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DISSERTATION / DOCTORAL THESIS

Titel der Dissertation /Title of the Doctoral Thesis

„ Functional and mechanistic characterization of novel selective modulators of retinoid X receptors “

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"*All my life through, the new sights of nature made me rejoice like a child*."

— Marie Curie

"*Every great and deep difficulty bears in itself its own solution. It forces us to change our thinking in order to find it.*"
-Niels Bohr

ABSTRACT

Retinoid X receptors (RXRs) are nuclear receptors displaying a variety of biological functions. They regulate physiological and developmental processes by acting as signal integrators to control the transcription of target genes.

Ligands of RXR are called rexinoids and they modulate nuclear receptor function either as agonist or as antagonist. This modulation is due to the ligand-receptor complex formation, the ability of RXRs to heterodimerize with other nuclear receptors or to form homodimeric receptor complexes, as well as the ability of the formed ligand-receptor complex to differentially recruit diverse co-activators and co-repressors to the respective target gene promoters. Some small molecule ligands exert anticancer activity and have a potential for the treatment of metabolic diseases but their usage is limited due to toxicity and unfavorable tissue and receptor subtype selectivity that cause various side effects.

This work describes the identification of selective small-molecule ligands for RXR that are structurally based on the partial RXR agonist and natural occurring rexinoid Honokiol. Honokiol is a biphenylic neolignan initially isolated from the bark of *Magnolia officinalis*. It has been shown that Honokiol promotes RXRα-, as well as PPARγ-dependent luciferase gene expression in human embryonic kidney cells (HEK293). By using receptor specific luciferase-based testing models, 5 asymmetric honokiol derivatives were identified that selectively transactivate RXRα-dependent but not PPARγ-, LXRα-, LXRβ-, and FXR-dependent luciferase gene expression in a dose-dependent manner. The calculated EC_{50} values for RXRα activation indicate a high potency of action of these derivatives and range from 170 to 850 nM in comparison to Honokiol (EC_{50}: 1038 nM). Selected candidates were further functionally characterized and their impact on macrophage cholesterol efflux in differentiated THP-1 cells as well as the impact on lipid accumulation in liver cells (HepG2 cells) was investigated.

Additionally and in line with its partial agonism, a microarray assay for real-time co-regulator-nuclear receptor interactions revealed that we have identified compounds that recruit certain co regulators with a higher selectivity than full agonists (e.g. Bexarotene, 9-cis retinoic acid) to the RXRα ligand-binding domain *in vitro.*

In summary, this work describes the functional and mechanistic characterization of novel selective modulators of retinoid X receptors.

ZUSAMMENFASSUNG

Retinoid-X-Rezeptoren (RXRs) sind Kernrezeptoren, die eine Vielzahl von biologischen Funktionen aufweisen. Sie regulieren physiologische Prozesse und spielen eine große Rolle in der Entwicklung von Organsimen, indem sie als Signalintegratoren wirken um die Transkription von Zielgenen zu kontrollieren.

Liganden von Retinoid-X-Rezeptoren werden als Rexinoide bezeichnet. Am Rezeptor gebunden, agieren Sie entweder als Agonist oder Antagonist. Liganden Bindung führt zur Bildung von Liganden-Rezeptor-Komplexen, die entweder Homodimere oder Heterodimer sind, bestehend aus RXR und einem Partnerkernrezeptor. Die Rezeptoraktivierung oder -hemmung hängt von verschiedenen Cofaktoren ab, die Liganden-abhängig oder –unabhängig mit dem Rezeptor assoziiert sind und zu den jeweiligen Promotorregionen der Zielgene rekrutieren werden.

RXR Liganden zeigen Aktivität gegen bestimmte Krebsarten und haben ein Potenzial zur Behandlung von Stoffwechselerkrankungen. Die Möglichkeiten zur Anwendung dieser RXR Liganden sind jedoch beschränkt durch einen hohen Toxizitätsgrad und, aufgrund des breiten Wirkungsspektrums in verschiedenen Geweben, das Auftreten Nebenwirkungen. Diese Arbeit beschreibt die Identifizierung von neuen RXR Liganden mit selektiveren Eigenschaften als volle Agonisten, die strukturell auf dem partiellen RXR-Agonisten und dem natürlich vorkommenden Rexinoid Honokiol basieren.

Honokiol ist ein Biphenyl Neolignan, welches aus der Rinde von *Magnolia officinalis* isoliert wurde. Es wurde gezeigt, dass Honokiol die RXRα-, sowie PPARγ-abhängige Luciferase-Genexpression in humanen embryonalen Nierenzellen (HEK293) erhöht. In einem zellbasierten Luciferase-Reportergensystem wurden asymmetrische Honokiolderivate identifiziert, die selektiv die RXRα-abhängige, aber nicht PPARγ-, LXRα-, LXRβ- und FXR-abhängige Luciferase-Genexpression in einer dosisabhängigen Weise transaktivieren. Die berechneten EC_{50}-Werte für die RXRα-Aktivierung zeigen eine hohe Potenz (170- 850 nM) im Vergleich zu Honokiol (EC_{50}: 1038 nM).

Die Wirkung dieser Derivate auf den Prozess des Cholesterin-Efflux in Makrophagen sowie die Auswirkung auf die Lipidakkumulation in Leberzellen (HepG2-Zellen) wurde genauer untersucht. Ein Mikroarray-Assay wurde durchgeführt, um die Interaktion von Coregulator-Kernrezeptor-Wechselwirkungen zu identifizieren. Zusammenfassend beschreibt diese Arbeit die funktionale und mechanistische Charakterisierung von neuartigen selektiven Modulatoren des Retinoid-X-Rezeptors.

CONTENTS

Introduction

1. Nuclear receptors

Nuclear receptors are a super-family of highly conserved transcription factors that activate receptor-specific and tissue-specific sets of target genes. They are also called hormone receptors because they were historically discovered in endocrinology and often bind small lipophilic ligands like hormones. Modulation of nuclear receptor action has been described in almost every aspect of development, cell-differentiation, metabolism, cell death and even cancer and other human diseases.[1]

Because they regulate a diverse set of biological function with key roles in metabolic and hormonal homeostasis virtually all nuclear receptors with identified ligands are well characterized targets for drug development to treat various diseases including obesity, diabetes, atherosclerosis, inflammation and endocrine disorders.[2]

1.1. The nuclear receptor superfamily

In human 48 genes encode for nuclear receptors and the superfamily can be sub-divided into 7 subfamilies including the receptors for lipophilic vitamins, cholesterol metabolites, thyroid hormones, and steroid hormones.[3,4]

A large number of nuclear receptors are classified as orphan receptors because their ligands are not discovered yet or are not characterized well.[5-7]

However, nuclear receptors with known, well-described ligands can be categorized as follows: Classic steroid receptors are nuclear receptors for steroid hormones and function as homodimers. The androgen receptor (AR), estrogen receptor (ER), glucocorticoid receptor (GR), the mineralocorticoid receptor (MR) and the progesterone receptor (PR) all belong to this category.[8]

Non-steroidal receptors function as hetero- or as homodimers to regulate downstream gene expression. The central hetero-or homo- dimerization partner in this category of nuclear receptors is the retinoid X receptor (RXR). The classic RXR heterodimer receptors play important roles in metabolic homeostasis, development and immunity.[9] Another category of nuclear receptors is the xenobiotic receptors that mainly has a role in the protection of toxic substances.[10,11] Table 1 summarizes the main categories.

Category	Name	Subtypes/ Isoforms	Natural Ligand	Therapeutic Relevance	Example of therapeutic Ligands
Classic RXR Heterodimer Receptors	Retinoid X Receptor	RXRα RXRβ RXRγ	All-*trans* retinoic acid	Subcutaneous T-cell lymphoma (Skin Cancer)	Bexarotene LG1069 (Targretin)
I. Permissive Nuclear Receptors	Retinoic Acid Receptor	RARα RARβ RARγ	Retinoic acid	Acne	Isotretinoin (Accutane)
	Liver X Receptor	LXRα LXRβ	24,25-Epoxycholesterol, 24-Hydroxycholesterol	Atherosclerosis (considered), Role in Lipid and Cholesterol synthesis	-
	Peroxisome proliferator-activated Receptors	PPARα PPARβ/δ PPARγ	Fatty acids, Eicosanoids	Dyslipidemia (PPARα), Diabetes and Insulin sensitization (PPARγ)	Fenofibrate (Tricor; PPARα), Thiazolidenediones(Avandia, Actos;PPARγ)
	Farnesoid Receptor	FXR	Chenodeoxycholic acid	Role in cholesterol maintenance, Cholestasis, Protects hepatocytes from bile toxicity	Obechitolic acid (Ocaliva)
II. Non Permissive Nuclear receptors	Vitamin D Receptor	VDR	Vitamin D, Bile acids	Hypocalcemia, Osteoporosis, Renal failure	Calcitriol (Rocaltrol)
	Thyroid hormone Receptor	TRα TRβ	Thyroid hormone	Thyroid deficiency	Levothyroixine (Synthroid)
Classic Steroid Receptors	Estrogen Receptor	ERα ERβ	Estrogens, Estradiol	Breast Cancer, Osteoporosis prevention, Menopausal symptoms	Tamoxifen, Raloxifene (Evista), Genestein, Diethylstilbestrol, Equine estrogenes (Premarin)
	Glucocorticoid Receptor	GR	Glucocorticoids, Cortisol	Asthma, Arthritis, Rhinitis, Cancer, Immune suppressant	Prednisone, Dexamethasone
	Mineralocorticoid Receptor	MR	Aldosterone, Deoxycorticosterone	Hypertension, Heart failure	Spironolactone (Aldactone), Epleronone (Inspra)

Category	Name	Subtypes/ Isoforms	Natural Ligand	Therapeutic Relevance	Example of therapeutic Ligands
	Progesterone Receptor	PR	Progestins, Progesterone	Abortifacient, Menstrual control	RU486 (Mifepristone)
	Androgen Receptor	AR	Androgens, Testosterone	Prostate cancer	Flutamide, Bicalutamide (Casodex)
Xenobiotic Receptors	Constitutive Androstane Receptor	PXR	Xenobiotics	Protection from toxic metabolites	St. John´s wort, Rifampicin
	Pregnane Receptor	CAR	Xenobiotics	Protection from toxic metabolites	Phenobarbitol
Orphan Receptors	Estrogen-related receptors	ERRα ERRβ ERRγ	unknown	Muscle fatty metabolism (EERα)	Tamoxifen, Diethylstilbestrol (EERγ)
	RAR-related receptors	RORα RORβ RORγ	Cholesterol, Cholesterol sulfate	Bone maintenance, circadian rhythm, cerebellum development	-
	Human nuclear receptors 4	HNF4α HNF4γ	Palmitic acid	Role in diabetes	-
	Reverse erbA	Rev-erbAα Rev-erbAβ	unknown	Circadian rhythm	-
	Testis receptors	TR2 TR4	unknown	unknown	-
	Tailless-like	TLX	unknown	Role in Neuronal development	-
	Photoreceptor-specific nuclear receptor	PNR	unknown	Role in Photoreceptor differentiation	-
	Chicken ovalbumin upstream promoter-transcription factor	COUP-TFI COUP-TFII COUP-TFIII	unknown	Role in neuronal development, vascular development	-
	NGF-induced factor B	NUR77	unknown	Role in thyomcyte apoptosis	-

Category	Name	Subtypes/ Isoforms	Natural Ligand	Therapeutic Relevance	Example of therapeutic Ligands
	Nur-related factor 1	NURR1	unknown	Role in dopaminergic neuron development	-
	Neuron-derived orphan receptor 1	NOR1	unknown	unknown	-
	Steroidogenic factor 1	SF1	Phospolipids	Role in sexual development	-
	Liver receptor homologous protein 1	LRH1	Phospholipids	Role in lipid-homeostasis, Cell-cycle control	-
	Germ cell nuclear factor	GCNF	unknown	Role in vertebrate embryogenesis	-
NR-like, DBD-less recptors	DSS-AHC critica region on the chromosome gene 1	DAX1	unknown		-
	Short heterodimer partner	SHP	unknown		-

Table 1. The nuclear receptor superfamily, their natural ligands and therapeutic relevance. *(adopted and modified from Moore et al.[10])*

1.2. Nuclear receptor architecture

Conceptually, the ligand-dependent nuclear receptors bind as trans-acting proteins to cis-acting DNA sequences (and chromatin) in promoter regions to initiate transcription of their target genes upon ligand binding. Thus, they integrate endocrine signals to produce a cellular output. The receptors have a modular structure consisting of six domains (A to F) including the highly conserved DNA-binding domain (DBD) and the ligand-binding domain (LBD).[12]
The modular structure of nuclear receptors is highly conserved in the nuclear receptor superfamily.

From the N- to the C- terminus the functional domains are as follow (Figure 1):

- The A/B domain contains the ligand-independent activation function 1 (AF-1) that serves as interaction interface for co-activator (CoA) proteins containing a LXXR motive.

- The C-domain or DNA binding domain (DBD) is required for the interaction with specific DNA sequences (half site response elements) within the promoter region of target genes.
- The D region is a hinge that connects the DNA binding domain with the Ligand-binding domain (LBD) of the receptor.
- The E domain or Ligandbinding domain (LBD) contains the ligand-dependent activation function 2 (AF-2) with the ligand binding pocket important for the interaction with coregulatory proteins (corepressors, CoRs and co-activators, CoAs). Essentially this domain consists of 12 α-helices that form a hydrophobic pocket.[13-16]

The domains C to E form the dimerization interface necessary to form homodimers or heterodimers with the retinoid X receptor (RXR).
Binding of a ligand to the LBD causes a change of the receptor conformation that changes the affinity to certain coregulatory molecules that may stimulate transcription (co-activators) or repress transcription (corepressors). This recruitment leads to a remodellig of the chromatin and a modification of the transcriptional machinery.[17,18]
The structural and functional domains are shown in Figure 1.

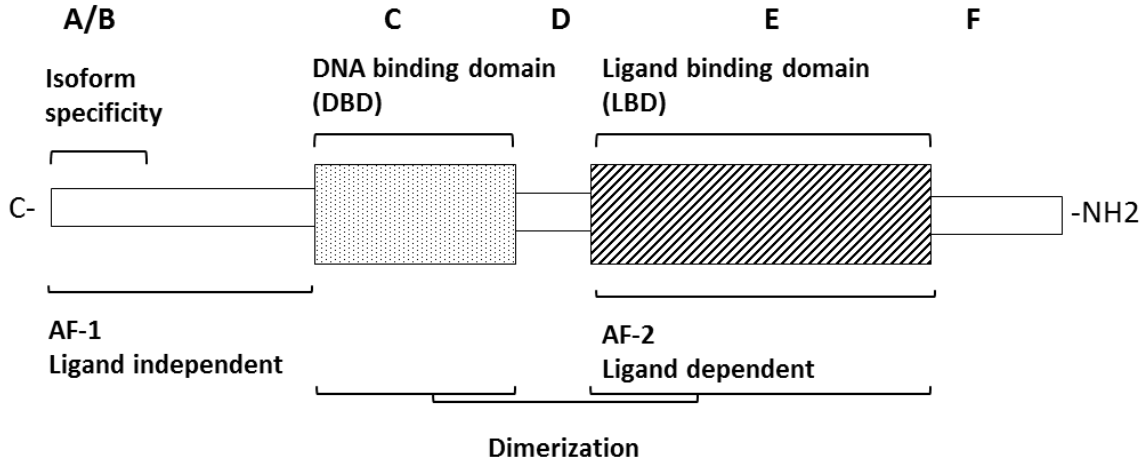


Figure 1. Nuclear receptor architecture with their functional domains.

1.3. Nuclear receptors as drug targets

Nuclear receptors have a history as drug targets and this has several reasons. They selectively bind drug like molecules and a single-receptor usually regulates a diverse set of biological functions that have key roles in development, homeostasis and many diseases such as obesity and diabetes,[19,20] cancer[21,22] and neurological disorders.[23]
Nuclear receptor ligands can be full agonists or antagonists and besides that they also bind partial agonists and antagonists that make them good candidates as drug targets. Partial (or mixed) ligands bind less efficient and with lower affinity to the receptor and thus induce a very unique conformational change that may lead to the recruitment of specific sets of coactivators. These partial ligands are selective modulators and are referred to as selective NR modulators (SNuRMs). Selective liver X receptor modulators (SeLRMs), selective peroxisome proliferator-activated receptor modulators (SPARMs) are examples of this kind of mixed ligands. [24-26] It is believed that such selective compounds act in a more specific way as drug targets overcoming certain side effects.[27] The principle paradigm behind the action of such SNuRMs is that they activate transcription of only selected target genes in a tissue specific manner to avoid side effects.
There are two critical points that have to be considered in the development of selective nuclear receptor modulators. First, one needs to identify the coregulators that are associated with a certain nuclear receptor and the other critical point is that the selective activity on certain promoter regions must be given and predictable. The first critical point in identifying SNuRMs is that the cofactors associated to the certain receptor in a certain tissue during development must be known. There has been some effort done to identify coregulators and to determine their tissue distribution and expression profiles.[28] In general there are 50-100 different cofactors associated with different nuclear receptors. Some cofactors bind nearly all nuclear receptors (e.g. steroid receptor co-activator SRC-1) while others are much more specific (e.g. peroxisome proliferator-activated receptor γ co-activator PGC-1). Some of the coregulators are tissue specific and/or transiently expressed during development.[29,30]
Another critical point for the development of SNuRMs is to find ligands that selectively activate the transcription of specific target genes or act in a tissue specific way to overcome unwanted secondary actions.[31,32]

1.4. The retinoid X receptor (RXR)

Retinoid X Receptors belong to the steroid/thyroid hormone superfamily designated as NR2B1-3[33]. There are three different RXR-isoforms: RXRα, RXRβ and RXRγ that are all encoded by different genes and are differentially expressed in different tissues.
RXRα is mainly expressed in epidermis, intestine, kidney, liver and macrophages. RXRβ is expressed ubiquitously and RXRγ is mainly expressed in the muscle and parts of the central nervous system.[34,35]

1.4.1. Hetero- and homo-dimerization

The Retinoid X Receptor can act as homodimer by the dimerization with itself or may form heterodimers together with other nuclear receptors such as the retinoic acid receptors RARα/β, the liver X receptors LXRα/β, the peroxisome proliferators-activated receptors PPARα/δ/γ, the farnesoid receptor FXR, the thyroid hormone receptor TRα/β and the vitamin D receptor VDR. There is no obvious preference for a certain RXR isoform for most of the heterodimerization partners.[5]
Furthermore, RXR may form tetramers that are transcriptionally active either in the presence or absence of a non-activating ligand (a *trans*-isomer of 9-cis retinoic acid).[36]
RXR thus builds a unique category in the nuclear receptor superfamily with an important role in a very diverse set of biological functions and nuclear receptor-mediated signalling pathways (Table 1).

1.4.2. RXR architecture and transactivation

Retinoid X Receptors have a modular structure to provide a surface for integrating intracellular signals to form a certain output. This concept is reflected in the architecture of the nuclear receptors that allows essentially receptor-protein, receptor-DNA and receptor-chromatin interactions induced by ligand binding via allosteric mechanism. The architecture of nuclear receptors is highly conserved and is sketched in Figure 1.
The main domains are the DNA-binding domain (DBD) and the ligand-binding domain (LBD). The LBD can be seen as an input-output processor. A certain input (ligand binding or modifications such as phosphorylation of certain amino acid residues) lead to an

allosteric change of the receptor surface that are actual docking sites for the transcription machinery, chromatin remodelling complexes that represent the output of the processor. Furthermore there are coregulators involved in the regulation of this communication in the cellular context and the ratio of corepressors (CoRs) and co-activators (CoAs) determine if a certain ligand acts as agonist or antagonist in a cell-specific or tissue specific manner.[32,37]

1.4.2.1. The RXR DNA-binding domain

The DNA-binding domain of nuclear receptors is highly conserved in the nuclear receptor superfamily and comprises two zinc finger motives and two α-helices. The human RXRα DBD (aa130-209) contains the zinc-finger domains at position aa135-155 and 171-190 each complexing a zinc (II) ion through four cysteines. The domain recognizes selectively DNA regulatory elements that are composed of certain tandem repeats that are in the case of RXR heterodimers sites containing the sequence AGGTCA arranged in a direct repeat configuration. Characteristic is that inter-half spacings are 1-5 bp long. These response elements are known as DR1-DR5, respectively. The direct repeat (DR) half sites separated by 1-5 base-paires to which the different RXR isoforms bind as homo- or heterodimers with their respective partner nuclear receptors are summarized in Table 2. Other members of the nuclear receptor superfamily recognize response elements containing inverted or everted repeats.[38,39]
In direct repeats, RXR generally occupies the 5'-element. RXR can also form RXR-RXR homodimers that bind to DR1.[40]

RXRE Direct Repeats

5´-AGGTCA(n)xAGGTCA-3´

3´-TCCAGT(n)xTCCAGT-5´

RXR Response Element	**RXR 3´Binding Partner**
DR-1	RARα, β, γ PPARα, β/δ, γ HNF4α, β COUP-TFI,II RXRα, β, γ
DR-2	RARα, β, γ PPARα, β/δ, γ RXRα, β, γ
DR-3	VDR
DR-4	TR LXRα, β RARα, β, γ
DR-5	RARα, β, γ TR3/Nur77/NGFI-B

Table 2. RXR-DNA-binding domain-DNA interaction. The half sites (5´-AGGTCA-3´) that are recognized by the different heterodimers or homodimers are listed. *They are all direct repeats with 1-5 bp spacings. N=undefined nucleotide, X= 1-5 base pairs. Sequences that are degenerated do also exist. (adopted from Dawson et al. 2012.[34])*

1.4.2.2. The RXR ligand-binding domain

The RXR ligand binding domain of all nuclear receptors consists of a single protein domain. Structural studies have brought more insights how ligand binding within this single protein domain leads to the recruitment of certain cofactors. The ligand binding domain has a certain tertiary structure that typically consists of α-helices that are arranged in three layers. This layers form a “sandwich”-like structure to from a hydrophobic lig-

and binding pocket for the interaction of the characteristic hydrophobic nuclear receptor ligands.[41] The globular structure of the ligand binding domain consists of 11 α-helices (helix 1-11) whereas helix 12 (H12) works as a mobile arm to the ligand binding pocket. The position of H12 is critical for interaction of cofactors that contain a so called NR box or LXXLL motif (L: leucine, X: any amino acid). The overall co-activator surface of the ligand binding domain is formed by helices 3, 5 and 12 (H3, H5 and H12). [42]
The NR box contains a leucine that is a hydrophobic amino acid that allows the interaction with the hydrophobic LBD while a highly conserved lysine (H3) and a highly conserved glutamic acid (H12) forms a clamp by forming hydrogen bonds of the peptide backbone of the flanking NR box.[43] The allosteric changes of the RXR ligand binding domain upon ligand binding can be illustrated by comparing the RXRα *apo* and RXRα *holo* structure. Figure 2 shows a comparison of the two different structures and illustrates that the biggest conformational changes undergoes helix 12 (H12). The *holo* RXRα LBD seals the ligand binding pocket whereas in the *apo* RXRα LBD helix 12 points away from the ligand binding pocket. Helix 12 contains amino acid residues important for the recruitment of CoAs and for transcriptional activation.[44,45]

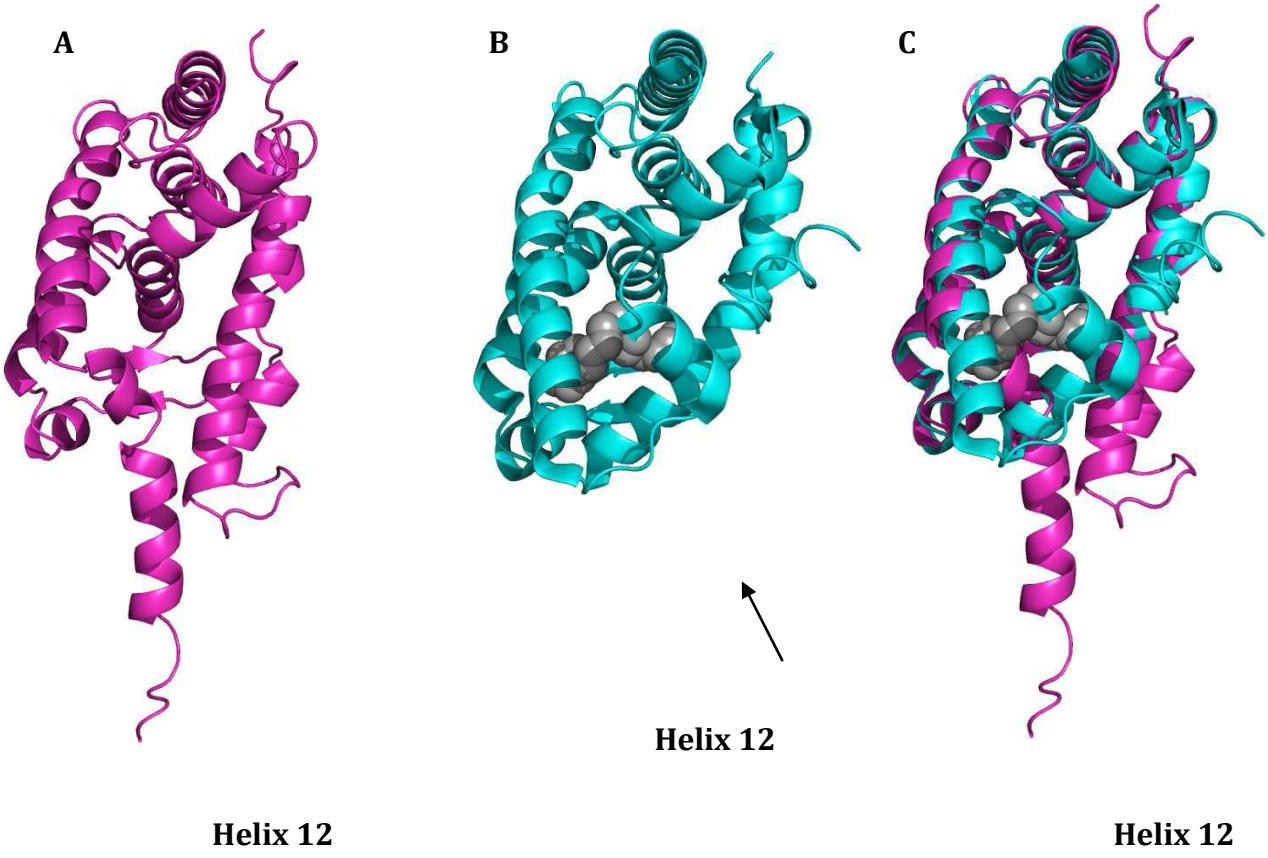


Figure 2. The RXRα apo and RXRα holo ligand-binding domain (LBD) *(**A**) The crystal structure of the unliganded RXRα apo LBD (PDB entry 1LBD[45]) (**B**) The co-crystal structure with a ligand (Docosahexaenoic acid, DHA) (PDB entry 1MV9[46]) (**C**) Overly of the RXRα apo and RXRα holo ligand-binding domain. Graphics adopted from Huber K., University of Innsbruck. [47]*

1.4.2.3. Permissive and non-permissive nuclear receptors

There are two main classes of RXR heterodimers:

- Permissive nuclear receptor heterodimers: LXRs, PPARs, FXR, PXR and CAR
- Non-permissive nuclear receptor heterodimers:VDR, TRs[9,48]

Permissive nuclear receptor heterodimers can be activated by ligands of RXR or ligands for its permissive heterodimerization partner. They can be furthermore activated by both ligands or in a cooperative, synergistic way. RXR is an active transcriptional partner in permissive heterodimers. Non-permissive nuclear receptor heterodimers can

only be activated by the heterodimerization partner but not via activation of RXR. In this case RXR is a silent partner (so called subordination).[34,49]

The RXR-RAR heterodimer is a special case since it is conditionally permissive. Ligand binding of RAR activates transcription and allows an RXR ligand to enhance transcription thus becoming permissive while being non-permissive in the absence of a RAR agonist. The RXR-RAR heterodimer has been termed "conditionally" permissive since it has been shown in a few cases that the heterodimer can be activated in the absence of a RAR ligand.[50]

1.4.3. RXR isotypes/isoforms and their tissue distribution

The human RXR genes of the three different RXR isoforms are located on chromosome 9, 6 and 1 (bands q34.3, 21.3 and q22-q23, respectively).[51]

The three different RXR-isoforms: RXRα, RXRβ and RXRγ are encoded by different genes that contain 10 exons and are differentially expressed in different tissues.[52]

The RXR promoter is considered to be a housekeeping gene promoter because of its high G+C content in the 5´ untranslated region. There have been nuclear receptor DR-sites (DR-0 1, 3 and 4) in the RXR promoter region identified indicating a possible feedback regulation.[53,54]

The expression levels also do differ during different developmental stages. The isoform RXRα has high expression levels in liver, lung, muscle, kidney, epidermis, intestine and skin and in macrophages. RXRβ is expressed ubiquitously and RXRγ is mainly expressed in the muscle and parts of the central nervous system.[55-57]

There is a certain polymorphism in the human population concerning different variants of RXR isoforms. In a study published in 2009, a RXRβ c.52C<T polymorphism was linked to metabolic dysfunctions (higher body mass and gallstone risk), a RXRγ (p.Gly14Ser) polymorphism was reported to be linked with hyperlipidaemia, and other RXR variants have been linked to high triglyceride and free fatty acid levels in type 2 diabetes.[58]

1.5. Retinoid X receptor ligands (rexinoids)

Ligands of the Retinoid X receptors are called rexinoid whereas ligands of the retinoid acid receptor are called retinoids.

Structural ligands for RXR bind to the ligand binding pocket within the ligand-binding domain of the receptor and alter its conformation. This induces a communication with the core of the activation function-2 (AF-2) and allows the formation of a binding grove for co-activators (CoAs).[34]

In the following RXR ligands will be discussed that are endogenous (section 1.5.1.), that are present in dietry (section 1.5.2.), synthetic (section 1.5.3.) or are natural products (section 1.5.4.).

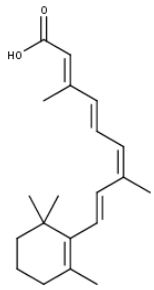
1.5.1. Endogenous RXR ligands

Endogenous rexinoids and retinoids derive from the vitamin A (retinol) metabolism. Retinol is absorbed via the blood stream and is taken up in the cell by retinol-binding protein. Dehydrogenases convert retinol to retinal that is finally converted from retinal to retinoic acid (RA). RA is bound by cellular retinoic-acid binding proteins (CRABP) that transport RA to the nucleus in order to act on the respective nuclear receptors.[59]

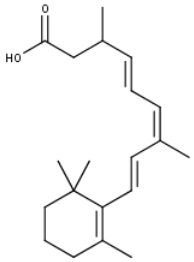
9-cis retinoic acid was considered to be an endogenous ligand for RXR but this is controversial, because under normal conditions this vitamin A- metabolite is not detectable in serum whereas all-*trans*-retinoic acid and 13-retinoic acid can be detected. Only when excess all-*trans* retinoic acid is administered in animal experiments 9-cis retinoic acid is detectable in serum because of the rapid isomerization from all-*trans* retinoic acid. [60]

However, 9-cis retinoic acid was initially described to be present in the liver and in kidney in higher concentrations compared to all-*trans* retinoic acid.[61]

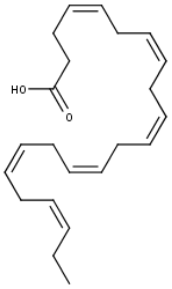
There are other *in vivo* vitamin A metabolites described that are endogenous ligands for RXR such as retinal or dehydro-retinoids. Dehydro-retinoids derive from all-trans-retinol to produce all-*trans*-13, 14-dihydroretinol that is finally converted to all-*trans*-13, 14-dihydroretinoic acid that acts on RXR/RAR heterodimers.[62] 9-cis-13, 14-dihydroretinoic acid is the first endogenous ligand for RXR described with a physiological relevance in mammals, although the metabolic pathway is unknown.[63]



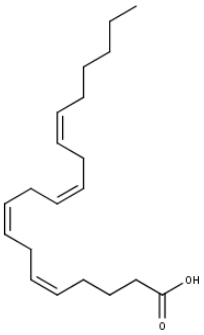
9-cis-retinoic acid



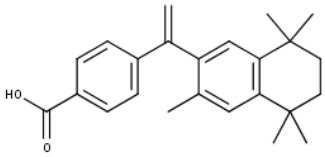
9-cis-13,14-dihydroretinoic acid



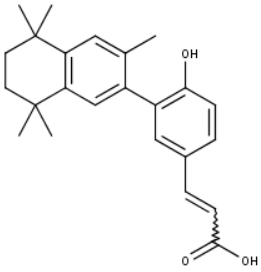
DHA



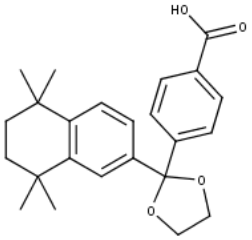
Arachidonic acid



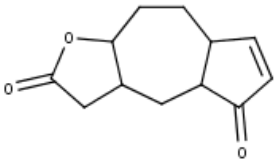
Bexarotene



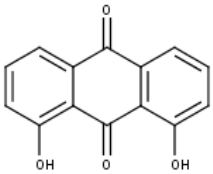
CD3254



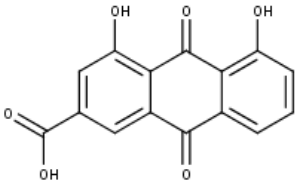
SR11237



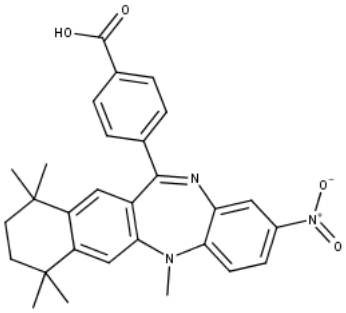
Bigelovin



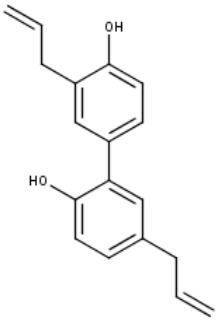
Danthron



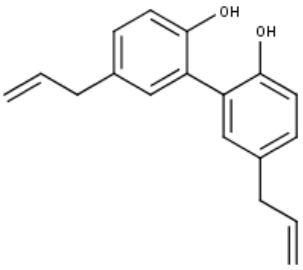
Rhein



HX531



Honokiol



Magnolol

Figure 3. Structures of selected retinoid X receptor ligands.

1.5.2. RXR ligands from dietary

Dietary- derived ligands for RXR are unsaturated fatty acids such as docosahexaenoic acid (DHA, 22:6), arachidonic acid (20:4) and oleic acid (18:1) and also other eicosanoids were found to activate RXR (Figure 3). These fatty acids are not selective for RXR but rather activate also other nuclear receptors. [32]

Common to these dietary ligands is that they all share a flexible skeleton that fits to the L-shaped ligand-binding domain of RXR.

The promiscuity of the nuclear receptor RXR to bind different metabolite dietary highlights the role of RXR as central sensor of the metabolism and its role in with other nuclear receptors such as the PPARs, LXR or FXR to maintain lipid-, bile acid- and glucose homeostasis in the body.[9]

1.5.3. Synthetic RXR ligands

A series of RXR ligands have been developed synthetically. One of them is the pan-RXR full agonist bexarotene (Targretin™, LGD109) that is used to treat subcutaneous T-cell lymphomas.[64,65] The highly conserved ligand-binding domain (LBD) of RXR makes it rather difficult to develop isoform-selective RXR ligands Furthermore, due to the existence of permissive heterodimers; synthetic RXR agonists may activate different nuclear receptor heterodimers and thus possess various pharmacological effects. This may lead to various side effects as seen in the case of Bexarotene. Observed side effects caused by bexarotene are high plasma triglyceride levels, hepatomegaly (via activation of LXR) and suppression of the thyroid hormone axis (via activation of TR).[66] In contrast, a heterodimer-selective ligand has been synthesized, LG101506, that activate PPARα and PPARγ but do not suppress thyroid hormone signaling and is used as insulin sensitizer. Other selective pan RXR-agonists are CD3254 and SR11237.

Examples of synthetic pan-antagonists are UVI3003 and the dibenzoazepine HX531 (Figure 3).[67,68]

1.5.4. Natural products and derivatives as rexinoids

Natural product derived rexinoids represent a very diverse set of molecules that bind the nuclear receptor RXR. The fact that such dissimilar structures are able to act as functional rexinoids reflects the conformational adaptability of the RXR ligand binding pocket.

The diterpenes hydrophene acid and methoprene acid have been shown to transactivate RXR in a cell-based transactivation model in Schneider cells. Methoprene acid is a metabolite of microorganisms that metabolize the pesticide methoprene used for mosquito control. Methoprene itself was designed structurally related to the juvenile hormone of insects (JHIII) and blocks metamorphosis of these insects. It has been considered that methoprene is safe although its action on RXR possibly affects retinoic acid- signalling during development. [69]

The natural product phytanic acid has been shown to transactivate RXR and also has been shown to bind PPARα. Phytanic acid is a metabolite of phytol that originates from chlorophyll metabolism and can be taken up from the diet. [70,71]

Naturally occurring RXR antagonists are β-apocarotenoids (cleavage products from β-carotene) such as β-apo-14´-carotenal that antagonizes RXR and PPAR activation. B-apo-13-caroteneone is a highly potent RXRα antagonist. It has been shown that this natural compound is able to induce RXR-tetramerization that silences this nuclear receptor.[72,73]

The sesquiterpene lactone Bigelovin isolated from the flowers of the plant *Inulla hupehensis* acts as an antagonist of the LXR/RXR heterodimer and on the other hand enhances PPARγ/RXR transactivation. This plant is used in traditional Chinese medicine and it was shown that this natural rexinoid inhibits cell growth of several cancer cell lines. [74]

The napthoquinones danthron and rhein are natural rexinoids isolated form another plant used in traditional medicine called rhubarb (Dahuang) that derives from the plant *Rheum plamatum*. Both rexinoids are specific RXRα antagonists.[75]

The neolignans honokiol and magnolol are representatives of another naturally occurring compound class that act as rexinoids. Neolignans in general are built up of two C6C3 units (two propylbenzenes) where the two propylbenzenes are linked at any carbon except the β-carbon of the propyl side chain.

These natural products derive from the bark of *Magnolia obovate* or *Magnolia officinalis* or other Magnolia species that is used in traditional Japanese medicine (Kampo prescription, Hou Po).[76,77]
The structures of selected rexinoids are illustrated in Figure 3.

1.5.4.1. Honokiol and magnolol and their pharmacology

Honokiol and magnolol are biphenylic neolignans and thus belong to the polyphenols. According to IUPAC honokiol is named 2-(4-hydroxy-3-prop-2-enyl-phenyl)-4-prop-2-enylphenol an magnolol 4-allyl-2-(5-allyl-2-hydroxy-phenyl) phenol.
Honokiol, as well as magnolol, have been initially isolated from the bark of *Magnolia officinalis* but can be found in various other Magnolia species.[78] Both molecules have two hydroxyl groups as phenyl side chains and are isomers as shown in Figure 3. Honokiol is a highly pleiotropic compound and possesses various pharmacological effects and is thus involved in a lot of different cellular pathways. The compound has main actions in the cardiovascular system, the central nervous system and the gastrointestinal system and has been shown to have anti-tumorigenic, anti-inflammatory, and antioxidant effects.[79,80] In cell-based luciferase gene transactivation models it was found that honokiol activates RXRα with higher potency compared to DHA and phytanic acid but was less potent in comparison to 9-cis retinoic acid.[81] In transactivation models shown by *Kotani et al 2010* honokiol does not activate RARα and β/δ and only weakly PPARγ. In cells it activates LXR/RXR heterodimers and enhances mRNA levels of the LXR/RXR target genes ABCA1 and ABCG1. Honokiol furthermore activated cholesterol efflux in peritoneal macrophages together with the endogenous LXR ligand (22R)-hydroxycholesterol in a synergistic way. Taken together, in the cellular context honokiol acts via the modulation of the LXR/RXR heterodimer.[82] Furthermore, honokiol has been described as very weak partial PPARγ agonist with non-adipogenic properties. In contrast, magnolol is a more potent PPARγ agonist in comparison to honokiol.[83] The neuro-modulatory effects of Magnolia bark extract are partly due to the action of honokiol (and magnolol) on the $GABA_A$ receptor. It is believed that it acts similar to benzodiazepines but with higher subunit selectivity.[84, 85] In another study it has been shown that honokiol affects the synthesis of GABA itself. [86]

Additionally, honokiol and magnolol are acting on cannabinoid receptors (cannabinoid receptors 1 and 2, CBR1 and CBR2). Magnolol is a partial CBR2 agonist, whereas honokiol is a CBR2 antagonist and a full CBR1 agonist. [87] Due to their properties to act on the $GABA_A$ and CB receptors honokiol and magnolol have been used in previous studies as lead structure for the design of honokiol derivatives with more selective and more effective properties for these receptors. [84,88-90]

1.5.4.2. Partial agonists of RXR

Partial agonists activate the receptor less efficient in comparison to full agonists and are sometimes considered to display both agonistic and antagonistic effects at the same time resulting in a decrease of overall efficacy.[91]

A study from *De Lera* and *Bourget 2007* addressed the question of how partial agonism may work in the RXR LBD. They compared the co-crystal structures with the full agonist CD3254 and with CD3254-derivatives (partial agonists) to reveal the amino acid side chains involved in the *agonist-partial agonist-to antagonist transition*.

They quantified the impact of ligand binding on the motion on helix 12 (H12) and found that the difference was a certain amino acid side chain in helix 11 (L436) that was affected. They showed that the interaction with this residue caused by partial agonists leads to a destabilisation of the *holo* conformation of H12 in solution that causes a decrease in the interaction with coactivators. Thus, full agonists in comparison to partial differ in their impact to stabilize *holo*-H12. [92] Therefore, what matters is how a ligand "senses" the intracellular co-regulator levels and thus may act as tissue selective modulator and may, depending on the context act as agonist or antagonist.[93]

It is hypothesized that partial RXR agonists (and antagonists) have a therapeutic potential since they display less side effects in comparison to full agonists.[94] This therapeutic potential is to date largely unexplored. In a study CBT-PMN, a partial selective RXR agonist, was investigated and compared to full agonists in mice in the context of type 2 diabetes and has been shown to produce less side effects in a mouse model of Type 2 diabetes in comparison to a full agonist (no increase of serum triglyceride levels, body weight and cholesterol levels in the blood).[95]

1.5.4.3. Rexinoids with heterodimer selectivity

Rexinoids that are selective for certain heterodimers are called selective RXR modulators (specific NR modulators, SNuRMs) and built an important class of ligands.
The selectivity and functional consequence of a ligand is determined by the conformational change induced in the LBD of the receptor. This conformational change lead to the recruitment or repulsion of different coregulators that interact with the LBD to regulate gene expression in a cell-type specific manner.
This class of compounds may act as agonists or partial agonists of selective permissive heterodimers or as synergists where they increase potency for the partner ligand or as selective antagonists where they can inhibit synergistic RXR agonist activity in permissive heterodimers.[96]
Table 3 summarizes some rexinoids that have been shown to display heterodimer selectivity.[97]

Selective RXR modulator	Heterodimer selectivity	References
LG100268	LXR/RXR agonist PPARγ/RXR agonist	98,99
LG101305	AR/RXR antagonist	100
LG101506	PPARγ/RXR agonist RXR/RXR partial agonist	101,102
L100754	PPARγ/RXR agonist RXR/RXR antagonist	103
PA024	LXR/RXR agonist PPAR/RXR agonist	104
HX630	PPARγ/RXR agonist	105

Table 3. Heterodimer selective RXR modulators.

1.6. Nuclear receptor coregulators

Coregulator proteins function as adaptors to bridge the communication of the nuclear receptor to the transcriptional machinery and mediate the regulation of transcription. The first nuclear receptor coregulators were identified in the 90ies in protein-protein interaction studies. They were classified as co-activators, such as SCR1and corepressor proteins, such as SMRT and NCoR. [106-108] A high number of coregulator proteins have been identified in the meantime and more than 350 of them are now listed in the "Nuclear Receptor Signalling Atlas" (www.NURSA.org).[109]

The presence or the absence of a certain ligand determines the coregulators recruited to the nuclear receptor and thus the repression or activation of transcription in a certain tissue. Most coactivators do this via a certain motif with the sequence LxxLL (NR box, see section 1.2.).[110] An analogous motif has been identified in corepressor proteins and is termed CoRNR box (LxxH/IxxI/L).[111,112] Which coregulator is recruited to the nuclear receptor is determined by the position of helix 12 (H12) in the ligand-binding domain of the respective nuclear receptor.[29] The initial proposed mechanism was that the agonist bound *holo*-receptor interacts with coactivators that possess a specific enzyme activity e.g. histone acetyl transferase activity (HAT activity) or possess a chromatin remodelling function to activate transcription. The non-ligand bound receptor (*apo*-receptor) interacts with corepressors, e.g. histone deactelyases (HDAC activity) to prevent transcription of target genes. However, some corepressors were described that are ligand-dependent. Furthermore, in some cases the *apo*-receptor can be modified by post-translational modifications to activate target gene transcription.[113-115]

The nuclear receptor coactivator 1 (NCOA1/SRC-1) is a coactivator possessing HAT activity and belongs to the p160/steroid receptor coactivator (SRC) family of coregulators.[116] Nuclear receptor coactivator 2 (NCOA2/SRC-2/TIF-2/GRIP-1/p160) and nuclear receptor coactivator 3 (NCOA3/SRC-3/RAC3/ACTR/pCIP/AIB-1) also belong to the SCR-family of coregulators that possess HAT activity.[117]

There are functional homologues of these proteins such as CREBP/CBP (CREB binding protein) and EP300/p300 that have HAT activity and have been shown to interact with SRC-1, RNA polymerase II and other transcription factors. [118]

The peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1) is a coactivator that modulates RNA processing and is involved in energy metabolism. [119]

Other coregulators are associated with the "mediator" complex such as MED1 (TRAP220/TRIP2/CRSP200) and function as coordinators of transcription factors and the basal transcriptional machinery. MED1 plays a crucial role in ligand-dependent chromatin remodelling and may be able to form loops within the DNA to recruit the machinery to enhancer sequences.[120]

TBP is a TATA-binding protein that serves as basal activator of transcription. Together with TAFs (TBP-associated factors), they form the TFIID complex that binds to the core promoter and helps positioning the polymerase and acts as a scaffold for the transcriptional complex.[121]

Main coregulators that interact with RXR are listed in Table 4.

Coregulator Name	**Activity**	**Specific**	**AF-2 dependent**	**References**
NCOA1	Co-activator	No	Yes	106,122
NCOA2	Co-activator	No	Yes	106,123,124
NCOA3	Co-activator	No	-	125,126
PGC1A	Co-activator	No	Yes	119
MED1	Co-activator	No	Yes	125,127
TBP	Co-activator	No	Yes	125,128
TAF4	Co-activator	No	Yes	125,128,129
TAF11	Co-activator	No	Yes	129
CREBP	Co-activator	No	Yes	130,131
EP300	Co-activator	No	Yes	130

Table 4. Main coregulators described for the retinoid X receptor.

2. Targeting retinoid X receptors in human disease

In the last years effort has been made to develop compounds targeting these nuclear signal integrators for therapeutic applications such as for the treatment of cancer, metabolic diseases or neurological disorders.[65,132]

A synthetic RXR ligand, the full RXR agonist Bexarotene (Targretin™, LGD109) is used to treat subcutaneous T-cell lymphomas and breast cancer but patients experienced severe side effects when the synthetic drug went through the clinical trials.[133,134]

Other rexinoids are being tested in preclinical settings for the treatment of atherosclerosis or insulin resistance.[135]

Overall, full retinoid X receptor agonists have been shown to induce hepatomegaly, to increase plasma triglyceride levels and to suppress the thyroid hormone axis. [136]

2.1. The role of nuclear receptors in macrophage biology

Macrophages play a major role in the innate immune system and have main roles in the first defence mechanisms against pathogens, the clearance of cellular debris, in tissue remodelling and also in the integration of lipid metabolism in different tissues.[137]

Furthermore macrophages play a critical role in diseases such as atherosclerosis, insulin resistance and neurologic disorders.[138-140]

The isoform RXRα is highly expressed in human blood monocytes and dendritic cells and RXRβ is expressed at lower levels in this cell types.

Furthermore blood monocytes express low levels of PPARα and moderate levels of PPARβ/δ, RARα and γ, the LXRs, VDR, Nur77 and Nurr1. Human dendritic cells express PPARβ/δ and PPARγ, RARα, the LXRs and VDR.[141]

Retinoid receptors play a major role in monocyte-macrophage differentiation and RXR target genes are critical for the maintenance of the homeostasis between self-renewal and differentiation. Furthermore, it has been shown that RXR controls differentiation and apoptosis in hematopoietic stem cells pointing out how important RXR function is for myeloid cell fates during the different stages of maturation.[141]

Heterodimerization with RAR and PPARγ controls self-renewal in the most primitive hematopoietic stem cells. RAR/RXR inactivation maintains self-renewal and PPARγ/RXR activation promotes differentiation of these cells. Selective RXR modulators can activate

different pathways that have been considered to treat certain pathological conditions such as myelodysplastic syndromes or atherosclerosis.[142]

In the context of immune function, the most important nuclear receptors are the PPARs and LXRs. However, RAR, VDR, PXR and FXR as well as Nur1 and Nur77 are involved mediating macrophage activation.[143-147]

Animal studies have shown the beneficial and clinically important effects of RXR agonists in models for chronic inflammatory diseases, such as atherosclerosis, insulin-resistant diabetes and neurodegeneration. [138-140]

2.2. Nuclear receptors in lipid and cholesterol metabolism

Macrophages regulate lipid-metabolism and are crucial for lipid homeostasis by up taking, storing and oxidizing lipids and executing cholesterol efflux. These mechanisms are crucial to prevent diseases and keep the homeostatic balance of lipid metabolism upright.[148]

Lipid-ligand activated transcription factors together with RXR regulate storage and release and elimination of lipids in human macrophages. The main receptors that are involved are the permissive heterodimer receptors PPARs and LXRs.

More recently, PXR, FXR, Nurr1 and Nur77 have been described to also play a role in energy metabolism but will not be described further here.[149-151]

2.2.1. The peroxisome proliferator-activated receptors (PPARs)

The three receptor subtypes of PPAR, PPARα, PPARβ and PPARγ (NRC1C1-NR1C3) are all encoded by different genes and also display distinct biological functions. Together with RXR as their heterodimer partner, they recognize DR-1 or DR-2 type recognition sequences within the promoter regions of their target genes (Table 2).

PPARα is expressed in tissues that are of high relevance for lipid and fatty acid catabolism, such as the liver, kidney, skeletal muscle and heart, brown fat and the intestine. [152] The action of this isoform reduces triglyceride levels and low-density lipoprotein levels (LDL) in the blood and elevates levels of high-density lipoprotein particles (HDL).[153,154]

Endogenous ligands of PPARα are saturated and unsaturated fatty acids such as arachidonic acid, palmitic acid, oleic acid or linoleic acid and important synthetic agonists are

fibrates for the treatment of insulin sensitivity, to improve blood glucose levels or hypertriglyceridemia. [26,155,156]

PPARβ/δ is also more broadly expressed and is found mainly in the brain, adipose tissue and the skin. The activity of this nuclear receptor subtype ameliorates glucose and lipid metabolism primarily in adipose tissue, heart and skeletal muscles.[157]

PPARγ is mainly expressed in adipocytes and the liver and at lower levels in skeletal muscles and the liver. Furthermore PPARα and PPARγ are expressed in monocytes/macrophages and vascular wall cells.[158,159]

Similar to PPARα, PPARγ plays a crucial role in lipid homeostasis as well as in glucose homeostasis. Moreover, PPARγ has major roles during the inflammatory response.[160,161]

2.2.2. The liver X receptors (LXRs)

The Liver X Receptors are another subclass that forms heterodimers with RXR.

LXRs exist in two different isoforms (LXRα and LXRβ termed NR1H3 and NR1H2, respectively) and the LXR/RXR heterodimer recognizes specific DNA sequences (DR-4) in the promoter and regulatory regions of target genes (Table 2).

The un-liganded LXR/RXR heterodimer binds constitutively to this recognition sequences in a repressed state due to interactions with corepressors that block transcription and recruit histone deactelyases.[162]

Endogenous ligands of the LXR/RXR heterodimer are oxysterols and intermediate metabolites of cholesterol biosynthesis. [163,164]

Ligand activation of LXR leads to the recruitment of specific co-activators (e.g. SRC-1) and the interaction of histone acetyl transferases to initiate transcription of target genes.[164] Besides genes important for maintaining cholesterol and lipid homeostasis LXR/RXR activation inhibits the transcription of certain pro-inflammatory genes.[165,166]

2.2.3. The retinoid X receptors (RXRs)

The retinoid X receptor controls cholesterol uptake, its efflux and cholesterol storage mainly due to the activation of permissive heterodimers (see section 1.4.2.3.).

Uptake of lipoproteins is regulated *via* scavenger receptors. Activation of RXR in macrophages with 9-cis retinoic acid or other rexinoids upregulates the expression of the

scavenger receptor CD36,[167] while other scavenger receptors are downregulated (SRA-II/II) that overall leads to an decreased lipid storage within cells.[167,168] It has been shown that CD36 regulation in macrophages is mediated by several RXR-heterodimers, such as PPAR/RXR, RAR/RXR and FXR/RXR.[169,170]

The regulation of SRA has been shown to be regulated by the permissive action of Nurr1/RXR and Nur77/RXR heterodimers.[171]

The efflux of cholesterol out of cells is a highly important mechanism to get rid of excess cholesterol. The efflux of cholesterol is mediated by the action of different ABC transporters.

Ligand activation of RXR by 9-cis retinoic acid, bexarotene or other rexinoids like LG100268 and HX630 or the natural occurring rexinoid honokiol have been shown to promote ABCA1 and ABCG1 expression from different cell lines, including human macrophage cell lines or primary mouse macrophages.[167,168,172,173]

Other RXR targets important for cholesterol efflux is the cholesterol transport protein ADP-ribosylation factor-like 7 (ARL4C) and a sterol eliminating enzyme CYP27A1.

The expression of these two proteins is mediated by the permissive action of PPARα/RXR, PPARγ/RXR and/or the LXRs/RXR.[167]

Furthermore, RXR activation is involved in the processing as well as the storage of lipids. In human macrophages, RXR activation leads to the induction of the expression of apolipoprotein E (ApoE) that promotes the efflux of lipids to apolipoproteins.

Another key regulator in cholesterol and lipid homeostasis is the transcription factor SREBP1 that induces a lipogenic gene program (e.g. induction of fatty acid synthase, acetyl-CoA-carboxylase) and leads to the transcription of genes involved in cholesterol biosynthesis and uptake. SREBP1 is a target of the LXR/RXR heterodimers.[174]

2.3 Atherosclerosis, lipid- and cholesterol metabolism

Atherosclerosis is a chronic inflammatory disease hallmarked by thickening and hardening of artery walls. The disease causes coronary and cerebrovascular diseases that are the two major morbidities worldwide.[175]

There are a lot of different environmental and genetic risk factors such as hypertension, obesity, insulin resistance or type 2 diabetes.[176] However, the disease is characterized by a local immune response that is caused by a deposition of cholesterol, lipid and cellular debris followed by a deposition of white blood cells into the sub endothelium. These depositions may become persistent and are then called atherosclerotic plaques.[138]

Nuclear receptors are heavily involved in lipid and cholesterol homeostasis as well in glucose homeostasis and the role of these receptors in the control of macrophage gene regulation and gene expression has been therefore studied extensively during the past years.[167,177] Indeed, the main constituent of such depositions are cholesterol and lipids and consequently atherogenesis occurs by the infiltration of the sub endothelial space by monocytes that subsequently start to differentiate into macrophages. Macrophages express lipoprotein receptors or scavenger receptors that mediate the uptake of lipids and cholesterol from these deposits. If the lipid accumulations exceed a critical point, the accumulation of these lipids in macrophages leads to the formation of foam cells that drives the lipid deposition further. Thus, macrophages are important cholesterol-accumulating cells in atherosclerosis and efflux of cholesterol out of these lipid-laden cells under pathologic conditions might protect against atherosclerosis.[178]

Reverse cholesterol transport (RCT) is the major physiological process that promotes cholesterol efflux from peripheral tissues by high-density lipoprotein (HDL) to deliver the excess cholesterol to the liver. From the liver cholesterol is partly eliminated through the bile and the feces. This process maintains the cholesterol homeostasis by maintaining the balance of cholesterol intake and *de novo* cholesterol synthesis.

In macrophages, multiple genes regulate RCT.

Modified lipoproteins are taken up by scavenger receptors in macrophages and are delivered to endosomes/lysosomes where the initial step of RCT occurs: the hydrolysis of cholesterol esters (CE) to free cholesterol (FC) and fatty acids. This initial step is mediated by lysosome acid lipase (LAL). Processed cholesterol is then integrated into the cellular membrane. Niemann Pick type C 1 and 2 proteins (NPC1 and NPC1) control these

intracellular mechanisms.[179] Excess cholesterol on the other hand is transported to the endoplasmic reticulum and is there re-esterified with fatty acids by acyl-CoA cholesterol acyltransferase 1 (ACAT1) and is stored as lipid droplets.[180]

Three main transporters play a critical role in macrophage cholesterol efflux. ATP-binding cassette transporter ABCA1, ABCG1, ABCG4 and the scavenger receptor type I(SR-BI). The main cholesterol transporters are expressed under the regulation of the action of RXR and LXR, mainly the permissive heterodimer LXR/RXR. [181] The transporters interact with the cholesterol acceptors HDL and Apo-AI and effluxed cholesterol is then carried out by HDL to the liver to be taken up by the liver SR-BI (termed direct pathway). Alternatively, free cholesterol is transferred by CEPT (cholesteryl ester transfer protein) to apoB-containing lipoproteins that are later cleared by the liver via receptors such as low-density lipoprotein receptor (LDLR) (termed indirect pathway). [178]

Under atherosclerotic conditions the lipid accumulation in macrophages take overhand, that lead to the formation of foam cells that remarkably influences the progress of the disease progression.[182]

For this reason, one strategy to treat atherosclerosis and prevent atherosclerotic genesis would be to enhance efflux of excess cholesterol out of peripheral cells(macrophages) to prevent cellular cholesterol retention by pharmacotherapy.[183,184]

2.3.1. Ligands for RXR in lipid-handling related diseases

It has been shown that rexinoids significantly reduces the progression and development of atherosclerosis in mouse models of dyslipidaemia and they do so by enhancing the capacity of cholesterol efflux within macrophages. [170,185]

Ligands for PPARs and LXR are reported to possess similar effects in disease models that indicate that the effect of rexinoids might be due to these receptor heterodimers *in vivo*.[132,145]

Rexinoids have been reported to have potential in the treatment of other lipid-handling related diseases as well, such as certain neurological conditions.[186,187]

Bexarotene was reported in 2012 by *Cramer et al* to clear β-amyloid plaques and improve cognitive deficits in a mouse model of Alzheimer's disease (AD).[188]

One hypothesis is that the therapeutic effect in AD models is due to the upregulation of ApoE and the increase in ABCA1 expression mediated by the activation of LXR/RXR and PPAR/RXR permissive heterodimers.[189]

Finally, ligands for retinoid X receptors have a therapeutic potential for treating lipid-related and metabolic diseases but their development is still challenging because of off-target effects of permissive RXR heterodimers, cytotoxicity and tissue availability of RXR. Observed side effects of activation of RXR is rising of plasma triglyceride levels and the suppression of the thyroid axis via the activation of permissive RXR heterodimers.[190,191] Therefore, development of novel RXR-selective ligands for therapy aims to develop heterodimer selectivity with improved action.[192]

3. Honokiol/magnolol derivatives used in this study

Semisynthetic honokiol derivatives synthesized at the Institute of Applied Synthetic Chemistry (Univ.Prof. Dipl.-Ing. Dr.techn. Marko Mihovilovic, Vienna University of Technology, Vienna)[193] and the Institute of Pharmaceutical Sciences (Priv. Doz. Dr. Wolfgang M. Schuehly, Karl-Franzens-University Graz, Graz)[84,194,195] were initially screened in a cell-based luciferase-reporter gene assay in HEK293 and HepG2 cells for nuclear receptor activation *in vitro* (see section 2.3.2.-2.3.5).

In total 55 neolignans were tested for transactivation of RXRα, the PPAR isoforms α, β/δ and γ, the LXR isoforms α and β and FXR. The screening was performed by Dr. Angela Ladurner, Dr. Clemens Malainer and former diploma students from the Department of Pharmacognosy, University of Vienna (Reem Selim, Amina Cocic and Andrea Holzer) and by myself.[196-198]

The structures of tested semisynthetic neolignans and the results of the screening are displayed and summarized in the Appendix (Appendix Figure 1 and Table 1).

Five derivatives selectively activated human full length RXRα in this cell-based luciferase testing model.

Two semisynthetic honokiol derivatives from the Institute of Applied Synthetic Chemistry (Group of Univ.Prof. Dipl.-Ing. Dr.techn.Marko Mihovilovic, University of Technology, Vienna) were finally selected for further functional characterization.

Compounds 2284 (LRK017) and 2817 (LRK363) were synthesized by Dr. Lukas Rycek and Dr. Dominik Dreier at the Vienna University of Technology.[199]

Aim of the work

Considering the important biological role of the retinoid X receptor the aim of this work was to identify new partial agonists for the retinoid X receptor that act as selective modulators structurally based on the natural compounds and naturally occurring rexinoids honokiol and magnolol.

Potent compounds that responded in an initial screen in a cellular luciferase-based testing model in HEK293 and HepG2 cells in a dose-dependent manner were selected and further investigated.

A coregulator interaction profile of the RXRα ligand-binding domain (LBD) of 154 peptides representing 64 possible interacting coregulators was performed and compared to co-regulator interaction profiles of full RXR agonists

In vitro binding studies and functional studies of potent RXRα agonists further lead to a pharmacological and bioactivity profile of active honokiol derivatives.

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Material and Methods

1. Materials

1.1. Products and supplier information

Name	*Supplier*
Cell Culture Material	
Benzylpenicillin	Lonza Group Ltd. (Basel, Switzerland)
Dulbecco´s Modified Eagle Medium (DMEM)	Lonza Group Ltd. (Basel, Switzerland)
Foetal bovine serum	Lonza Group Ltd. (Basel, Switzerland)
L-glutamine	Lonza Group Ltd. (Basel, Switzerland)
Penicillin (potassium salt)-Streptomycin sulphate	Lonza Group Ltd. (Basel, Switzerland)
Trypsin	Invitrogen (CA, USA)
Biologicals and Chemicals	
Amphicillin (sodium salt)	Sigma (MO, USA)
Adenosin-5´triphosphate disodium salt	Sigma (MO, USA)
Agar	Sigma (MO, USA)
ApoA1 recombinant	Sigma (MO, USA)
Bodipy®493/503	Molecular Probes (O, US)
Bovine serum albumine	New England Biolabs (MA, USA)
Coenzyme A (trilithium salt)	Sigma (MO, USA)
CompleteTM	Roche Diagnostics, (Penzberg,Germany)
Dithiothreitol (DTT)	Fluka (MO, USA)
D-Luciferin (sodium salt)	Synchem (Flesberg, Germany)
Dexmethasone	Sigma (MO, USA)
DMSO	Fluka (MO, USA)
Insulin	Sigma (MO, USA)
IBMX	Sigma (MO, USA)
Kanamycin	Sigma (MO, USA)
LB broth	Sigma (MO, USA)
Lipofectamine®2000	Invitrogen (CA, USA)
Oil red O	Fluka (MO, USA)
PMA	Sigma (MO, USA)
p-coumaric acid	Sigma (MO, USA)
PMSF	Sigma (MO, USA)
Reporter lysis 5X buffer	Promega (Wi, USA)
Resazurin (sodium salt)	Sigma (MO, USA)
Roti®Quant	Carl Roth (Karlsruhe, Germany)
TEMED	Fluka (MO, USA)
Tritium labeled Cholesterol	PerkinElmer (MA, USA)

Triton® X-100	Sigma (MO, USA)
Unesterified Cholesterol (UC)	Sigma (MO, USA)
[1,2-^{3}H(N)]-Cholesterol 49.0 Ci/mmol	Perkin Elmer (Boston, MA, USA)
Compounds	
9-cis retinoic acid	Cayman (MI,USA)
Bexarotene	Sigma (MO, USA)
GW3965	Sigma (MO,USA)
HX351	Tocris Bioscience (Washington DC, USA)
Honokiol	University of Vienna, J.Rollinger (Austria)
Magnolol	University of Vienna, J. Rollinger (Austria)
Pioglitazone	Molekula (Shaftesbury, UK)
Rosiglitazone	Cayman (MI,USA)
T0901317	Sigma (MO, USA)

Table 5. Cell culture material, biologicals and chemicals.

1.2. Cell culture media and supplements

Name	Components	Amount
HEK293 and HepG2 growth medium (DMEM complete)	DMEM high glucose (phenol red free) Penicillin (potassium salt) Streptomycin sulphate L-glutamine Foetal bovine serum (heat inactivated)	500mL 100 U/mL 100 µg/mL 2 mM 10 %
DMEM$_{stripped}$ medium	DMEM high glucose (phenol red free) Penicillin (potassium salt) Streptomycin sulphate L-glutamine Foetal bovine serum, stripped (FBS$_{stripped}$)	500mL 100 U/mL 100 µg/mL 2 mM 10 %
FBS$_{stripped}$	Foetal bovine serum (heat inactivated) Charcoal powder T70-Dextran powder Sucrose powder HEPES buffer stock pH 7.6 $MgCl_2$ stock	0.0025% w/v 0.25 M 1M 1M

Name	Components	Amount
$DMEM_{starvation}$ medium	DMEM high glucose (phenol red free)	500mL
	L-glutamine	2 mM
THP-1 medium (RPMI complete)	RPMI high glucose (phenol red)	500 mL
	Penicillin (potassium salt)	100 U/mL
	Streptomycin sulphate	100 µg/mL
	L-glutamine	2 mM
	Foetal bovine serum (heat inactivated)	10 %
3T3-L1 medium (Adipocte growth medium)	DMEM high glucose (phenol red free)	500 mL
	Penicillin (potassium salt)	100 U/mL
	Streptomycin sulphate	100 µg/mL
	L-glutamine	2 mM
	Calf serum (heat inactivated)	10 %
Medium for Luciferase Assays	DMEM (high glucose)	500 mL
	L-glutamine	2 mM
Adipocyte differentiation medium	DMEM high glucose (phenol red free)	500mL
	Penicillin (potassium salt)	100 U/mL
	Streptomycin sulphate	100 µg/mL
	L-glutamine	2 mM
	Foetal bovine serum (heat inactivated)	10 %
	Insulin	1 µg/mL
	Dexamethasone	250 nM
	IBMX	250 µM
Adipocyte medium	DMEM high glucose (phenol red free)	500mL
	Penicillin (potassium salt)	100 U/mL
	Streptomycin sulphate	100 µg/mL
	L-glutamine	2 mM
	Foetal bovine serum (heat inactivated)	10 %
	Insulin	1 µg/mL

Table 6. Cell culture media and supplements.

1.3. Commercially available kits

Name	Supplier
LanthaScreen™ TR- FRET RXRalpha Coactivator Assay Kit, goat	Invitrogen (CA, USA) (Catalogue number PV4797)
pegGOLD Plasmid Midiprep Kit	PeqLab (Erlangen, Germany)
PureYield™ Plasmid Midiprep System	Promega (Wi, USA)
RNA Isolation Kit	PeqLab (Erlangen, Germany)
Superscript™ First-Strand Synthesis System	Invitrogen (CA, USA)
qPCR Green Master Mix	Roche Diagnostics, (Penzberg,Germany)

Table 7. Commericailly available kits.

1.4. Reagents and buffers

Name and Components	Amount
<u>Solutions for transient transfection:</u> **(Calcium phosphate method)**	
Calcium phosphate- $NaHPO_4$ (stock solution) CaCl2 2M	5.2 g in 500mL H2O
2 x HBS	8.0 g NaCl, 6.5 g HEPES, 10 mL Na-HPO4 stock solution pH adjusted to 7.4 with diluted NaOH or HCL and filles up to 500 mL
<u>Luciferase Measurement:</u>	
<u>Luciferase lysis buffer</u>	1.2 mL 5 x Reporter lysis buffer (Promega) 4.8 mL ddH_20 6 µl 270 mM Coenzyme A [270 µM] 6 µl 1M DTT [10.1.mM]

Luciferin	
Luciferin	32 mg/mL
Tricine	21 mM, pH 7.8
ATP	
Tricine pH 7.8	20 mM, pH 7.8
$MgCl_2$ 21.5 mM	21.5 mM
Adenosin-5´triphosphate disodium salt	3.8 mM
Cell Viability Assay:	
10x Resazurin stock	1 mg/ml in sterile PBS Prior to use, the stock was diluted in sterile PBS 1:10 and further diluted 1:10 with the growth medium in the well plates (DMEM or RPMI + 1% Glutmine)
Western blot solutions and buffers:	
Protein extraction	
stock solution RIPA lysis buffer 50mM	50 mM Tris/HCl pH 7.4 500 mM NaCl 5.0 mM NP40 12.06 mM Na-Deoxycholate 3.47 mM SDS 7.7 mM NaN_3 dissolved in H_2O freshly added prior to use: 4 % Complete (protease inhibitor cocktail, Roche Diagnostics, Penzberg Germany) 1 mM PMSF 1 mM NaF 1 mM NaVO3
Protein quantifcation	1 : 4.75 Roti® -Quant (Roth, Karslruhe, Germany) in Aqua dest
Immunoblotting:	
Western blot sample buffer 3x	187.5 mM Tris/HCl pH 6.8 0.2 M SDS 30% Glycerol 0.2 mM Bromphenol blue 15% β-Mercaptoethanol in 1000 mL ddH_2O

Resolving gel	7.5-10 % PAA (Rotiphorese® Gel30) 375 mM Tris/HCl pH 8.8 0.1% SDS 0.1% TEMED 0.05% APS 7.5 mL ddH_2O
Stacking gel	5 % PAA (Rotiphorese® Gel30) 125 mM Tris/HCl pH 6.8 0.1% SDS 0.1% TEMED 0.05% APS 3.75 mL ddH2O
SDS Running buffer 10x	248 mM Tris-base 1.9 M Glycine 35 mM SDS in 1000 mL ddH2O
Blotting Buffer 5x	125 mM Tris-base 971 mM Glycine in 1000 mL H_2O
TBS-T pH 8.0 10x	248 mM Tris-base 1.9 M NaCl 1 % Tween-20 in 1000 mL H_2O
ECL-solution	100mM Tris-base pH 8.5 1.24 mM Luminl 0.2 mM p-Coumaric acid 0.018% H_2O_2 In 10 mL H_2O
Solutions for Confocal microcopy: **Formaldehyde solution 3%**	Formaldehyde stock solution (37%) 33ml, PBS solution 374 ml
BSA/PBS solution (0.2%)	3g BSA in 1500 mL PBS solution 3 g 1500 ml (mixed overnight and sterile-filtered)
Triton solution (0.2%)	800 µl Triton in 400 ml BSA/PBS solution (0.2%) (mixed overnight and sterile-filtered)

Table 8. Reagents and buffers.

1.5. Plasmid DNA

Name of plasmid	Source	Antibiotic resistance	Used restriction enzymes
Expression plasmids			
EGFP (pEGFP-N1)	Clontech (Mountain View, CA, USA)	Kanamycin	Nhell, EcoRI
Human RXRα, full length (pcDNA3.1+)	Missouri S&T cDNA Res (Rolla, Missouri, USA)	Ampicillin	EcoRI, Xhol
Human LXRα, full length (pCMV)	Missouri S&T cDNA Res (Rolla, Missouri, USA)	Ampicillin	ApaI, KpnI
Human LXRβ, full length (pcDNA3.1+)	Missouri S&T cDNA Res (Rolla, Missouri, USA)	Ampicillin	ApaI, KpnI
Human PPARγ Full length (pSG5-PL-hPPAR γ1)	Univ. Prof. Walter Wahli and Univ. Prof. Beatrice Desvergne	Ampicillin	EcoRI, HindIII
GAL4 hRXRαLBD Chimeric Expression of GAL4-LBD (pCMX)	Univ. Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA)	Ampicillin	Functionally varyfied
GAL4 hPPARγ LBD Chimeric Expression of GAL4-LBD (pCMX)	Univ. Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA)	Ampicillin	unctionally varyfied
pET15b-His6 human-RXRα (pET15b)	Dr. Marcel Scheepstra (Technische Universiteit, Eindhoven, The Netherlands)	Ampicillin	Protein sequenced
Reporterplasmids			
RXR_RE (RXR(2) Luciferase Reporter Vector) (pTL Luc)	Missouri S&T cDNA Res (Rolla, Missouri, USA)	Ampicillin	HindIII, BamHI

Name of plasmid	Source	Antibiotic resistance	Used restriction enzymes
ABCA1promoter -703/-38 Luciferase Reporter Vector (pGL4.14)	Dr. Irena Ignatova (University of Virginia Health System, Charlottesville, Virginia, USA)	Ampicillin	KpnI, SacI
SREBP1c promoter - 556/+42 Luciferase Reporter Vector (pGL3)	Dr. Gyun Sik Oh (Asan Medical Center, Seoul, South Korea)	Ampicillin	KpnI, XhoI
PPAR_RE Luciferase Reporter Vectors (tk-PPREx3-luc)	Univ. Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA)	Ampicillin	EcoRI, HindIII
tk(MH1000) 4x LUC GAL4 responsive Luciferase Reporter Vector	Univ. Prof. Ronald M. Evans (Howard Hughes Medical Institute, La Jolla, CA)	Ampicillin	Functionally varyfied

Table 9. Plasmid DNA.

1.6. Primer and antibodies

Primer	Supplier/gene code
Primer name (Gene)	**QuantiTect Primer Assay, Qiagen (Hilden, Germany)**
ABCA1	[Human] (ENSG00000165029)
ABCG1	[Human] (ENSG00000160179)
SRBI	[Human] (ENSG00000073060)
FASN	[Human] (ENSG00000169710)
SREBP	[Human] (ENSG00000072310)
ACC	[Human] (ENSG00000132142)
18S	[Human] (ENSG00000225840)

Table 10. Primers for quantitative RT-PCR.

Antibodies		**Antibodies**	
Target (size in kDa)	**Source**	**Dilution**	**Provider (Catalog number)**
ABCA1(220kDa)	rabbit, pcAb	1:1000	Novus Biologicals (B400-105)
SR-BI (82kDa)	rabbit, pcAb	1:1000	Novus Biologicals, (NB400-104)
mature SREBP1c (68kDa)	rabbit, pcAb	1:1000	Novus Biologicals, (NB100-2215)
precursor SREBP1c (125kDa)	rabbit, pcAb	1:1000	Novus Biologicals, (NB100-2215)
FAS (273kDa)	rabbit, pcAb	1:1000	Cell Signal, 31895
ACC (280 kDa)	rabbit, pcAb	1:1000	Cell Signal, 3662
actin (42kDa)	mouse, mcAb	1:500	MP Biomedicals, Eschwege, Germany (69100)
anti-mouse IgG	goat, pcAb	1:2500	Cell Signal (7074)
anti-rabbit IgG	goat, pcAb	1:500	Promega (W4011)

Table 11. Antibodies.

1.7. Technical equipment

Name	**Supplier**
Bacterial incubator	Edmund Buehler (Hechingen, Germany)
C1000 Thermal Cycler	BIO RAD Laboratories (CA, USA)
DNA/RNA UV Cleaner	Biosan (Vienna, Austria)
Eluator™ Vacuum Elution Device	Promega (Wi, USA)
FACSCalibur™	BD Biosciences Pharmingen (CA, USA)
Fluorescence Microscope Olympus BX51	Olympus Europa GmbH (Hamburg, Germany)
Leica DMi8 (Confocal Microscope)	Leica Microsystems (Vienna, Austria)
Light Cycler 480®	Roche (Basel, Switzerland)

Name	Supplier
Light Microscope Olympus CKX31	Olympus Europa GmbH (Hamburg, Germany)
Mini Trans-BlotTM Electrophoretic Transfer Cell	BIO RAD Laboratories (CA, USA)
NanoDrop 2000	Thermo Scientific (MA, USA)
Power supply Power Pac™ HC	BIO RAD Laboratories (CA, USA)
Tecan GENios Pro	Tecan (Mannedorf, Switzerland)
Tecan Infinite200Pro	Tecan (Mannedorf, Switzerland)
Vac-Man® Laboratory Vacuum Manifold	Promega (Wi, USA)
Vi-CellTM XR Cell Viability Analyzer	Beckman Coulter (CA, USA)

Table 12. Technical equipment.

1.8. Scientific software

Name	Supplier
Adobe Photoshop CS5	Adobe systems Software (Ireland)
Cell Quest Pro version 5.2 BD	Biosciences (San Diego, CA, USA)
GraphPad PRISMTM, version 4.03	GraphPad Software, Inc. (CA, USA)
EndNote X, version 1.01	Thomson ResearchSoft (CA, USA)
ImageReader LAS3000	Fujifilm (Tokyo, Japan)
Irfan View for windows version 4.27	Irfan Skiljan (Wiener Neustadt, Austria)
Leica LASX	Leica Microsystems (Vienna, Austria)
MDL ISIS Draw 2.5	Symyx Technologies (CA, USA)
Microsoft Excel 2010	Microsoft (WA, USA)
MultiGaugeV3.0 Science Lab 2005	Fujifilm (Tokyo, Japan)
Tecan iControl	Tecan (Mannedorf, Switzerland)
Vi-CellTM XR 2.03	Beckman Coulter (CA, USA)

Table 13. Computer software for data acquisition and analysis.

2. Methods

2.1. Plasmid DNA preparation

Plasmid DNA preparation was performed by amplification in competent *E.coli* cells (JM109, Sigma-Aldrich).
Before amplification in larger scale, plasmids were validated by a miniprep procedure (pegGOLD Plasmid Prep, PegLab) and subsequent restriction analysis using appropriate restriction enzymes. Digested Plasmid DNA fragments were observed by electrophoresis.
Amplification of Plasmid DNA in a larger scale by maxiprep was performed by using a vacuum-based purification procedure with PureYield™ Plasmid Midiprep System (Promega).
The exact preparation steps were performed according to the manufacturer´s protocol.

2.2. General cell culture conditions and maintenance of cell lines

Adherent cells were grown as adherent monolayer cultures.
For general cultivation they were kept in 75 cm^2 cell culture flasks (Sarstedt, Nümbrecht, Germany) in complete growth medium. Cells were incubated in a humidified incubator at 37°C and 5%CO_2. Sub-culturing was performed 3 times a week upon reaching 80% confluence and growth was examined with a microscope as well as by measuring cell viability (%) with Trypan Blue staining using a cell counter device (Vi-Cell, Beckman Coulter, Brea, CA).
Passaging of cells was performed with Trypsin digestion for 1-3 min at 37°C depending on the cell type. Cells were washed once with PBS beforehand and after trypsinisation resuspended in complete medium to stop the digestion. 6-8 Mio. cells were seeded in complete growth medium in a new 75 cm^2 cell culture flask and stored in an incubator (37 °C, 5 % CO_2).
Experiments were performed in 6-, 12-, 24- or 96 well plates at a confluence of 70-90 %. Cells were treated with compounds dissolved in DMSO whereas the DMSO concentration never exceeded 0.2%, in most cases a final concentration of 0.1% DMSO was used.

Suspension cells (THP1 monocytes) were cultured in 175 cm2 flasks (Sarstedt, Nümbrecht, Germany) at 37°C and 5% CO_2 and were split every 2-3 day to ensure that the cell number never exceeded 0.8 x 10^6/mL and to assure a cell viability of <95%.
For splitting the cell suspension was carefully centrifuged at 150 g for 4 min and the cell pellet was resuspended in fresh growth medium. Cell number and viability was assessed with a cell counter (ViCell, Beckman Coulter, Brea, CA) by Trypan Blue staining. A cell suspension of 0.2 x 10^6/mL cells was then incubated at 37°C and 5%CO_2 for further cultivation.

2.2.1. Freezing and thawing of cells

Cells were tryspinized and resuspended in complete medium containing 20% fetal calf serum and 20% DMSO. 1 ml of 1 Mio./mL cells were pipetted in a cryovial and kept on ice and then were cooled down for 1°C/hour until -80°C. Cells were then stored in long term in liquid nitrogen (-196°C).
For thawing cell cryovials were thawed and the cell supsension was immediately transferred in 10 mL prewarmed normal growth medium. Cells were then centrifuged to remove the excess DMSO for 4 min at 150 g. Resuspended cells were then kept in 15 ml fresh complete medium and on every second following day the medium was exchanged until they reached 80% confluency. Cells were passaged at least once before further experiments were performed.

2.2.2. Preparation of charcoal-stripped serum

For preparation of stripped Foetal bovine serum (FBS), 0.25% w/v of charcoal and 0.0025% w/v T-70 dextran were freshly prepared in a buffer containing 0.25 M sucrose, 1.5 mM $MgCL_2$ and 10mM HEPES (pH 7.6). The charcoal/dextran solution was then gently rotated over night at 4°C. Next day the solution was centrifuged at 410 g for 10 minutes. The decanted solution was then mixed with an equal volume of heat-inactivated FBS and rotated over night at 4°C.
The day after the mixture was again centrifuged at 410 g for 10 minutes and filtered through a Millipore flask filter.

2.3. Experiments in HEK293 cells

2.3.1. HEK293 cell line

Human Embryonic Kidney 293 cells (HEK293 cells) were used in this study for screening of natural products and natural product-derived derivatives in a cell-based luciferase gene transactivation assay and to further assess potency and specificity of candidate compounds (nuclear receptor agonists).
The HEK293 cell line was derived 1973 by transformation by exposing embryonic kidney cells to sheared DNA of adenovirus type 5 (AD5).[200] HEK293 cells are the most frequently used cells after HeLa in cell biological studies and after CHO cells in biotechnology.[201] The cell line is easy to handle, easy to transfect and was for this reason selected for this experimental approach.

2.3.2. Transient transfection of HEK293 cells

Transient transfection of HEK293 cells was performed using the Calcium phosphate method.[202]
6 x 10^6 cells were seeded in 15 cm petri dishes in 20 mL complete medium and were grown over night at 37°C, 5% CO_2. Next day cells were transfected with 1.5 mL transfection mix per petri dish. Transfection was performed by using 6 µg of expression plasmid, 6 µg of the respective reporter plasmids and 3 µg EGFP expression plasmid per petri dish. After 6 hours of transfection cells were reseeded on diluted compounds and solvent control in 96 well plates (50.000 cells per well). Non transfected cells were used as negative control. Here 1 x 10^6 cells were seeded in 10 cm petri dishes in parallel to the cells that were transfected.

2.3.3. Luciferase reporter gene assay in HEK293 cells

HEK293 cells were transiently transfected as described above with a reporter plasmid containing the respective nuclear receptor response element followed by a luciferase reporter gene, nuclear receptor expression plasmids encoding for the different nuclear receptors and with an EGFP expression plasmid as internal transfection control.

All plasmids that were used in this study are listed in section 1.5. (Plasmid DNA). Transfected HEK293 cells were incubated with the compounds diluted in $DMEM_{stripped}$ medium together with the appropriate controls for 18 hours if not otherwise stated. The medium was then removed by using a vacuum pump and cells were frozen at -80°C for at least an hour before cell lysis for luciferase measurement.
Cell Lysis was performed in 50 µl per well (96-well plate) Lysis Buffer for Luciferase Measurement on a plate shaker for 10 minutes.
40 µl of each lysate was then transferred to a black non-transparent 96-well plate and luminescence (luciferase activity) and fluorescence (EGFP) was measured on a TECAN Genios Pro plate reader or on a TECAN Infinit200 plate reader.
Parameters for measurement were as follows:

Luminescence measurement	**Fluorescence measurement**
Measurement mode : Luminescence	Measurement mode : Fluorescence
Plate definition file : GRE96fb	Excitation wavelength : 485 nm
Integration time (manual) : 2000 ms	Emission wavelength : 520 nm
Attenuation : None	Gain : Optimal
Time between move and integration : 50 ms	Number of reads : 1
Well kinetic number : 1	Integration time : 1000 µs
	Lag time: 0 µs
Injector A volume : 50 µl	Mirror selection : 40 ms
Injector A speed : 200 µl/s	
Injector B volume : 50 µl	
Injector B speed : 200 µl/s	
Injection mode : Standard	

Table 14. Settings for luminescence and fluorescence measurements in 96 well format.

Luminescence and fluorescence values were obtained in quadruplicates (4 wells per condition tested) and values of untransfected cells for both measurements (auto-luminescence and auto fluorescence) were background subtracted before further analysis.

2.3.4. Luciferase reporter gene assay with human full-length receptor genes

This cell-based transactivation assay in HEK293 was performed by using plasmids encoding the respective full-length human nuclear receptor genes. This experimental setting was used for dose-response experiments in order to be able to assess efficacy and potency of selected honokiol derivatives upon their action on the full receptor.

Retinoid X receptor alpha (NR2B1) was cloned into the pcDNA3.1+ from Invitrogen by Missouri S&T cDNA Resource Center. Human Kidney cDNA (Biochain) was used to amplify the open reading frame of NR2B1 and subsequently cloned at EcoRI(5´) and XhoI(3´) into pcDNA3.1+(Invitrogen). The size of the insert was 1389 bp.

First, HEK293 cells were transiently transfected (see 2.3.2.) using 3 µg luciferase reporter vector DNA, 6 µg human full-length nuclear receptor vector DNA and 3 µg of EGFP expression vector DNA (see 1.5. plasmid DNA) in petri dishes containing 20 mL of complete growth medium.
After 6 hours of transfection cells were washed, harvested and reseeded into 96-well plates in a density of 50.000 cells per well containing the respective dilution of testing compounds in $DMEM_{stripped}$ medium.
Untransfected cells were used as negative control and results were normalized to the solvent control (0.1% DMSO if not otherwise stated).

2.3.5. Mammalian one-hybrid assay (GAL4 binding assay)

Another luciferase reporter gene assay was used for further assessment of specificity for RXRα- and PPARγ- ligand binding domains of tested honokiol derivatives. Nuclear receptor transactivation was investigated in a „one -hybrid"system.
In a „one-hybrid" system the interaction of the DNA-binding domain of the yeast transcription factor GAL4 to the GAL4 upstream activating sequence (UAS) is combined with a specific nuclear receptor ligand binding domain. In this experimental setting modulation of transcriptional transactivation of the luciferase reporter gene is due to binding of

the ligand to the LBD only and the system is independent of certain response elements and heterodimer partners.

The plasmid DNA for the UAS-reporter construct (UAS-tk-luc) and the construct encoding the chimeric GAL4 DNA-binding domain:nuclear receptor ligand-binding domain was a kind gift of Dr. Ronald Evans (Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, CA) see section 1.5.

For transactivation studies HEK293 cells were transiently transfected (see 2.3.2.) with 10 µg of the respective expression plasmids encoding the chimeric fusion protein of GAL4 DNA-binding domain and the nuclear receptor ligand-binding domain and 2 µg of the responsive reporter plasmid containing a Renilla luciferase gene under the control of several UAS elements. Furthermore, cells were transfected with 2 µg of a third plasmid encoding EGFP as internal transfection control.
After 6 hours of transfection, cells were washed in PBS harvested and reseeded into 96 well plates in a density of 50.000 cells per well containing dilutions of testing compounds in $DMEM_{stripped}$ medium.
Untransfected cells were used as negative control and results were normalized to the solvent control (0.1% DMSO if not otherwise stated).

2.3.6. Data analysis

Data analysis of Luciferase reporter gene assays was performed in Microsoft Office Excel of at least three independent experiments. Measurements were performed in quadruplicate for each condition. Relative luminescence units and relative fluorescence units were background subtracted by values obtained from untransfected cells.
Then the ratio of luminescence and fluorescence values was calculated and normalized to the solvent control DMSO (0.1% if not otherwise stated).
Final figures and statistical analysis was performed in GraphPad Prism 4.03.
To calculate EC_{50} values and to make concentration response curves the test compounds were measured at different concentrations, data were logarithmically transformed and a sigmoidal dose response with a variable slope was performed.

2.4. RXRα co-activator recruitment assay

A coactivator recruitment assay was performed using LanthaScreen® TR-FRET RXR alpha Coactivator Assay Kit (Invitrogen) according to the manufacturers' protocol. In this assay the recruitment of a fluorescin-labelled coactivator peptide (PGC1α) to the RXRα ligand binding domain (hRXRα LBD) is measured *in vitro*.
Several dilutions of the test compounds were incubated with 10 nM of a Glutathione-S-transferase (GST)-tagged hRXRα LBD (GST-hRXRα LBD), 500 nM fluorescin-PGC1α peptide and 5 nM-anti-GST antibody. The binding of agonist to the ligand binding domain of the receptor causes a conformational change that results in a higher affinity for the coactivator peptide and this results in an energy transfer from the terbium-labelled anti-GST antibody to the fluorescin label on the coactivator peptide when excited at 340 nm resulting in an emission at 520 nm.
The time resolved FRET signal is measured as an emission ratio of 520:495 nm in the agonist mode. Emission was measured on a GeniosPro multiplate reader from Tecan. Following instrument setting were used for the measurement:

Fluorescence measurement
Measurement mode: Fluorescence
Excitation: 340 nm filter (30 nm bandwidth)
Emission: 520 nm filter (25 nm bandwidth)
Emission: 490 or 495 nm filter (10 nm bandwidth)
Gain: Optimal
Number of reads: 10
Delay Time: 100 µs
Integration Time: 200 µs
Mirror selection: dichroic

Table 15. Settings for fluorescence measurements for FRET.

Measurements were performed in a 384 well format and a final concentration volume of 20 µl in duplicate. 9-cis retinoic acid was used as positive control.

2.4.1. Data analysis

The TR FRET ratio of 520:495 was calculated in Excel and the calculated fluorescence ratios for the test compounds in different concentrations were used to determine the EC_{50} values in GraphPad Prism4.0 by logarithmically transforming the x-values (compound concentrations) and subsequent calculation of a sigmoidal dose response curve with a variable slope.

2.5. Real-time coregulator- nuclear receptor interaction

Micro array coregulator-nuclear receptor interactions (MARCoNI) were investigated and compared between full RXR agonists (e.g. bexarotene) and honokiol derivative 2284. The technology allows the identification of possible coregulator interactions that occur via amino-acid recognition motives of the regulator protein recruited by a ligand upon ligand-binding to the ligand-binding domain of the nuclear receptor.
This short α-helical amino acid sequences are in the case of co-activator interactions LXXLL-motives whereas in case of corepressors are LXXXIXXXL-motives.
These binding motifs, in which L (or I) indicate an (iso-) leucine while X indicates any amino acid, form a short α-helical structure that interacts with the activation function-2 (AF-2) located in the ligand-binding domain (LBD) of NRs. Selectivity between NRs and coregulator is determined by flanking, and intra-(iso-)leucine amino acids (X). Moreover, the affinity between a NR-box and the AF-2 domain of the NR is modulated by ligand binding and subsequent allosteric conformational change, involving reposition of helix-12 of the NR.
The micro array was performed by PamGene® (PamGene International B. V., 's-Hertogenbosch, The Netherlands) under the project number #200-124.
The micro array contains 154 peptides containing the respective recognition sequences that are immobilized on the array. The 154 peptides represent 64 coregulator proteins on this array.
The immobilized peptides on the array are then incubated with an isolated ligand-binding-domain of the nuclear receptor RXR together with the ligand. Fluorophore labels on the peptides as well as on an antibody detecting the RXR ligand-binding domain

(AF-2) allows to measure the receptor-peptide interaction with a time-resolved fluorescence resonance energy transfer (FRET) signal.
5 nM of GST-tagged RXRα ligand binding domain (LBD) (#PV4798, Invitrogen), 25 nm Alexa Fluor 488-conjugated anti-GST antibody (A11131, Invitrogen), 5 mM DTT, 2 % DMSO and ligand (Bexarotene and 2284 in 11 concentrations -4.2 logM and lower) in FRET-buffer were prepared on ice.
The profiling was performed on a PamStation®-96 controlled by EvolveHT software (PamGene International B. V., 's-Hertogenbosch, The Netherlands).
Nuclear Receptor PamChip® Arrays (PamGene International B.V.) contained 154 peptides. The arrays are made of a porous metal oxide carrier to which the peptides are spotted. The assay mixtures were incubated at the array and tiff. images were obtained at different cycles and further analysed by taking the median signal of each spot and that of the surrounding area (background) to be able to quantify the signal.
Dose responses were performed for bexarotene and 2284 and analysed in Graph Pad Prism 4.03 using a non-linear regression with a sigmoidal dose response (variable slope).

2.6. Functional studies

2.6.1. Experiments in differentiated THP-1 macrophages

2.6.1.1. THP-1 cell line

The THP-1 cell line is a human leukemia cell line initially isolated from the peripheral blood of a 1-year old patient suffering from acute monocytic leukemia. THP-1 monocytes as well as differentiated THP-1 macrophages are a common used model system to study both states of these cells of the innate immune system. [203] As monocytes they grow in suspension and are easy to cultivate. In this study differentiated THP-1 macrophages were used as a model system to investigate the action of selected honokiol derivatives on cholesterol efflux.

2.6.1.2. Differentiation of THP-1 monocytes into macrophages

THP-1 monocytes were maintained in RPMI-$1640_{complete}$ medium. To differentiate the THP-1 monocytes into THP-1 macrophages cells were seeded at a density 0.2 x 10^6 cells/mL in the presence of 200 nM phorbol myristate acetate (PMA) for 72 hours.

2.6.1.3. Cholesterol efflux in differentiated THP-1 macrophages

The ability of honokiol derivatives and control compounds to promote cholesterol efflux was determined in differentiated THP-1 macrophages with radioactively labelled [^{3}H]-Cholesterol as previously described with slight modifications.[204]
First, THP-1 monocytes were differentiated for 72 hours into macrophages. 24-well plates were seeded at a density of 0.2 x 10^6 cells/mL in $RMPI_{complete}$ medium containing 200 nM of PMA as differentiation reagent.
After 72 hours differentiated THP-1 macrophages were washed once in PBS and labelled for 24 hours in serum-free RPMI medium containing 0.1% BSA, 0.2 µCi/mL [^{3}H]-Cholesterol and 20µg/ml un-esterified cholesterol together with the indicated compounds dissolved in DMSO or DMSO as solvent control (0.1% DMSO).

After 24 hours cells were washed twice in PBS and treated a second time with the compounds or the solvent DMSO control in serum-free RPMI medium in the presence or the absence of a cholesterol acceptor (10 µg/mL recombinant ApoAI or 1% human Plasma). After 4-6 hours the supernatant as well as cell lysates (cell lysis was done by using 0.1 M NaOH for 10 min) were measured with a scintillation counter.
Each assay was performed in at least three independent experiments performed in duplicate.

2.6.1.4. Co-treatment experiments in differentiated THP-1 macrophages

To investigate the RXR-dependence of the identified RXRα agonists from the cell based-transactivation studies in HEK293 cells in the functional assays in macrophages co treatments with a specific RXR-antagonist were performed.
Cholesterol efflux was experimentally assessed as described in section 2.5.1.3. (Cholesterol Efflux in differentiated THP-1 Macrophages) with the exception that cells were pretreated 30 minutes with 1 µM of the RXR antagonist HX531[205] before the test compound treatment.

2.6.2. Experiments in HepG2 cells

2.6.2.1. HepG2 cell line

The HepG2 cell line is derived from an epithelial hepatoblastoma originating from a 15-year-old Caucasian male.[206] The cell line was established in 1979 by Barbara Knowles and colleagues but has mistakenly described as hepatocellular carcinoma for a long time although they are of epithelial origin.[207]
HepG2 cells are adherent, have an epithelial morphology and 55 chromosome pairs. They grow in monolayers but tend to form small aggregates. They have been used successfully *in vitro* to study polarized hepatocytes because of their relative high degree of morphological and functional differentiation and this is of importance for using this cell line as model for human liver diseases.
In this work HepG2 cells were used as a model system for liver steatosis (non-alcoholic fatty liver) to investigate selected honokiol derivatives whether they are able to induce the lipogenic transcriptional program and thus lipid accumulation in the liver or not.[208]

2.6.2.2. Quantification of lipids in HepG2 cells

Quantification of intracellular lipids in HepG2 cells was performed by using Oil Red O staining with subsequent isopropanol extraction and the measurement of the absorbance of the dye at 550 nm and 490 nm.
Furthermore, the fluorescent dye Bodipy®493/503 was used to stain neutral lipids and thus lipid droplets in HepG2 cells. Lipid droplets were visualized using confocal microscopy and quantified using flow cytometry.

2.6.2.2.1. Staining for lipids in HepG2 cells

Oil Red O staining is a standard assay performed to stain adipogenic cultures and to detect neutral lipids and lipid accumulation within cells. The assay is very easy and allows a fast quantification of intracellular lipids. The disadvantage is that Oil Red O does not

distinguish between different lipid species within lipid droplets because it does only stain the most hydrophobic and neutral lipids.[209]
HepG2 cells were grown in 24 well plates until they reached a confluence of around 80%.
The treatment with the compounds and appropriate controls was done in $DMEM_{complete}$ medium for 72 hours.
Cells were then fixed with 10% formalin solution at room temperature for one hour. After fixation cells were washed twice in PBS followed by one washing step with 60% isopropanol. Cells were then stained with a working solution of Oil Red O containing 0.1% Oil Red O in 60% isopropanol for 10 minutes at room temperature.
After staining the Oil Red O solution was removed and cells were carefully washed in deionized water. Finally, Oil Red O was extracted with 99% isopropanol and absorbance was measured at 550 nm and 490 nm on a Sunrise plate reader.

2.6.2.2.2. Quantification of lipid droplets using flow cytometry

Flow cytometry allows analysing particles according to their size and granularity as well as their fluorescence intensity in the presence of a fluorophore.
The principle is that particles pass a laser beam and scatter the light beam. The scattering properties depend on the physical properties of the particles. Proportional to the particle size and cell surface area is the so called forward-scattered light (FSC). The granularity and complexity of a particle or a cell is proportional to the side-scattered light (SSC). Immunostaining or staining with specific dyes of surface markers or intracellular organelles or proteins allows the additional detection orthogonal to the light stream by detectors that collect specific emission spectra of the used fluorophore.
In this work, lipid droplets were stained by using Bodipy®493/503 that specifically stains neutral lipids/non polar lipids and is thus suitable to detect lipid droplets. An accumulation of lipid droplets results in a higher fluorescence intensity of the used dye.
All measurements were performed on a FACS Calibur™ flow cytometer by illuminating the cells with an argon laser (488 nm) to detect Bodipy®493/503 emission.
In all flow cytometry measurements, a population of 10000 cells was examined.
HepG2 cells were seeded either in 12 or 24 well plates in $DMEM_{complete}$ and incubated until they reached 80-90% confluence. HepG2 cells were then treated with the test com-

pounds and appropriate controls (T0901317 and Bexarotene) in $DMEM_{stripped}$ medium and incubated for 72 hours.
Treated HepG2 cells were then washed once with PBS and collected by after tryspin digestion and resuspension in $DMEM_{complete}$ medium. Cells were then centrifuged for 4 min and 1400 rpm and the supernatant was removed. Lipid staining was performed with 1µg/mL Bodipy®493/503 in PBS for 15 minutes in the dark and investigated by flow cytometry.

2.6.2.3. Confocal microscopy of HepG2 cells

In addition to flow cytometric analysis of lipid droplets in HepG2 cells, cells were stained with Bodipy®493/503 and investigated microscopically using a confocal microscope (Leica DMi8, Leica Microsystems).
HepG2 cells were seeded on coverslips in 6-well plates at a density of 10^6 cells/well and were treated at a confluence of 80-90 % with the compounds for 72 hours.
Cells were washed twice in PBS and then fixed with 3% formaldehyde in PBS for 15 minutes. Cells were then washed in a 0.2% BSA solution in PBS three times and were then permeabililized for 2 minutes in 0.2% Triton X-100 in BSA/PBS. Lipids in HepG2 cells were then stained with 1 µg/ml Bodipy® 493/503 in PBS and counterstained with 300 nM DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) in PBS.
Imaging was performed using 488 nm (Bodipy®493/503) and 405 nm (DAPI) excitation wavelength and by using appropriate band pass filter settings.

2.6.3. Experiments in 3T3 L1 cells

2.6.3.1. 3T3 L1 cell line

3T3 L1 cells are an immortalized fibroblast cell line derived from mouse embryonic tissue (Swiss Albino mouse).[210] The cell line is a widely used pre-adipose cell line in lipid and glucose research.
3T3 L1 cells can be easily differentiated into adipocytes.[211] Adipogenesis is one of the best characterized differentiation processes.[212] Adipogenesis involves the activity of different RXR dimer compositions (RXR-Homodimers and PPARγ/RXR –Heterodimers). The different dimers activate target genes involved in glucose and lipid metabolism.[213,214]

2.6.3.2. Adipogenesis in 3T3 L1 cells

Adipogenesis experiments were started by seeding 0.1 x 10^6 3T3 L1 cells/well in 12-well plates
Cells were grown in growth medium (20% calf serum) until they reached confluence. After another 2 days the medium was changed to *adipocyte differentiation medium* containing 10% fetal calf serum, insulin, dexamethasone and IBMX (see 1.2.).
After 2 days the medium was changed to *adipocyte medium* together with the treatments of the compounds dissolved in DMSO (0.1%). The medium together with the treatments was changed every second day and on day 10 cells were stained for lipids using Oil-Red-O (see 2.6.3.3.)

2.6.3.3. Staining for lipids in adipocytes (3T3 L1)

Adipocytes were first fixed with 10% formalin solution at room temperature for one hour. After fixation cells were washed twice in PBS followed by one washing step with 60% isopropanol.
Lipid staining was done using a working solution of Oil Red O containing 0.1% Oil Red O in 60% isopropanol for 10 minutes at room temperature.

After staining the Oil Red O solution was removed and cells were carefully washed in deionized water. Finally, Oil Red O was extracted with 99% isopropanol and absorbance was measured at 550 nm and 490 nm on a Sunrise plate reader.

2.7. Cell viability assay in different cell lines (Resazurin Assay)

To assess possible cytotoxic effects of honokiol derivatives in this study a resazurin conversion assay was employed. Resazurin conversion is a relative sensitive method to determine cell viability. Resazurin is a blue non-fluorescent agent that can be chemically reduced to the fluorescent pink resofurin. Resazurin conversion depends on redox equivalents provided by the cellular metabolism. Thus, the conversion (fluorescence signal) is direct proportional to the number of viable cells and thus to cell viability.[215]

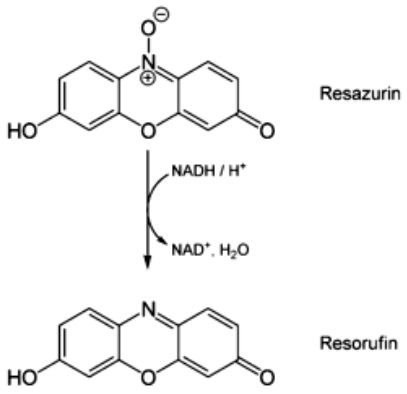

Figure 4. Conversion of Resazurin by Redoxäquivalents.

THP-1 cells were seeded at a density of 0.2 x 10^6 cells per well in 96-well plates and were then differentiated into THP-1 macrophages by incubating them in 200 nM PMA for 72 hours in RPMI-$1640_{complete}$ medium. After differentiation cells were washed in PBS and then treated with the compounds in RPMI$164_{starvation}$ medium supplemented with 0.1% BSA and 10 µg/ml unesterified cholesterol.

HEK293 and HepG2 cells were plated at a density of 40.000 cells per well in 96-well plates and incubated in $DMEM_{complete}$ medium for 24 hours. The next day cells were treated with the test compounds in DMSO.

For all assays 50 µg/ml Digitonin was used as positive control.

After 24 hours of incubation with the test compounds cells were washed again in PBS and then incubated with 10 µg/mL resazurin solution in PBS. After 4 hours plates were read on a Tecan GeniosPro plate reader using an excitation wavelength of 535 nm and an emission wavelength of 580 nm.

2.8. RNA detection by qPCR

2.7.1. Total RNA extraction

Total RNA extraction was performed by using a total RNA kit from PeqGOLD® (Peglab, Germany) according to the manufacturer´s protocol.
THP-1 cells were differentiated and treated as described in section 2.5.1.3. in 6-well plates for 72 hours and treated with the compounds and controls before RNA isolation.
HepG2 cells were seeded at a density of 10^6 cells per well in 6-well format and treated with the compounds on the next day for 24 hours in $DMEM_{complete}$ medium.
For all steps RNAse-free filter tips were used.
Cells were lysed with 400 µl of the provided lysis buffer and lysates were transferred to a DNA removing column and centrifuged for 1 min at 12.000 g.
The flow-through lysate was then mixed with an equal volume of 70% ethanol and transferred to a RNA binding column and centrifuged again for 1 min at 10.000 g to bind the RNA to the column.
After two washing steps with the provided washing solution RNA was eluted in 60 µl RNAse free water. The quality of RNA was monitored on a 1% agarose gel according to standard procedures (stained with the cyanine dye SYBR® Green I). To do so, 10 µl RNA of RNA/loading buffer mixture was loaded on the gel and electrophoresis was conducted at 150 Volt for 30 min. mRNA was considered to be present sufficiently at a ratio of 28S:18S ribosomal RNA at 2:1.
Quantification of RNA was done using a Nano Drop 2000 UV-Vis Spectrophotometer (Thermo Fisher Scientific).
RNA was considered to be "pure" at a ratio of absorbance at 260 nm to 280 nm of ~2.
RNA was stored at -80 °C until further use.

2.7.2. Reverse transcription into cDNA

cDNA synthesis was performed using a High Capacity cDNA Reverse Transcription Kit® from Applied Biosystems (Thermo Fisher Scientific) including RNAse inhibitor according to the protocol supplied by the manufacturer.
1 µg of RNA was used in a reaction volume of 20 µl.

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 °C	37 °C	85 °C	4 °C
Time	10 min	120 min	5 min	∞

Table 16. Thermocycler conditions.

After reverse transcription 30 µl of PCR-grade water was added to the samples thus getting a concentration of 20 ng/µl cDNA assuming a 100% efficiency of reverse transcription.

2.7.3. Real-time quantitative PCR (RT-qPCR)

Quantitative polymerase chain reaction (qPCR) allows the amplification of a few copies or a single copy of DNA exponentially in several orders of magnitude. In cycles of repeated heating and cooling, DNA strands are melted and enzymatic amplification of the DNA strands occurs by using specific primer sets. Quantitative PCR uses fluorescent dyes such as SYBR Green or fluorophore-containing DNA probes to measure the amount of amplified DNA in real time.

To quantitatively amplify genes of interest a Light Cycler™ LC480 SYBR Green I Master Kit (Roche Diagnostics, Vienna, Austria) was used.

The reaction mix volume was 15 µl containing 7.5 µl of Green master Mix, 1.5 µl Primer (QuantiTect Primer Assay, Qiagen/Germany), 4 µl PCR-grade water and 2 µl cDNA that was prepared according to the protocol described in section 2.7.2.

The primers used in this work are listed in section 1.6.

Quantitative real-time PCR was performed on a LigtCycler™LC480 from Roche Diagnostics and contained one denaturation step at 95°C for 10 min and 50 amplification steps, including an annealing step for 30 seconds at 55°C and an elongation step for 15 seconds at 72°C.

Data was analysed using the LigtCycler™LC480 Software and melting curves were used to make sure that the PCR resulted in amplification of only one product. Quantitative PCR was analysed according to the ΔCdt method.[216]

2.9. Protein detection by western blotting

Protein detection was performed in differentiated THP-1 macrophages and in HepG2 cells.

If not otherwise stated cells were treated for 24 hours with the indicated compounds and whole cell protein lysates were performed.

THP-1 cells were differentiated as described in section 2.5.1.2.

Briefly, 0.2 x10^6 cells/mL were seeded in 6 well plates containing 200 nM PMA and incubated for 72 hours. Differentiated THP-1 cells were then washed in PBS and treated 24 hours with the indicated compounds in serum free RPMI-medium containing 0.1% BSA and 20 µg/mL unesterified cholesterol. Whole cell lysis was performed in 280 µl freshly prepared ice cold NP-40 containing lysis buffer for 30 min while smoothly shakning at 4°C. Cells were scraped with a cell scraper and transferred into an eppedorf tube. After centrifugation at 4° (16000 g, 20 minutes) supernatants were stored in a fresh Eppendorf tube at -80°C.

HepG2 cells were seeded in 6 –well plate (1.10^6 cells/well) in DMEM complete medium and treated the next day with the indicated compounds for 24 hour in serum free DMEM medium. Whole cell lysis was performed in freshly prepared ice cold RIPA buffer and sonicated 2 times for 10 seconds at 100% power with a BANDELIN™Sonoplus (VW 2070) sonicator. After centrifugation at 4° (13.0000rpm, 20 minutes) supernatants were stored in a fresh Eppendorf tube at -80°C.

2.9.1. Protein quantification

The Bradford method was used to quantify the protein content in the whole cell lysates.[217]

An acidic solution of Coomassie Brilliant Blue G250 has an absorption maximum at 479 nm and this maximum shifts to higher wavelength (595 nm) when the solution reacts with aromatic side chains of proteins. The lysates obtained from whole cell lysis were diluted in distilled water 1:10 and mixed with the Bradford reagent 1:20 in a 96 well plate in triplicate and absorption was measured on a plate reader (sunrise). To calculate the protein content a calibration curve was made with rising concentrations of a bovine serum albumin (BSA) solution.

2.9.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

20-30 µg of protein were subjected to standard gel electrophoretic techniques using SDS-PAGE (Bio-Rad Laboratories GmbH, Vienna).
7.5-10% containing polyacrylamide (PAA)-containing gels were prepared with Rotiphorese® Gel 30 (containing 30 % acrylamide solution with 0.8 % bis-acrylamide at the ratio 37.5: 1). In each lane the same amount of protein was loaded and proteins were separated using a Mini-PROTEAN™ 3 Cell System connected to a power supply for 60 minutes and 200 Volt.

2.9.3. Immunoblotting and protein detection

Immunoblotting was performed using standard tank blotting techniques in 1y blotting buffer for 90 min at 100 mA using a Mini Trans-Blot™ Electrophoretic Transfer Cell system. Proteins were transferred to a PVDF membrane and then blocked in blocking solution. Blocking solutions contained either 5% non-fat milk powder in TBS-T or 5% BSA and blocking was performed under smooth shaking for at least 1 hour. After three washing steps, each at least 5 minutes, membranes were incubated with specific primary antibodies overnight at 4°C under smooth shaking (see section 1.6.). Membranes were again washed three times for 5 minutes and horseradish-peroxidase conjugated antibodies were added and incubated for 1 hour at room temperature. Membranes were washed and protein bands were visualized with an ECL solution and detected with a LAS-3000™ Luminescent Image Analyser. Bands were quantified semi-quantitatively using AIDA™ and the MultiGauge V3.0 software.

2.10. Data analysis and statistics

Final data analysis was performed in Microsoft Office Excel of at least three independent experiments if not otherwise stated.
Experimental data analysed initially in Microsoft Excel were then further analysed statistically and graphs were made using Graph Pad Prism 4.03.

Results and Discussion

1. Nuclear receptor transactivation by honokiolderivatives

1.1. Selective RXRα-dependent luciferase gene transactivation by honokiol derivatives

In total 55 neolignans (Appendix Figure 1) were screened initially in cell-based transactivation models in HEK293 cells and HepG2 cells for nuclear receptor transactivation. The following full-length receptors were tested: RXRα, PPARα, PPARβ/δ, PPARγ, LXRα, LXRβ and FXR.[196-198] (Appendix Table1).

We identified five honokiol derivatives selective for the nuclear receptor RXRα that did not significantly activate the other receptors above the stated screening thresholds up to a concentration of 10µM (Table 17).

	RXRα	PPARγ	PPARα	PPARβ/δ	LXRα	LXRβ	FXR
	fold activation	fold activation	fold activation	fold activation	fold activation	fold activation	fold activation
NR agonists	9-cis RA (5µM)	Pioglitazone (5µM)	GW 7647 (1µM)	GW 0742 (1µM)	GW 3965 (1µM)	GW 3965 (1µM)	GW 4064 (1µM)
	37.00	6.90	2.95	22.09	5.49	4.01	31.23
Honokiol (10 µM)	17.90	1.44	1.67	3.60	1.07	0.66	1.03
752 (10 µM)	23.46	1.67	1.31	3.00	1.67	1.35	1.19
762 (10 µM)	20.36	1.91	1.28	2.46	0.66	0.50	0.98
781 (10 µM)	18.38	1.58	1.67	3.00	1.07	0.74	1.40
2284 (10 µM)	12.10	1.18	0.91	3.90	0.78	0.91	1.03
2817 (10 µM)	22.04	2.21	1.00	3.80	1.81	1.88	1.07

Table 17: Nuclear receptor-mediated luciferase reporter gene transactivation
We identified honokiol derivatives that preferentially activated human full length RXRα in a luciferase-based transactivation model. HEK293 cells (and HepG2 cells in case of LXR) were transfected with an expression plasmid encoding the respective human nuclear receptor gene and a reporter plasmid carrying a promoter with the appropriate nuclear receptor response element coupled to a luciferase gene, and EGFP as internal control. Cells were treated for 18 h with 10 µM of the test compounds and the nuclear receptor agonist controls as shown above. Luciferase activity was normalized to EGFP and results are expressed as fold induction compared to the solvent DMSO (0.1%). Data are shown as means of at least three independent experiments performed in quadruplicate. Activation thresholds/cut-offs were set according to the sensitivity of the assay according to the response of the respective used positive controls (response in fold-activation compared to the solvent control). (PPARγ>2.5 fold, RXRα>10.0 fold, PPARα>1.5 fold, PPARβ/δ> 4.0 fold, LXR and FXR>2.0 fold).

1.1.1. Selectivity of honokiol derivatives with respect to reported receptor targets of honokiol

Honokiol has pleiotropic effects with variable pharmacological features and acts on different biological receptors.[80,218]

The naturally occurring rexinoid honokiol enhances chloride-mediated currents through the chloride channel $GABA_A$ of different subunit compositions in *Xenopus laevis* oocytes and modulates cannabinoid receptor signalling. Moreover, 4´-O-methylhonokiol (752) has been shown to activate the G-protein coupled receptor GPR55.[86,219]

The ability of the identified RXR-selective (versus other receptors) honokiol derivatives in this study to modulate $GABA_A$-mediated chloride currents was investigated for compounds 752 (4´-O-methylhonokiol) and 762 by our collaboration partners at the University of Graz. Both derivatives modulate the $GABA_A$ receptor. Compound 2284 was investigated by Lukas Rycek at the Vienna University of Technology and does not activate this chloride channel in *Xenopus laevis* oocytes.[88,219]

Furthermore, compounds 752, 762, 2284 and 2817 have been investigated towards their ability to activate the cannabinoid receptor subtypes 1 and 2 (CBR1 and CBR2).[219]

4´-O-methylhonokiol (752) and derivative 762 modulate one or both cannabinoid receptor subtypes. Derivatives 2284 and 2817 have been tested on CBR1 and CBR2 in the Gertsch laboratory by Patricia Schenker (University of Bern) and do not activate either of the subtypes at 10 µM (unpublished data).

In summary, derivatives 2284 and 2817 possess the most selective biological profile with respect to receptor activities described for honokiol.

We therefore focused in this work on honokiol derivatives 2284 and 2817.

Table 18 summarizes the receptor activity profile of honokiol in comparison to honokiol derivatives 752, 762, 782, 2284 and 2817.

	$GABA_A$	CBR	GPR55	References
Honokiol	EC_{50}: 39.3 µM	Ki: 1.8 µM (CBR2) Ki: 6.4 µM (CBR1)	nd	87,219,220
752	EC_{50}: 27.2. µM	Ki: 43.9 nM (CB2, inverse agonist) Ki: 2.4 µM (CB1) Ki: 43.3. nM (CB2)	IC_{50}: 7.7 µM	87,219,220
762	EC_{50}: 16.9 µM	Ki: 84 nM (CBR2)	nd	219,220
781	nd	nd	nd	-
2284	inactive	Inactive *	nd	89
2817	nd	Inactive *	nd	-

Table 18: Activity of honokiol and honokiol derivatives on the $GABA_A$ receptor, the Cannabinoid receptors (CBR) 1 and 2 and the G-protein coupled receptor GPR55.
*The $GABA_A$ receptor activation was studied on α1β2γ2 $GABA_A$ receptors from rat expressed in Xenopus laevis oocytes. Cannabinoid receptor activities were studied in CHO membranes. *unpublished data: Activation of CBR1 and CBR2 by honokiolderivatives 2284 and 2817 was assessed at 10 µM in CHO membranes. (Patricia Schenker, Gertsch Laboratory, University of Bern). nd: not determined*

1.2. Luciferase gene transactivation of human RXRα by honokiol derivatives 2284 and 2817 in HEK293 cells

The potency and efficacy as reflected by the EC_{50} and E_{max} values of the selected honokiol derivatives was compared to the natural occurring rexinoid honokiol and to a full agonist (9-cis retinoic acid) in concentration response experiments. Concentration response curves were calculated with increasing concentrations of the compounds.
The sigmoidal concentration-response curve indicates that compounds 2284 and 2817 are partial agonists in RXRα luciferase gene transactivation experiments as compared to the natural ligand 9-cis retinoic acid.
Concentration response curves of compounds 2284 and 2817 in comparison to honokiol and the full RXR-agonist 9-cis retinoic acid are shown in Figure 5.

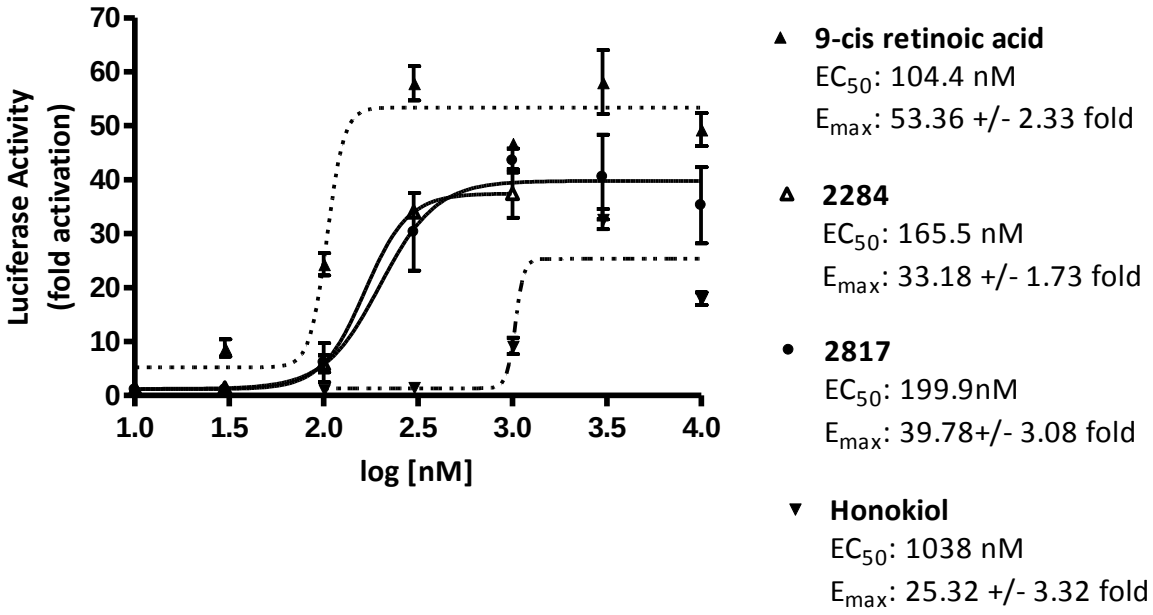

Figure 5. Concentration response curves of human full-length RXRα transactivation by honokiol and derivatives 2284 and 2817. *HEK293 cells, transfected with an expression plasmid encoding the full length RXRα receptor, a reporter plasmid containing a synthetic RXR response element followed by a luciferase gene and an EGFP expression plasmid as internal control, were treated for 18 h with increasing concentrations of the compounds and RXRα-dependent luciferase gene expression was measured. Results are expressed as fold induction compared to the solvent DMSO (0.1%). Data are shown as means ± SD of at least three independent experiments performed in quadruplicate. To calculate the EC_{50} values data were curve fitted and non-linear transformed (sigmoidal dose response with variable slope) in GraphPad™Prism 4.0.*

1.3. Luciferase gene transactivation of RXRα by 2284 and 2817 is abolished by the RXR-antagonist HX531

To further prove the RXR-dependency in the cell-based nuclear receptor luciferase reporter model in HEK293 cells, co-treatment experiments with the pan RXR-antagonist HX531 were performed.[205] The diazepinylbenzoic acid HX531 is a potent RXR antagonist that acts on all RXR-isoforms (RXRα, β and γ). HEK293 cells were treated with a receptor saturating concentration of HX531 (1 μM) and then with the indicated compounds at receptor saturating concentrations (1 μM). The conditions used in this experiment did not show any obvious impairment to the HEK293 cells when comparing the fluorescence values from GFP with the treated versus untreated or solvent control treated cells (0.1% DMSO). Luciferase activity was significantly reduced upon co-treatment indicating that the response in the cell-based assay was specific due to RXRα activation (Figure 6).

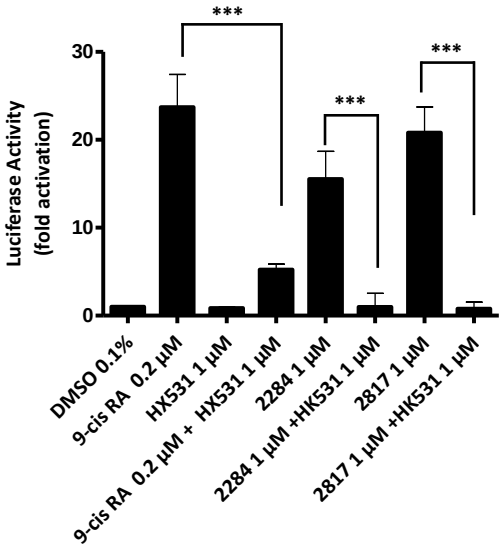

Figure 6. Co-treatment with the pan RXRα antagonist HX531 and indicated compounds.
*HEK 293 cells were transfected with an expression plasmid encoding for the full length RXRα, a reporter plasmid carrying a Renilla luciferase gene under the control of RXR response elements and an expression vector encoding EGFP as internal control. HEK293 cells were pre-treated for 30 min with 1μM HX531. Cells were then co-treated with the compounds as indicated above for 18 hours and luciferase activity was measured as described (see section 2.3.3.). Results are expressed as means ± SD of three independent experiments performed in quadruplicate. Statistical significance was calculated using One-Way ANOVA (Bonferroni post-test). p<0.001 ***, 9-cis RA: 9-cis retinoic acid.*

1.4. Nuclear receptor LBD - GAL4-DBD/UAS-dependent luciferase gene transactivation

Binding to the ligand binding domain of the receptor was investigated in a GAL4-binding assay (Mammalian one-hybrid assay). [221]

Expression plasmids encoding chimeric proteins of the yeast-specific transcription factor DNA –binding domain GAL4 and the receptor ligand-binding domain of the nuclear receptors RXRα and PPARγ, respectively. The DNA sequences in these vectors are fused in frame producing fully functional chimeric proteins. As reporter in the cell-based luciferase assays a vector is used carrying the luciferase gene under the control of an upstream activating sequence that is recognized by the GAL4-DBD. [221]

1.4.1. Transactivation of human RXRα LBD

The ability to transactivate the human RXRα ligand-binding domain was investigated for all five honokiol derivatives and their potency (EC_{50} value) and efficacy (E_{max} value) was calculated in concentration response experiments (see Appendix Table 2).

Concentration responses of compounds 2284 and 2817 are shown in Figure 8.

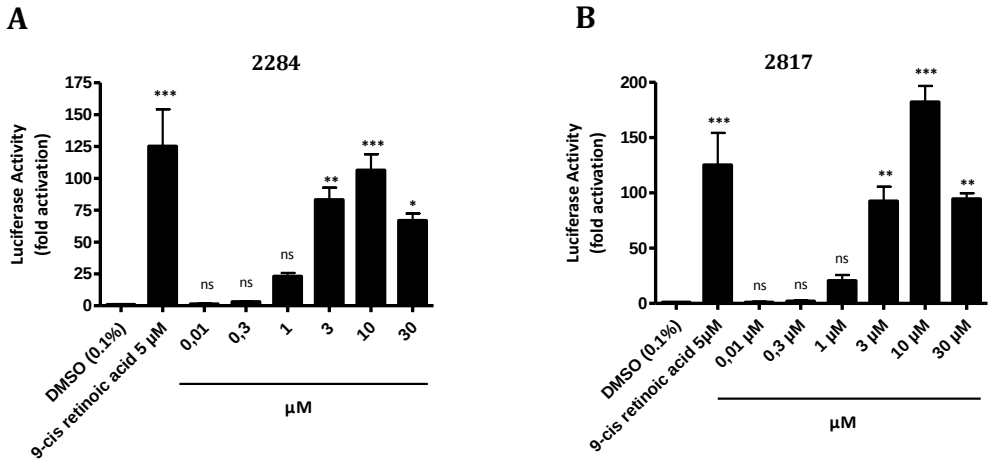

Figure 8. Concentration-dependent reporter gene expression by (A) 2284 and (B) 2817 in the hRXR-LBD- GAL4/UAS luciferase assay

*HEK 293 cells were transfected with an expression plasmid encoding a chimeric protein consisting of the yeast Gal4-DBD fused in frame to the hRXRα-LBD. The yeast GAL4 DBD recognizes specific upstream activating sequences (UAS) and the reporter (luciferase gene) used in this system is therefore fused to several UAS-elements (see 2.3.5.). Results are expressed as means ± SD of three independent experiments performed in quadruplicate. Statistical significance was calculated using One-Way ANOVA (Bonferroni post-test). p<0.05 *, p<0.001**, p<0.0001 ***, ns not significant.*

1.4.2. Transactivation of human-PPARγ LBD

The ability of 2284 and 2817 to bind the PPARγ LBD was investigated in a similar experiment as in 1.4.1. to further assess their specificity for the retinoid X receptor.
An expression vector encoding the chimeric GAL4DBD-hPPARγLBD protein was used.
Figure 9 shows the transactivation of 2284 and 2817 in comparison to honokiol and magnolol as well as the full PPAR-agonist pioglitazone at 10 µM.
There was no significant transactivation activation of the PPARγ LBD by honokiol, 2284 and 2817, whereas the positive controls pioglitazone (35.2 ±3.6 fold) and magnolol (4.1 ±0.9 fold) activates luciferase gene expression in this assay.[222]

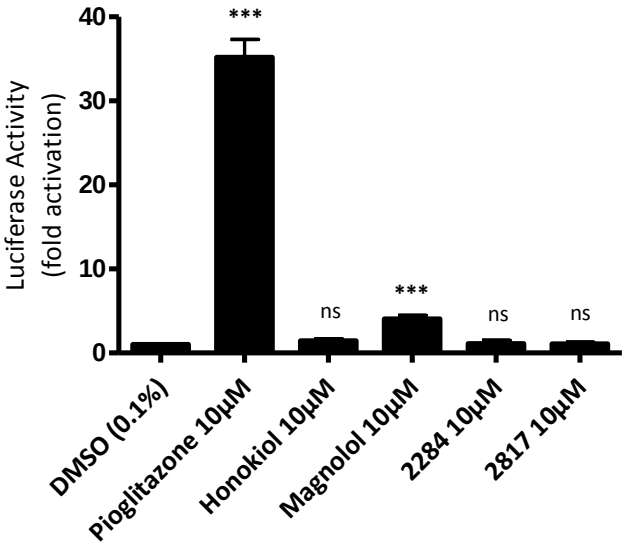

Figure 9. Transactivation of hPPARγ LBD- GAL4/UAS luciferase gene expression
HEK 293 cells were transfected with an expression plasmid encoding a chimeric protein consisting of the yeast Gal4-DBD fused in frame to the hPPARγ-LBD. The yeast GAL4 DBD recognizes the UAS sequence in the respective reporter vector containing a luciferase gene as reporter. Compounds were tested at 20 µM and incubated with transfected HEK293 cells for 18 hours. The full PPAR-agonist pioglitazone was used as positive control. Results are expressed ±SEM of three independent experiments performed in quadruplicate. Statistical significance was calculated using an unpaired t-test.

1.4.3. Concentration-dependent transactivation of RXRα- and RXRβ LBD by 2284

Concentration-dependent transactivation of the RXR ligand binding domains of the isoforms α and β in the GAL4-binding assay by 2284 was investigated and concentration-response curves are displayed in Figure 10.

First, 2284 and honokiol are not isoform specific upon binding to their LBDs. When comparing the action of honokiol and 2284 on both isoforms in terms of their E_{max} values no difference was observed between honokiol and 2284 (E_{max} of 108.3± 8.33 vs122.0± 45.69 fold for RXRα LBD and E_{max} of 50.34±2.58 versus 55.34± 6.47 fold for RXRβ LBD). However, 2284 binds with higher potency on both isoforms in comparison to honokiol. Figure 10 shows concentration response curves of 2284 in comparison to honokiol in transactivation experiments with RXRα LBD and RXRβ LBD.

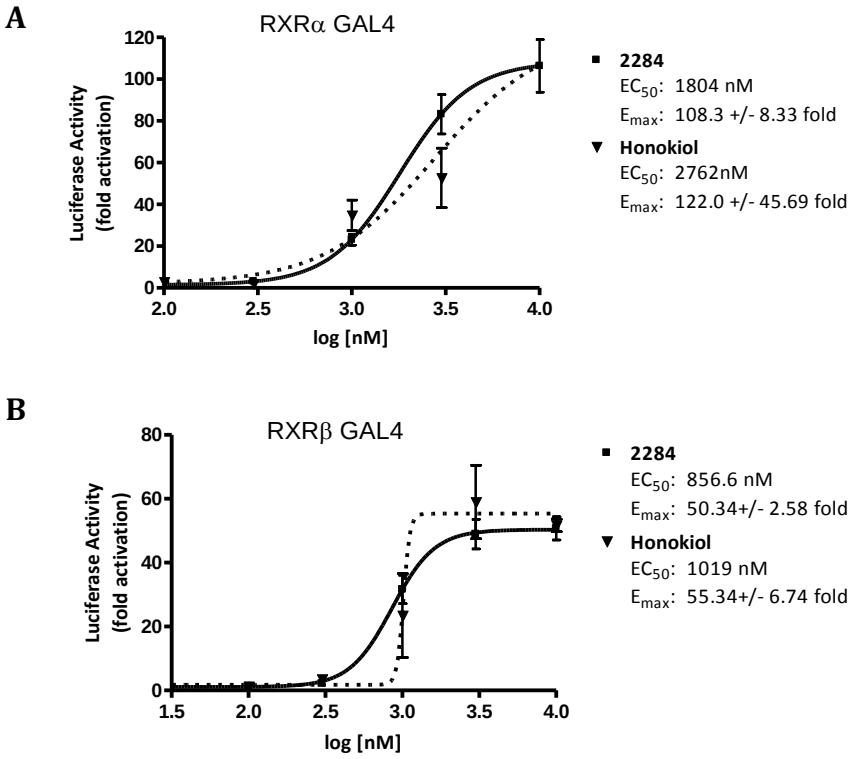

Figure 10. Concentration response curves by 2284 in comparison to honokiol in the (A) hRXRα-LBD-GAL4/UAS and (B) hRXRβ-LBD-GAL4/UAS Luciferase assay

HEK 293 cells were transfected with an expression plasmid encoding for a chimeric protein consisting of the yeast Gal4-DBD fused in frame to the hRXRα-LBD.or the hRXRβ-LBD.The yeast GAL4 DBD recognizes specific upstream activating sequences (UAS) and the reporter (luciferase gene) used in this system is therefore fused to several UAS-elements (see 2.3.5.). Results are expressed as means ± SEM of three independent experiments performed in quadruplicate.

1.5. Summary and Discussion

By using receptor specific luciferase-based testing models, we identified honokiol derivatives that transactivate RXRα-dependent but not LXRα-, LXRβ-, PPARα, β/δ, or γ– and FXR-dependent luciferase reporter gene expression (Appendix Table 1 and Figure 1) by acting as partial agonist on this receptor (Figure 5). Five of the identified honokiol derivatives transactivate reporter gene expression in an RXR-dependent way in HEK293 cells. Derivatives 752, 762, 781, 2284 and 2817 showed high potency for RXR with EC_{50} values below 1 μM in this cell-based testing model (Table 1). Derivate 2284 possesses the highest potency and is ~6-fold more potent than honokiol (EC_{50}: 1.04 μM) with an EC_{50} value of 0.17 μM. Compound 2817 is ~ 5-fold more potent then honokiol with an EC_{50} value of 0.20 μM (Figure 5). Kotani et al have identified honokiol as novel natural product rexinoid in 2010.[82] Later, it has been shown that honokiol has subsidiary roles in the activation of RXR heterodimers.[223] In *in vitro* binding studies, honokiol has been shown to act as a weak PPARγ agonist.[224] Therefore, we aimed to investigate direct binding of the derivatives to the nuclear receptor ligand binding domains (LBD) in a cell-based assay in HEK293 cells. We tested the binding of honokiol, 2284 and 2817 to the LBD of two RXR isoforms (RXRα and RXRβ) and PPARγ by using a mammalian one-hybrid assay. Here we used plasmids encoding for chimeric proteins of Gal4-RXRα LBD, Gal4-RXRβ LBD or Gal4-PPARγ LBD to transfect HEK293 cells using a Gal4-specific reporter plasmid (see section 1.6.). Compounds 2284 and 2817 and honokiol dose-dependently transactivate Gal4-driven reporter gene expression by binding to the RXR LBD (Figure 8), but do not transactivate Gal4-PPARγ LBD (Figure 9). Magnolol, another bioactive neolignan isolated from *Magnolia* species, is described as dual agonist for RXRα and PPARγ and transactivates activates Gal4-PPARγ LBD-dependent reporter gene expression in the mammalian one hybrid assay (Figure 9).[225,226] Although, honokiol has been described as weak agonist of the PPARγ *in vitro*,[224] it did not significantly transactivate the Gal4-PPARγ LBD-dependent reporter gene expression (Figure 9). Furthermore, we show that derivative 2284 transactivates both RXR isoforms, α and β, as it is described for honokiol (Figure 10).[173] Since honokiol and certain honokiol derivatives have been described to act on other receptors in the nervous system we aimed to investigate the receptor specificities of selected honokiol derivatives.[80,89,220]

Honokiol derivatives 2284 and 2817 do not activate the $GABA_A$ receptor or act on the cannabinoid receptors CB1 or CB2 up to 10 μM as it is the case for the natural compounds honokiol and 4´-O-methylhonokiol (752)(Table 17). Making it very unlikely that the newly identified honokiol derivatives may act on the GABAergic system or cross react with the cannabinoid system.[87, 88,219]

Therefore, based on this data, this work describes novel partial RXR agonists that act specifically on the retinoid X receptor we aimed to further investigate the most selective candidates (2284 and 2817) in the context of lipid metabolism.

2. Co-regulator nuclear receptor interaction studies with human RXRα LBD

2.1. Recruitment of PGC1α to human RXRα LBD by 2284 *in vitro*

To evaluate the ability of 2284 to recruit a coactivator peptide (PGC1α) to the nuclear receptor LBD of RXRα an *in vitro* a co-recruitment assay was performed with a recombinant GST-tagged RXRα LBD (Lanthascreen® TR-FRET Coactivator assay Kit).
The RXRα LBD is incubated with increasing concentrations of test compounds followed by a mixture of fluorescein-labelled coactivator peptide (PGC1α) and an anti-GST antibody labelled with terbium (Tb). Ligand-binding to the LBD induces a conformational change and thus a recruitment of the coactivator peptide to the LBD producing a FRET-signal. The ratio of 520/495 nm can then be calculated and an assessment of binding can be done by calculating the EC_{50} values out of concentration response curves.
The full RXR-agonist 9-cis retinoic acid has an EC_{50} value of 97.24 nM in this assay whereas 2284 is a partial agonist with much lower potency compared to 9 –cis retinoic acid (EC_{50} of ~1 µM)

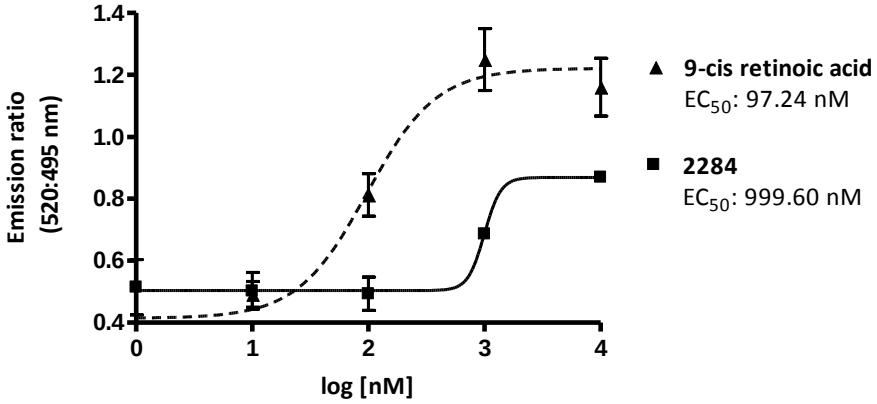

Figure 11. Recruitment of fluorescein-PGC1α coactivator by 2284 and 9-cis retinoic acid to the ligand binding domain of RXRα in vitro. *Compounds were serially diluted in DMSO and incubated with purified human RXRα LBD tagged with GST (10nM), a terbium-labeled anti-GST antibody (5nM), and fluorescently labelled PGC1α coactivator peptide (500nM). Compounds that bind to the RXRα LBD lead to the recruitment of fluorescently labelled PGC1α and thus to a fluorescence resonance energy transfer (FRET-signal). Binding is estimated from the increase of the emission ratio 520nm/495nm upon excitation at 34 nm. Data points are represented as mean ± SEM from three independent experiments performed in duplicate. EC_{50} values were calculated using a sigmoidal dose response with variable slope (GraphPad Prism 4.0).*

2.2. Coregulator-nuclear receptor interaction profiling of 2284 in comparison to full RXR agonists

A RXR-coregulator-interaction profile was made in order to be able to compare full RXR agonists (bexarotene and 9-cis retinoic acid) with honokiol derivatives that are partial agonists for RXR.

The RXR-coregulator interaction profile upon incubation with receptor-saturating concentrations of agonists was compiled by a micro array assay for real-time coregulator-nuclear receptor binding (MARCoNI, PamGene® International B. V., 's-Hertogenbosch, The Netherlands).

154 immobilized peptides, representing 64 coregulators, on the array were incubated with an isolated ligand-binding-domain of the nuclear receptor RXRα together with the ligand. Fluorophore labels on the peptides as well as on an antibody detecting the RXR ligand-binding domain (AF-2) allows to measure the receptor-peptide interaction with a time-resolved fluorescence resonance energy transfer (FRET) signal. Binding to certain peptide motifs represented on the peptide array was normalized to net binding values (solvent control DMSO) and a modulation index (MI) was calculated that is the compound-induced log-fold change of nuclear receptor binding.

A comparison of coregulator-nuclear receptor profiles of honokiol derivative 2284 and the full RXR agonists bexarotene and 9-cis retinoic acid at receptor-saturating concentrations is shown in Figure 12.

Furthermore, concentration-response experiments were performed for 2284 and bexarotene and analyzed in GraphPad Prism 4.03 using a non-linear regression with a sigmoidal dose response (variable slope). EC_{50} values were calculated for each peptide interaction and compared to bexarotene. A comparison of EC_{50} values for each peptide interaction is shown in Appendix Figure 2.

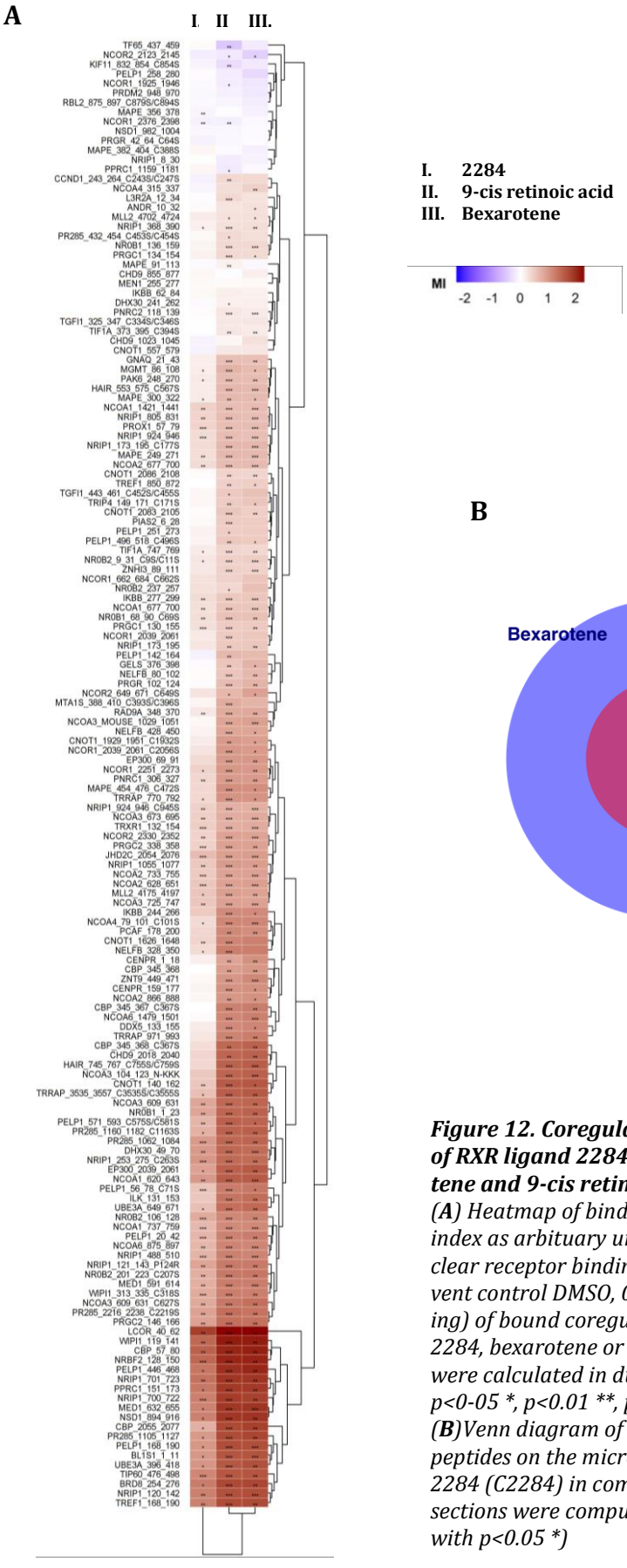

Figure 12. Coregulator-interaction profiling of RXR ligand 2284 in comparison to bexarotene and 9-cis retinoic acid

*(**A**) Heatmap of binding values (MI: modulation index as arbituary units of fold log-change of nuclear receptor binding in comparison to the solvent control DMSO, 0:no binding, >1: high binding) of bound coregulator peptides recruited by 2284, bexarotene or 9-cis retinoic acid MI values were calculated in duplex ±SEM, unpaired t-test, p<0-05 *, p<0.01 **, p<0.001***)*

*(**B**)Venn diagram of coregulators represented by peptides on the micro array bound by compound 2284 (C2284) in comparison to bexarotene (Intersections were computed upon significant binding with p<0.05 *)*

2.3. Summary and Discussion

Compound 2284 showed the highest potency for RXRα and showed the best selectivity profile with respect to reported receptor targets of honokiol (Table 17 and 18). The partial agonist 2284 was therefore further investigated towards the ability to recruit coregulators to the AF-2 of the RXR LBD.

First, to evaluate and confirm the binding of 2284 to the nuclear receptor RXRα LBD a FRET-based coactivator recruitment assay was performed (LanthaScreen® TR-FRET RXR alpha Coactivator Assay) using a PGC1-α peptide. Binding to the RXRα ligand-binding domain was assessed in a concentration-dependent manner and was compared to the full agonist 9-cis retinoic acid. Concentration-response curves show that 2284 partially recruits PGC1-α coactivator peptide (E_{max}: 0.87±0.08) whereas 9-cis retinoic acid as full agonist recruits the peptide more efficient (E_{max}: 1.22±0.06). Furthermore 2284 is around 10-fold less potent as the full agonist (Figure 11).

Additionally, a micro array assay for real-time coregulator-nuclear receptor binding (MARCoNI, PamGene® International B. V, 's-Hertogenbosch, The Netherlands) was performed to get more insights in the possible interaction partners recruited by honokiol derivative 2284. The nuclear receptor- coregulator interaction profile of 2284 was compared to the profile of full RXR agonists (bexarotene and 9-cis retinoic acid).

2284 significantly modulated RXRα interaction with multiple coregulator motifs. Interestingly, 2284 interactions represent a subset of interactions modulated by the reference agonist bexarotene and 2284 does not modulate the binding of coregulators that are not modulated by binding of bexarotene.

Bexarotene appeared to modulate the binding of 58 coregulator (represented on the microarray by 124 different coregulator peptides), whereas 2284 modulated the binding of 42 coregulators (represented by 80 coregulator peptides on the microarray since some coregulator have several interaction motifs).

14 coregulators, represented by 44 different peptides, were significantly ($p<0.05$) modulated only by the full RXR agonist bexarotene (Figure 12B). The 14 coregulators selective modulated by bexarotene are listed in the Appendix Table 3.

Furthermore, as reflected in the cell-based luciferase gene expression in HEK293 cells, 2284 appears to be less potent then bexarotene and for some coregulators the interaction appears to be partial as assessed in dose-response experiments (Figure 12, Appendix Figure 2).

3. Functional studies

The identified RXR-selective honokiol derivatives were further characterized functionally in different cell types with a main focus set on their function in human macrophages (THP-1 derived macrophages) and in a liver cell line (HepG2).
In macrophages, the ability of selected candidates to promote cholesterol efflux was examined. The cell model system used to investigate cholesterol efflux was a human monocyte-derived cell line (THP-1) and efflux of cholesterol was measured with radioactively labelled unesterified cholesterol.
Furthermore, the action of honokiol derivatives 2284 and 2817 in liver cells was studied to investigate a possible action on mainly the LXR/RXR and PPARγ/RXR regulated target genes and cellular functions. Here, a human hepatocellular carcinoma cell line (HepG2 cells) was used to assess the potential risk of activating the lipogenic gene program and thus lipid accumulation in this model.[227,228]
Functional assays were performed followed by a more detailed assessment of nuclear receptor target genes on the mRNA level as well as on the protein level.

3.1. Honokiol derivatives 2284 and 2817 promote cholesterol efflux in differentiated THP-1 macrophages

Cholesterol efflux in differentiated THP-1 macrophages was measured with radioactively labelled cholesterol as described (2.6.1.3.).
ApoA1-mediated cholesterol efflux in differentiated THP-1 macrophages was measured upon treatment for 24 hours with honokiol derivatives or a full RXR-agonist as control (Appendix Figure 4). Cholesterol efflux, mediated by 2284 or 2817and the full RXR-agonist bexarotene as control is shown in Figure 13 (A).
To prove the RXR-dependency of this effect co-treatment with the pan-RXR antagonist HX531 were performed.
In this experiments cells were pre-treated 30 min with the pan-RXR-antagonist HX531 (1 µM) before the actual treatment with the indicated compounds for 24 hours (Figure 13 (A)).

Furthermore, human plasma-mediated cholesterol efflux was measured as described (2.6.1.3.) in a similar experiment to imitate more physiological conditions in this functional cell model.

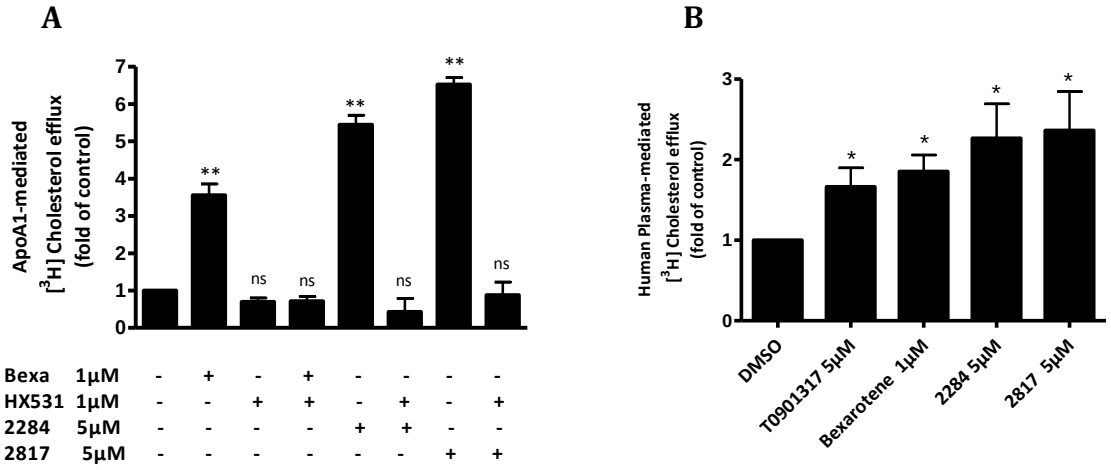

Figure 13. Honokiol derivatives 2284 and 2817 promote cholesterol efflux in human THP-1 macrophages. *(**A**) Differentiated THP-1 macrophages were treated for 24 hours with the indicated compounds and cholesterol efflux was measured with radioactively labelled unesterified cholesterol. Co-treatment experiments with the pan RXR-antagonist revealed that the efflux effect is mediated by RXR.*
Results are shown as fold activation compared to the solvent control DMSO (0.2%) and are expressed as average of three independent experiments ±SEM performed in duplex (One-Way ANOVA, Dunnett´s statistical test; p<0.05, p<0.01 **, ns not significant) (**B**) Human Plasma-mediated Cholesterol efflux in differentiated THP-1 macrophages. Human Plasma-mediated cholesterol efflux was measured in a similar experiment as in (A) after treatment with the indicated compounds for 24 hours by using human plasma (1%) as acceptor. Results are displayed as fold activation compared to the solvent control DMSO (0.1%) and are expressed ±SEM performed in duplicate (One-Way ANOVA, Dunnett´s statistical test; p<0.05*, p<0.01 **, ns not significant) T0901317: full LXR-agonist, Bexarotene: full RXR agonist.*

3.1.1. Influence of 2284 and 2817 on mRNA levels of RXR target genes in differentiated THP-1 macrophages

The sterol transporters ABCA1 and ABCG1 and the scavenger receptor SR-BI are the main transporters responsible for mediating cholesterol efflux in macrophages. The transcription of these transporters is activated by permissive nuclear receptor heterodimers with liver X receptors and PPARγ.[204] Previous studies have shown that honokiol activates ABCA1 transcription in a reporter gene assay in in U251-MG, a human glioma cell line that stably expresses an ABCA1-promoter luciferase reporter.[173]
Therefore, and because of the potent cholesterol efflux promotion in the functional assay, the activation of the target ABCA1, ABCG1 and SR-BI were investigated by quantitative PCR.
THP-1 monocytes were differentiated into macrophages for 72 hours and then treated for 24 hours with saturating concentrations of honokiol derivatives 2284 and 2817 (5 µM). The full LXR-agonist T0901317 (1 µM) and the full RXR-agonist bexarotene (1 µM) served as positive controls.
Results are shown in Figure 14 and are expressed as fold activations normalized to the solvent control condition (DMSO 0.1%).

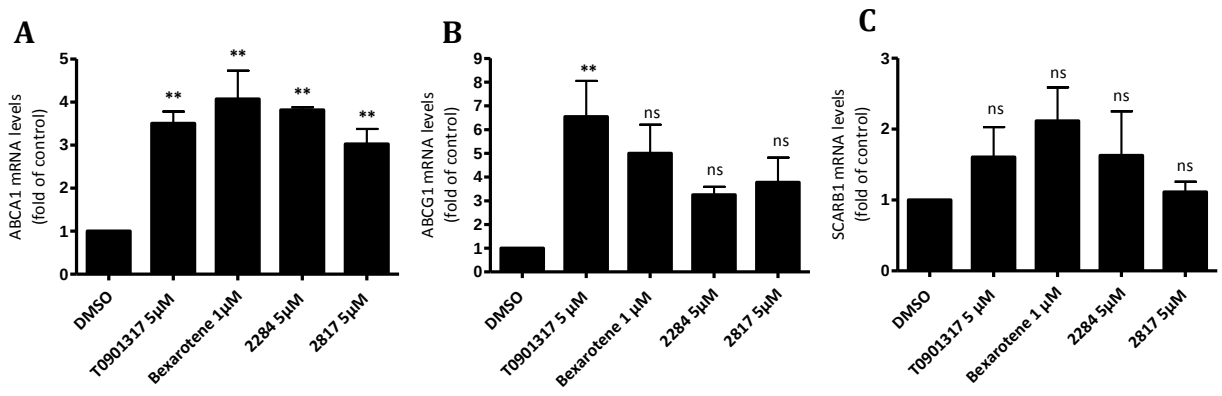

Figure 14. Honokiol derivatives 2284 and 2817 increase mRNA levels of the sterol transporter ABCA1(A) but not ABCG1 or SR-BI/SCARB1 (B and C) after 24 hours
Differentiated THP-1 macrophages were treated for 24 hours with 5 µM 2284 or 2817 and with the indicated positive controls (LXR agonist T0901317 5 µM and RXR agonist Bexarotene 1µM).
mRNA was quantified after RNA extraction and reverse transcription into cDNA. Results are displayed normalized to 18S ribosomal RNA. Graphs show mean values ±SEM relative to the solvent control DMSO (0.1%). (***A***) *ABCA1 mRNA levels,* (***B***) *ABCG1 mRNA levels,* (***C***) *SCARB1 mRNA levels. (One Way-ANOVA, Dunnett´s statistical test; p<0.01 **, ns not significant).*

3.1.2. 2284 and 2817 increase protein levels of the cholesterol transporters ABCA1 and SR-BI in differentiated THP-1 macrophages

The potent efflux of cholesterol by 2284 and 2817 seems to be due to an increase of protein level of the primary sterol transporter ABCA1 (Figure 15).
After 24 hours treatment with the indicated compounds (5 µM), protein levels of this transporter are increased around 3-fold comparable to the full LXR-agonist T0901317 (5 µM) and the full RXR-agonist bexarotene (1 µM).
Furthermore, the protein levels of the scavenger receptor SR-BI were slightly upregulated (around 1.5-fold) after 24 hours.
The sterol transporter ABCG1 was not investigated in this experiments due to the lack of an available specific ABCG1-antibody for western blot experiments.

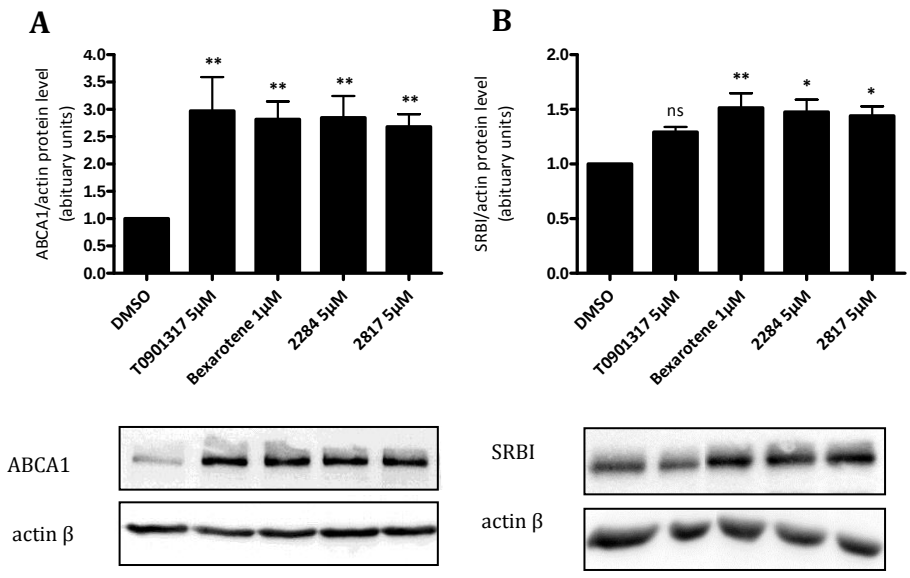

Figure 15. Honokiol derivatives 2284 and 2817 increase protein levels of the sterol transporter ABCA1 (A) and the scavenger receptor SRBI (B) after 14 hours
Differentiated THP-1 macrophages were treated for 24 hours with 5 µM 2284 or 2817 and with the indicated positive controls (LXR agonist T0901317 5 µM and RXR agonist bexarotene 1 µM).
Cells were lysed and protein levels were quantified by Western Blots analysis. Results are normalized to β-actin as loading control. Graphs show mean values ±SEM relative to the solvent control DMSO (0.1%).
(A)ABCA1,(B) SR BI protein levels. (One-Way ANOVA, Dunnett´s statistical test; p<0.05, p<0.01 **, ns not significant)*

3.2. Liver steatosis model in HepG2 cells

The regulation of hepatic gene expression in lipid and cholesterol metabolic functions is largely due to the action of permissive nuclear receptor heterodimers with the liver X receptors. The activation of LXR/RXR in hepatic cells plays an important role in cholesterol homeostasis in the liver. The activation of LXR/RXR in liver cells induces the transcription of genes involved in the mobilisation and transport of cholesterol such as the ATP-binding cassette (ABC) transporters ABCA1 or ABCG1. [229,230] Besides the cholesterol homeostatic gene program, activation of LXR/RXR induces a lipogenic gene program that lead to the synthesis of triglycerides within those cells via activation of certain lipogenic transcription factors such as SREBP1c.[231]

Furthermore, also direct activation of genes that encode for enzymes involved in lipogenesis are regulated by LXR/RXR. Examples are FASN or ACC.[232,233]

The pharmacological activation of this permissive heterodimer *in vivo* has been shown to result in the development of hyperglyceridemia and fatty liver (liver steatosis) that is an unwanted side effect.[234,235]

Functional investigations of the identified RXR-selective honokiolderivatives 2284 and 2817 were performed to assess the possible capacity of these rexinoids to induce a lipogenic gene program via the activation of permissive nuclear receptor heterodimers and hence lipid droplet accumulation in liver cells.[236]

Lipid droplet accumulation was experimentally assessed with different staining methods to account for the neutral fat content as well as the whole fat content.

HepG2 cells were treated with the indicated compounds and treated for 72 hours under $DMEM_{complete}$ conditions. First, neutral lipids were stained with the fluorophore Bodipy®493/503 and DAPI to stain cell nuclei and were then investigated by confocal microscopy. (Figure 16A).

Overall neutral lipid content was then quantified by using FACS by measuring fluorescence intensity of the lipid dye Bodipy493/503 (Figure 16B).

Finally, an Oil-red O staining was performed in HepG2 cells treated for 72 hours with the compounds (Fugure 16C).

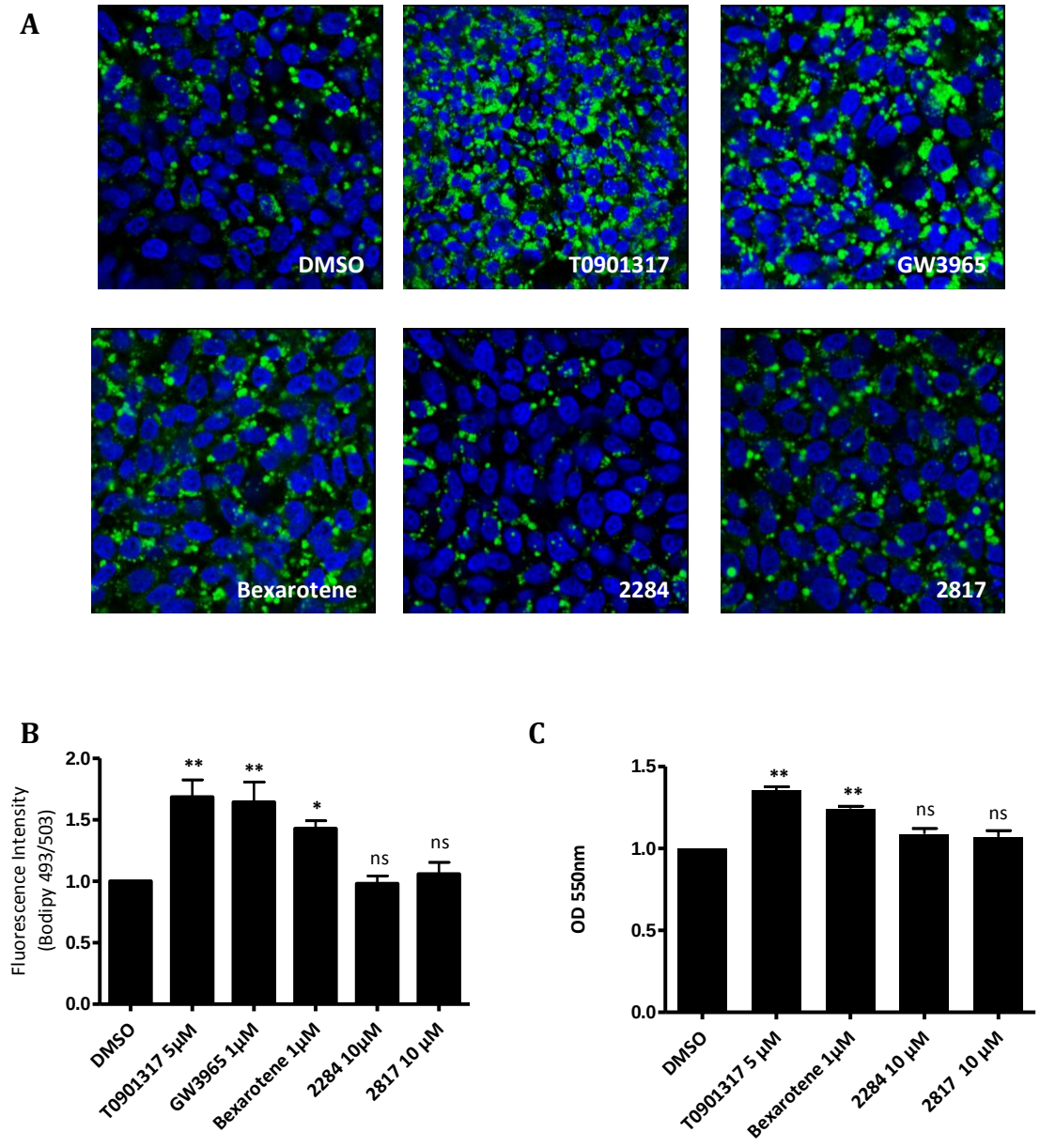

Figure 16. Lipid droplet accumulation in HepG2 cells upon treatment with full LXR-agonists (T0901317, GW3965) and a full RXR-agonist (Bexarotene) in comparison to honokiol derivatives 2284 and 2817

*(**A**) HepG2 cells were treated for 72 hours with the indicated compounds (0.1% DMSO as solvent, T0901317 5 µM, GW3965 and bexarotene 1 µM, 2284 and 2817 10 µM) and lipid droplets were stained with the lipid dye and green fluorophore Bodipy®493/503. Counterstaining was peformed with DAPI that stains nuclei. Images were taken on a Leica Confocal microscope and representative images are shown. (**B**) HepG2 cells were treated for 72 hours with the indicated compounds and stained with the lipid dye Bodipy®493/503 and lipid droplets were measured with FACS. Results are expressed as mean±SEM normalized to the solvent control DMSO (0.1%). (**C**) HepG2 cells were treated for 72 hours and an Oil-red O staining was performed. Stained Oil-red O was then extracted with 2- propanol and absorbance at 550 nm was measured. Results are expressed as measn±SEM compared to the solvent control (One Way-ANOVA, Dunnett´s statistical test: p<0.05*, p<0.01 **, ns not significant)*

3.2.1. Influence of 2284 and 2817 on mRNA levels of permissive heterodimer LXR/RXR target genes in HepG2 cells

Target genes of permissive LXR/RXR heterodimers were selected to investigate the mechanism of action of honokiol derivatives 2284 and 2817. RNA levels of selected target genes were quantified in HepG2 cells upon treatment with 2284 and 2817 and compared with the activation of LXR/RXR target genes by the full RXR agonist bexarotene and the full LXR agonist T0901317.

HepG2 cells were treated for 24 hours with the indicated compounds, RNA was extracted, and reverse transcribed into cDNA. RT-qPCR was performed with the appropriate primers for selected target genes.

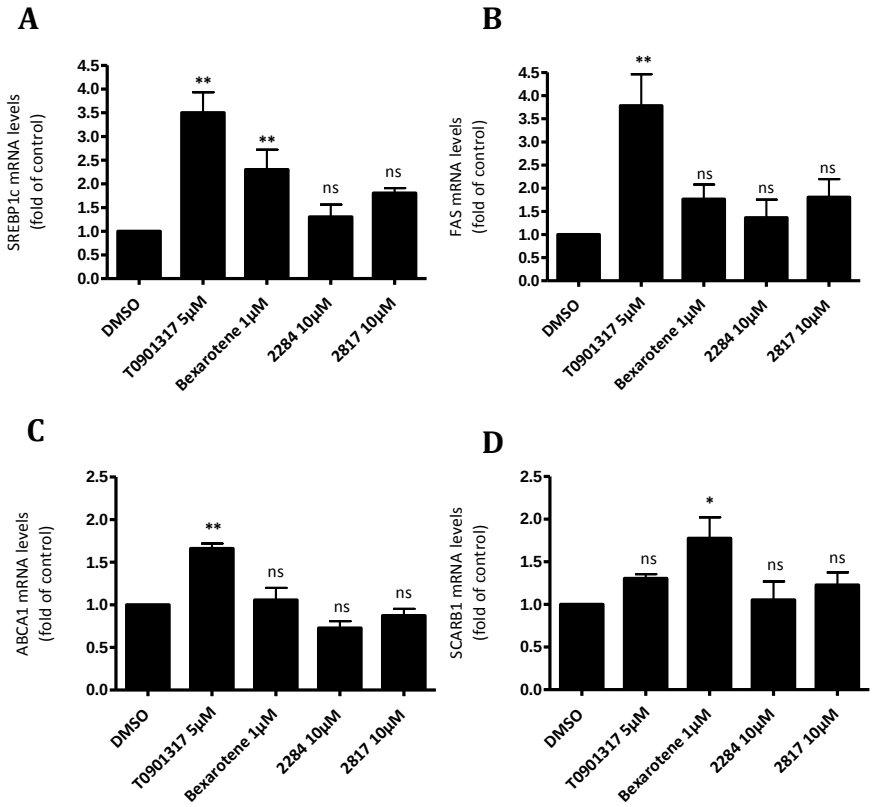

Figure 17. Honokiol derivatives 2284 and 2817 do not increase mRNA levels of the lipogenic transcription factor SREBP1 (A and B) and non-lipogenic genes (C and D)

HepG2 cells were treated for 24 hours with 10 µM 2284 or 10 µM 2817 and with the indicated positive controls (LXR agonist T0901317 5 µM and RXR agonist Bexarotene 1 µM).
mRNA was quantified after RNA extraction and reverse transcription of cDNA. Results are displayed normalized to 18S ribosomal RNA. Graphs show mean values ±SEM relative to the solvent control DMSO (0.1%).
*(**A**) SREBP1 mRNA levels, (**B**) FAS mRNA levels, (**C**) ABCA1 mRNA levels and (**D**) SCARB1 mRNA levels. (One Way-ANOVA, Dunnett´s statistical test; p<0.01 **, ns not significant).*

3.2.2. Influence of 2284 and 2817 on protein levels of permissive heterodimer LXR/RXR target genes in HepG2 cells

In order to investigate the mechanism of action of honokiol derivatives 2284 and 2817 in hepatocellular lipid metabolism, the protein expression of SREBP-1c, the key transcription factor of lipogenic genes, was investigated in more detail.
Proteolytic cleavage of membrane bound precursor SREBP-1 to nuclear mature SREBP1 is required for activation of lipogenic target genes by this transcription factor. Mature SREBP1 activates transcription of genes that control fatty acid and TAG-synthesis as well as cholesterol synthesis.[237]
HepG2 cells incubated with 2284 had no effect on SREBP1 protein levels, neither the precursor nor the mature protein, while 2817 increased protein levels of this transcription factor modestly. However, bexarotene did not show any effect on protein levels either under these conditions (24-hour treatment) (Figure 18).

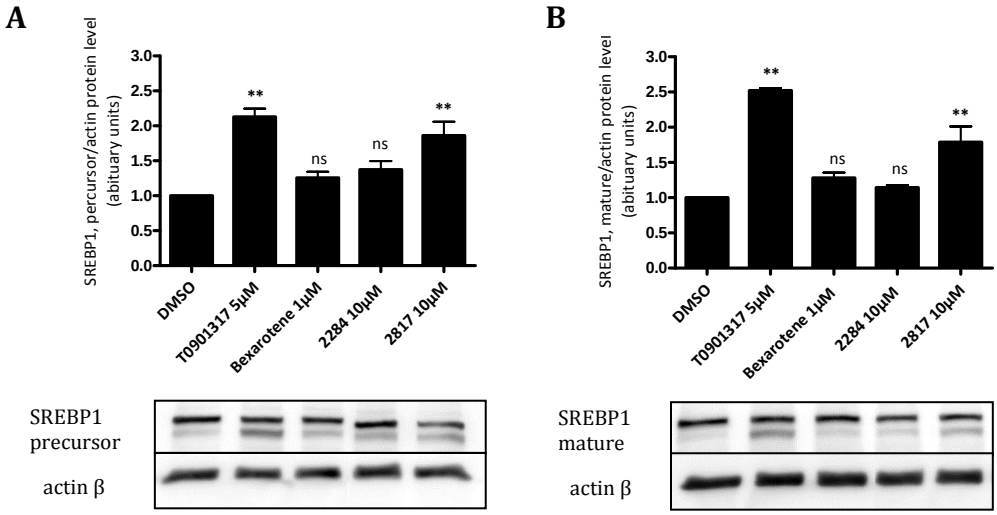

Figure 18. Impact of. Honokiol derivatives 2284 and 2817 on the protein levels of SREBP1 precursor (A)and mature SREBP1(B)
HepG2 cells were treated for 24 hours with 10µM 2284 or 2817 or with the indicated positive controls (LXR agonist T0901317 5 µM and RXR agonist Bexarotene 1 µM).
Cells were lysed and protein levels were quantified with Western Blot analysis. Results are normalized to β-actin as loading control. Graphs show mean values ±SEM relative to the solvent control DMSO (0.1%).
SREBP1 protein levels were quantified with one antibody that detects both precursor (125kDa) and mature (68 kDa) SREBP1. (One-Way ANOVA, Dunnett´s statistical test; p<0.05, p<0.01 **, ns not significant)*

3.2.3. RXR/LXR driven transactivation of the SREBP promoter by 2284 and 2817

The ability of honokiol derivatives 2284 and 2817 to drive the promoter of SREBP1 was further investigated in transactivation experiments in HepG2.
HepG2 cells or HEK293 cells were transfected with a reporter plasmid carrying a luciferase gene under the control of the SREBP1 promoter and an expression plasmid for EGFP as internal control.
HepG2 cells and HEK293 cells were then incubated with the honokiolderivatives 2284 and 2817 at 10µM and the indicated positive controls (the full synthetic agonists for LXR, T0901317, and RXR, bexarotene) for 24 hours and 18 hours, respectively.
After cell lysis, luciferase activity was measured and is expressed in Figure 19 normalized to the internal control EGFP.

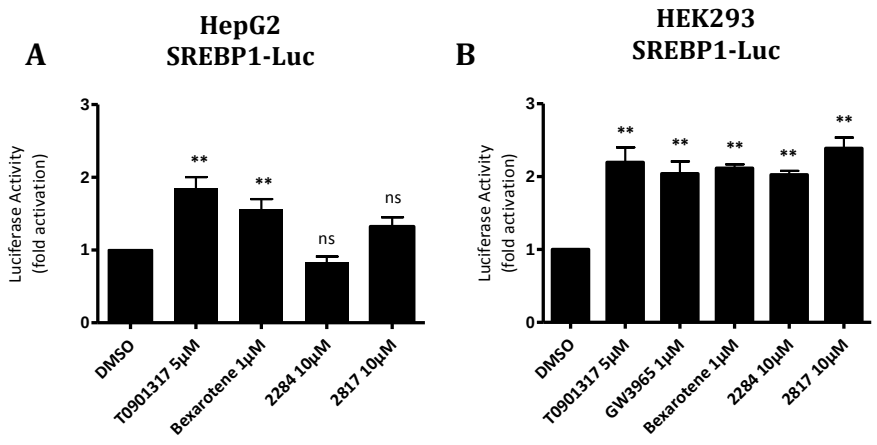

Figure 19. Stimulation of the SREBP1 promoter by honokiol derivatives 2284 and 2817 in HepG2 cells(A) and HEK293 cells (B)
HepG2 cells and HEK293 cells were transfected with a luciferase reporter construct containing the wild type SREBP1 promoter. Cells were then treated with 10 µM 2284 or 2817 or with the indicated positive controls (LXR agonist T0901317 5 µM and RXR agonist Bexarotene 1 µM).
Cells were lysed and luciferase activity was measured and normalized to the internal control EGFP.
Results are displayed as fold activation as mean values ±SEM relative to the solvent control DMSO (0.1%).
*(One-Way ANOVA, Dunnett´s statistical test, p<0.01 **, ns not significant)*

3.3. Adipogenesis in 3T3-L1 cells

Honokiol has been shown to act as a weak partial PPARγ agonist with no adipogenic potential.[224]

The honokiol derivatives selected for this study did not show significant transactivation of PPARγ in HEK293 cells as shown in Table 17.

However, the derivatives 2284 and 2817 were functionally tested whether they possess any adipogenic potential in murine 3T3-L1 cells to exclude any tissue selective activation of PPAR/RXR permissive heterodimers.

3T3-L1 preadipocytes were grown to superconfluency and were induced to differentiate into adipocytes and accumulate lipid droplets in a PPAR-dependent way upon treatment with insulin (1µg/mL) and the indicated compounds as shown in Figure 20.

Cells were then stained with the lipophilic dye Oil Red O and bound dye was 2-propanol extreacted and measured spectrophotometrically at 550nm.

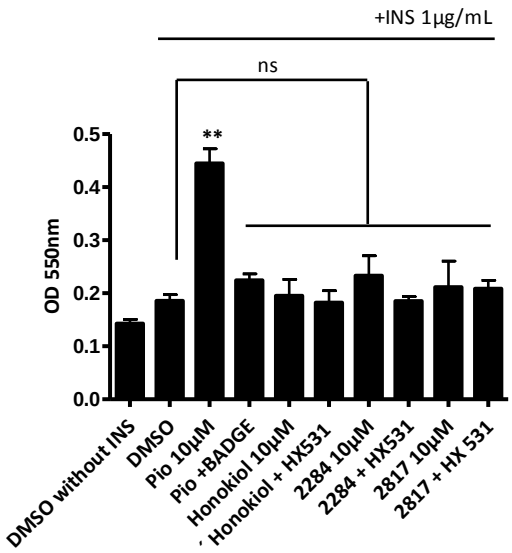

Figure 20. Adipogic potential of honokiol derivatives 2284 and 2817 in comparison to honokiol and the full PPARγ agonist pioglitazone (Pio)

*3T3-L1 preadipocytes, murine fibroblasts, were differentiated into adipocytes and lipid accumulation was induced in a PPAR-dependent way by treatment with insulin (1 µg/mL). Pioglitazone at 10 µM (Pio) was used as positive control. A set of cells was pre-treated with the respective antagonists for 30 minutes to assess the nuclear receptor dependency. BADGE (50µM) is a PPARγ antagonist and HX531 (1 µM) is a pan RXR-antagonist. After 10 days, adipogenesis was measured by measuring lipid accumulation within those cells using the lipogenic dye Oil red O. Results are displayed as fold activation as mean values ±SEM relative to the solvent control DMSO (0.2%). (One-Way ANOVA, Dunnett´s statistical test, p<0.01 **, ns not significant)*

3.4. Influence of honokiol derivatives on the cell viability in different cell lines

In order to assess possible cytotoxic effects of honokiol derivatives used in this study a Resazurin conversion assay was performed.

Cell lines that were used for functional and mechanistic studies (differentiated THP1-cells, HepG2 cells and HEK293 cells) were treated for 24 hours with the derivatives up to 30 µM and Resazurin reduction was measured by measuring the red fluorescence of Resofurin.

Cell viability of differentiated THP-1 macrophages, HepG2 cells and HEK293 cells upon treatment with derivative 2284 for24 hours is shown in Figure 21.

Differentiated THP-1 cells did not tolerate higher concentrations of 2284 as 10 µM for 24 hours and showed a reduction in cell viability greater than 80% at 30 µM. 2284, however, did not alter significantly cell viability up to 30 µM in the other cell types.

Cell viability data for all five honokiol derivatives are shown in the Appendix (Appendix Figure 5).

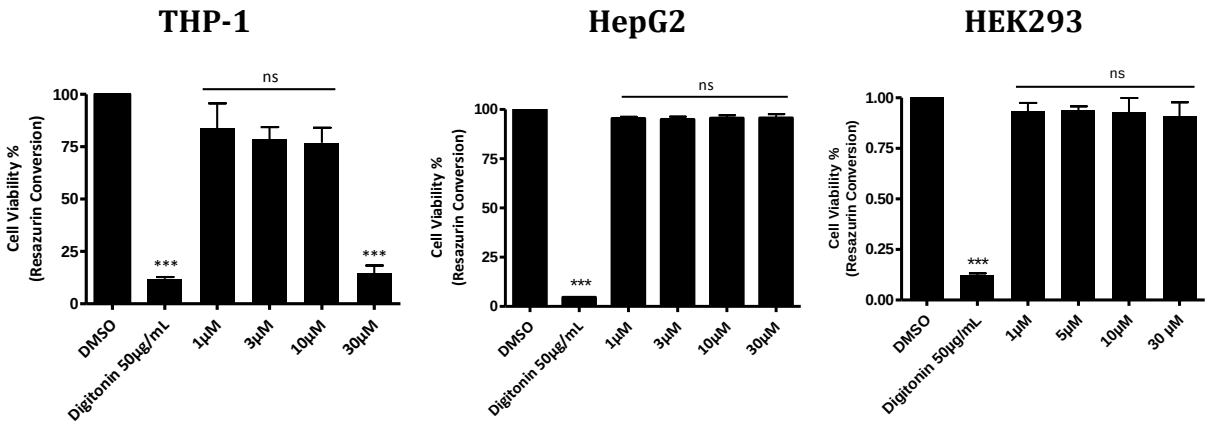

Figure 21.Influence of Honokiolderivative 2284 on cell viability as measured by resazurin conversion in differentiated THP-1 macrophages HepG2 cells and HEK293 cells

*The different cell lines were treated for 24 hours with derivative 2284 at different concentrations and resazurin conversion was measured. Digitonin (50 µg/mL) was used as a positive control. Data are expressed as mean values ±SEM normalized to the solvent control DMSO (0.1%). (unpaired t-test, p<0.001 ***, ns not significant)*

3.5. Summary and Discussion

Cholesterol homeostasis in macrophages plays a crucial role in metabolic diseases and macrophages are the most prominent cells that accumulate cholesterol in atherosclerosis to form foam cells that drive inflammation and the disease.[238,239]
Cholesterol efflux out of macrophages is one proposed strategy to treat and prevent cardiovascular diseases.[239] One possibility to promote reverse cholesterol transport in macrophages would be to target nuclear receptors such as the retinoid X receptor.[240]
In this study we aimed to identify novel RXR agonists that promote cholesterol efflux out of macrophages with an improved side effect profile since full RXR agonists have been shown to induce hypertriglyceridemia and steatosis in the liver *via* the activation of the permissive LXR/RXR heterodimer.[241,242]
To evaluate the biological function of honokiol derivatives identified as potent, partial RXR-selective agonists (section 1) functional assays in human monocyte derived form THP-1 macrophages and a human liver cell line (HepG2 cells) were performed.
Four of five honokiol derivatives (752, 781, 2284 and 2817) significantly promote cholesterol efflux in THP-1 derived macrophages (Appendix Figure 4).
Compound 2284 at 5 µM increases ApoAI-mediated cholesterol efflux in this functional model ~6-fold compared to the solvent control DMSO upon treatment for 24 hours, whereas compound 2817 at 5 µM was even stronger and increases ApoAI-mediated cholesterol efflux ~7-fold compared to the solvent control. This effect was dependent on the activation of RXR in this cell model, since co-treating the cells with the pan RXR-antagonist HX531 at 1 µM completely abolished this effect (Figure 13A). Furthermore, both derivatives, 2284 and 2817, significantly promote human plasma-mediated cholesterol efflux in this functional cell model. (Figure 13 B).
To exclude possible cytotoxic effects of the honokiol derivatives in THP-1 derived macrophages a sensitive cell viability assay was performed (Figure 21, Appendix Figure 5). Compounds 2284 and 2817 did not significantly alter cell viability as measured by resazurin conversion in differentiated THP-1 macrophages up to a concentration of 10 µM and 0.1% DMSO (solvent). At 30 µM both derivatives decreased the ability of converting resazurin more than 80 %. However, concentrations of honokiol derivatives used for functional assays in THP-1 derived macrophages never exceeded 5 µM.
The biological evaluation of derivatives 2284 and 2817 was assessed on the transcriptional and translational level of selected target genes of permissive nuclear receptor di-

mers in human THP-1-derived macrophages. Both honokiol derivatives at 5 µM significantly increased gene transcription of the primary sterol transporter ABCA1 after 24 hours treatment around 3-4 fold (2284: 3.81 ± 0.13 fold , 2817: 3.03 ±0.70 fold) in a similar manner as the full RXR-agonist bexarotene at 1 µM (3.47 ±1.56 fold) and the full LXR-agonist T0901317 at 1 µM (3.23 ±0.87 fold). The sterol transporter ABCG1 and the scavenger receptor SCARB1/SR BI mRNA was not significantly affected by 2284 or 2817 (Figure 14). The influence of 2284 and 2817 was furthermore reflected on the protein level of ABCA1. Here, the ABCA1 protein levels were increased around 3-fold upon treating THP-1 macrophages for 24 hours with 5 µM of 2284 or 2817.

Interestingly, although compounds 2284 or 2817 increased transcription of the primary sterol transporter mRNA to a similar extend compared to full agonists of RXR or LXR, the increase in ApoA1-mediated cholesterol efflux by these honokiol derivatives was remarkably higher compared to the full agonist bexarotene. Derivative 2284 was around 50% more efficient in promoting ApoA1-mediated cholesterol efflux and 2817 around 80% more efficient under these conditions. This indicates that it is very likely that another mechanism of action of the two honokiol derivatives 2284 and 2817 might be involved in promoting cholesterol efflux within THP-1 macrophages. However, human plasma-mediated cholesterol efflux was not significantly different affected by 2284 and 2817 at 5 µM (2.13 ±0.83 and 2.19 ±0.92 fold efflux of solvent control, respectively) in comparison to bexarotene at 1 µM (1.75 ±0.49 fold efflux of solvent control).

Investigation of the protein levels of the scavenger receptor B1 (SR BI) might explain the stronger functional effects of 2284 and 2817 (Figure 15). Both derivatives increase the levels of SR BI significantly (2284: 48 ±19 %, 2817: 44 ±15%) after 24 hours.

One possible mechanism might be that the derivatives activate permissive PPAR heterodimers in THP-1 cells leading to activation of the transcription of the scavenger receptor SR BI, since the scavenger receptor B1 is a known PPAR-target gene. [243,244]

However, other mechanisms of action cannot be excluded. Secondly, the biological evaluation of derivatives 2284 and 2817 in functional assays was performed in a liver cell line. (HepG2 cells). Activation of the permissive heterodimer LXR/RXR in the liver increases cholesterol secretion and bile acid production via the activation of CYP7A1 but also increases lipogenesis via the activation of other factors such as SREBP1 and ChREBP.[245] The pharmacological activation of this lipogenic genes via the permissive

nuclear receptor heterodimer LXR/RXR induces fat accumulation and thus leads to fatty liver (liver steatosis).[214,215]

2284 and 2817 did not induce significant accumulation of lipid droplets (neutral lipids) or overall intracellular fat in a steatosis model in HepG2 cells at 10 µM over 72 hours in comparison to bexarotene or the full LXR agonist T0901317 (Figure 16).

On the transcriptional level mRNA levels of the lipogenic transcription factor SREBP1 or the direct LXR/RXR target gene FAS (fatty acid synthase) were not upregulated by 2284 nor 2817 after 24 hours, whereas this was the case by treating HepG2 cells with full agonists for LXR or RXR (Figure 17).

However, having a closer look on the protein levels of either the precursor or the mature SREBP1 protein levels upon treatment of HepG2 cells for 24 hours with 2817 showed a slight upregulation of this lipogenic transcription factor whereas 2284 did not show any effect on SREBP1 protein levels (Figure 18).

In transactivation experiments in HepG2 cells using a SREBP1-promoter luciferase reporter vector both derivatives, 2284 and 2817 did not significantly transactivate reporter gene expression in comparison to bexarotene or T0901317. However, 2817 showed a higher tendency of activation, although this was not significant in this assay.

Furthermore, performing the same experiment in HEK293 cells, both honokiol derivatives induced luciferase reporter gene expression significantly compared to the solvent control (Figure 19). This contradictory results of the same assay in two different cell lines (HepG2 cells and HEK293 cells) indicate that transactivation of permissive RXR-heterodimers by these honokiol derivatives depends on the cell type, most likely on the absence or presence of coregulators or other factors.

HEK293 cells transfected with either the permissive LXR/RXR or PPARγ/RXR heterodimer treated with a full agonist for the partner receptor together with 2284 (T0901317 for LXR and pioglitazone for PPARγ) were performed. 2284 synergistically activated PPARγ/RXR –dependent reporter gene expression together with pioglitazone. No synergistic effect was observed by 2284 together with the full LXR agonist T0901317 on the LXR/RXR heterodimer (Appendix Figure 6). This was surprising, since 2284 activates ABCA1 target gene expression in THP-1 derived macrophages and ABCA1 is described to be a target gene of the LXR/RXR heterodimer in human macrophages.

Although 2284 synergistically transactivates the permissive PPARγ/RXRα heterodimer in HEK293 cells, compounds 2284 and 2817 did not show any adipogenic effect in 3T3-

L1 cells as this is the case for full PPARγ agonists (pioglitazone) indicating that there is no activation of the permissive heterodimer PPARγ/RXR.
This further shows that the action of this selective RXR modulators strongly depends on the cell type.

Summary and Conclusion

In summary, this work describes novel selective RXR modulators structurally based on the natural compound honokiol. Receptor-specific luciferase-based testing models in HEK293 and HepG2 cells led to the identification of 5 asymmetric honokiol derivatives that selectively transactivate RXRα-dependent gene expression. The identified derivatives possess a much higher potency for the receptor compared to honokiol (EC_{50}: 1.04 µM) as partial agonists. Compound 2284 is the most potent derivative (EC_{50}: 0.17 µM), showing a ~6-fold higher potency than honokiol. Additionally, compound 2284 showed the highest selectivity towards other receptors that are activated by honokiol such as certain subunit-compositions of the $GABA_A$ receptor or the cannabinoid receptors. In fact, 2284 did not act on any receptor honokiol has been described to act on up to 10 µM.[199] Selected honokiol derivatives were functionally investigated in human macrophages (differentiated THP-1 monocytes) and a human liver cell line (HepG2). Pharmacological activation of the permissive nuclear receptor heterodimers LXR/RXR leads to increased ABCA1 expression and thus promotes cholesterol efflux within macrophages.[223,246] This is one proposed strategy for the treatment or prevention of atherosclerosis.[247,248] Four of five honokiol derivatives potently promote cholesterol efflux in differentiated THP-1 macrophages. 2284 increases ApoA1-mediated cholesterol efflux around 6-fold compared to the solvent control and compound 2817 was even more effective increasing cholesterol efflux in this cell model around 7-fold compared to the solvent control. Interestingly, the two honokiol derivatives 2284 and 2817 more potently promote cholesterol efflux in comparison to the full synthetic pan RXR-agonist bexarotene at receptor saturating concentrations indicating that the derivatives might increase cholesterol efflux in those cells by an additional mechanism. This observed difference in ApoA1-mediated cholesterol efflux was not observed using human plasma as acceptor in this assay (Figure 13B). Cholesterol efflux in THP-1 macrophages is increased by honokiol derivatives investigated in this study by increasing ABCA1 mRNA levels and protein levels (Figure 14A), most likely due to the activation of the permissive heterodimer LXR/RXR in this cell type.[249]

Furthermore, protein levels of the scavenger receptor class B, type I (SR BI/SCARB1) were upregulated (Figure 14C). An increase of protein levels of scavenger receptor may facilitate passive diffusion of cholesterol out of the cell.[243]

Pharmacological transactivation of permissive heterodimers of LXR/RXR by full agonists (e.g. the full LXR-agonist T0901317 or the full RXR-agonist bexarotene) in the liver may lead to the development of a fatty liver (liver steatosis) due to the activation of a lipogenic gene program.[241,250,251] This is due to the LXR/RXR-dependent activation of membrane-bound transcription factors belonging to the family of sterol-regulatory element binding proteins that induce other genes involved in hepatic lipid metabolism.[237,252]

For this reason, honokiol derivatives 2284 and 2817 were investigated in a liver cell-line (HepG2) for their ability to induce lipid droplet accumulation within those cells. Both derivatives did not significantly induce lipid droplet accumulation in HepG2 cells as it is the case for the full LXR-agonist T0901317 or the full agonist bexarotene (Figure 16). Furthermore, both derivatives did not significantly induce SREBP1 mRNA or protein levels in HepG2 cells at 10 µM (Figures 17 and 18). Finally, 2284 and 2187 did not induce reporter gene expression *via* the SREBP1 promoter in HepG2 cells (Figure 19A). However, this was the case in HEK293 cells (Figure 19B) indicating that the ability to transactivate nuclear receptor-dependent gene expression via RXR is cell type dependent and/or dependent .[253]

Selective nuclear receptor modulators ideally act in a tissue/cell type selective way to overcome potential side effects and this is proposed to be mediated by a selective interaction profile for certain coregulators that are present or absent in certain tissues/cell types. [12,253]Coregulator-RXR interactions were investigated for honokiol derivative 2284 using a microarray assay for nuclear receptor- interactions. The coregulator-interaction profile of 2284 was compared to the profile of full RXR-agonists (bexarotene and 9-cis retinoic acid). Indeed, 2284 significantly modulated RXRα-coregulator interaction of 42 coregulators (represented by 80 coregulator peptides on the array) representing only a subset of RXRα-coregulator interactions modulated by the full agonist bexarotene that facilitated RXRα-coregulator interactions of 58 coregulators (represented by 124 peptides on the array). 14 coregulators were significantly modulated only by bexarotene in comparison to 2284 (Figure 12).

In general, the coregulator interactions modulated by honokiol derivative 2284 was less potent compared to bexarotene and concentration-response experiments revealed that these interactions are partial compared to bexarotene (Appendix Figure 2).
Interestingly, coactivators involved in lipid metabolic processes such as CHD9, that has been described to act as coactivator for SREBP gene expression, are not recruited upon 2284 binding to the RXR LBD that might explain the non-lipogenic effect of this derivative (Appendix Figure 3). We are currently investigating in protein-protein interaction studies which coregulator are differentially recruited to the RXR LBD by 2284 in comparison to full RXR agonists in the human liver cell line HepG2.
In summary, this work describes novel selective RXR modulators based on the structure of the natural compound honokiol with an improved receptor selectivity compared to honokiol with derivative 2284 possessing the highest selectivity and potency for RXR compared to the natural product honokiol. Compound 2284 is a partial RXR agonist with a coregulator-interaction profile representing a subset of that of full RXR-agonists (e.g. bexarotene). The derivatives 2284 and 2817 potently promote ApoA1- and human plasma- mediated cholesterol efflux in human THP-1 derived macrophages by increasing ABCA1 mRNA and ABCA1 protein levels. Compounds 2284 and 2817 did not induce lipid accumulation in a liver cell line (HepG2 cells) or induced a lipogenic gene program in this cell type. Finally no cytotoxic effects were observed up to 10 μM in three different cell lines, making them potential new drug targets for the treatment or prevention of metabolic diseases.

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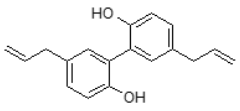
Appendix



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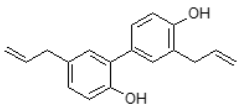

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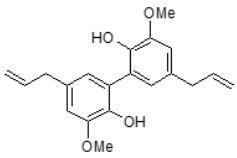

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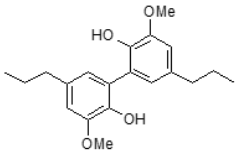

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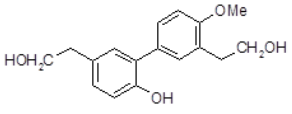

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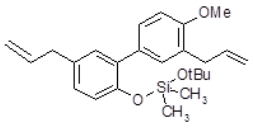

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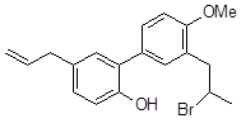

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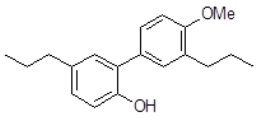

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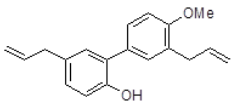

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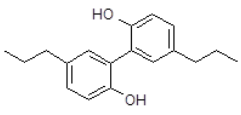

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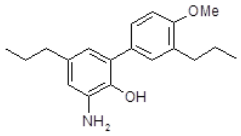

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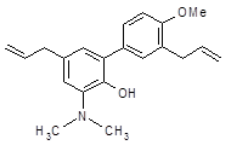

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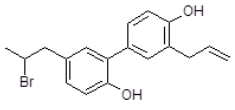

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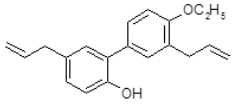

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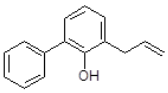

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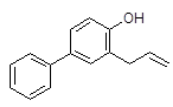
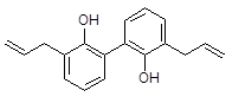
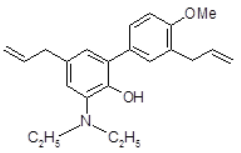


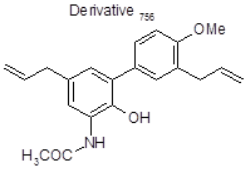
Derivative $_{767}$



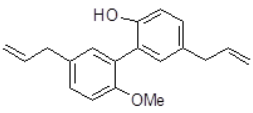
Derivative $_{768}$



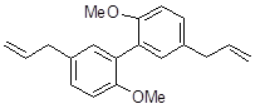
Derivative $_{756}$



Derivative $_{757}$



Derivative $_{758}$



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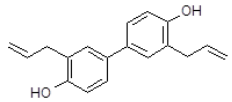
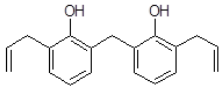
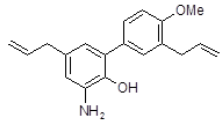


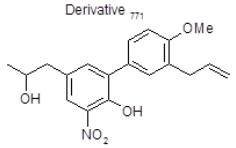
Derivative $_{769}$



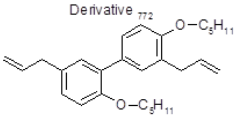
Derivative $_{770}$



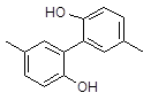
Derivative $_{771}$



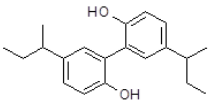
Derivative $_{772}$



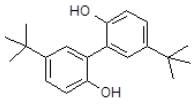
Derivative $_{773}$



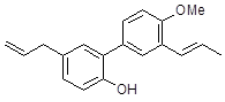
Derivative $_{774}$



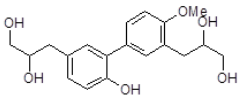
Derivative $_{775}$



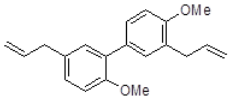
Derivative $_{776}$



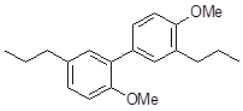
Derivative $_{777}$



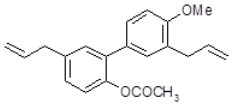
Derivative $_{778}$



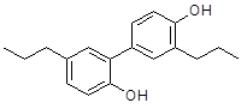
Derivative $_{779}$



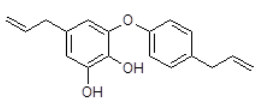
Derivative $_{780}$



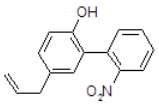
Derivative $_{781}$



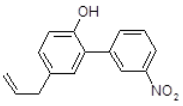
Derivative $_{782}$



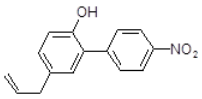
Derivative $_{783}$



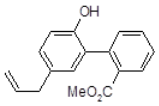
Derivative $_{2283}$



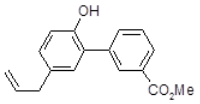
Derivative $_{2284}$



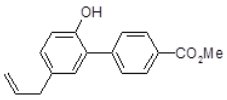
Derivative $_{2285}$



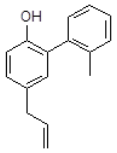
Derivative $_{2286}$



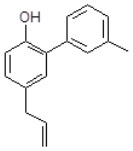
Derivative $_{2287}$



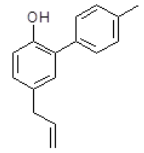
Derivative $_{2288}$



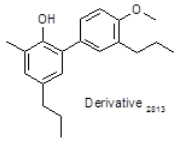
Derivative $_{2956}$



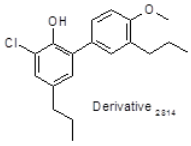
Derivative $_{2957}$



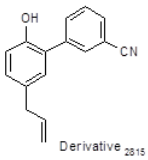
Derivative $_{2958}$



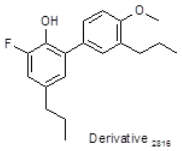
Derivative $_{2813}$



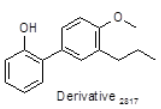
Derivative $_{2814}$



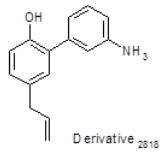
Derivative $_{2815}$



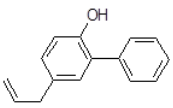
Derivative $_{2816}$



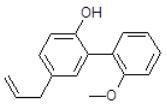
Derivative $_{2817}$



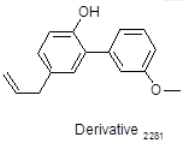
Derivative $_{2818}$



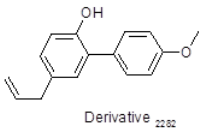
Derivative $_{2279}$



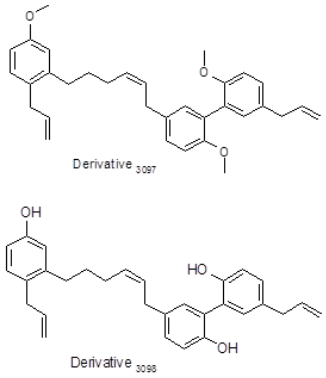
Derivative $_{2280}$



Derivative $_{2281}$



Derivative $_{2282}$



Appendix Figure 1**. **Structures of Neolignans (Honokiol/Magnolol derivatives) used in this study.

Compounds 727-783 were provided and synthesised by Dr. Wolfgang M. Schuehly (Karl-Franzens-University, Graz), compounds 2813-3098 were provided and synthesized by Dr. Marko Mihovilovic, Dr. Lukas Rycek and Dr. Dominik Dreier (Vienna University of Technology, Vienna).

DNTI-ID	Internal ID	Activation of PPARγ in HEK293 cells			Activation of RXRα in HEK293 cells			Activation of LXRα in HepG2 cells	Activation of LXRβ in HepG2 cells
		activity at 10 uM	EC_{50} (uM)	E_{max} (fold activation)	activity at 10 uM	EC_{50} (uM)	E_{max} (fold activation)	activity at 10 uM	activity at 10 uM
	9-cis retinoic acid (5µM)	-	-	-	**37.00**	**0.05**	**37.98**	-	-
	Pio-glitzone (5µM)	**6.9**	**0.19**	**9.25**	-	-	-	-	-
	GW 3965 (1µM)	-	-	-	-	-	-	**5.49**	**4.01**
PP03-Mag-off-co-A1_2 (Magnolol)	727	2.65	1.62	3.03	27.83	3.39	24.00	0.87	0.58
PP03-Mag-off-co-A1_3 (Honokiol)	728	1.44	3.90	2.00	17.90	1.07	21.53	1.07	0.66
PP03-4433-08-3_1 (Dieugenol)	729	3.57	0.62	3.58	1.29	n.d.	n.d.	1.24	0.79
PP03-13991-42-9_1	730	3.61	0.33	3.34	1.14	n.d.	n.d.	1.13	0.99
1241 PP03-WS-1-A_1	**752**	**1.67**	**n.d.**	**n.d.**	**23.46**	**0.69**	**24.32**	**1.67**	**1.35**
1242 PP03-WS-10-A_1	753	2.83	3.55	2.80	19.46	3.69	18.10	1.10	0.85
1243 PP03-WS-101-A_1	754	2.99	3.38	2.77	1.47	n.d.	n.d.	0.92	0.71
1244 PP03-WS-102b-A_1	755	1.44	n.d.	n.d.	1.32	n.d.	n.d.	0.94	0.84
1245 PP03-WS-103b-A_1	756	1.25	n.d.	n.d.	1.48	n.d.	n.d.	0.75	0.96
1246 PP03-WS-104-A_1	757	1.25	n.d.	n.d.	1.84	n.d.	n.d.	0.55	0.59
1247 PP03-WS-11-A_1	758	1.94	n.d.	n.d.	4.61	12.70	26.40	0.59	0.94
1248 PP03-WS-12-A_1	759	1.71	n.d.	n.d.	1.74	n.d.	n.d.	0.50	0.73
1249 PP03-WS-15-A_1	760	1.15	n.d.	n.d.	1.06	n.d.	n.d.	0.88	0.70
1250 PP03-WS-16-A_1	761	0.98	n.d.	n.d.	1.01	n.d.	n.d.	0.81	0.83
1251 PP03-WS-19b-A_1	**762**	**1.91**	**n.d.**	**n.d.**	**20.36**	**0.73**	**20.93**	**0.66**	0.50
1252 PP03-WS-2-A_1	763	2.20	n.d.	n.d.	20.78	1.98	20.89	0.65	0.50
1253 PP03-WS-20a-A_1	764	1.14	n.d.	n.d.	2.32	n.d.	n.d.	0.73	0.76
1254 PP03-WS-21b-A_1	765	1.43	n.d.	n.d.	18.47	9.38	33.09	0.68	0.75
1255 PP03-WS-22a-A_1	766	1.82	n.d.	n.d.	0.83	n.d.	n.d.	0.73	0.75
1256 PP03-WS-22b-A_1	767	1.40	n.d.	n.d.	2.42	n.d.	n.d.	0.83	0.66
1257 PP03-WS-23a-A_1	768	1.60	n.d.	n.d.	3.63	n.d.	n.d.	0.93	1.46
1258 PP03-WS-23b-A_1	769	1.27	n.d.	n.d.	4.45	n.d.	n.d.	0.69	1.51
1259 PP03-WS-24a-A_1	770	1.06	n.d.	n.d.	0.86	n.d.	n.d.	0.74	1.35
1260 PP03-WS-25-A_1	771	1.08	n.d.	n.d.	1.36	n.d.	n.d.	0.87	1.02
1261 PP03-WS-26bc-A_1	772	1.73	n.d.	n.d.	2.83	n.d.	n.d.	0.70	1.09
1262 PP03-WS-29c-A_1	773	1.75	n.d.	n.d.	1.13	n.d.	n.d.	0.89	1.66
1263 PP03-WS-31-A_1	774	1.72	n.d.	n.d.	8.57	n.d.	n.d.	0.82	1.31
1264 PP03-WS-32-A_1	775	2.03	n.d.	n.d.	7.15	n.d.	n.d.	0.73	1.06
1265 PP03-WS-33-A_1	776	2.65	2.45	2.70	2.16	n.d.	n.d.	0.57	0.36

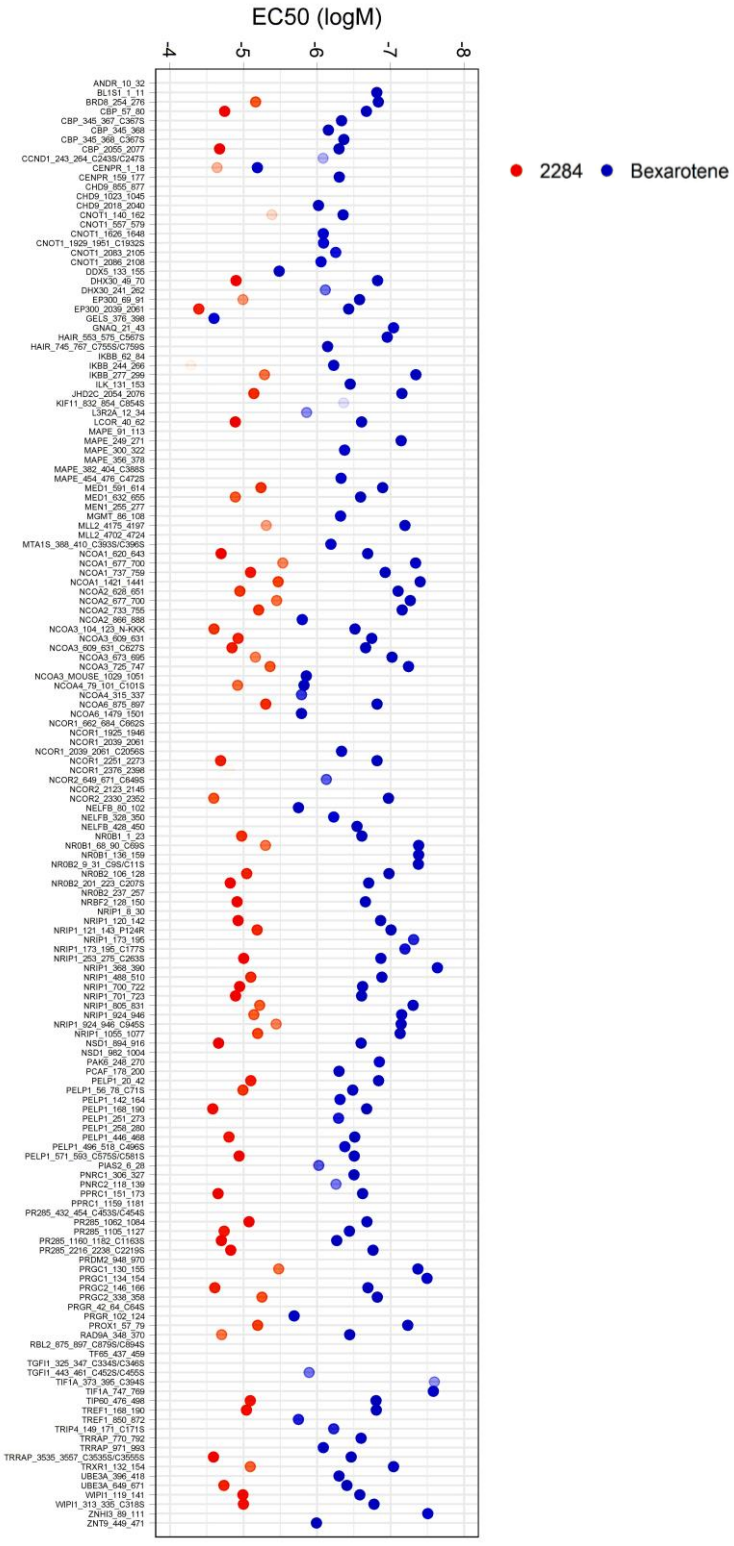
DNTI-ID	Internal ID	Activation of PPARγ in HEK293 cells			Activation of RXRα in HEK293 cells			Activation of LXRα in HepG2 cells	Activation of LXRβ in HepG2 cells
		activity at 10 uM	EC_{50} (uM)	E_{max} (fold activation)	activity at 10 uM	EC_{50} (uM)	E_{max} (fold activation)	activity at 10 uM	activity at 10 uM
	9-cis retinoic acid (5µM)	-	-	-	**37.00**	**0.05**	**37.98**		
	Pioglita-zone (5µM)	**6.9**	**0.19**	**9.25**	-	-	-		
	GW 3965 (1µM)	-	-	-	-	-	-	**5.49**	**4.01**
1270 PP03-WS-7a-A_1	781	1.58	n.d.	n.d.	18.38	0.47	21.62	1.07	0.74
1271 PP03-WS-9-A_1	**782**	**0.96**	**n.d.**	**n.d.**	**25.13**	**3.81**	**25.1**	**1.02**	**0.64**
1272 PP03-WS-obovatol-A_1	783	1.19	n.d.	n.d.	2.63	n.d.	n.d.	1.00	0.97
LRK372	2813	1.48	n.d.	n.d.	3.69	n.d.	n.d.	0.69	0.80
LRK403	2814	1.84	n.d.	n.d.	1.49	n.d.	n.d.	0.75	1.07
AH17	2815	1.49	n.d.	n.d.	19.32	1.03	21.76	0.97	0.51
LRK364	2816	3.02	3.36	2.87	22.28	5.24	23.81	1.00	0.77
LRK363	**2817**	**2.21**	**n.d.**	**n.d.**	**22.04**	**0.59**	**21.97**	**1.81**	**1.88**
AH018	2818	1.67	n.d.	n.d.	7.71	n.d.	n.d.	1.00	0.82
LRK045	2279	1.19	n.d.	n.d.	4.70	n.d.	n.d.	1.05	0.72
LRK079	2280	1.09	n.d.	n.d.	6.48	n.d.	n.d.	0.88	0.65
LRK073	2281	1.51	n.d.	n.d.	23.46	6.35	27.20	0.41	0.86
LRK081	2282	1.27	n.d.	n.d.	2.02	n.d.	n.d.	0.46	0.82
LRK069	2283	0.95	n.d.	n.d.	1.23	n.d.	n.d.	0.89	0.68
LRK071	**2284**	**1.18**	**n.d.**	**n.d.**	**12.10**	**0.41**	**15.20**	**0.78**	**0.91**
LRK070	2285	1.01	n.d.	n.d.	27.40	4.21	27.40	0.67	1.07
LRK076	2286	0.68	n.d.	n.d.	1.42	n.d.	n.d.	0.85	0.88
LRK077	2287	1.10	n.d.	n.d.	19.91	2.19	20.11	1.00	1.19
LRK078	2288	1.28	n.d.	n.d.	1.36	n.d.	n.d.	0.94	1.28
LRK583	2956	1.10	n.d.	n.d.	1.11	n.d.	n.d.	0.87	1.12
LRK584	2957	2.45	n.d.	n.d.	24.82	1.08	26.77	1.20	0.84
LRK585	2958	1.33	n.d.	n.d.	23.12	7.86	33.41	0.85	0.74
protected sesqui-magnolol	3097	2.65	4.27	3.02	0.96	n.d.	n.d.	1.00	1.06
sesqui-magnolol	3098	4.11	0.88	4.39	0.98	n.d.	n.d.	0.53	0.74

Appendix Table 1. Transactivation by Neolignans of human full length receptors PPARγ, RXRα, LXRα and LXRβ in HEK293 and HepG2 cells.

Neolignans were screened for nuclear receptor activity in cell-based luciferase transactivation models in HEK293 cells and HepG2 cells. Fold activities are displayed by treating cells for 18 hours with 10 µM of the indicated compounds. Active compounds were further tested in different concentrations (1, 3, 10, 30 µM) to calculate EC_{50} values.[196] Five derivatives (752, 762, 781, 2284 and 2817 in bold) were identified to be RXRα selective compared to the PPARs and LXRs with EC_{50} values lower than 1 µM.

Compound	**EC_{50} [µM]**	**E_{max} [fold activation]**
Honokiol	2.76	122.0 ±45.7
9-cis retinoic acid	0.44	118.0 ±19.5
2284	1.80	108.3 ±8.3
2817	2.69	161.9 ± 12.9
752	2.53	112.4 ±6.8
762	7.62	40.0 ±1.8
781	3.11	184.6 ±10.8

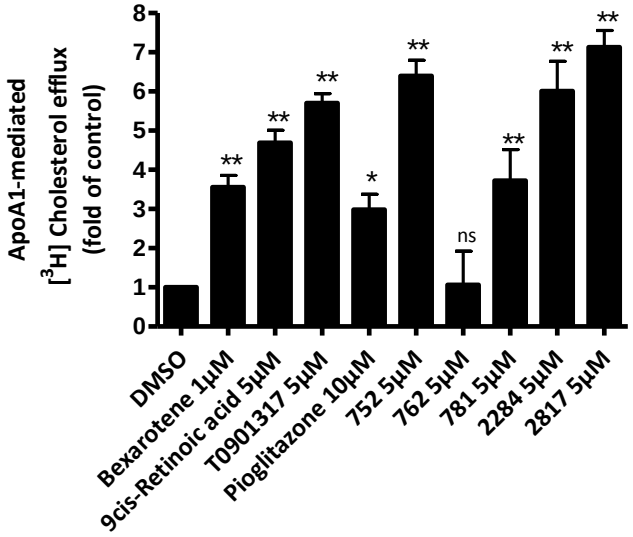
Appendix Table 2. GAL4-Binding Assay in HEK293 cells.
A Mammalian one- hybrid assay with GAL4 constructs was used to verify the ability of the honokiol derivatives to transactivate the ligand-binding domain of RXRα. HEK 293 cells were transfected with an expression plasmid encoding for a chimeric protein consisting of the yeast GAL4 DNA-binding domain (Gal4-DBD) domain fused to the human RXRα ligand-binding domain (hRXRα-LBD). The yeast GAL4 DBD recognizes specific upstream activating sequences (UAS) and the reporter (luciferase gene) used in this system is therefore fused to several UAS-elements (see 2.3.5.). Results are expressed as means ± SD of three independent experiments performed in quadruplicate.



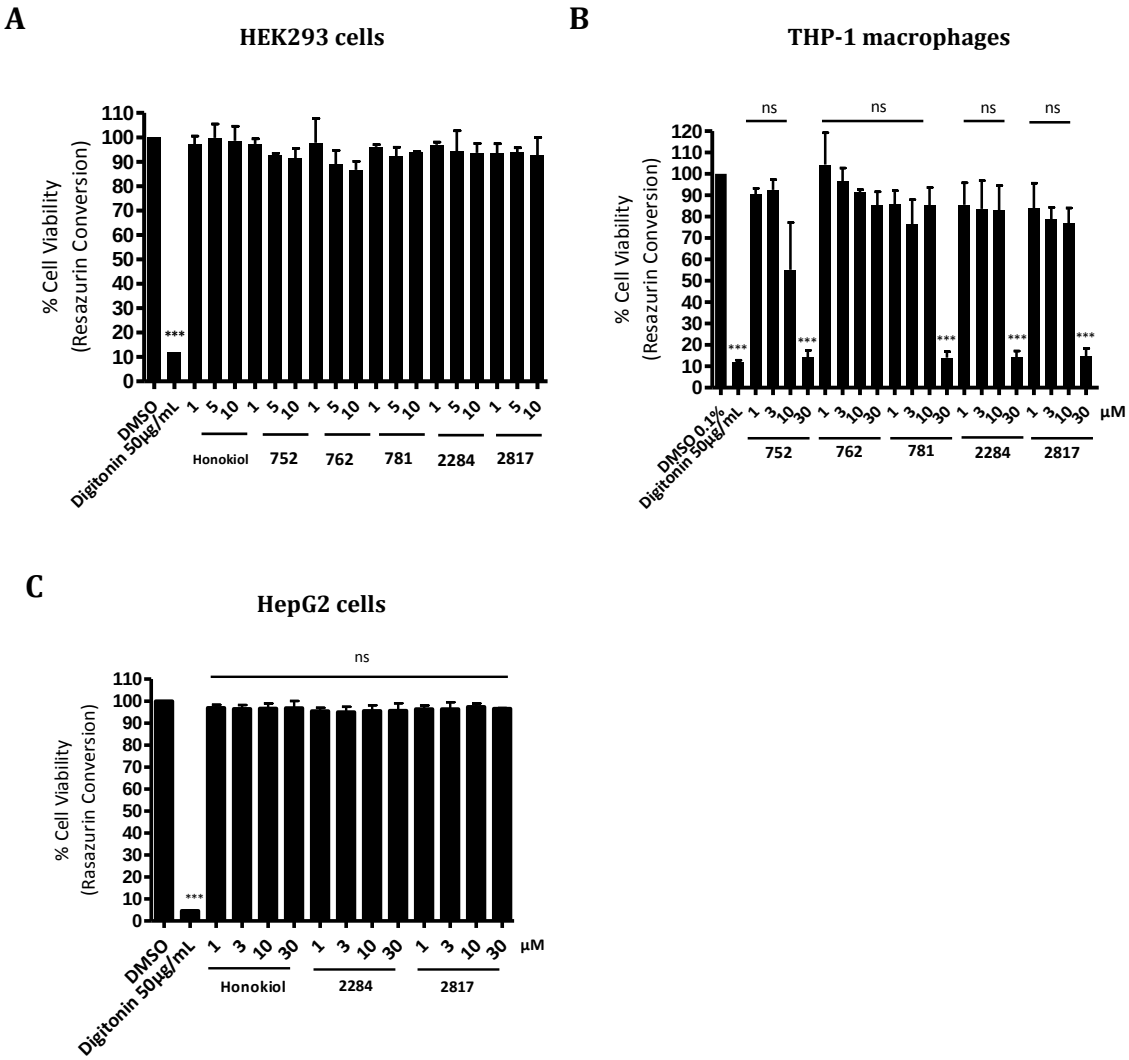
Appendix Figure 2. RXR Coregulator Profiling
Comparison of coregulator peptide-binding potencies (EC_{50} values) upon binding of 2284 and bexarotene to the RXRα LBD.

Coregulator	Uniprot accesion	Name	Description	Function	
ANDR	P10275	Androgen receptor	Androgene receptor signalling	Nuclear receptor	No modulation by 2284
CENPR	Q13352	Centromere protein R	Involved in the coactivation of nuclear receptors for retinoid X (RXRs) and thyroid hormone (TRs) in a ligand-dependent fashion	Coactivator	No modulation by 2284
CHD9	Q3L8U1	Chromodomain-helicase-DNA-bidning-protein 9	Transcriptional coactivator for PPARα, lipid metabolic process, activation of gene expression via SREBP	Coactivator	No modulation by 2284
DDX5	P17844	ATP-dependent RNA helicase	Androgene and Estrogene receptor binding	Coactivator	No modulation by 2284
GELS	P06396	Gelsolin precursor	Calcium-regulated, actin-modulating protein	Coactivator?	No modulation by 2284
GNAQ	P50148	Guanine nucleotide-binding protein G(q) subunit alpha	Signal transducer	Coactivator	No modulation by 2284
HAIR	Q43593	Protein hairless	Histone demethylase	Coactivator/corepressor	No modulation by 2284
ILK	Q13418	Integrin-linked protein kinase	59 kDa serine/threonine-protein kinase	Coactivator	No modulation by 2284
PCAF	Q92831	Histone acetyltransferase KAT2B	P300/CBP-associated factor (P/CAF)	Coactivator	No modulation by 2284
PNRC2	Q9NPJ4	Proline-rich nuclear receptor coactivator2	Involved in nonsense-mediated mRNA decay, energy balance in catabolic processes	Coactivator	No modulation by 2284
PRGR	P06401	Progesterone receptor	Progesterone receptor signalling	Nuclear receptor	No modulation by 2284
TRIP4	Q15650	Activating signal cointegrator 1	Thyroid receptor interacting protein 4 (ASC-1)	Coactivator	No modulation by 2284
ZNHI3	Q15649	Zinc finger HIT domain-containing protein 3	Thyroid receptor interacting protein 3 (TRIP-3)	Coactivator	No modulation by 2284
ZNT9	Q6PML9	Zinc transporter 9	Humanembryonic lung protein (HUEL)	Coactivator	No modulation by 2284

Appendix Figure 3. Coregulators that are not modulated by 2284 in comparison to bexarotene
Significant modulation of coregulator peptide binding (modulation index MI p<0.05)was compared between 2284 and bexarotene. 14 coregulators represented by 44 different coregulator peptides on the microarray were not significantly modulated by 2284. The 14 coregulators and their function are listed.*

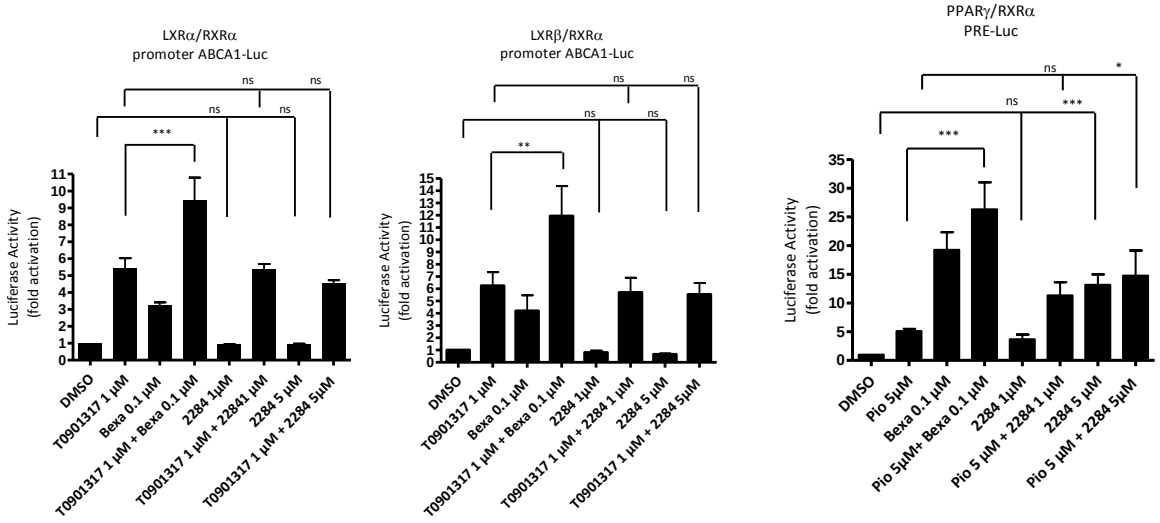


Appendix Figure 4. Honokiol derivatives promote cholesterol efflux in human THP-1 macrophages. *Four honokiol derivatives 752, 781, 2284 and 2817 significantly promote cholesterol efflux in THP-1 macrophages. Differentiated THP-1 macrophages were treated for 24 hours with the indicated compounds and cholesterol efflux was measured with radioactively labelled unesterified cholesterol. Results are shown as fold activation compared to the solvent control DMSO (0.1%) and are expressed as average of at least two independent experiments ±SEM performed in duplicate (One-Way ANOVA, Dunnett´s statistical test; p<0.05*, p<0.01 **, ns not significant). Bexarotene and 9-cis retinoic acid are full RXR agonists, T0901317 is a full LXR-agonist, Pioglitazone is a full PPARγ agonist.*



Appendix Figure 5. Influence of Honokiol derivatives on cell viability as measured by resazurin conversion in (A) HEK293 cells (B) differentiated THP-1 macrophages and (C) HepG2 cells.

*The different cell lines were treated for 24 hours with increasing concentrations of honokiol derivatives and a Resazurin conversion assay was performed. Digitonin (50 µg/mL) was used as a positive control. Data are expressed as mean values of at least two independent experiments ±SEM normalized to the solvent control DMSO (0.1%). (unpaired t-test, p<0.001 ***, ns not significant).*



Appendix Figure 6. Synergistic transactivation of permissive heterodimers by 2284 in HEK293 cells. *HEK293 cells were transfected for 6 hours with an expression plasmid encoding the full length RXRα receptor and the respective heterodimer partner receptor (LXRα, LXRβ or PPARγ), a reporter plasmid and an EGFP expression plasmid as internal control. The luciferase reporters used were plasmids containing the ABCA1 promoter region followed by a luciferase gene (ABCA1-LUC) or a plasmid containing several PPAR-response elements followed by a luciferase gene (PRE-LUC). Transfected HEK293 cells were treated for 18 h with the indicated compounds and nuclear receptor-dependent luciferase gene expression was measured. Results are expressed as fold induction compared to the solvent DMSO (0.3%). Data are shown as means ± SEM of at least three independent experiments performed in quadruplicate. One-Way ANOVA (Bonferroni-post test. T0901317: full LXR-agonist, Bexa: Bexarotene, full RXR-agonist, Pio: Piolitazone, full PPARγ agonist.*

Abbreviations

3

3T3 L1 Mouse immortalized fibroblast cell line

A

ABC transporter ATP-binding casette transporter

ABCA1 ATP-binding cassette transporter A1, ATP binding cassette transporter A1

ABCG1 ATP binding cassette transporter G1

ACAT1 Acyl-CoA cholesterol acyltransferase 1

ACC Acetyl coenzyme A carboxylase

AD Alzheimer´s disease

AF-1 Activation function 1

AF-2 Activation function 2

ANOVA Analysis of variance

ApoAI Apolipoprotein A1

ApoE Apolipoprotein E

AR Androgen receptor

C

CBR Cannabinoid receptor

CE cholesterol ester

CEPT Cholesteryl ester transfer protein

CoA Co-activators

CoR Corepressor

CoRNR box Co regulator nuclear receptor box

CRABP Cellular retinoic acid binding protein

D

DBD DNA binding domain

DMSO Dimethylsulfoxide

DR1-DR5 direact repeat 1-5

E

ER Estrogene receptor

F

FASN Fatty acid synthase

FC free cholesterol

FRET Fluorescence resonance energy transfer

FXR Farnesoid X receptor

G

$GABA_A$ *G*amma-aminobutyric acid receptor

GAL4 Yeast transcription factor Gal4

GPR55 G-protein coupled receptor 55

GR Glucocorticoid receptor, Glucocorticoid receptor

GST Glutathion-S-transferase

H

H12 helix 12

HAT activity Histone acetyl transferase activity, Histone acetyl transferase activity

HDAC activity Histone deacetylase activity

HDL High density lipoprotein, high density lipoprotein

HEK293 Human embryonic kidney 293 cells, human embryonic kidney 293 cells

HepG2 Hepatocellular carcinoma cell line, Hepatocellular carcinoma cell line, Hepatocellular carcinoma cell line

L

LAL Lysosome acid lipase

LBD Ligand-binding domain, Ligand-binding domain

LDLR Low-density lipoprotein receptor

LXR Liver X receptor

M

MARCoNI Micro array for nuclear for real-time coregulator-nuclear receptor interaction

MI Modulation index

MR Mineralocorticoid receptor

N

NCOA Nuclear receptor coactivator

NPC Niemann Pick type C protein

NR Nuclear receptor

NR box nuclear receptor box (LXXL motif)

P

PGC-1 Peroxisome proliferator-activated receptor gamma coactivator 1

PMA Phorbol myristate acetate

PPAR Peroxisome proliferator-activating receptor

R

RA Retinoic acid

RAR Retinoic Acid receptor

RCT Reverse cholesterol transport, Reverse cholesterol transport, Reverse cholesterol transport

RT-qPCR Real Time quantitative polymerase chain reaction

RXR Retinoid X receptor

S

SNuRMs Selective nuclear receptor modulators

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis 2.9.2. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SRC Steroid receptor coactivator

SREBP1c Serum rsponse element binding protein 1c

T

THP-1 human leukemia cell line

U

UAS Upstream activating sequence

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Pan SP, Kunert O, Kretschmer N, Latkolik SL,Rappai J, Dirsch VM, Bochkov V, Bauer R. C13 megastigmane derivatives from *Epipremnum pinnatum*– β-damascenone inhibits COX-2 and IL-8 gene expression. Research Article: submitted to Journal of Natural Products

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Functional and Mechanistic Aspects of a Novel and Highly Selective RXR Modulator. ÖPhG Conference. Pharma 2030-Current and Future Challenges. Innsbruck, Austria, 22nd of April, 2017.

Poster Presentation: Latkolik SL, Dirsch VM, Mihovilovic MD, Ladurner A, Rycek L, Heiss EH, Malainer C, Schwaiger S, Stuppner H, Schuehly W, Atanasov AG. Honokiol derivatives as RXRα modulators. EMBO conference on Nuclear Receptors: From Moleculaes to Humans. Corsica, France, 2015.

Poster Presentation: Latkolik SL, Dirsch VM, Mihovilovic MD, Ladurner A, Rycek L, Heiss EH, Malainer C, Schwaiger S, Stuppner H, Schuehly W, Atanasov AG. Honokiol derivatives as RXRα modulators. DNTI-meeting (Drugs from Nature targeting inflammation Natural products and Drug Discovery-Future perpectives.International Symposium, Vienna University of Technology, Austria, 2014.

Poster Presentation: Latkolik SL, Lasztoczi B, Klausberger T. Organisation of $GABA_A$ receptor α 3 subunits in the hippocampus. Oxford Science Days, Anatomical Neuropharmacology Unit, England, 2012.

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(Laboratory of Molecular Systematics, Museum of Natural History Vienna), SMBE2008, Barcelona.

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"One never notices what has been done; one can only see what remains to be done."
-Marie Curie