity in a ROR α -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/sf 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 22S-OHC and 27-OHC did not influence ROR α or ROR γ activity.^{100,103}

Given the role of RORs in bile acid metabolism,⁴⁵ 7 α -hydroxycholesterol produced by CYP7A1, the key enzyme in bile acid synthesis,¹¹⁰ and other related 7-oxysterols (7-OS, 7 β -hydroxycholesterol and 7-ketocholesterol) were investigated on ROR α/γ .¹⁰³ A competitive radioligand binding assay performed against tightly bound 25-[³H]OHC indeed showed that all these 7-OS bind with high affinity to the ROR α/γ LBD. Furthermore, 7-OS decreased transcriptional activity in a ROR α/γ -Gal4 and full-length ROR α/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 7 α -OHC confirmed the ROR α 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 ROR α/γ being in a constitutively active (and thus co-



activator-bound) state, where inverse agonists such as 7-OS can interfere with this process. Finally, in a more functional setting using ROR α / γ siRNA in murine hepatocytes, the metabolic effects of 7- α OS (decrease in *G6pc/Peptck* 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 ROR α .⁹⁶

Later additional oxysterols were investigated.¹⁰¹ In ROR γ -Gal4, full-length ROR γ and ROR γ t 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 7 β , 27-OHC. Notably, in this assay 7 α , 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 ROR γ or ROR α inverse agonists or agonists^{101,103,112} including 25-epoxycholesterol and 7-ketocholesterol were only weakly to moderately active. Cholestenic acid derivatives of 27-hydroxylated sterols were only barely active, emphasizing the importance of the hydroxyl group at carbon 27 for ROR γ t agonism. Many of the tested oxysterols had activities on other nuclear receptors, however, 7 β , 27-OHC and 7 α , 27-OHC seemed to be the most selective ROR γ t agonists. Direct binding of oxysterols was investigated in thermal shift assays with ROR α / β / γ LBD and the co-activator peptide NCOA1, where 27-OHC oxysterols bound most potently to the ROR γ LBD (7 β , 27-OHC > 7-keto, 27-OHC > 27-OHC > 7 α , 27-OHC). Notably, 27-OHC binding to the ROR γ LBD was significantly lower in the presence of NCOA1, suggesting that this oxysterol is no endogenous ROR γ agonist.¹⁰¹ In a functional approach 7 β , 27-OHC and 7 α , 27-OHC, but not 7 α , 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. ROR γ t dependency was confirmed with ROR γ t-deficient mouse CD4⁺ T cells. In addition, it was confirmed that 7 β , 27-OHC and 7 α , 27-OHC do not activate ROR α , as functional ROR α is expressed in ROR γ t 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 7 β , 27-OHC restored this effect, suggesting a physiological role of 27-OHC oxysterols in this process. The importance of CYP27A1 and 7 β / 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 ROR γ t agonist.¹⁰¹

Another oxysterol studied in more detail is 24S-OHC (cerebrosterol), mostly found in the brain.^{112,113} It showed similar effects on ROR α and ROR γ transcriptional activity in Gal4 assays as 7-oxysterols. Using ChIP-reChIP assays with the coactivator NCOA2, 24S-OHC was demonstrated to reduce recruitment of this peptide to ROR α . Notably, also 24(S),25-epoxycholesterol, found in micromolar concentrations in the

liver and brain,^{114,115} and 24R-OHC act as specific partial inverse agonists for ROR γ with an IC₅₀ 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-[³H]OHC, respectively, with no activity on ROR α .¹¹² The high abundance of cerebrosterol in young children¹¹³ and its ROR α 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 detected² 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)D₃, 20,23(OH)₂D₃, and 1,25(OH)₂D₃ 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 ROR α or ROR γ overexpressed, 20(OH)D₃ and 20,23(OH)₂D₃ inhibited doxycycline-induced transactivation of both receptors, while 20(OH)D₂, 1,25(OH)₂D₃, and vitamin D₂ were considerably less potent. In a mammalian two-hybrid system, used to evaluate the interaction of the ROR α or ROR γ LBD with the coactivator peptide EBIP96, 20(OH)D₃ dose-dependently decreased this interaction. In full-length transactivation assays with the *Bmal1* or *G6pase* promoter, 20(OH)D₃, 20,23(OH)₂D₃ and 20(OH)D₂ acted inhibitory, while 1,25(OH)₂D₃ and vitamin D₂ had no or much weaker activity. Moreover, 20(OH)D₃ and 20,23(OH)₂D₃ 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)D₃ and 20,23(OH)₂D₃ produced scores for binding to ROR α and ROR γ and binding pose similar to 25OHC.¹¹⁹ In a follow up study IC₅₀ values of several CYP11A1-derived secosteroids were determined in the RORE-driven ROR α and ROR γ reporter assay. Notably, all tested compounds (20(OH)D₃, 1,20(OH)₂D₃, 20,23(OH)₂D₃, 1,20,23(OH)₃D₃, 20,24(OH)₂D₃, 1,20,24(OH)₃D₃, 20,25(OH)₂D₃, 1,20,25(OH)₃D₃, 20,26(OH)₂D₃, 1,20,26(OH)₃D₃, 17,20,23(OH)₃D₃, 1,25(OH)₂D₃), elicited similar IC₅₀ values in the range of 0.1 to 0.01 nM. Additionally, molecular docking studies predicted binding of these vitamin D analogues to ROR α and ROR γ .¹²⁰ Taken together, the studied secosteroids acted as inverse agonists on ROR α and ROR γ , which might explain the multitude of effects elicited by vitamin D.

3.1.5 Neoruscogenin. Neoruscogenin, a steroidal saponin, which can be found in *Ruscus aculeatus* (Asparagaceae), was identified as an agonist of ROR α .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 ROR α LBD is quantified using luminescence, was able to identify ROR α ligands within fractionated plant extracts. Using this method combined with subsequent isolation and chromatographic



purification steps led to the identification of 25S-ruscogenin (from *Dalbergia cambodiana*, Fabaceae) as a potent ROR α agonist. Due to its better availability, neoruscogenin, which was found to be even more potent in the HTS method than 25S-ruscogenin, was subjected to further studies, which proved the agonistic properties of neoruscogenin in a ROR α -Gal4 luciferase assay and by a significant increase in gene expression of ROR α 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 ROR α 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 ROR γ t.¹²³ It was able to inhibit Th17 differentiation of naïve CD4⁺ T cells (murine, human) and down-regulated IL-17 but not ROR α / γ t 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 ROR α / γ t were transfected in HEK293T cells and (iii) co-activator peptide binding to the LBDs (TR-FRET assay), revealed selective inhibition of ROR γ t 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 study¹²⁴ ursolic acid lessened the incidence and severity of collagen-induced arthritis in mice while decreasing the expression of the proinflammatory cytokines TNF- α , IL-1 β , 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 ROR γ t. 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 ROR γ t 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 ROR γ t.^{3,61,132} Furthermore, ursolic acid is

a known inhibitor of the NF- κ B pathway.¹³³ Inhibition of NF- κ B 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 ROR γ also indirectly *via* NF- κ B 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 ROR γ -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, ROR γ t selectivity (over ROR α and ROR β) was evaluated by an AlphaScreen® assay.¹³⁹

Since ursolic acid has many other targets besides ROR γ t (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 3 β ,28-dihydroxy-lupan-29-oic acid were discovered as agonist and inverse agonist of ROR γ t, respectively. Betulinaldehyde was able to enhance co-activator and suppress co-repressor binding to the ROR γ t LBD in an AlphaScreen® and HTRF® assay, respectively, while 3 β ,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 (3 β ,28-dihydroxy-lupan-29-oic was the more effective one, even at lower concentrations), suggesting a direct interaction between the substances and the ROR γ t LBD. K_d values were determined by SPR and the selectivity of the substances for the ROR γ t 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 sulfate⁹) through hydrophobic interactions in the case of betulinaldehyde but was disrupted when 3 β ,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 ROR γ t.⁹ X-ray analysis of ROR γ t 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 ROR γ t 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



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 EC₅₀ 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 past¹⁴²) 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 ROR γ transactivation, and displaces a co-activator peptide.^{138,143} Co-crystal structures with rockogenin, the ROR γ -LBD and the co-repressor peptide SMRT22 could determine that rockogenin interacts with the ROR γ -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 ROR β LBD and completely replace the fortuitous pseudoligand stearate, which copurifies with the ROR β 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 ROR β 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 [³H] all-trans retinoic acid specifically binds to the LBD of ROR β . Moreover, in a Gal4 reporter assay in HT22 cells, all-trans retinoic acid inhibited ROR β and ROR γ transactivation, while being inactive on ROR α . 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 ROR γ t at 10 μ g 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 μ M) have been reported to dose-dependently enhance ROR α - and ROR γ -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 ROR α - or ROR γ 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 ROR α / γ - and STAT3-dependently and to enhance the interaction between ROR γ t 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 ROR γ t 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-[³H]OHC nobiletin showed robust competitive binding to the LBDs of ROR α and ROR γ , but with higher affinity to ROR γ . In addition, nobiletin was active in ROR α - and ROR γ -Gal4 mammalian one-hybrid assays, indicating direct binding of nobiletin to the ROR α and ROR γ LBD. Moreover, nobiletin dose-dependently and ROR α / γ -dependently increased ROR α and ROR γ transactivation of the *Bmal1* promoter in Hepa1-6 cells and the expression of ROR target genes such as *Cyp7b1*, *Ikb*, 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 ROR α and ROR γ 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 ROR γ using a ROR γ -Gal4



mammalian one hybrid assay in insect cells.¹⁵⁵ Digoxin acted as an inverse agonist, decreasing the transcriptional activity of ROR γ (but not ROR α or other nuclear receptors, *e.g.* hAR or LXR) when pretreated with 22-OHC at 10 μ M 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 ROR γ (t) inhibitory activity while the aglycone of digoxin, digoxigenin, was inactive. Interestingly, according to the authors,¹⁵⁵ digoxigenin does not even bind to the ROR γ t LBD, a notion challenged by later observations.¹⁵⁶ Treatment of murine CD4⁺ cells with digoxin during polarization resulted in a selectively reduced expression of ROR γ t-controlled genes (*e.g.* *Il17a/f*, *Il23r*). Reduction of Th17 differentiation upon digoxin treatment was specific, since other cell lines (*e.g.* Th1, Treg) were not affected.^{6,155} Moreover, only naïve murine CD4⁺ cells transduced with ROR γ t (and not ROR α ⁶⁶) experienced a decrease in *Il17* expression upon digoxin treatment, as shown using flow cytometry. Gene expression profiling (GEP) revealed that digoxin treatment and ROR γ t deficiency mostly (>90%) impacted the same genes. This led to the suggestion that ROR γ t is the dominant target of this cardenolide.¹⁵⁵ Moreover, ROR γ t gene expression itself was left unaltered by digoxin, indicating a direct inhibitory effect. Using ChIP analysis, digoxin was shown to inhibit ROR γ t-binding to key gene loci (*Il17a/f*, *Il23r*) of Th17 cells. Furthermore, co-activator binding to the LBD of ROR γ t 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 ROR γ t inhibition.¹⁵⁵ Interestingly, digoxin was effective only in relatively high concentrations (≥ 1 μ M).⁶ Moreover, the co-crystal of the human ROR γ t 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 μ M in a later study.¹⁵⁷

After digoxin was established as an inhibitor of ROR γ (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 AAA¹⁶⁴), 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 ROR γ 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



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 ROR γ t 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 α 1 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 ROR γ (t) inhibition in general.

3.4.1.2 Digoxin as ROR γ (t)-agonist? Surprisingly, another group reported agonism of digoxin on ROR γ (t) in a ROR γ 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 μ M 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 ROR γ -regulated genes *G6PC* and *NPAS2* in HepG2 cells following digoxin treatment. In agreement with previous studies, a mammalian one-hybrid assays with ROR γ -LBD:Gal4-DBD and ROR α -LBD:Gal4-DBD constructs showed a ROR γ specific activity of digoxin.^{6,155,171} Interestingly, when overexpressing human and mouse ROR γ (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 ROR γ t and NCOA-1 (but decreased NCOA-2) binding in the promoter region of ROR γ -regulated genes (*G6PC*, *NPAS2*, *IL17*).¹⁷¹ It would be interesting to see whether the increase in co-activator occupancy is dependent on ROR γ *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 ROR γ LBD was used and NCOA-2 was absent. Of note, docking of digoxin to the ROR γ t 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 ROR γ t 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 ROR γ (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 ROR β 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 ROR α has not been withdrawn.¹⁷³ In a more recent study,⁹⁴ melatonin was shown to decrease ROR α 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 μ M and to decrease the ability of ROR α to bind to its response elements, as shown in a transfection assay and a gel mobility shift assay, respectively, without affecting ROR α protein levels. It has been shown before that increased [Ca²⁺]_i levels and enhanced Ca²⁺/calmodulin (CaM)-dependent protein kinase IV activity stimulates ROR α 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 ROR α transactivation similarly than melatonin.⁹⁴ Melatonin is known to modulate [Ca²⁺]_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 [Ca²⁺]_i levels, suggesting that melatonin influences ROR α activity *via* CaM antagonism.⁹⁴ In *in vitro* cultured goat spermatids, 0.1 μ M melatonin increased ROR α



Table 2 Selected indirect modulators of RORs

Substance class	Examples	Effect on RORs	Study results
Steroids and terpenoids	Dioscin, pristimerin, 3 β -acetyloxy-oleanolic acid, saikosaponin A	<ul style="list-style-type: none"> Decrease of RORγt mRNA^{189,190} and protein^{180,183} levels Inhibition of RORγt transcriptional activity¹⁹⁰ 	<ul style="list-style-type: none"> 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	<ul style="list-style-type: none"> Increase of RORα transcriptional activity¹⁹¹ and <i>Rora/RORA</i> expression^{191,192} Decreased expression of RORγt^{193,194} 	<ul style="list-style-type: none"> Increased expression of RORα target genes (e.g. <i>BMAL1</i>, <i>Fgf21</i>)^{191,192} Decrease of proinflammatory cytokines (e.g. IL-17)^{193,194} Decrease in Th17 cell differentiation^{193,194} Protection from liver damage in rodents¹⁹⁴
Cardiac glycosides	Uscharin, calcein, calotropin, digoxigenin, dihydroouabain, strophanthidine	<ul style="list-style-type: none"> Decrease of RORγt transcriptional activity¹⁵⁷ Decrease of RORγt protein levels¹⁵⁷ Increase of RORγ transcriptional activity¹⁵⁶ Direct interaction with RORγt suggested by docking^{156,157} 	<ul style="list-style-type: none"> Decrease in Th17 cell differentiation¹⁵⁷ Increased expression of RORγ(t) target genes (e.g. <i>G6PC</i>, <i>IL-17</i>)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)	<ul style="list-style-type: none"> Decrease of RORγt mRNA^{184,186,195–198} and protein^{185,198} levels Decrease of RORγt transcriptional activity¹⁹⁹ 	<ul style="list-style-type: none"> Amelioration of inflammatory diseases^{184,185,195,196,198} and emphysema¹⁸⁶ 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	<ul style="list-style-type: none"> Increase of cyclic <i>RORA</i> expression²⁰⁰ Increase of RORα transcriptional activity in a Gal4 system, thus direct interaction suggested²⁰⁰ Decrease of RORγt mRNA levels^{181,182} 	<ul style="list-style-type: none"> Amelioration of inflammatory diseases in rodents¹⁸¹ Decrease of proinflammatory cytokines (e.g. IL-17)¹⁸¹ Decrease in Th17 cell differentiation¹⁸¹

mRNA and protein levels. However, in CHO cells and human keratinocytes, melatonin was not able to inhibit ROR α or ROR γ transactivation. In addition, relatively low docking scores in *in silico* modeling have been obtained for ROR α or ROR γ , 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 ROR γ t protein expression *via* inhibition of NF- κ B 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 decoction¹⁸¹ and ginger extract¹⁸² on ROR. Other proposed mechanisms include the STAT5-dependent modulation of IFN- γ , or PPAR γ expression, ultimately decreasing ROR γ t levels (e.g. primisterin,¹⁸³ arctigenin,¹⁸⁴ astragalus polysaccharide¹⁸⁵) and the downregulation of the transcription factor HIF-1 α *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-ROR α (expressed in *E.*



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 ROR γ . 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 IC₅₀ value,¹⁵⁵ its benefits were examined in numerous preclinical studies in rodents. The cardenolide was deemed both, inverse agonist^{6,155} and agonist¹⁷¹ of ROR γ (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 IC₅₀ 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 ROR γ 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 ROR γ (t) in humans, pharmacokinetic issues arising for instance from kidney impairments²⁰³ 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 μ M while still inhibiting ROR γ with an IC₅₀ of 12 μ M (*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.

7 Acknowledgements

This work was supported by the Vienna Anniversary Foundation for Higher Education (H-268438/2018). Special thanks to Andrea Szabo for drawing the figures.

8 References

- 1 F. R. Santori, P. Huang, S. A. Van De Pavert, E. F. Douglass, D. J. Leaver, B. A. Haubrich, R. Keber, G. Lorbek, T. Konijn, B. N. Rosales, D. Rozman, S. Horvat, A. Rahier, R. E. Mebius, F. Rastinejad, W. D. Nes and D. R. Littman, *Cell Metab.*, 2015, **21**, 286–298.
- 2 X. Hu, Y. Wang, L. Y. Hao, X. Liu, C. A. Lesch, B. M. Sanchez, J. M. Wendling, R. W. Morgan, T. D. Aicher, L. L. Carter, P. L. Toogood and G. D. Glick, *Nat. Chem. Biol.*, 2015, **11**, 141–147.
- 3 A. M. Jetten and D. N. Cook, *Annu. Rev. Pharmacol. Toxicol.*, 2020, **60**, 371–390.
- 4 A. Wärnmark, E. Treuter, A. P. H. Wright and J. Å. Gustafsson, *Mol. Endocrinol.*, 2003, **17**, 1901–1909.
- 5 Y. Zhang, X. Y. Luo, D. H. Wu and Y. Xu, *Acta Pharmacol. Sin.*, 2015, **36**, 71–87.
- 6 S. Fujita-Sato, S. Ito, T. Isobe, T. Ohyama, K. Wakabayashi, K. Morishita, O. Ando and F. Isono, *J. Biol. Chem.*, 2011, **286**, 31409–31417.
- 7 A. Aranda and A. Pascual, *Physiol. Rev.*, 2001, **81**, 1269–1304.
- 8 J. A. Kallen, J. M. Schlaeppli, F. Bitsch, S. Geisse, M. Geiser, I. Delhon and B. Fournier, *Structure*, 2002, **10**, 1697–1707.
- 9 J. Kallen, A. Izaac, C. Be, L. Arista, D. Orain, K. Kaupmann, C. Guntermann, K. Hoegenauer and S. Hintermann, *ChemMedChem*, 2017, **12**, 1014–1021.
- 10 X. Li, M. Anderson, D. Collin, I. Muegge, J. Wan, D. Brennan, S. Kugler, D. Terenzio, C. Kennedy, S. Lin, M. E. Labadia, B. Cook, R. Hughes and N. A. Farrow, *J. Biol. Chem.*, 2017, **292**, 11618–11630.
- 11 P. J. Watson, L. Fairall and J. W. R. Schwabe, *Mol. Cell. Endocrinol.*, 2012, **348**, 440–449.



- 12 C. A. Snyder, M. L. Goodson, A. C. Schroeder and M. L. Privalsky, *Mol. Cell. Endocrinol.*, 2015, **413**, 228–235.
- 13 M. L. Privalsky, *Annu. Rev. Physiol.*, 2004, **66**, 315–360.
- 14 D. P. Marciano, M. R. Chang, C. A. Corzo, D. Goswami, V. Q. Lam, B. D. Pascal and P. R. Griffin, *Cell Metab.*, 2014, **19**, 193–208.
- 15 Y. W. He, M. L. Deftos, E. W. Ojala and M. J. Bevan, *Immunity*, 1998, **9**, 797–806.
- 16 D. N. Cook, H. S. Kang and A. M. Jetten, *Nucl. Recept. Res.*, 2015, **2**, 101185, DOI: 10.11131/2015/101185.
- 17 D. J. Kojetin and T. P. Burris, *Nat. Rev. Drug Discovery*, 2014, **13**, 197–216.
- 18 L. A. Solt, P. R. Griffin and T. P. Burris, *Curr. Opin. Lipidol.*, 2010, **21**, 204–211.
- 19 N. Sun, H. Guo and Y. Wang, *Expert Opin. Ther. Pat.*, 2019, **29**, 663–674.
- 20 V. Amirkia and M. Heinrich, *Front. Pharmacol.*, 2015, **6**, 1–8.
- 21 J. A. Beutler, *Curr. Protoc. Pharmacol.*, 2019, **86**, e67.
- 22 A. L. Harvey, R. Edrada-Ebel and R. J. Quinn, *Nat. Rev. Drug Discovery*, 2015, **14**, 111–129.
- 23 D. J. Newman and G. M. Cragg, *J. Nat. Prod.*, 2016, **79**, 629–661.
- 24 A. M. Jetten, S. Kurebayashi and E. Ueda, *Prog. Nucleic Acid Res. Mol. Biol.*, 2001, **69**, 205–247.
- 25 A. M. Jetten, *Nucl. Recept. Signaling*, 2009, 7(1), e003, DOI: 10.1621/nrs.07003.
- 26 L. A. Solt and T. P. Burris, *Trends Endocrinol. Metab.*, 2012, **23**, 619–627.
- 27 R. L. Sidman, P. W. Lane and M. M. Dickie, *Science*, 1962, **137**, 610–612.
- 28 K. Herrup and R. J. Mullen, *Brain Res.*, 1979, **172**, 1–12.
- 29 B. A. Hamilton, W. N. Frankel, A. W. Kerrebrock, T. L. Hawkins, W. FitzHugh, K. Kusumi, L. B. Russell, K. L. Mueller, V. Van Berkel, B. W. Birren, L. Kruglyak and E. S. Lander, *Nature*, 1996, **379**, 736–739.
- 30 M. Steinmayr, E. André, F. Conquet, L. Rondi-Reig, N. Delhaye-Bouchaud, N. Auclair, H. Daniel, F. Crépel, J. Mariani, C. Sotelo and M. Becker-André, *Proc. Natl. Acad. Sci. U. S. A.*, 1998, **95**, 3960–3965.
- 31 C. I. Jarvis, B. Staels, B. Brugg, Y. Lemaigre-Dubreuil, A. Tedgui and J. Mariani, *Mol. Cell. Endocrinol.*, 2002, **186**, 1–5.
- 32 M. Akashi and T. Takumi, *Nat. Struct. Mol. Biol.*, 2005, **12**, 441–448.
- 33 N. Schaeren-Wiemers, E. André, J. P. Kapfhammer and M. Becker-André, *European Journal of Neuroscience*, 1997, **9**, 2687–2701.
- 34 E. André, F. Conquet, M. Steinmayr, S. C. Stratton, V. Porciatti and M. Becker-André, *EMBO J.*, 1998, **17**, 3867–3877.
- 35 Z. Sun, D. Unutmaz, Y. R. Zou, M. J. Sunshine, A. Pierani, S. Brenner-Morton, R. E. Mebius and D. R. Littman, *Science*, 2000, **288**, 2369–2373.
- 36 S. Kurebayashi, E. Ueda, M. Sakaue, D. D. Patel, A. Medvedev, F. Zhang and A. M. Jetten, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 10132–10137.
- 37 T. K. Sato, S. Panda, L. J. Miraglia, T. M. Reyes, R. D. Rudic, P. McNamara, K. A. Naik, G. A. Fitzgerald, S. A. Kay and J. B. Hogenesch, *Neuron*, 2004, **43**, 527–537.
- 38 C. L. Partch, C. B. Green and J. S. Takahashi, *Trends Cell Biol.*, 2014, **24**, 90–99.
- 39 J. P. DeBruyne, D. R. Weaver and S. M. Reppert, *Nat. Neurosci.*, 2007, **10**, 543–545.
- 40 Y. Takeda, H. S. Kang, M. Angers and A. M. Jetten, *Nucleic Acids Res.*, 2011, **39**, 4769–4782.
- 41 Y. Takeda, R. Jothi, V. Birault and A. M. Jetten, *Nucleic Acids Res.*, 2012, **40**, 8519–8535.
- 42 V. Mongrain, X. Ruan, H. Dardente, E. E. Fortier and N. Cermakian, *Genes Cells*, 2008, **13**, 1197–1210.
- 43 Y. Takeda, H. S. Kang, J. Freudenberger, L. M. DeGraff, R. Jothi and A. M. Jetten, *PLoS Genet.*, 2014, **10**(5), e1004331, DOI: 10.1371/journal.pgen.1004331.
- 44 Y. Takeda, H. S. Kang, F. B. Lih, H. Jiang, W. S. Blaner and A. M. Jetten, *Nucleic Acids Res.*, 2014, **42**, 10448–10459.
- 45 S. K. Hong, M. Angers, Y. B. Ju, X. Wu, J. M. Gimble, T. Wada, W. Xie, J. B. Collins, S. F. Grissom and A. M. Jetten, *Physiol. Genomics*, 2007, **31**, 281–294.
- 46 J. Fan, Z. Lv, G. Yang, T. ting Liao, J. Xu, F. Wu, Q. Huang, M. Guo, G. Hu, M. Zhou, L. Duan, S. Liu and Y. Jin, *Front. Immunol.*, 2018, **9**, 1187, DOI: 10.3389/fimmu.2018.01187.
- 47 G. Xiong, C. Wang, B. M. Evers, B. P. Zhou and R. Xu, *Cancer Res.*, 2012, **72**, 1728–1739.
- 48 H. Kim, J. M. Lee, G. Lee, J. Bhin, S. K. Oh, K. Kim, K. E. Pyo, J. S. Lee, H. Y. Yim, K. Il Kim, D. Hwang, J. Chung and S. H. Baek, *Mol. Cell*, 2011, **44**, 797–810.
- 49 Y. Wang, L. A. Solt, D. J. Kojetin and T. P. Burris, *PLoS One*, 2012, 7(4), e34921, DOI: 10.1371/journal.pone.0034921.
- 50 X. Pan, J. Zhao, W. N. Zhang, H. Y. Li, R. Mu, T. Zhou, H. Y. Zhang, W. L. Gong, M. Yu, J. H. Man, P. J. Zhang, A. N. Li and X. M. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2009, **106**, 3788–3793.
- 51 J. S. Tzartos, M. A. Friese, M. J. Craner, J. Palace, J. Newcombe, M. M. Esiri and L. Fugger, *Am. J. Pathol.*, 2008, **172**, 146–155.
- 52 E. Lubberts, M. I. Koenders, B. Oppers-Walgreen, L. Van Den Berselaar, C. J. J. Coenen-De Roo, L. A. B. Joosten and W. B. Van Den Berg, *Arthritis Rheuma*, 2004, **50**, 650–659.
- 53 J. Yang, Y. Chu, X. Yang, D. Gao, L. Zhu, X. Yang, L. Wan and M. Li, *Arthritis Rheuma*, 2009, **60**, 1472–1483.
- 54 S. Kagami, H. L. Rizzo, J. J. Lee, Y. Koguchi and A. Blauvelt, *J. Invest. Dermatol.*, 2010, **130**, 1373–1383.
- 55 L. Novelli, M. S. Chimenti, A. Chiricozzi and R. Perricone, *Autoimmun. Rev.*, 2014, **13**, 64–69.
- 56 S. Molet, Q. Hamid, F. Davoine, E. Nutku, R. Taha, N. Pagé, R. Olivenstein, J. Elias and J. Chakir, *J. Allergy Clin. Immunol.*, 2001, **108**, 430–438.
- 57 I. Agache, C. Ciobanu, C. Agache and M. Anghel, *Respir. Med.*, 2010, **104**, 1131–1137.
- 58 W. Zou and N. P. Restifo, *Nat. Rev. Immunol.*, 2010, **10**, 248–256.
- 59 C. M. Wilke, I. Kryczek, S. Wei, E. Zhao, K. Wu, G. Wang and W. Zou, *Carcinogenesis*, 2011, **32**, 643–649.



- 60 R. P. Singh, S. Hasan, S. Sharma, S. Nagra, D. T. Yamaguchi, D. T. W. Wong, B. H. Hahn and A. Hossain, *Autoimmun. Rev.*, 2014, **13**, 1174–1181.
- 61 I. I. Ivanov, B. S. McKenzie, L. Zhou, C. E. Tadokoro, A. Lepelley, J. J. Lafaille, D. J. Cua and D. R. Littman, *Cell*, 2006, **126**, 1121–1133.
- 62 K. Oboki, T. Ohno, H. Saito and S. Nakae, *Allergol. Int.*, 2008, **57**, 121–134.
- 63 G. L. Stritesky, N. Yeh and M. H. Kaplan, *J. Immunol.*, 2008, **181**, 5948–5955.
- 64 R. Nurieva, X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten and C. Dong, *Nature*, 2007, **448**, 480–483.
- 65 C. Dong, *Nat. Rev. Immunol.*, 2008, **8**, 337–348.
- 66 X. O. Yang, B. P. Pappu, R. Nurieva, A. Akimzhanov, H. S. Kang, Y. Chung, L. Ma, B. Shah, A. D. Panopoulos, K. S. Schluns, S. S. Watowich, Q. Tian, A. M. Jetten and C. Dong, *Immunity*, 2008, **28**, 29–39.
- 67 W. J. Chen, W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady and S. M. Wahl, *J. Exp. Med.*, 2003, **198**, 1875–1886.
- 68 L. Zhou, J. E. Lopes, M. M. W. Chong, I. I. Ivanov, R. Min, G. D. Vitoria, Y. Shen, J. Du, Y. P. Rubtsov, A. Y. Rudensky, S. F. Ziegler and D. R. Littman, *Nature*, 2008, **453**, 236–240.
- 69 P. R. Mangan, L. E. Harrington, D. B. O'Quinn, W. S. Helms, D. C. Bullard, C. O. Elson, R. D. Hatton, S. M. Wahl, T. R. Schoeb and C. T. Weaver, *Nature*, 2006, **441**, 231–234.
- 70 T. Korn, E. Bettelli, W. Gao, A. Awasthi, A. Jäger, T. B. Strom, M. Oukka and V. K. Kuchroo, *Nature*, 2007, **448**, 484–487.
- 71 L. Zhou, I. I. Ivanov, R. Spolski, R. Min, K. Shenderov, T. Egawa, D. E. Levy, W. J. Leonard and D. R. Littman, *Nat. Immunol.*, 2007, **8**, 967–974.
- 72 I. I. Ivanov, L. Zhou and D. R. Littman, *Semin. Immunol.*, 2007, **19**, 409–417.
- 73 E. Raspé, H. Duez, P. Gervois, C. Fiévet, J. C. Fruchart, S. Besnard, J. Mariani, A. Tedgui and B. Staels, *J. Biol. Chem.*, 2001, **276**, 2865–2871.
- 74 P. Lau, R. L. Fitzsimmons, S. Raichur, S. C. M. Wang, A. Lechtken and G. E. O. Muscat, *J. Biol. Chem.*, 2008, **283**, 18411–18421.
- 75 P. Lau, R. L. Fitzsimmons, M. A. Pearen, M. J. Watt and G. E. O. Muscat, *Diabetologia*, 2011, **54**, 1169–1180.
- 76 N. Kumar, D. J. Kojetin, L. A. Solt, K. G. Kumar, P. Nuhant, D. R. Duckett, M. D. Cameron, A. A. Butler, W. R. Roush, P. R. Griffin and T. P. Burris, *ACS Chem. Biol.*, 2011, **6**, 218–222.
- 77 P. Madsen and N. Westergaard, *Expert Opin. Ther. Pat.*, 2001, **11**, 1429–1441.
- 78 P. Delerive, D. Monté, G. Dubois, F. Trottein, J. Fruchart-Najib, J. Mariani, J. C. Fruchart and B. Staels, *EMBO Rep.*, 2001, **2**, 42–48.
- 79 A. Mamontova, S. Séguret-Macé, B. Esposito, C. Chanial, M. Bouly, N. Delhay-Bouchaud, G. Luc, B. Staels, N. Duverger, J. Mariani and A. Tedgui, *Circulation*, 1998, **98**, 2738–2743.
- 80 T. Meyer, M. Kneissel, J. Mariani and B. Fournier, *Proc. Natl. Acad. Sci. U. S. A.*, 2000, **97**, 9197–9202.
- 81 M. M. Roforth, G. Liu, S. Khosla and D. G. Monroe, *J. Bone Miner. Res.*, 2012, **27**, 891–901.
- 82 C. Gege, T. Schlüter and T. Hoffmann, *Bioorg. Med. Chem. Lett.*, 2014, **24**, 5265–5267.
- 83 R. Patouret, C. Doebelin, R. D. Garcia-Ordóñez, M. Ra Chang, C. Ruiz, M. D. Cameron, P. R. Griffin and T. M. Kamenecka, *Bioorg. Med. Chem. Lett.*, 2018, **28**, 1178–1181.
- 84 M. R. Chang, V. Dharmarajan, C. Doebelin, R. D. Garcia-Ordóñez, S. J. Novick, D. S. Kuruvilla, T. M. Kamenecka and P. R. Griffin, *ACS Chem. Biol.*, 2016, **11**, 1012–1018.
- 85 X. Hu, X. Liu, J. Moisan, Y. Wang, C. A. Lesch, C. Spooner, R. W. Morgan, E. M. Zawadzka, D. Mertz, D. Bousley, K. Majchrzak, I. Kryczek, C. Taylor, C. Van Huis, D. Skalitzky, A. Hurd, T. D. Aicher, P. L. Toogood, G. D. Glick, C. M. Paulos, W. Zou and L. L. Carter, *Oncoimmunology*, 2016, **5**(12), e1254854, DOI: 10.1080/2162402x.2016.1254854.
- 86 D. Mahalingam, J. S. Wang, E. P. Hamilton, J. Sarantopoulos, J. Nemunaitis, G. Weems, L. Carter, X. Hu, M. Schreeder and H. J. Wilkins, *Clin. Cancer Res.*, 2019, **25**, 3508–3516.
- 87 D. I. Gabrilovich, *Cancer Immunol. Res.*, 2017, **5**, 3–8.
- 88 T. Condamine, I. Ramachandran, J. I. Youn and D. I. Gabrilovich, *Annu. Rev. Med.*, 2015, **66**, 97–110.
- 89 L. Strauss, S. Sangaletti, F. M. Consonni, G. Szebeni, S. Morlacchi, M. G. Totaro, C. Porta, A. Anselmo, S. Tartari, A. Doni, F. Zitelli, C. Tripodo, M. P. Colombo and A. Sica, *Cancer Cell*, 2015, **28**, 253–269.
- 90 M. Takaishi, M. Ishizaki, K. Suzuki, T. Isobe, T. Shimoizato and S. Sano, *J. Dermatol. Sci.*, 2017, **85**, 12–19.
- 91 J. Wang, J. X. Zou, X. Xue, D. Cai, Y. Zhang, Z. Duan, Q. Xiang, J. C. Yang, M. C. Louie, A. D. Borowsky, A. C. Gao, C. P. Evans, K. S. Lam, J. Xu, H. J. Kung, R. M. Evans, Y. Xu and H. W. Chen, *Nat. Med.*, 2016, **22**, 488–496.
- 92 D. Cai, J. Wang, B. Gao, J. Li, F. Wu, J. X. Zou, J. Xu, Y. Jiang, H. Zou, Z. Huang, A. D. Borowsky, R. J. Bold, P. N. Lara, J. J. Li, X. Chen, K. S. Lam, K. F. To, H. J. Kung, O. Fiehn, R. Zhao, R. M. Evans and H. W. Chen, *Nat. Commun.*, 2019, **10**(1), 4621, DOI: 10.1038/s41467-019-12529-3.
- 93 C. S. Constantinescu, N. Farooqi, K. O'Brien and B. Gran, *Br. J. Pharmacol.*, 2011, **164**, 1079–1106.
- 94 J. Dai, P. T. Ram, L. Yuan, L. L. Spriggs and S. M. Hill, *Mol. Cell. Endocrinol.*, 2001, **176**, 111–120.
- 95 M. Becker-André, I. Wiesenberger, N. Schaeren-Wiemers, E. André, M. Missbach, J. H. Saurat and C. Carlberg, *J. Biol. Chem.*, 1994, **269**, 28531–28534.
- 96 F. Bitsch, R. Aichholz, J. Kallen, S. Geisse, B. Fournier and J. M. Schlaeppli, *Anal. Biochem.*, 2003, **323**, 139–149.
- 97 J. P. Renaud and D. Moras, *Cell. Mol. Life Sci.*, 2000, **57**, 1748–1769.
- 98 O. René, B. P. Fauber, G. D. L. Boenig, B. Burton, C. Eidenschenk, C. Everett, A. Gobbi, S. G. Hymowitz, A. R. Johnson, J. R. Kiefer, M. Liimatta, P. Lockey,



- M. Norman, W. Ouyang, H. A. Wallweber and H. Wong, *ACS Med. Chem. Lett.*, 2015, **6**, 276–281.
- 99 C. Doebelin, R. Patouret, R. D. Garcia-Ordenez, M. R. Chang, V. Dharmarajan, D. S. Kuruvilla, S. J. Novick, L. Lin, M. D. Cameron, P. R. Griffin and T. M. Kamenecka, *ChemMedChem*, 2016, **11**, 2607–2620.
- 100 L. Jin, D. Martynowski, S. Zheng, T. Wada, W. Xie and Y. Li, *Mol. Endocrinol.*, 2010, **24**, 923–929.
- 101 P. Soroosh, J. Wu, X. Xue, J. Song, S. W. Sutton, M. Sablad, J. Yu, M. I. Nelen, X. Liu, G. Castro, R. Luna, S. Crawford, H. Banie, R. A. Dandridge, X. Deng, A. Bittner, C. Kuei, M. Tootoonchi, N. Rozenkrants, K. Herman, J. Gao, X. V. Yang, K. Sachen, K. Ngo, W. P. Fung-Leung, S. Nguyen, A. De Leon-Tabaldo, J. Blevitt, Y. Zhang, M. D. Cummings, T. Rao, N. S. Mani, C. Liu, M. McKinnon, M. E. Milla, A. M. Fourie and S. Sun, *Proc. Natl. Acad. Sci. U. S. A.*, 2014, **111**, 12163–12168.
- 102 C. Stehlin-Gaon, D. Willmann, D. Zeyer, S. Sanglier, A. Van Dorselaer, J. P. Renaud, D. Moras and R. Schüle, *Nat. Struct. Biol.*, 2003, **10**, 820–825.
- 103 Y. Wang, N. Kumar, L. A. Solt, T. I. Richardson, L. M. Helvering, C. Crumbley, R. D. Garcia-Ordenez, K. R. Stayrook, X. Zhang, S. Novick, M. J. Chalmers, P. R. Griffin and T. P. Burris, *J. Biol. Chem.*, 2010, **285**, 5013–5025.
- 104 J. Kallen, J. M. Schlaeppli, F. Bitsch, I. Delhon and B. Fournier, *J. Biol. Chem.*, 2004, **279**, 14033–14038.
- 105 O. Hanyu, H. Nakae, T. Miida, Y. Higashi, H. Fuda, M. Endo, A. Kohjitani, H. Sone and C. A. Strott, *Biochem. Biophys. Res. Commun.*, 2012, **428**, 99–104.
- 106 X. Zhang, J. Jin, X. Peng, V. S. Ramgolam and S. Markovic-Plese, *J. Immunol.*, 2008, **180**(10), 6988–6996, DOI: 10.4049/jimmunol.180.10.6988.
- 107 S. I. Kagami, T. Owada, H. Kanari, Y. Saito, A. Suto, K. Ikeda, K. Hirose, N. Watanabe, I. Iwamoto and H. Nakajima, *Int. Immunol.*, 2009, **21**(6), 679–689, DOI: 10.1093/intimm/dxp037.
- 108 Z. K. Tuong, P. Lau, J. C. Yeo, M. A. Pearen, A. A. Wall, A. C. Stanley, J. L. Stow and G. E. O. Muscat, *Endocrinology*, 2013, **154**(1), 140–149, DOI: 10.1210/en.2012-1889.
- 109 Z. K. Tuong, P. Lau, X. Du, N. D. Condon, J. M. Goode, T. G. Oh, J. C. Yeo, G. E. O. Muscat and J. L. Stow, *PLoS One*, 2016, **11**, 1–16.
- 110 J. Y. L. Chiang, *F1000Research*, 2017, **6**, 2029, DOI: 10.12688/f1000research.12449.1.
- 111 A. R. Chopra, J. F. Louet, P. Saha, J. An, F. DeMayo, J. Xu, B. York, S. Karpen, M. Finegold, D. Moore, L. Chan, C. B. Newgard and B. W. O'Malley, *Science*, 2008, **322**, 1395–1399.
- 112 Y. Wang, N. Kumar, C. Crumbley, P. R. Griffin and T. P. Burris, *Biochim. Biophys. Acta, Mol. Cell Biol. Lipids*, 2010, **1801**, 917–923.
- 113 D. Lütjohann, O. Breuer, G. Ahlborg, I. Nennesmo, Å. Sidén, U. Diczfalussy and I. Björkhem, *Proc. Natl. Acad. Sci. U. S. A.*, 1996, **93**, 9799–9804.
- 114 J. Wong, C. M. Quinn, G. Guillemin and A. J. Brown, *J. Neurochem.*, 2007, **103**(5), 1764–1773, DOI: 10.1111/j.1471-4159.2007.04913.x.
- 115 T. A. Spencer, A. K. Gayen, S. Phirwa, J. A. Nelson, F. R. Taylor, A. A. Kandutsch and S. K. Erickson, *J. Biol. Chem.*, 1985, **260**(25), 13391–13394.
- 116 J. M. Lehmann, S. A. Klier, L. B. Moore, T. A. Smith-Oliver, B. B. Oliver, J. L. Su, S. S. Sundseth, D. A. Winegar, D. E. Blanchard, T. A. Spencer and T. M. Willson, *J. Biol. Chem.*, 1997, **272**, 3137–3140.
- 117 S. Hirayama, S. Nakagawa, S. Soda, Y. Kamimura, E. Nishioka, T. Ueno, Y. Fukushima, K. ichi Higuchi, M. Inoue, U. Seino, H. Ohmura, S. Yamato and T. Miida, *Atherosclerosis*, 2013, **230**(1), 48–51, DOI: 10.1016/j.atherosclerosis.2013.06.016.
- 118 A. J. Brown and W. Jessup, *Atherosclerosis*, 1999, **142**(1), 1–28.
- 119 A. T. Slominski, T. K. Kim, Y. Takeda, Z. Janjetovic, A. A. Brozyna, C. Skobowiat, J. Wang, A. Postlethwaite, W. Li, R. C. Tuckey and A. M. Jetten, *FASEB J.*, 2014, **28**, 2775–2789.
- 120 A. T. Slominski, T. K. Kim, J. V. Hobrath, A. S. W. Oak, E. K. Y. Tang, E. W. Tieu, W. Li, R. C. Tuckey and A. M. Jetten, *J. Steroid Biochem. Mol. Biol.*, 2017, **173**, 42–56.
- 121 S. Helleboid, C. Haug, K. Lamotte, Y. Zhou, J. Wei, S. Daix, L. Cambula, G. Rigou, D. W. Hum and R. Walczak, *J. Biomol. Screening*, 2014, **19**, 399–406.
- 122 Y. Ikeda, A. Murakami and H. Ohigashi, *Mol. Nutr. Food Res.*, 2008, **52**, 26–42.
- 123 T. Xu, X. Wang, B. Zhong, R. I. Nurieva, S. Ding and C. Dong, *J. Biol. Chem.*, 2011, **286**, 22707–22710.
- 124 S. Y. Baek, J. Lee, D. G. Lee, M. K. Park, J. Lee, S. K. Kwok, M. La Cho and S. H. Park, *Acta Pharmacol. Sin.*, 2014, **35**, 1177–1187.
- 125 K. Kim, E. A. Shin, J. H. Jung, J. E. Park, D. S. Kim, B. S. Shim and S. H. Kim, *Int. J. Mol. Sci.*, 2018, **20**(1), 114, DOI: 10.3390/ijms20010114.
- 126 M. Zhai, J. Guo, H. Ma, W. Shi, D. Jou, D. Yan, T. Liu, J. Tao, J. Duan, Y. Wang, S. Li, J. Lv, C. Li, J. Lin, C. Zhang and L. Lin, *Atherosclerosis*, 2018, **271**, 128–135, DOI: 10.1016/j.atherosclerosis.2018.02.022.
- 127 M. K. Shanmugam, T. H. Ong, A. P. Kumar, C. K. Lun, P. C. Ho, P. T. H. Wong, K. M. Hui and G. Sethi, *PLoS One*, 2012, **7**(3), e32476, DOI: 10.1371/journal.pone.0032476.
- 128 S. Prasad, V. R. Yadav, B. Sung, S. Reuter, R. Kannappan, A. Deorukhkar, P. Diagaradjane, C. Wei, V. Baladandayuthapani, S. Krishnan, S. Guha and B. B. Aggarwal, *Clin. Cancer Res.*, 2012, **18**(18), 4942–4953, DOI: 10.1158/1078-0432.ccr-11-2805.
- 129 S. Prasad, V. R. Yadav, B. Sung, S. C. Gupta, A. K. Tyagi and B. B. Aggarwal, *Oncotarget*, 2016, **7**(11), 13182–13196, DOI: 10.18632/oncotarget.7537.
- 130 X. Ma, Y. Zhang, Z. Wang, Y. Shen, M. Zhang, Q. Nie, Y. Hou and G. Bai, *Mol. Nutr. Food Res.*, 2017, **61**(12), 1700332, DOI: 10.1002/mnfr.201700332.



- 131 T. Liu, H. Ma, W. Shi, J. Duan, Y. Wang, C. Zhang, C. Li, J. Lin, S. Li, J. Lv and L. Lin, *Int. J. Oncol.*, 2017, **51**(2), 555–562, DOI: 10.3892/ijo.2017.4035.
- 132 T. J. Harris, J. F. Grosso, H.-R. Yen, H. Xin, M. Kortylewski, E. Albesiano, E. L. Hipkiss, D. Getnet, M. V. Goldberg, C. H. Maris, F. Housseau, H. Yu, D. M. Pardoll and C. G. Drake, *J. Immunol.*, 2007, **179**(7), 4313–4317, DOI: 10.4049/jimmunol.179.7.4313.
- 133 S. Shishodia, S. Majumdar, S. Banerjee and B. B. Aggarwal, *Cancer Res.*, 2003, **63**(15), 4375–4383.
- 134 N. Wang, H. Liang and K. Zen, *Front. Immunol.*, 2014, **5**, 614.
- 135 R. Catlett-Falcone, T. H. Landowski, M. M. Oshiro, J. Turkson, A. Levitzki, R. Savino, G. Ciliberto, L. Moscinski, J. L. Fernández-Luna, G. Nuñez, W. S. Dalton and R. Jove, *Immunity*, 1999, **10**(1), 105–115, DOI: 10.1016/S1074-7613(00)80011-4.
- 136 Z. Zhong, Z. Wen and J. E. Darnell, *Science*, 1994, **264**(5155), 95–98, DOI: 10.1126/science.8140422.
- 137 T. Liu, L. Zhang, D. Joo and S. C. Sun, *Signal Transduction Targeted Ther.*, 2017, **2**, 17023, DOI: 10.1038/sigtrans.2017.23.
- 138 M. Noguchi, A. Nomura, K. Murase, S. Doi, K. Yamaguchi, K. Hirata, M. Shiozaki, S. Hirashima, M. Kotoku, T. Yamaguchi, Y. Katsuda, R. Steensma, X. Li, H. Tao, B. Tse, M. Fenn, R. Babine, E. Bradley, P. Crowe, S. Thacher, T. Adachi and M. Kamada, *Genes Cells*, 2017, **22**, 535–551.
- 139 F. Yang, R. Zhang, D. Ni, X. Luo, S. Chen, C. Luo and W. Xiao, *Fitoterapia*, 2019, **137**, 9–10.
- 140 Ł. Woźniak, S. Skąpska and K. Marszałek, *Molecules*, 2015, **20**, 20614–20641.
- 141 X. Zhou, H. Chen, F. Wei, Q. Zhao, Q. Su, Y. Lei, M. Yin, X. Tian, Z. Liu, B. Yu, C. Bai, X. He and Z. Huang, *Immunol. Invest.*, 2020, **49**(6), 632–647, DOI: 10.1080/08820139.2019.1698599.
- 142 S. M. Fu, C. Dai, Z. Zhao and F. Gaskin, *F1000Research*, 2015, **4**, 939.
- 143 S. Thacher, X. Li, R. Babine and B. Tse, *US Pat.*, US8389739, 2013.
- 144 C. Stehlin, J. M. Wurtz, A. Steinmetz, E. Greiner, R. Schüle, D. Moras and J. P. Renaud, *EMBO J.*, 2001, **20**, 5822–5831.
- 145 C. L. Zhao, M. S. Sarwar, J. H. Ye, C. F. Ku, W. F. Li, G. Y. Luo, J. J. Zhang, J. Xu, Z. F. Huang, S. W. Tsang, L. T. Pan and H. J. Zhang, *Acta Crystallogr., Sect. C: Struct. Chem.*, 2018, **74**, 635–640.
- 146 N. Li, X. Wu, W. Zhuang, L. Xia, Y. Chen, R. Zhao, M. Yi, Q. Wan, L. Du and Y. Zhou, *Mol. Nutr. Food Res.*, 2020, **64**(4), e1900751.
- 147 H. Kojima, Y. Takeda, R. Muromoto, M. Takahashi, T. Hirao, S. Takeuchi, A. M. Jetten and T. Matsuda, *Toxicology*, 2015, **329**, 32–39, DOI: 10.1016/j.tox.2015.01.007.
- 148 M. Takahashi, R. Muromoto, H. Kojima, S. Takeuchi, Y. Kitai, J. Ichi Kashiwakura and T. Matsuda, *Biochem. Biophys. Res. Commun.*, 2017, **489**, 503–508.
- 149 A. T. Dias, S. B. R. de Castro, C. C. de Souza Alves, M. G. Evangelista, L. C. da Silva, D. R. L. Reis, M. A. Machado, M. A. Juliano and A. P. Ferreira, *Inflammation Res.*, 2018, **67**(7), 597–608, DOI: 10.1007/s00011-018-1146-7.
- 150 M. N. Mansoori, A. M. Tyagi, P. Shukla, K. Srivastava, K. Dev, R. Chillara, R. Maurya and D. Singh, *Menopause*, 2016, **23**(5), 565–576, DOI: 10.1097/gme.0000000000000646.
- 151 F. Zhang, Z. Wang, M. Li, Y. Lan, Y. Chen and C. Wang, *Int. Immunopharmacol.*, 2015, **28**(1), 546–553, DOI: 10.1016/j.intimp.2015.07.023.
- 152 B. He, K. Nohara, N. Park, Y. S. Park, B. Guillory, Z. Zhao, J. M. Garcia, N. Koike, C. C. Lee, J. S. Takahashi, S. H. Yoo and Z. Chen, *Cell Metab.*, 2016, **23**, 610–621.
- 153 K. Nohara, V. Mallampalli, T. Nemkov, M. Wirianto, J. Yang, Y. Ye, Y. Sun, L. Han, K. A. Esser, E. Mileykovskaya, A. D'Alessandro, C. B. Green, J. S. Takahashi, W. Dowhan, S. H. Yoo and Z. Chen, *Nat. Commun.*, 2019, **10**(1), 3923, DOI: 10.1038/s41467-019-11926-y.
- 154 K. Nohara, T. Nemkov, A. D'Alessandro, S. H. Yoo and Z. Chen, *Int. J. Mol. Sci.*, 2019, **20**(17), 4281, DOI: 10.3390/ijms20174281.
- 155 J. R. Huh, M. W. L. Leung, P. Huang, D. A. Ryan, M. R. Krout, R. R. V. Malapaka, J. Chow, N. Manel, M. Ciofani, S. V. Kim, A. Cuesta, F. R. Santori, J. J. Lafaille, H. E. Xu, D. Y. Gin, F. Rastinejad and D. R. Littman, *Nature*, 2011, **472**, 486–490.
- 156 K. Karaś, A. Sałkowska, A. Walczak-Drzewiecka, K. Ryba, J. Dastyh, R. A. Bachorz and M. Ratajewski, *Toxicol. Lett.*, 2018, **295**, 314–324.
- 157 J. Liu, L. P. Bai, F. Yang, X. Yao, K. Lei, C. W. Kei Lam, Q. Wu, Y. Zhuang, R. Xiao, K. Liao, H. Kuok, T. Li and L. Liu, *Mol. Pharm.*, 2019, **16**, 798–807.
- 158 V. Gorbacheva, R. Fan, X. Li and A. Valujskikh, *Am. J. Pathol.*, 2010, **177**, 1265–1273.
- 159 S. Itoh, N. Kimura, R. C. Axtell, J. B. Velotta, Y. Gong, X. Wang, N. Kajiwar, A. Nambu, E. Shimura, H. Adachi, Y. Iwakura, H. Saito, K. Okumura, K. Sudo, L. Steinman, R. C. Robbins, S. Nakae and M. P. Fischbein, *Circulation*, 2011, **124**(11 Suppl.), S187–S196, DOI: 10.1161/circulationaha.110.014852.
- 160 M. A. Antonysamy, W. C. Fanslow, F. Fu, W. Li, S. Qian, A. B. Troutt and A. W. Thomson, *J. Immunol.*, 1999, **162**, 577–584.
- 161 J. Wu, C. Zhou, W. Chen, A. Xie, J. Li, S. Wang, P. Ye, W. Wang and J. Xia, *Transplantation*, 2013, **95**, 434–441.
- 162 Z. Wei, Y. Wang, K. Zhang, Y. Liao, P. Ye, J. Wu, Y. Wang, F. Li, Y. Yao, Y. Zhou and J. Liu, *Arterioscler., Thromb., Vasc. Biol.*, 2014, **34**, 2429–2438.
- 163 A. K. Sharma, G. Lu, A. Jester, W. F. Johnston, Y. Zhao, V. A. Hajzus, M. Reza Saadatizadeh, G. Su, C. M. Bhamidipati, G. S. Mehta, I. L. Kron, V. E. Laubach, M. P. Murphy, G. Ailawadi and G. R. Upchurch, *Circulation*, 2012, **126**(11 Suppl. 1), S38–S45, DOI: 10.1161/circulationaha.111.083451.



- 164 A. Wanhainen, F. Verzini, I. Van Herzele, E. Allaire, M. Bown, T. Cohnert, F. Dick, J. van Herwaarden, C. Karkos, M. Koelemay, T. Kölbel, I. Loftus, K. Mani, G. Melissano, J. Powell, Z. Szeberin, G. J. de Borst, N. Chakfe, S. Debus, R. Hinchliffe, S. Kakkos, I. Koncar, P. Kolh, J. S. Lindholt, M. de Vega, F. Vermassen, M. Björck, S. Cheng, R. Dalman, L. Davidovic, K. Donas, J. Earnshaw, H. H. Eckstein, J. Golledge, S. Haulon, T. Mastracci, R. Naylor, J. B. Ricco and H. Verhagen, *Eur. J. Vasc. Endovasc. Surg.*, 2019, **57**, 8–93.
- 165 J. Lee, S. Baek, J. Lee, J. Lee, D. G. Lee, M. K. Park, M. La Cho, S. H. Park and S. K. Kwok, *Int. Immunopharmacol.*, 2015, **26**, 103–111.
- 166 H. Shi, X. Mao, Y. Zhong, Y. Liu, X. Zhao, K. Yu, R. Zhu, Y. Wei, J. Zhu, H. Sun, Y. Mao and Q. Zeng, *Br. J. Pharmacol.*, 2016, **173**, 1517–1528.
- 167 S. Tani, R. Takano, S. Tamura, S. Oishi, M. Iwaizumi, Y. Hamaya, K. Takagaki, T. Nagata, S. Seto, T. Horii, I. Kosugi, T. Iwashita, S. Osawa, T. Furuta, H. Miyajima and K. Sugimoto, *Inflammatory Bowel Dis.*, 2017, **23**, 728–738.
- 168 S. Johansson, P. Lindholm, J. Gullbo, R. Larsson, L. Bohlin and P. Claeson, *Anticancer Drugs*, 2001, **12**, 475–483.
- 169 E. M. Price and J. B. Lingrel, *Biochemistry*, 1988, **27**, 8400–8408.
- 170 R. Thomas, P. Gray and J. Andrews, *Adv. Drug Res.*, 1990, **19**, 311–562.
- 171 K. Karaś, A. Sałkowska, M. Sobalska-Kwapis, A. Walczak-Drzewiecka, D. Strapagiel, J. Dastyh, R. A. Bachorz and M. Ratajewski, *Front. Pharmacol.*, 2019, **9**, 1460, DOI: 10.3389/fphar.2018.01460.
- 172 N. Kumar, L. a. Solt, J. J. Conkright, Y. Wang, M. a. Istrate, S. a. Busby, R. D. Garcia-ordonez, T. P. Burris and P. R. Griffin, *Mol. Pharmacol.*, 2010, **77**, 228–236.
- 173 I. Wiesenberger, M. Missbach, J. pierre Kahlen, M. Schröder and C. Carlberg, *Nucleic Acids Res.*, 1995, **23**, 327–333.
- 174 C. D. Kane and A. R. Means, *EMBO J.*, 2000, **19**(4), 691–701, DOI: 10.1093/emboj/19.4.691.
- 175 G. Benítez-King, L. Huerto-Delgadillo and F. Antón-Tay, *Life Sci.*, 1993, **53**(3), 201–207, DOI: 10.1016/0024-3205(93)90670-x.
- 176 L. Brydon, F. Roka, L. Petit, P. De Coppet, M. Tissot, P. Barrett, P. J. Morgan, C. Nanoff, A. D. Strosberg and R. Jockers, *Mol. Endocrinol.*, 1999, **13**(12), 2025–2038, DOI: 10.1210/mend.13.12.0390.
- 177 J. Vanecek and D. C. Klein, *Endocrinology*, 1992, **130**(2), 701–707, DOI: 10.1210/endo.130.2.1733718.
- 178 N. Zisapel, *Br. J. Pharmacol.*, 2018, **175**, 3190–3199.
- 179 M. C. Beker, B. Caglayan, A. B. Caglayan, T. Kelestemur, E. Yalcin, A. Caglayan, U. Kilic, A. T. Baykal, R. J. Reiter and E. Kilic, *Sci. Rep.*, 2019, **9**(1), 19082, DOI: 10.1038/s41598-019-55663-0.
- 180 C. H. Piao, C. H. Song, E. J. Lee and O. H. Chai, *Chem.-Biol. Interact.*, 2020, **315**, 1–8.
- 181 M. Xu, X. Y. Duan, Q. Y. Chen, H. Fan, Z. Chao Hong, S. J. Deng, Z. Nan, H. Wu, Y. L. Dong, Y. J. Liu and C. Z. Zhou, *Biomed. Pharmacother.*, 2019, **109**, 2396–2408.
- 182 M. Kardan, A. Rafiei, J. Ghaffari, R. Valadan, Z. Morsaljahani and S. T. Haj-ghorbani, *Allergol. Immunopathol.*, 2019, **47**, 378–385.
- 183 L. Tong, S. M. Nanjundaiah, S. H. Venkatesha, B. Astry, H. Yu and K. D. Moudgil, *Clin. Immunol.*, 2014, **155**, 220–230.
- 184 W. Li, Z. Zhang, K. Zhang, Z. Xue, Y. Li, Z. Zhang, L. Zhang, C. Gu, Q. Zhang, J. Hao, Y. Da, Z. Yao, Y. Kong and R. Zhang, *Mol. Neurobiol.*, 2016, **53**, 5356–5366.
- 185 H. M. Zhao, Y. Wang, X. Y. Huang, M. F. Huang, R. Xu, H. Y. Yue, B. G. Zhou, H. Y. Huang, Q. M. Sun and D. Y. Liu, *World J. Gastroenterol.*, 2016, **22**, 3175–3185.
- 186 H. Zhang, X. Zhou, X. Chen, Y. Lin, S. Qiu, Y. Zhao, Q. Tang, Y. Liang and X. Zhong, *Inflammation Res.*, 2019, **68**, 957–968.
- 187 E. V. Dang, J. Barbi, H. Y. Yang, D. Jinasena, H. Yu, Y. Zheng, Z. Bordman, J. Fu, Y. Kim, H. R. Yen, W. Luo, K. Zeller, L. Shimoda, S. L. Topalian, G. L. Semenza, C. V. Dang, D. M. Pardoll and F. Pan, *Cell*, 2011, **146**, 772–784.
- 188 W. Ren, J. Yin, J. Duan, G. Liu, B. Tan, G. Yang, G. Wu, F. W. Bazer, Y. Peng and Y. Yin, *Eur. J. Immunol.*, 2016, **46**, 291–299.
- 189 Y. J. Cao, Y. Xu, B. Liu, X. Zheng, J. Wu, Y. Zhang, X. S. Li, Y. Qi, Y. M. Sun, W. B. Wen, L. Hou and C. P. Wan, *Am. J. Chin. Med.*, 2019, **47**, 423–437.
- 190 X. Zhou, H. Chen, F. Wei, Q. Zhao, Q. Su, J. Liang, M. Yin, X. Tian, Z. Liu, B. Yu, C. Bai, X. He and Z. Huang, *J. Immunol. Res.*, 2019, 2431617, DOI: 10.1155/2019/2431617.
- 191 Y. Dang, S. Ling, J. Ma, R. Ni and J. W. Xu, *J. Evidence-Based Complementary Altern. Med.*, 2015, 920431, DOI: 10.1155/2015/920431.
- 192 T. Hirai, K. Nomura, R. Ikai, K. ichi Nakashima and M. Inoue, *Biomed. Pharmacother.*, 2019, **109**, 503–510.
- 193 G. D. Kang and D. H. Kim, *J. Ethnopharmacol.*, 2016, **189**, 175–185.
- 194 C. B. Wei, K. Tao, R. Jiang, L. Di Zhou, Q. H. Zhang and C. S. Yuan, *Int. Immunopharmacol.*, 2017, **53**, 73–82.
- 195 S. J. Yu, R. Jiang, Y. Z. Mazzu, C. B. Wei, Z. L. Sun, Y. Z. Zhang, L. Di Zhou and Q. H. Zhang, *Am. J. Chin. Med.*, 2016, **44**, 1221–1236.
- 196 K. Li, Y. Chen, R. Jiang, D. Chen, H. Wang, W. Xiong, D. Li, Z. Liu, X. Li, J. Li and K. Yuan, *Mol. Med. Rep.*, 2017, **16**, 1207–1215.
- 197 Z. Yan, Z. Xiaoyu, S. Zhixin, Q. Di, D. Xinyu, X. Jing, H. Jing, D. Wang, Z. Xi, Z. Chunrong and W. Daoxin, *Sci. Rep.*, 2016, **6**, 1–11.
- 198 A. Ma, Y. Yang, Q. Wang, Y. Wang, J. Wen and Y. Zhang, *Mol. Med. Rep.*, 2017, **15**, 3615–3622.
- 199 X. Zhou, H. Chen, F. Wei, Q. Zhao, Q. Su, Y. Lei, M. Yin, X. Tian, Z. Liu, B. Yu, C. Bai, X. He and Z. Huang, *Int. J. Rheum. Dis.*, 2020, **23**, 74–83.
- 200 A. Ribas-Latre, J. M. Del Bas, L. Baselga-Escudero, E. Casanova, A. Arola-Arnal, M. J. Salvadó, C. Bladé and L. Arola, *J. Funct. Foods*, 2015, **13**, 336–344.
- 201 P. Ponikowski, A. A. Voors, S. D. Anker, H. Bueno, J. G. F. Cleland, A. J. S. Coats, V. Falk, J. R. González-



- Juanatey, V. P. Harjola, E. A. Jankowska, M. Jessup, C. Linde, P. Nihoyannopoulos, J. T. Parissis, B. Pieske, J. P. Riley, G. M. C. Rosano, L. M. Ruilope, F. Ruschitzka, F. H. Rutten, P. Van Der Meer, H. S. Sisakian, E. Isayev, A. Kurlianskaya, W. Mullens, M. Tokmakova, P. Agathangelou, V. Melenovsky, H. Wiggers, M. Hassanein, T. Uetoe, J. Lommi, E. S. Kostovska, Y. Juilliere, A. Aladashvili, A. Luchner, C. Chrysoshoou, N. Nyolczas, G. Thorgeirsson, J. M. Weinstein, A. Di Lenarda, N. Aidargaliyeva, G. Bajraktari, M. Beishenkulov, G. Kamzola, T. Abdel-Massih, J. Celutkienė, S. Noppe, A. Cassar, E. Vataman, S. AbirKhalil, P. van Pol, R. Mo, E. Straburzynska-Migaj, C. Fonseca, O. Chioncel, E. Shlyakhto, M. Zavatta, P. Otasevic, E. Goncalvesova, M. Lainscak, B. D. Molina, M. Schaufelberger, T. Suter, M. B. Yilmaz, L. Voronkov and C. Davies, *Eur. Heart J.*, 2016, **37**, 2129–2200.
- 202 Z. D. Goldberger and A. L. Goldberger, *Am. J. Cardiol.*, 2012, **109**, 1818–1821.
- 203 E. Iisalo, *Clin. Pharmacokinet.*, 1977, **2**, 1–16.
- 204 M. Dal Prà, D. Carta, G. Szabadkai, M. Suman, Y. Frión-Herrera, N. Paccagnella, G. Castellani, S. De Martin and M. G. Ferlin, *Bioorg. Med. Chem.*, 2018, **26**, 1686–1704.
- 205 H. Toyama, M. Nakamura, M. Nakamura, Y. Matsumoto, M. Nakagomi and Y. Hashimoto, *Bioorg. Med. Chem.*, 2014, **22**, 1948–1959.
- 206 B. P. Fauber and S. Magnuson, *J. Med. Chem.*, 2014, **57**, 5871–5892.

