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DEPARTMENT OF PHARMACOGNOSY

CHARACTERIZATION OF A SYNTHETIC LEOLIGIN DERIVATIVE, WITH AGONISTIC FXR AND ENHANCING MACROPHAGE CHOLESTEROL EFFLUX ACTIVITY

DIPLOMA THESIS

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Consultant: Dr. Angela Ladurner

Hradec Králové, 2016 **Kovářová Lenka**

Hereby I declare that this thesis is my original authorial work. All literature and other sources which I used are listed in the complete references and properly cited in the thesis. The work has not been used to gain other or identical academic degree.

Prohlašuji, že tato práce je mým původním autorských dílem. Veškerá literatura a další zdroje, z nichž jsem při zpracování čerpala, jsou uvedeny v seznamu použité literatury a v práci jsou řádně citovány. Práce nebyla použita k získání jiného nebo stejného titulu.

V Hradci Králové, dne Lenka Kovářová

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Table of contents

ABSTRAKT

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Název diplomové práce: Charakteristika syntetického derivátu leoliginu, agonisty FXR zvyšujícího cholesterolový eflux z makrofágů

Ateroskleróza je patologický a multifaktoriální proces způsobující vznik kardiovaskulárních onemocnění, které jsou hlavními příčinami úmrtí v západním světě. Iniciální fáze aterosklerózy je charakterizována akumulací lipidních částic, převážně LDL cholesterolu a pěnových buněk původně získaných z makrofágů, ve velkých arteriích, což vede k postupnému ztluštění cévní stěny. Tyto progresivní změny vyvolávají tvorbu plaku, následovaného rupturou, trombózou a vedoucí až k vzniku kardiovaskulární příhody.

Reverzní transport cholesterolu je důležitý preventivní mechanismus, který zajišťuje odstranění nadbytku aterogenních lipoproteinů z makrofágů. Tento eflux je zprostředkován ABC transportéry v makrofázích, hlavně ABCA1 a ABCG1 a zčásti scavenger receptorem (SR-B1).

Několik nukleárních receptorů, včetně PPARγ, LXRα a LXRβ, upreguluje tyto transportéry a ovlivňuje tak cholesterolový metabolismus, zejména prostřednictvím PPAR-LXR-ABCA1 dráhy. RXRα je schopný tvořit funkční heterodimery spolu s LXR a FXR. Je zajímavé, že farnesoidní receptor X je exprimován v játrech a střevech, ale také v makrofázích. Ligandy tohoto receptoru jsou diskutovány jako potenciální zástupci pro lipidové poruchy.

V této práci jsme se zaměřili na charakterizaci derivátu leoliginu, který v předchozí studii prokázal aktivaci farnesoidního receptoru. Kromě toho bylo prokázáno, že tato

látka zvyšuje cholesterolový eflux z pěnových buněk původně získaných z makrofágů. Proto byla možná modulace ABCA1, ABCG1 a také SR-B1 exprese touto látkou zkoumána pomocí metody Western blotting. Aktivita této látky na dalších nukleárních receptorech byla hodnocena pomocí Luciferase gene reporter assay a Gal-4 assay byla využita ke stanovení specifity této látky.

Tyto experimenty odhalily, že testovaný derivát leoliginu je schopný v závislosti na dávce zvyšovat hladiny ABCA1 proteinu a potvrdily také specifickou aktivaci farnesoidního receptoru, ostatní sledované nukleární receptory (LXRα, LXRβ, PPARα, PPARγ, RXRα) nebyly aktivovány. Kromě derivátu leoliginu byly zkoumány také FXR agonista, chenodeoxycholová kyselina (CDCA) a FXR modulátor, guggulsterone. Je zajímavé, že obě látky byly schopné zvýšit hladinu ABCA1 proteinu. Kromě toho, guggulsterone prokázal dávkově závislou transaktivaci LXRα and LXRβ.

Zde prezentovaná data nestačí k vytvoření vazby mezi cholesterolovým efluxem z pěnových buněk původně získaných z makrofágů a farnesoidním receptorem. Další experimenty jsou nutné pro objasnění základního mechanismu, kterým tyto látky zvyšují cholesterolový eflux.

ABSTRACT

Charles University, Faculty of Pharmacy in Hradec Králové, Department of Biological and Medical Sciences

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Title of the diploma thesis: Characterization of a synthetic leoligin derivative, with agonistic FXR and enhancing macrophage cholesterol efflux activity

Atherosclerosis is a pathologic multifactorial process triggering the development of cardiovascular diseases, which are the leading causes of death in the western world. The initial phase of atherosclerosis is characterized by the accumulation of lipid particles, mainly low-density lipoproteins (LDL) and macrophage-derived foam cells in large arteries, leading to the gradual thickening of the vessel wall. These progressive alterations elicit plaque formation, followed by rupture, thrombosis and finally can lead to a cardiovascular event. Reverse cholesterol transport is an important preventive mechanism, which ensures removal of excessive atherogenic lipoproteins from macrophages. This efflux is facilitated by ATP binding cassette transporters, mainly ABCA1 and ABCG1 and in part by scavenger receptor B1 (SR-B1).

Several nuclear receptors, including PPARγ, LXRα and LXRβ are known to upregulate these transporters and influence cholesterol metabolism, especially through a PPARγ-LXR-ABCA1 pathway. RXRα is able to form functional heterodimers amongst others with LXRs and FXRs. Interestingly, FXR is expressed in the liver and the intestine, but also in macrophages. Ligands of this receptor are discussed as potential therapeutic agents for lipid disorders.

In this work we focused on the characterization of a leoligin derivative, which was shown to activate FXR in a previous study. In addition this compound was shown to

increase cholesterol efflux from macrophage-derived foam cells. Therefore a possible modulation of ABCA1, ABCG1 and also SR-B1 expression by this compound was investigated via Western blotting. Activity of this compound on other nuclear receptors was evaluated via luciferase reporter gene assay and a Gal-4 assay to determine the specifity of the compound.

These experiments revealed that the tested leoligin derivative dose-dependently increased ABCA1 protein levels and confirmed a specific activation of FXR, as other investigated nuclear receptors (LXRα, LXRβ, PPARα, PPARγ, RXRα) were not activated. In addition to the leoligin derivative the FXR agonist, chenodeoxycholic acid (CDCA), and the FXR modulator, guggulsterone were investigated. Interestingly, both compound were able to enhance ABCA1 protein levels. Additionaly, guggulsterone was shown to dose-dependently transactivate LXRα and LXRβ.

The here presented data is not sufficient to establish a link between cholesterol efflux from macrophage-derived foam cells and FXR. Further experiments are required to elucidate the underlying mechanism of the increased cholesterol efflux elicited by these compounds.

1. INTRODUCTION

1.1 Atherosclerosis

Atherosclerosis is an inflammatory and degenerative disorder, endangering mainly Western societies. It is considered to be one of the most frequent underlying causes for cardiovascular diseases (CVD), which belong to the main causes of death in industrialized countries [1]. The most common manifestations of CVD are coronary heart disease, ischemic stroke and peripheral vascular disease [2].

The slowly progressing process of atherosclerosis starts already in childhood. Some studies suggest the beginning of this treatable disorder already in foetal development. However threats to human health and complications occur mainly in middle age. The main characteristic of atherosclerosis is the thickening of the innermost layer of the vessel wall in medium-sized and large arteries [3]. Indeed the subendothelial accumulation of lipids and fibrous elements cause luminal narrowing, which restricts blood flow and reduces essential oxygen and nutrient supplementation to vital organs and tissues [4].

1.1.1 Risk factors

Multiple heterogeneous modifiable or non-influenceable factors contributing to the development and progression of atherosclerosis and CVD have been discussed. The most appreciated epidemiologic studies for CVD, are the Framingham Heart Study and the Reynolds study. Together they revealed the major risk factors for the disease [5, 6]. Risk factors are divided into two groups – factors with a strong genetic component, which are presented in **Table 1** and environmental factors. Environmental factors include mainly high-fat diet, smoking and tobacco exposure, low antioxidant levels, lack of exercise related with obesity, air pollution and infectious agents, especially *Chlamydia pneumoniae* [7]. Interference between individual risk factors could also play a major role in progression of atherosclerosis.

Table 1: Atherosclerotic risk factors

LDL, low-density lipoprotein; VLDL, very low-density lipoprotein; HDL, high density lipoprotein

Adapted from [7]

Factors with significant genetic components
Elevated levels of LDL/VLDL, homocysteine, hemostatic factors
Reduced levels of HDL
Hypertension
Diabetes mellitus
Obesity, metabolic syndrome
Systemic inflammation
Depression
Family history
Gender – male

1.1.2 The artery wall

Blood vessels consist of three concentric layers with diverging composition and function (see **Figure 1**). The tunica intima (interna) is characterized by a monolayer of endothelial cells and a small amount of connective tissue, forming a semipermeable barrier between blood and the underlying subendothelial area.

Metabolically active endothelium has a decisive role in the maintenance of vascular homeostasis and tone. It is able to produce different types of signaling molecules that depend on incoming mechanical or chemical impulses. A balance between vasodilatory factors such as nitric oxide (NO), endothelium-derived hyperpolarizing factor and prostacyclins, which are responsible for inhibition of platelet activation, antioxidant and anti-inflammatory effects, is required for suitable behavior of this process. Vasoconstrictors, mainly endothelin-1, angiotensin II and thromboxane A2 are associated with the opposite effects and are responsible for increased oxidative stress and proliferation of vascular smooth muscle cells [8].

The dividing line between the tunica intima and the tunica media is an elastic membrane, called the internal elastic lamina. The middle tunica media consists of smooth muscle cells and elastic fibres regulating the diameter of the vessel lumen, thereby controlling the blood flow and pressure. The tunica adventitia (externa) follows as the outermost layer, which is mainly composed of connective tissue, perivascular nerves, collagen and elastic fibers. In large vessels the vasa vasorum forms an additional layer and serves with its small blood vessels as nutritional supplier, but in the case of atherosclerotic lesions may have a causative role in plaque instability [9].

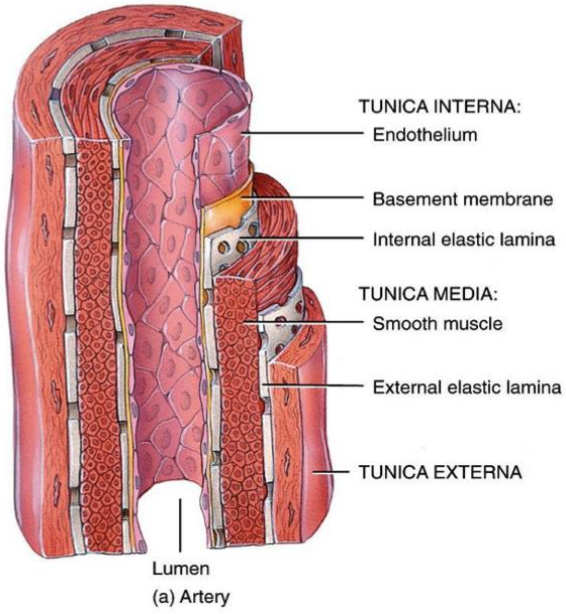

Figure 1: Structure of an artery wall

Picture adapted from [10]

1.1.3 Pathogenesis

Atherosclerosis as slowly progressing disease is characterized by many complicated processes, which include early lesions linked to initial endothelial dysfunction, retention of lipid inclusions and fibrous elements and leukocyte infiltration. Afterwards advanced and complicated atheromata with lipid-rich necrotic cores may frequently lead to acute thrombotic vascular disease and its fatal consequences, which is described in **Figure 2** (see page 17) [11].

1.1.3.1 Endothelial dysfunction and first lesions

Constant exposure to the mentioned risk factors and oxidative stress through reactive oxygen species (ROS) are leading causes for endothelial dysfunction and structural alterations of the artery wall. Additionally the associated reduction in endothelial NO bioavailability leads to insufficient vasodilatation. Decreased NO levels may be caused by an increased production of superoxide and the reaction of NO with superoxide leads to the creation of peroxynitrite, amplifying further oxidation. Similarly changed shear stress in the arterial wall contributes to widening of blood vessels [12, 13].

High serum levels of triglycerides, cholesterol and other atherogenic lipoproteins, mainly low-density lipoproteins (LDL) are indicated as main players in the initiation and also progression of atherosclerosis. LDL levels are evidently linked to the risk of CVD events [14]. Accordingly two apolipoprotein B (apoB) subclasses are the essential structural components of all atherogenic particles and thereby apoB levels exactly reflect the concentration of these lipoproteins in the blood stream. Apolipoprotein B48 is expressed in the intestine and forms chylomicrons, which are transformed by lipolysis in remnant lipoproteins. While apolipoprotein B100 is expressed in the liver binding very low-density lipoproteins (VLDL) gradually changing them into intermediate-density lipoprotein (IDL) and LDL [15].

Most LDLs leave the blood and accumulate in the innermost layer of the arterial wall, where they are oxidized and thereby form the more atherogenic oxidized-LDL particles. This modification is related to ROS and enzymes such a myeloperoxidase, sphingomyelinase and phospholipase [7]. In conjunction with hemodynamic strain, an impaired endothelium is responsible for chronic innate and adaptive immune responses and production of signaling molecules like vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1) and E- and P-selectin, which play important roles in endothelial-leukocyte migration and adhesion [16].

Consequently leukocytes are recruited to the intimal layer in response to these chemoattractant cytokines and adhesion molecules and induce tissue damage. One of the principal chemokines is monocyte chemoattractant protein-1 (MCP-1), which enables monocytes to infiltrate tissues towards stored lipoproteins [17].

Afterwards monocytes start to differentiate into macrophages and via expression of the scavenger receptors cluster of differentiation 36 (CD36) and scavenger receptor class A type 1 (SR-A1) are loaded mainly with oxidized LDL and other lipids [18].

Further types of participating pattern recognition receptors are toll-like receptors (TLR), innate immune system sensors, which trigger activation of nuclear factor-kappa B (NF-κB) and mitogen activated protein kinase pathways. In contrast to scavenger receptors, TLRs facilitate the recruitment of leukocytes and the generation of ROS, thereby amplifying the inflammatory process [19, 20]. NF-κB is an important transcription factor involved in the regulation of proinflammatory and anti-inflammatory genes, but also apoptosis and cell proliferation. Activation of the NF-kB pathway is induced by several extracellular stimuli such as the cytokines, tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), viral and bacterial components and also through TLRs [21]. The activated form was observed mainly in macrophages and smooth muscle cells in atherosclerotic lesions [22].

Intracellular lipid accumulation transforms macrophages into foam cells thereby developing plaques - fatty streaks (xanthomas), which are symptomless and common in young people. Activation of foam cells provokes recruitment and proliferation of smooth muscle cells and further monocytes, causing sequential damage of endothelial functions [23].

1.1.3.2 Advanced lesions

Xanthomas appear particularly in atherosclerosis-prone areas of the arteries and do not progress further, but evolve into relevant progressive atheromata. These formed atherosclerotic lesions constitute acellular lipid pools, which are transformed via infiltration of macrophages and foam cells into early necrotic cores [24].

Concurrently this leads to further necrosis and apoptosis of foam cells, smooth muscle cells and endothelial cells. These apoptotic cells produce tissue factors and additional procoagulants, which may in relation with platelets, induce thrombus formation. Predominantly extracellular lipids elicit intimal disorganization, impairment of the artery wall and additional development of necrotic atheromatous cores. Smooth muscle cells are recruited into the tunica intima and proliferate via platelet-derived growth factor. Extracellular matrix molecules – collagen and elastin are produced by them and form a fibrous cap together with macrophages, which prevents contact between the atherogenic lipid-rich core and blood [25]. Calcification is very frequent in atheromatous lesions and is indicated as coronary artery calcium score, a marker of coronary plaque load [26].

Neoangiogenesis, together with expansive vascular remodelling occur frequently in advanced atherosclerotic plaques and could contribute to their further growth. Clinical manifestations associated with enlarged lesions include ischemic symptoms caused by narrowing of the vessel lumen and plaque rupture leading to thrombosis and thereby to an acute cardiovascular event [27].

Vulnerable plaques with thin fibrous caps, large necrotic cores and also erosion of endothelium amplify the production of inflammatory cytokines, mainly interferon-γ (INF-γ) and TNF-α and procoagulant molecules. These unstable plaques often rupture and consequently high thrombogenic contents and tissue factors are exposed to the blood. The triggering of the coagulation cascade and platelet adherence causes the formation of a thrombus. Severe changes in vessel walls are partially unpredictable and can lead to significant clinical consequences, such as myocardial infarction, unstable angina, sudden cardiac death and stroke [28]. Data from literature confirmed that 76 % of all fatal heart attacks are caused by coronary thrombosis elicited by plaque rupture [29].

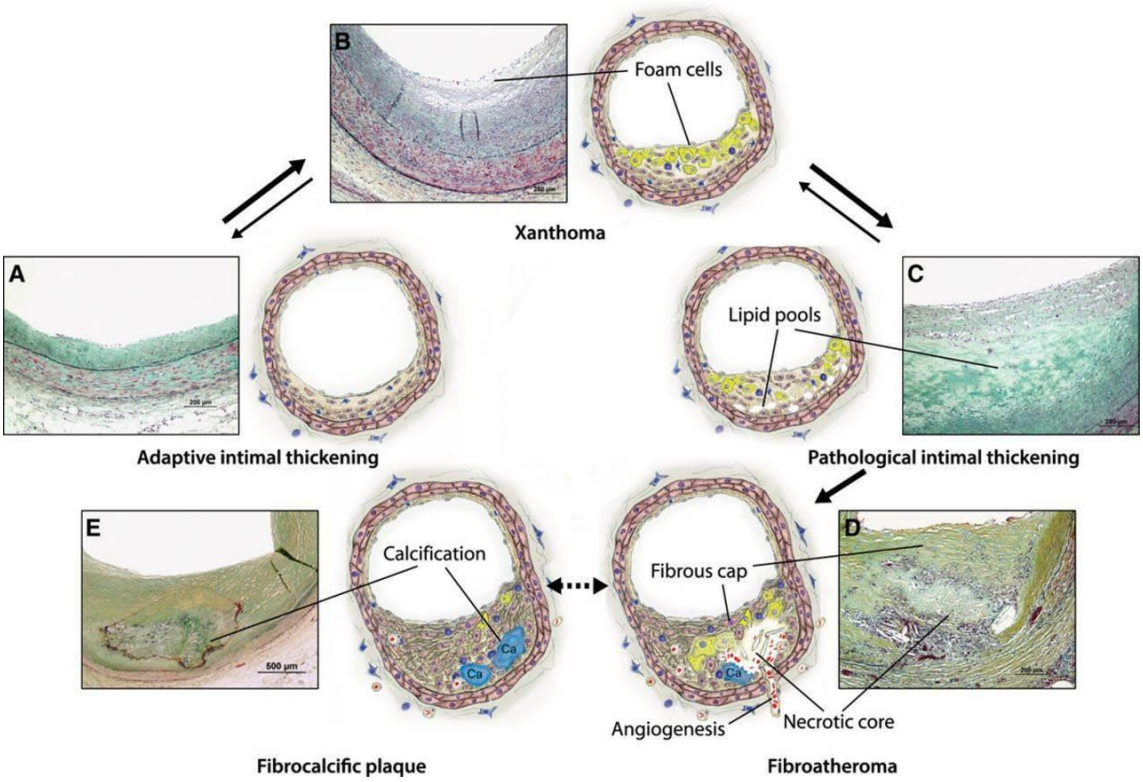

Figure 2: Atherosclerotic lesion types and their proposed progression

A. Adaptive intimal thickening characterized by smooth muscle cell accumulation in preferred sites. B. Specific accumulation of foam cells within the intima (xanthoma). C. Pathological intimal thickening including extracellular lipid pools, which can grow later into confluent necrotic cores. D. Fibroatheroma indicating the presence of a lipid-rich core, which can be calcified and form fibrocalcific plaque connected with significant luminal stenosis shown in E. Picture taken from [23]

1.2 Mononuclear phagocyte system

1.2.1 Characterization

Monocytes, macrophages and dendritic cells are originally derived from myeloid precursor cells in fetal liver and bone marrow [30].

Therefore each cell type has its own specific morphology, functional abilities and transcriptional profiles. Macrophage colony-stimulating factor (CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-34 are able to activate diverse macrophage functions and are irreplaceable for their proliferation and differentiation [31]. Macrophages are very heterogeneous cell populations, which achieve required phenotypes in response to variant tissue microenvironments [32]. Likewise, phagocytosis leads to alterations in their physiology and thereby to expression of specific surface proteins [33].

Formation of macrophages proceeds during embryonic and adult hematopoiesis in lymphoid organs. Interestingly, it is believed that the major part of adult tissue macrophages is generated within embryogenesis and not from circulating monocytes. Macrophages are specifically important for tissue homeostasis and repair as well as for the regulation of immune processes. Therefore they are important therapeutic targets whose origin and mechanisms obtain gradually more attention [34].

1.2.2 Development of macrophages and their function

During the last years, many different publications discuss the characterization and background of the macrophage origin. Apart from the hypothesis that the major part of tissue macrophages is formed during embryogenesis, as mentioned before, they can also be recruited from monocytes [30].

The binary classification of macrophages is based on their inflammatory states. These categories represent activated and alternatively activated macrophages, referred to as M1 and M2 subsets shown in **Figure 3**. During inflammatory processes macrophages differentiate through GM-CSF into M1 monocyte-derived inflammatory macrophages

with anti-pathogen activity and tissue protective effect. M1 macrophages are specific for high antigen presentation, production of IL-12, IL-23, reactive oxygen and nitrogen species. On the other side, CSF-1 stimulation elicits the anti-inflammatory cytokine profile of M2 macrophages, which is characterized by their ability to scavenge apoptotic cells and debris and their role in tissue repair and healing. M2 macrophages produce high levels of the anti-inflammatory cytokine IL-10 and transforming growth factor β (TGF-β) [35].

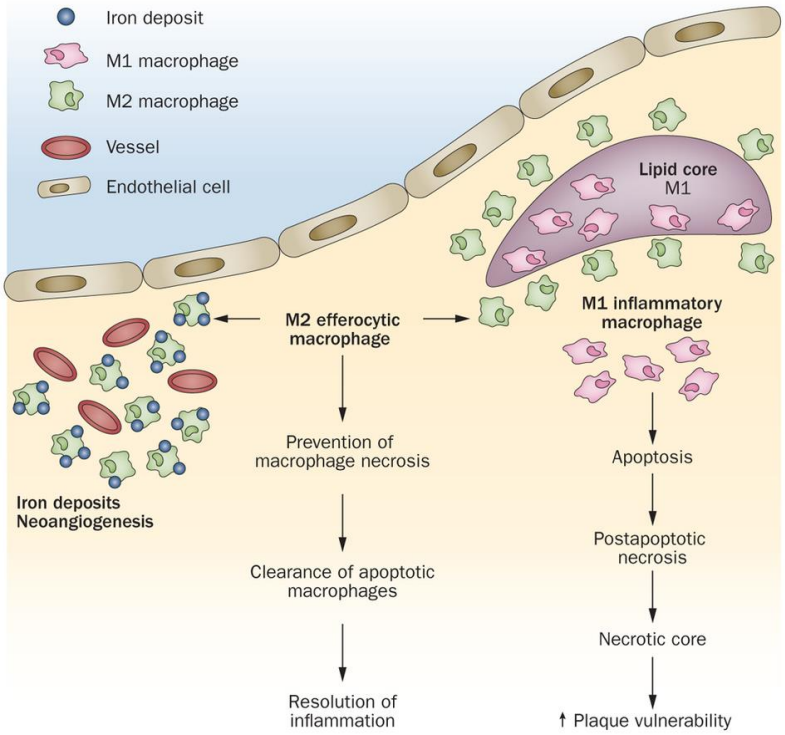

Figure 3: Role of M1 and M2 subtypes of macrophages in atherosclerotic processes

Illustration of the role of M1 and M2 macrophages.

Picture obtained from [36]

1.2.3 Role of macrophages in atherosclerosis

Interference in inflammatory processes, respectively of antigen-independent and adaptive immune responses is a principal macrophagic feature.

Moreover, macrophages are predominantly known as professional phagocytes, meaning that they recognize invading particles and organisms via pathogen-associated molecular patterns (PAMP) and endogenous danger signals. After engulfment these apoptotic cells, pathogens, cellular debris and other body-foreign particles activate macrophages to release pro-inflammatory cytokines TNF-α, IL-1, IL-6, complement factors, proteases, reactive nitrogen and oxygen species. These products lead to regional immune responses and in many times also to tissue injury [37].

In addition the ability of macrophages to accumulate and process foreign components is relevant for metabolic diseases. Cholesterol is the dominant apoptotic cell-derived constituent and macrophages have to be able to discharge it. Therefore macrophages are protected against cholesterol toxicity through the conversion of free cholesterol (FC) to cholesteryl ester, which is connected with effluxing of cellular cholesterol via ATP-binding cassette (ABC) transporters. This mechanism has a decisive role in maintaining lipid homeostasis [38] and is described in more detail in chapter 1.4. Cholesterol efflux. *In vitro* studies have shown, that apoptotic cells affect efflux because of increased levels of ABCA1 protein expression induced in the phagocytes [39].

1.3 Reverse cholesterol transport

Regulation of cholesterol plasma levels is one of the most critical issues in the prevention of atherosclerotic CVD. Clinical studies confirm the major role of high-density lipoproteins (HDLs) and its major component, apolipoprotein A-1 (apo A-1) in maintaining cholesterol balance thereby protecting against this disorder [40].

HDL cholesterol has multiple anti-atherogenic attributes, which include inhibition of platelet aggregation, anti-inflammatory and anti-adhesive effects and also NO promoting activity. Furthermore the main role of HDL is mediating reverse cholesterol transport (RCT), which is upregulated by ABC transporters A1 and G1 [41].

RCT is a complicated process, which involves delivery of excess cholesterol from peripheral cells and tissues, mainly foam cells to the liver for biliary excretion and finally excretion to the feces. This mechanism is closely described below and shown in **Figure 4**. The initial step of RCT is macrophage cholesterol efflux including lipidation of lipid-free apo A-1 via ABCA1 transporters, which is described in more detail in the next chapter.

FC collected by nascent HDL is esterified by the enzyme lecithin:cholesterol acyltransferase (LCAT) into cholesteryl ester (CE). CEs are transferred through scavenger receptor class B 1 (SR-B1) transporters directly to the liver or via cholesterol ester transfer protein (CETP) to apoB-containing lipoproteins. Subsequently LDL and VLDL particles are taken up by the liver through LDL receptors. Furthermore mature HDLs can be modified to smaller particles through the hepatic lipase (HDL triglycerides) or endothelial lipase (HDL phospholipids). Finally hepatic cholesterol is excreted into the bile directly as unesterified cholesterol (UC) or through conversion to bile acids via ABCG5/8 and ABCB11-mediated pathways. The last step is excretion in the feces [42, 43].

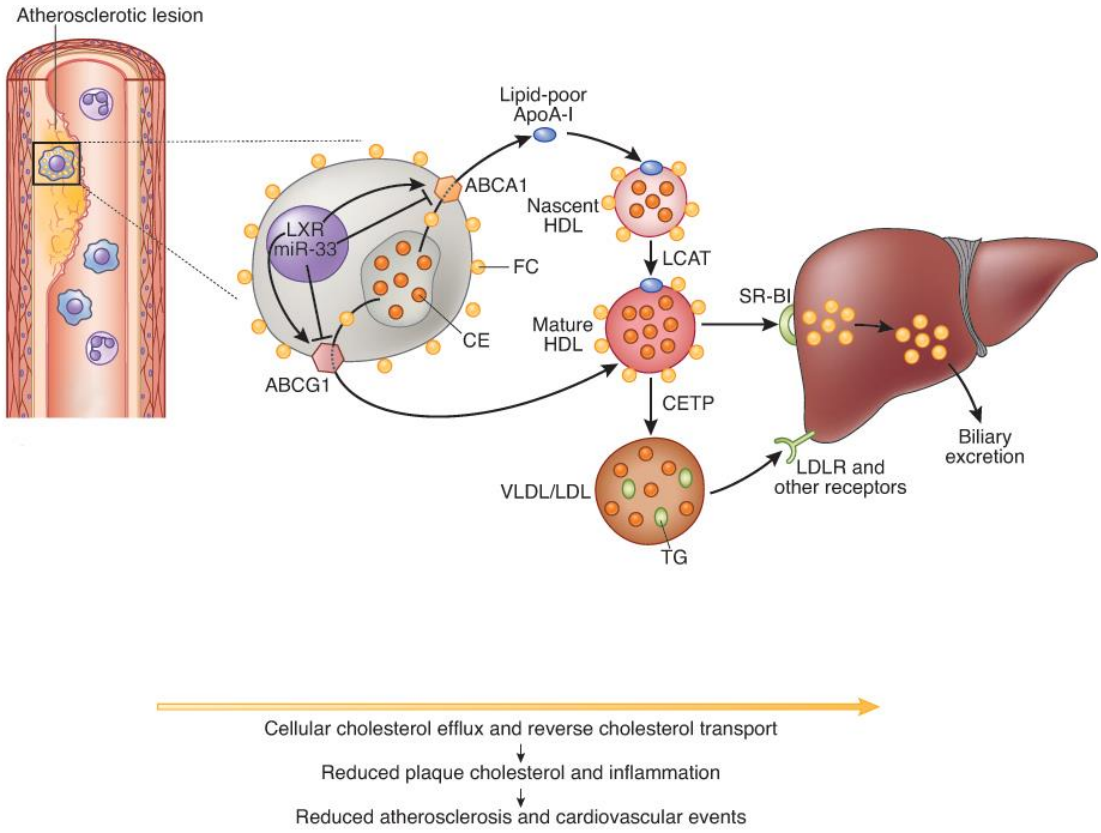

Figure 4: Reverse cholesterol transport and its initial mechanism cholesterol efflux

One of the most important parts of reverse cholesterol transport is HDL metabolism. Apo A-1 forms discoidal nascent HDL particles through lipidation that are easily modified and transported to the liver for excretion.

ABCA1, ATP-binding cassette transporter A1; ABCG1, ATP-binding cassette transporter G1; apo A-1, apolipoprotein A1; CE, cholesteryl ester; CETP, cholesterol ester transfer protein; FC, free cholesterol; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; LDL, low-density lipoprotein; LDLR, low density lipoprotein receptor; LXR, liver X receptor; SR-B1, scavenger receptor class B type 1; TG, triacylglycerol; VLDL, very low-density lipoprotein

Picture obtained from [43]

1.4 Cholesterol efflux

Efflux of UC from macrophage-derived foam cells to lipoprotein particles is the initial process of reverse cholesterol efflux. In total four different pathways are involved into removal of excess cholesterol by HDL and thereby play a role in protecting cells against the cytotoxic influences of cholesterol. The impairment of this pathways lead to the overloading of foam cells with cholesterol and thus favours the development and additional progress of atherosclerotic lesions [38].

The aqueous diffusion and facilitated diffusion mediated by scavenger receptor class B1 are part of passive pathways. But more important are active processes, which are facilitated through ABC transporters – ABCA1 and ABCG1 [38].

Recent studies reported that in humans ABCA1 and controversially also ABCG1 transporters are essential in translocating cholesterol into the extracellular space. It has been estimated that ABCA1 is responsible for 80 % and ABCG1 for the remaining 20 % of cholesterol efflux from macrophage-derived foam cells. In addition results from several laboratories confirmed that ABCG1 transporters can promote cholesterol efflux only through mature spherical HDLs. In comparison the SR-B1 pathway has a minor role [44].

Macrophages are able to export UC to extracellular HDLs, in particular to lipid-free apo A-1 acceptors supported by ABCA1 transporters. UC is transferred to the endoplasmic reticulum, where it is esterified by acyl-coenzyme A:cholesterol acyltransferase (ACAT). This leads to a reduced toxicity of cholesterol, which if not esterified could cause apoptosis. Cytosolic CEs are stored in lipid droplets, whose accumulation lead to the formation of foam cells. CEs can be hydrolysed by a neutral cholesteryl ester hydrolase and can later be transferred again to the endoplasmic reticulum for reesterification. The process is discontinued by the presence of cholesterol acceptors, predominantly HDL. Enhanced serum concentration of HDL supports elimination of cholesterol and thereby has a positive effect on RCT [42].

The balance of this whole pathway is very important, as loading of foam cells with cholesterol, decreased cholesterol efflux capacity and low plasma levels of HDL are connected with an increased incidence of CVD [45].

1.5 ATP binding cassettes

The ABC transporters are cell membrane proteins and members of the largest transporter gene family. ABC systems obtain energy through utilization of ATP and different subtypes are able to transport a huge variety of molecules against a concentration gradient across extra- and intracellular membranes [46].

Generally the structure of ABC proteins is defined by the presence of two hydrophilic ATP-binding domains (nucleotide binding domains (NBD)), located in the cytoplasm and two sets of hydrophobic transmembrane domains (TMD). The NBDs are responsible for hydrolysing ATP thus generating the driving force for active transport. The transmembrane domains on the other hand are important in recognition and translocating diverse substrates including ions, peptides, lipids and other metabolites across lipid membranes [47].

Nowadays 49 human ABC genes are known [48], which are divided into 7 structural subfamilies, labelled A to G. Each subunit has a specific role in physiological processes. The ABCA and ABCG transporters are the most common classes encountered in lipid trafficking pathways [49].

Additionally mutations in ABC genes are linked to multidrug resistance and several inherited disorders, including Tangier disease (ABCA1), familial HDL deficiency (ABCA1), Stargardt disease (ABCA4), progressive familial intrahepatic cholestasis (ABCB11), cystic fibrosis (ABCC7), sitosterolaemia (ABCG5 and ABCG8), etc [49].

1.5.1 ABCA1

The ABC transporter A1 is a member of subfamily A, which contains in total 12 genes. ABCA1 has a significant role in the promotion of macrophage phospholipid and FC efflux and interestingly participates also in elimination of oxidized phospholipids, proatherogenic particles from vascular smooth muscle cells.

This cell membrane protein translocates lipophilic molecules to apo A-1 located in the extracellular space. In cholesterol-loaded human macrophages the interaction

between ABCA1 transporters and cholesterol-deficient and phospholipid-depleted apo A-1 is predominant and leads to the formation of nascent HDL particles [50].

Mutation in the human ABCA1 gene is typical for Tangier disease, an autosomal recessive disorder, which is characterized by almost total absence of plasma HDL and apo A-1 as well as the enlargement of the spleen, lymph nodes and tonsils because of cholesteryl ester deposition. Therefore an essential role of ABCA1 in HDL synthesis could be established [51].

ABCA1 expression is induced by cyclic adenosine monophosphate (cAMP) analogs and loading of human macrophages with cholesterol. Recent experiments have confirmed that ligands of nuclear hormone receptors, including the liver X receptor (LXR) family and its heterodimeric partner retinoid X receptor (RXR) regulate macrophage ABCA1 transporter at the transcriptional level [52, 53].

1.5.2 ABCG1

Another ABC transporter, from the subfamily G is particularly involved in the process of cholesterol removal from peripheral tissues. But in contrast to ABCA1, the ABCG1 transporter is not able to promote cholesterol efflux to lipid-free apolipoproteins A-1, but straight to mature HDL particles as was mentioned earlier [54].

Recent studies performed with ABCG1 transporters showed controversial results in triggering atherosclerotic lesions. Studies with ABCG1 knockout mice suggested the possibility that this transporter is at least involved in the promotion of cholesterol efflux. On the other hand another study with human cholesterol-loaded macrophages led to the conclusion, that absence of this transporter is not able to decrease efflux to HDL [55]. This publication also points out the fact, that increased expression of both transporters, ABCA1 and ABCG1, can be induced by activation of different nuclear receptors (NRs), included RXR and LXR [55, 56].

1.6 Nuclear receptors

NRs are transcription factors, which regulate transcription of genes controlling an extensive area of physiological and pathophysiological processes. Pathways in which NRs have an essential role are cell development, proliferation and metabolic homeostasis [57]. NRs are able to negatively or positively regulate gene expression of their respective target genes [58]. Although NRs act in the nucleus, it was suggested that they have additional regulatory functions within the cytoplasm [59]. Key modulators of cholesterol and lipid homeostasis are LXRs, pregnane X receptor, subfamily of peroxisome proliferator-activated receptors (PPARs) and farnesoid X receptors (FXRs) [60].

The general NRs are ligand-dependent transcription factors, which are regulated by hormonal and metabolic substances, such as steroid hormones, retinoic acids, thyroid hormones, fatty acids, leukotrienes and prostaglandins [61]. For several NRs, so called orphan receptors, a natural or synthetic regulatory ligands have not been found yet. The identification of ligands for these receptors is very desirable as they could be potential pharmaceutical targets for the treatment of diverse diseases such as cancer, osteoporosis, atherosclerosis, diabetes etc. [62]. A recent study showed a significant role of pro-inflammatory cytokines, TNFα and INF-γ, and early transcription factors, such as NF-κB, signal transducer and activator of transcription 1 and 3 (STAT-1 and 3) in expression of NRs thereby affecting metabolic disorders [63].

The characteristic structure of NRs is defined by five or six domains (see **Figure 5**). The amino-terminal region (A/B domain) contains the transactivation region AF-1 and several autonomous transactivation domains (AD). The DNA binding domain (DBD) with P, D, T, A boxes is highly conserved and responsible for the recognition of highly specific DNA sequences, so called response elements (REs) in promoters, and also for dimerization of NRs. The DBD is often a target for post-translational modifications. The ligand binding domain (LBD) is located in the C-terminal part of the receptor. The

LBD is characterized by a set of allosteric signalling domains with four completely diverse surfaces. The dimerization surface, the ligand binding pocket, the coregulator binding surface and the activation function-2 (AF-2) region, whose conformation depend on bound ligand [64, 65]. Conformational changes in the LBD triggered by ligands influence coactivator and corepressor binding [66]. The hinge region is located between region C and E and holds nuclear localization signals. The C-terminal domain is form by a variable F region, whose functions are so far poorly understood [65].

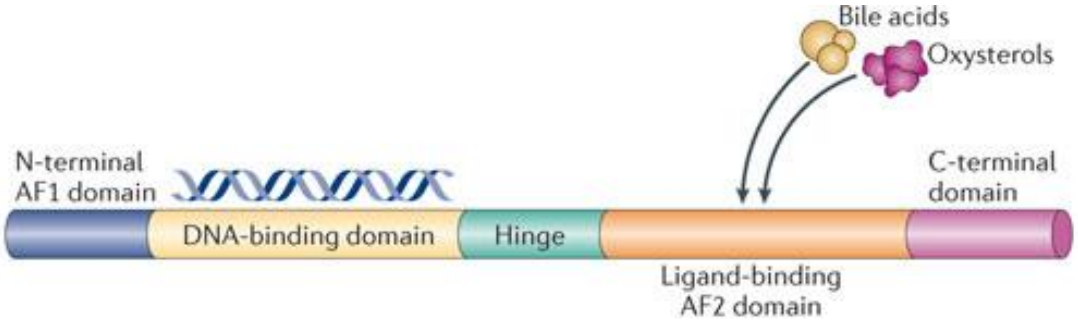

Figure 5: The primary structure of a nuclear receptor

AF1 domain, activation function 1 domain; AF2 domain, activation function domain 2

Picture taken from [67]

Receptors occur as monomers, homodimers or heterodimers and bind to DNA REs in enhancer or promoter regions of target genes. In dependence on the mode of action NRs are divided into three subclasses (**Figure 6**). The first subclass includes estrogen and androgen NRs. After ligand binding, these receptors homodimerize and enter into the nucleus. The ligand-receptor complex, together with coactivators, enables the activation of target genes [68]. The second subclass consists of thyroid (TR), retinoic acid (RAR), PPARs and LXRs. These NRs are characterized by binding to DNA REs even without ligands thus facilitating repressive functions. In the case of bound ligand these corepressors are substituted with a coactivator complex that leads to gene activation. Receptors from this subclass mainly form heterodimers with RXR. The third subclass,

called the orphan receptors, which typically forms monomers and consists of TLX (NR2E1), G-protein coupled receptors (GPCR), etc. [69].

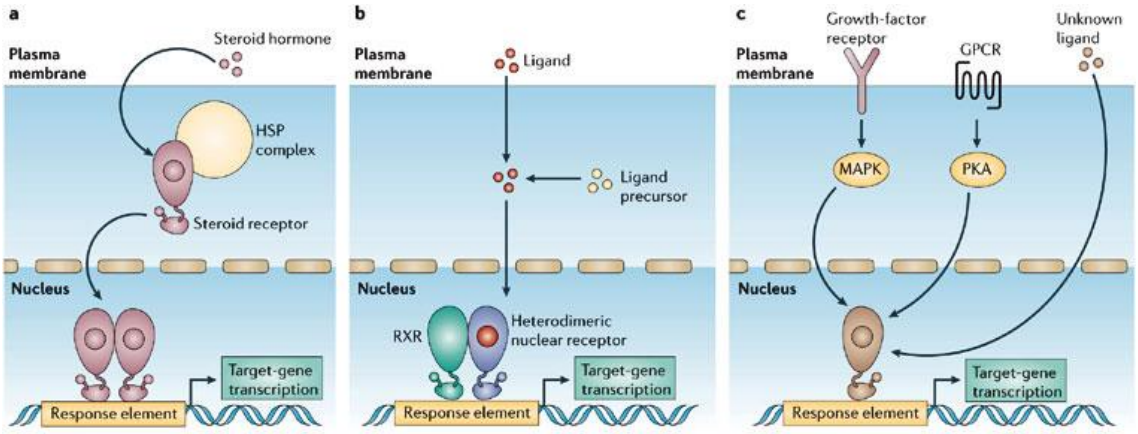

Figure 6: Mechanisms of DNA binding and transcriptional activation

A) Classical steroid-hormone NRs. B) Heterodimeric nuclear receptors, such as PPARs and LXRs, which bind to DNA with retinoid x receptors. C) Orphan nuclear receptors.
GPCR, G-protein coupled receptor; HSP, heat-shock protein; MAPK, mitogen-activated protein kinase; PKA, cAMP-dependent protein kinase; RXR, retinod X receptor

Picture adapted from [70]

1.6.1 Farnesoid X receptor

FXR is a member of the nuclear hormone superfamily, which is mainly expressed in liver, intestine, kidney and adrenals, but also in immune cells (macrophages). Recent studies with this transcription factor have established its role in the regulation of cholesterol and glucose metabolism, bile acid transport and inflammation response. FXR is expressed in diverse healthy, but also pathologic human tissues. In conjunction with RXR, FXR binds as heterodimer to DNA (see **Figure 7**), but is also able to regulate gene expression as a monomer [63, 71].

Initial studies performed with FXR knockout mice revealed a proatherogenic profile with elevated levels of cholesteryl esters, phospholipids and triglycerides. On the other

hand, HDL levels were significantly increased [72]. FXR has additionally been reported to modulate inflammation responses via interference with the NF-κB signalling pathway.

Strong natural activators of FXR are primary and secondary bile acids, the final products of cholesterol metabolism. As increased levels of chenodeoxycholic, lithocholic and deoxycholic acids have toxic effects, their homeostasis should be regulated. Guggulsterone acts as highly efficacious FXR modulator. On the other side the most active synthetic FXR ligands are GW4064 and INT-767 [73].

1.6.2 PPAR and LXR pathway

LXRs include two members, LXRα and LXRβ, which are activated by oxysterols. Several studies show their important role in controlling whole body cholesterol metabolism, cholesterol efflux and biosynthesis of VLDL. The inhibition of inflammation by NF-κB antagonism and decreased vascular smooth muscle cell proliferation belong to anti-atherosclerotic effects of both LXR isoforms. Additionally LXRs bind to ABCA1 and ABCG1 promotors and thus upregulate their expression levels and promote cholesterol efflux [74].

In addition the PPAR family include three subtypes – PPARα, PPARβ and PPARγ, which can be activated by fatty acids and pharmacological known synthetic compounds such as fibrates for PPARα and thiazolidinediones (pioglitazone) for PPARγ. PPARs have a role in regulation of lipid and lipoprotein metabolism, adipogenesis and glucose homeostasis [75]. Some groups established that these NRs are able to influence lipid metabolism and inflammation also in macrophages, endothelial cells and smooth muscle cells. Important role of PPARs in atherosclerosis support their ability to inhibit expression of VCAM-1 and MCP-1, which are engaged in leukocyte migration [76]. Interestingly, it was established that PPARγ receptors elevate ABCA1 expression and cholesterol elimination from macrophages via a transcriptional cascade mediated by LXRα [77].

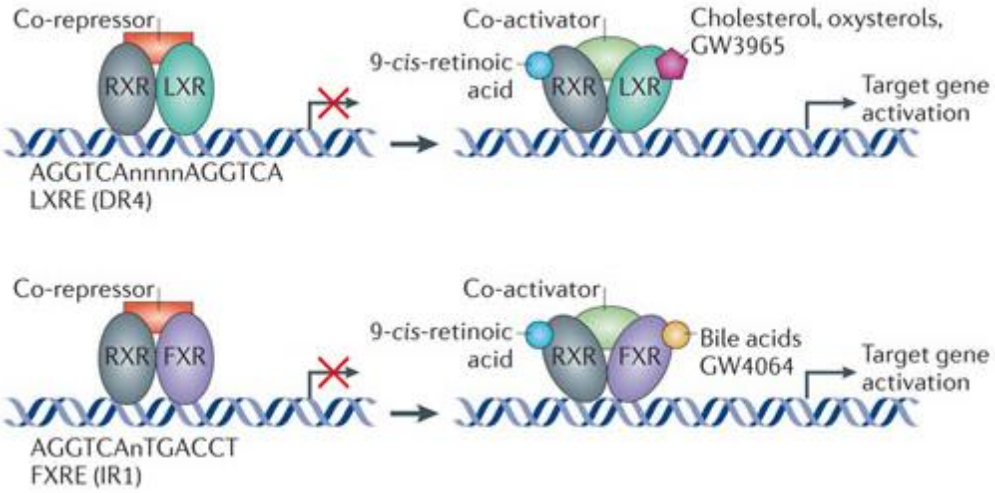

Figure 7: LXR/RXR and FXR/RXR heterodimers

Analogously, LXR or FXR forms a heterodimer with retinoid X receptor that binds to relevant response elements, LXRE or FXRE, in the regulatory regions. Ligand binding elicits conformational changes thereby releasing corepressors and recruiting coactivators to the heterodimer.

GW3965, ligand of liver X receptor; GW4064, ligand of farnesoid X receptor; FXR, farnesoid X receptor; FXRE, farnesoid X response element; LXR, liver X receptor; RXR, retinoid X receptor

Picture obtained from [67]

1.6.3 RXR receptors

RXRα, RXRβ, RXRγ are three different subtypes of RXRs whose most known ligands are 9-cis-retinoic acid (9-cis-RA) and fatty acids. RXR can modulate gene expression as homodimer, but forms heterodimers with a lot of NRs, including FXRs, LXRs and PPARs, PXRs etc. [78]. Their role lies mainly in regulation of metabolic signalling pathways, influencing glucose, fatty acid and cholesterol metabolism. Through activation of several NR complexes RXRs interfere multiple physiological processes.

RXR heterodimer complexes are categorized as either permissive or non-permissive based on their activation, which can be caused by bound RXR ligand or ligand of the heterodimeric partner receptor. The dimers with FXR, LXR and PPAR are considered to form permissive complexes. On the other hand non-permissive RXR-RAR or RXR-TR dimers are able to release corepressors and coactivators only in case of bound ligand of the partner. In consequence RXR agonists are now discussed for their protective cardiovascular potential [79].

1.7 Used natural products

1.7.1 Leoligin

Leoligin [(2S,3R,4R)-4-(3,4-dimethoxybenzyl)-2-(3,4-dimethoxyphenyl)tetrahydrofuran-3-yl]methyl (2Z)-2-methylbut-2-enoat], is a major lignan from *Leontopodium nivale ssp. alpinum* and belongs to the Asteraceae family. This alpine plant is widely known as Edelweiss and used in traditional medicine for centuries. Recent studies showed its anti-inflammatory and analgesic effects [80, 81]. A newly confirmed function of leoligin is its activation of CETP expression, which is particularly responsible in the regulation of HDL metabolism [82]. In this work the leogilin derivative 2780 was tested in different concentrations (chemical structure of leoligin derivative 2780 and leoligin is shown in **Figure 8** and **9)**. These compounds were synthetized and obtained from the group of Prof. Marko Mihovilovic, Institute of Applied Synthetic Chemistry, University of Technology in Vienna.

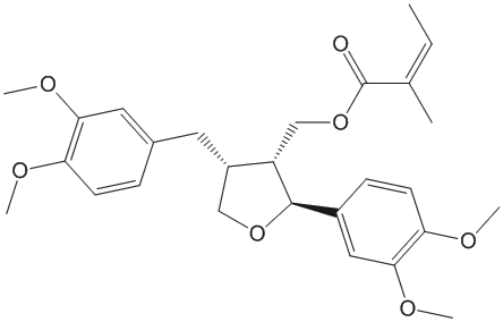

Figure 8: Structure of leoligin

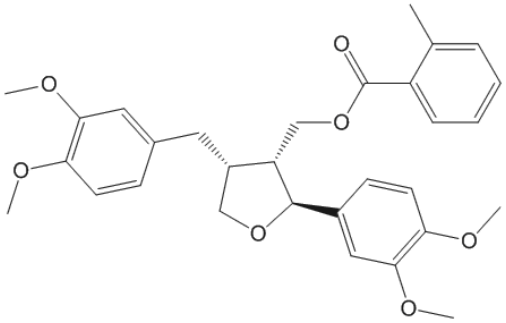

Figure 9: Structure of leoligin derivative 2780

1.7.2 Guggulsterone

The tree gum resin of *Commiphora mukul* with its bioactive constituent guggulsterone [4,17(20)-pregnadiene-3,16-dione] is used in traditional Indian medicine to treat a wide variety of illnesses, including obesity, hypercholesterolemia, atherosclerosis and rheumatism. Guggulsterone is a highly effective modulator of FXR *in vitro* and *in vivo*, and has been shown to decrease levels of LDLs and triglycerides in humans [73, 83].

However, guggulsterone upregulates the expression of the bile salt export pump (BSEP), which is important in the elimination of cholesterol metabolites from the liver. Additionally guggulsterone possesses anti-inflammatory activities presumably caused by inhibition of NF-κB [84]. All these activities can be linked to hypolipidemic activities of guggulsterone.

The trans-isomer of this compound, (*Z*)-guggulsterone (see **Figure 10**), was used in this work as an interesting tool compound, probably connecting FXR and lipid metabolism.

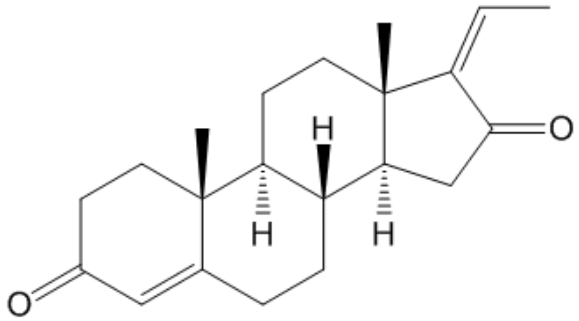

Figure 10: Structure of (*Z*)-guggulsterone

1.8 Preliminary research

In previous studies at our department the natural compound leoligin and also leoligin derivative 2780 were identified to increase cholesterol efflux mediated by apo A-1 in THP-1 cells. All tested concentrations of leoligin had no negative effect on cell viability as determined by ressazurin conversion assay (performed by Limei Wang, PhD student at the Department of Pharmacognosy, University of Vienna).

In addition the natural compound leoligin and several leoligin derivatives were tested on their ability to activate FXR. These studies were performed by Alexandra Holzer, diploma student at the Department of Pharmacognosy, University of Vienna. From this study one synthetic leoligin derivative emerged that had a strong activity on FXR transactivation (EC_{50}= 6.36 µM, fold activation 4.79 ± 0,92). This derivative is called 2780. This compound was also tested for a possible cytotoxic effect in the used cell model (HEK-293 cells) but fortunately had no negative influence on cell viability. Additionally leoligin was only able to slightly transactivate FXR. Based on this data, we aimed to further characterize this leoligin derivative 2780 to gain insights into the previously described effects.

2. AIMS OF THIS WORK

Worldwide the number of people suffering from cardiovascular diseases increases continually [85]. One of the most important risk factors for CVD is hyperlipidemia, which enormously enhances cardiovascular morbidity and mortality. Therefore the initial processes for the development for CVD as well as its progression are in the focus of many research teams.

As previously mentioned the leoligin study was initiated sooner and yielded very promising results in enhancing cholesterol efflux from macrophage-derived foam cells. Moreover the leoligin derivative 2780, which was also able to promote cholesterol efflux, was identified to be able to transactivate FXR. Therefore the aim of this thesis was to study the influence of this derivative in addition to other well-known FXR agonist on the expression of cholesterol transporters in THP-1 macrophage-derived foam cells. Additionally a modulator of FXR, the natural compound guggulsterone, which is used in traditional medicine over several thousands of years for lipid disorders, was investigated. In particular the experimental work was focused on the determination of the expression levels of the ABC transporters, ABCA1 and ABCG1 and scavenger receptor B1 (SR-B1) by western blotting.

Moreover the specifity of the leoligin derivative 2780 in the transactivation of other NRs was determined in the course of this work. The following NRs were evaluated via Luciferase reporter gene assay in HEK-293 cells: RXRα, LXRα, LXRβ, PPARα, PPARγ. Furthermore the binding of compound 2780 to FXR was confirmed in an FXR-Gal4-transactivation assay.

3. MATERIALS AND METHODS

3.1 Cell lines

All cell culture work was performed in a laminar flow cabinet under aseptic conditions. For the prevention of contamination all materials were sprayed with 70% ethanol before use. The cell lines used in this study were cryopreserved in liquid nitrogen and therefore had to be thawed and resuspended in complete medium. The 75 cm^2 or 175 cm^2 cell culture flasks were incubated at 37°C and 5 % CO_2 and were checked for contamination via light microscopy. When the cells reached a confluence of approximately 90% cells were passaged. All reagents used were prewarmed in a 37 °C water bath (detailed information regarding used media and solutions in **Table 6**, chapter Materials, page 49).

The used technical equipment is given in **Table 14**, chapter Materials, page 56 and for details about software see **Table 15**, chapter Materials, page 57.

3.1.1 HEK-293 cells

This cell line was originally derived from human embryonic kidney cells that were then transformed with the human adenovirus type 5. It is an adherent cell line that is commonly used in biological research.

The HEK-293 cells were passaged three times per week and were used until passage 40. Cells were regularly checked under the microscope for contamination and confluency assessment. When the cells were passaged the complete DMEM medium was discharged and cells in a 75 cm^2 were washed with 10 ml PBS and trypsinized with 2 ml trypsin/ethylene diamine tetraacetic acid (EDTA) solution. The tissue flask was incubated 1 minute at 37°C in an incubator to detach all cells from the surface. Afterwards 10 ml complete DMEM medium was added to stop trypsinization. Then the cell suspension was transferred into a new falcon tube. After resuspension, approximately 1 ml of this suspension was counted in a Vi-Cell™ XR Cell Viability Analyzer. The required amount of cells was calculated to obtain 5 x 10^6 cells in 12 ml

complete DMEM medium. This suspension was added into a new 75 cm^2 cell culture flask and incubated at 37°C and 5 % CO_2.

3.1.2 THP-1 cells

THP-1 is a human monocytic cell line that was derived from the peripheral blood of an acute monocytic leukemia patient. This cell line grows in suspension in a cell culture incubator at 37°C and 5 % CO_2 and needs to be passaged twice a week.

For subcultivation complete RPMI-1640 medium was used, which was supplemented with 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin and 50 ml heat-inactivated FBS. All of the supplements were sterile filtered before addition.

The passaging process should start always at the same time to get the best reproducibility. To consider these cells for an experiment cell viability should not be below 96%. First the cell suspension was transferred from the cell culture flask into a new 50 ml falcon tube, followed by centrifugation for 4 min at 150 x g. The supernatant was discarded and the pellet was resuspended in the same amount of new complete RPMI-1640 medium. Subsequently 1 ml of this cell suspension was measured with a Vi-Cell™ XR Cell Viability Analyzer. For subcultivation, 0.2 x 10^6 viable cells per ml, in total 4 x 10^6 cells in 20 ml complete RPMI-1640 medium were seeded into a new 175 cm^2 cell culture flask.

3.2 Luciferase Reporter Gene Assay

The Luciferase reporter gene assay is a widely used method facilitating the analysis of transcriptional activity. The advantages of this system are high detection sensitivity and simple quantification.

To perform this assay, cells were transfected with the expression plasmid of the investigated NR, the reporter plasmid with the corresponding hormone RE linked to a luciferase gene and an enhanced green fluorescent protein plasmid. Upon binding of the ligand the respective NR is activated. This activated NR is then able to bind to the RE of the target promotor and modulate the expression of the luciferase reporter gene.

The Gal-4 reporter gene assay is a different system. It is an efficient method for the detection of specific protein-ligand and protein-protein interaction. In comparison to conventional luciferase reporter gene assay, the Gal-4 assay determines the binding of the compound to the LBD of the investigated NR. Thereby the Gal-4 assay characterizes the compound as agonist or antagonist of the respective NR. This one-hybrid assay uses three plasmids: the expression plasmid containing the Gal-4 DBD fused to the NRs LBD, the plasmid containing a Gal-4 response element linked to the luciferase gene and an EGFP plasmid, as the transfection control. The conformational changes in the LBD triggered by the bound ligand lead to the activation of the Gal-4 response element and thereby to the expression of the luciferase gene [86].

Luciferase, an enzym originated from the firefly *Photinus pyralis,* catalyzes the conversion of luciferin to oxyluciferin in the presence of ATP and magnesium. Subsequently, this reaction emits visible yellow-green light with a wavelength of 562 nm (**Figure 11**). The light intensity of the bioluminescence correlates with the amount of luciferase transcribed which correspond to the activation of the upstream promotor.

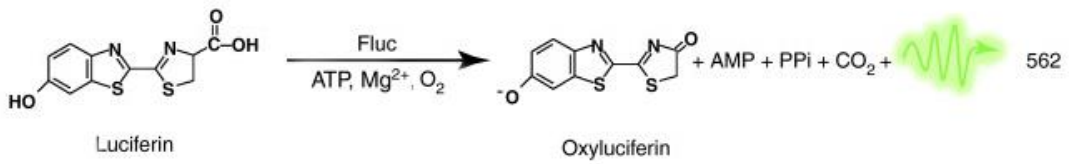

Figure 11: Bioluminescence reaction of luciferin to oxyluciferin catalyzed by luciferase

AMP, adenosine monophosphate; ATP, adenosine triphosphate; Fluc, firefly luciferase; PPi, inorganic pyrophosphate

Picture adapted from [87]

The bioluminescent proteins, aequorin and green fluorescent protein (GFP), are originated from the jellyfish *Aequorea victoria*. In the presence of calcium the photoprotein aequorin undergoes a conformational change resulting in the emission of blue light (λ_{max} = 470 nm). Then the GFP is able to absorb the light emitted by aequorin and fluoresce green (λ_{max} = 510 nm). In the experiments performed in the course of this work enhanced green fluorescent protein (EGFP), a mutant version of GFP, with optimized conditions (higher expression and brightness), was used. The transfection of cells with EGFP serves as an internal control determining the efficiency of the transfection and indicates a possible toxicity of the treatment [88].

3.2.1 Seeding and transfection

For luciferase reporter gene assays, cells were seeded twice a week in a concentration of 6 x 10^6 cells per 15 cm dish and 4 x 10^6 cells per 10 cm dish. Cells were incubated for 16 hours before the transfection was started.

After 16 hours cells were transfected using the calcium phosphate transfection method. The transfection mix was prepared in a total volume of 1500 µl and contained 5 µg of the tested NR expression plasmid (RXRα, LXRα, LXRβ, PPARα, PPARγ), 5 µg reporter plasmid with the corresponding hormone REs linked to a luciferase gene (RXRE, ABCA1, PPARE) and 3 µg EGFP plasmid. For Gal-4 assays 1 µg of expression plasmid encoding Gal-4 (FXR-Gal4), 5 µg of Gal-4 response element linked to a luciferase gene (tk(MH100)4Luc2) and 5 µg EGFP were used. The exact volumes were

dependent on stock concentrations and were filled up to 656 µl with water. Then 750 µl 2x HBS and 94 µl 2M calcium chloride were added and the mix was immediately vortexed (detailed information on used plasmids and solutions in **Table 7**, chapter Materials, page 50). The eppendorf tube was left 20 minutes at room temperature to allow the complexes to form. Afterwards the solution was added drop wise to the cells in the 15 cm dishes. The 10 cm dish with untransfected cells served as a control. Dishes were checked under the light microscope and incubated for 6 hours.

3.2.2 Preparation of compounds

50 µl of compound solutions in stripped DMEM medium were put in an empty 96 well-plate in quadruplicates. The tested samples included, leoligin derivative and guggulsterone as well as respective positive controls (see **Table 2**). As a negative control 0.1% dimethylsulfoxide (DMSO) was used. All experiments were performed at least three times.

Table 2: Agonists of diverse nuclear receptors

Nuclear receptors	Positive controls
RXRα	9-cis retinoic acid 5 µM, bexarotene 1 µM
LXRα	GW3965 1 µM
LXRβ	GW3965 1 µM
PPARα	GW7647 50 nM
PPARγ	Pioglitazone 5 µM, 10 µM

3.2.3 Reseeding

After 6 hours of incubation the medium with the transfection mix was aspirated from the dishes and the cells were washed with 20 ml PBS. To detach the cells from the surface trypsin/EDTA was added for 1 minute (2 ml for the 15 cm dish and 1 ml for the 10 cm dish). Trypsinization was stopped with the addition of 18 ml (15 cm dish) or 9 ml

(10 cm dish) complete DMEM medium. Then the obtained cell suspension was centrifuged at 1100 x g for 4 minutes. The supernatant was discarded and the pellet was resuspended in 10 ml (15 cm dish) or 5 ml (10 cm dish) stripped DMEM medium. After counting of the cells in a Vi-Cell™ XR Cell Viability Analyzer 50 000 cells per 150 µl were seeded per well in the already prepared 96-well plate. Finally the plate was gently shaken for better distribution and left in the incubator for 18 hours.

3.2.4 Measurement

On the next day the cells were checked under the fluorescent microscope to see if the EGFP transfection was successful. Then the plate was aspirated and stored at -80°C for at least one hour. Before the measurement ATP and Luciferin solutions were thawed and kept in a dark place. Additionally the multimode reader Tecan GENios™ Pro was prepared for the measurement. The parameters and settings for the measurement are described in **Table 3** and **4**.

After addition of 50 µl lysis buffer per well the 96-well plate was shaken for 10 min on a plate shaker. Then 40 µl of the cell lysate was transferred to a black 96-well plate and additionally shaken for 1 minute to remove air bubbles. Then the fluorescence and luminescence was measured in the multimode Tecan GENios™ Pro using auto injection of ATP and luciferin solutions (see recipes in **Table 8**, chapter Materials, page 51).

Table 3: Parameters for fluorescence measurement with the Tecan GENios™ Pro

Measurement mode – Fluorescence	
Excitation wavelength	485 nm
Emission wavelength	520 nm
Gain	Optimal
Number of reads	1
Integration time	1000 µs
Lag time	0 µs
Mirror selection	Dichroic 3
Time between move and flash	40 ms

Table 4: Parameters for luminescence measurement with Tecan GENios™ Pro

Measurement mode – Luminescence	
Integration time	2000 ms
Attenuation	None
Time between move and integration	50 ms
Well kinetic number	1
Well kinetic interval	2020 ms
Injector A delay	-250 ms
Injector A volume	50 µl
Injector A speed	200 µl/s
Injector B delay	-250 ms
Injector B volume	50µl
Injector B speed	200 µl/s
Injector mode	Standard

3.2.5 Evaluation

After subtraction of the background obtained from the untransfected cells the luminescence values were normalized against the fluorescence values. Results are presented as the ratio RLU/RFU (relative luminescence unit / relative fluorescence units) normalized to the solvent control (DMSO) also called fold activation.

3.3 Western blotting

Western blotting is an analytical technique widely used for the detection of proteins in cell lysates.

3.3.1 Seeding and treatment

THP-1 cells were seeded in a concentration of 0.2 x 10^6 viable cells/ml in a total volume 4 ml in a 6-well plate. In every well 6 µl 1 mM phorbol-12-myristate-13-acetate (PMA) was added yielding a concentration of 200 nM. Afterwards the plate was incubated for 72 hours to allow PMA-induced differentiation of the THP-1 monocytes into macrophages.

Compounds were diluted in serum-free RPMI-1640 medium with 0.1% BSA and 20 µg/ml UC (details on stock solution are given in **Table 9**, chapter Materials, page 52). After washing once with 4 ml PBS 2 ml of the prepared dilutions were added to the respective wells (**Table 5**). The plates were then incubated for 24 hours.

Table 5: Compound used for treating THP-1 cells

Compound	Stock	Concentration
Leoligin derivative 2780	30 mM	30 µM, 10 µM, 3 µM, 1 µM
CDCA	50 mM	50 µM, 30 µM, 10 µM, 3 µM
Lithocholic acid (LCA)	30 mM	10 µM
GW4064	30 mM	10 µM, 3 µM
Guggulsterone	50 mM	30 µM, 10 µM, 3 µM, 1 µM
Pioglitazone	30 mM	10 µM

3.3.2 Cell lysis and protein extraction

All following steps were performed on ice to avoid protein degradation. The medium was carefully aspirated with a Pasteur pipette and then cells were washed once with cold PBS. Afterwards 280 µl freshly prepared cold NP40 lysis buffer (see recipe in **Table 10**, chapter Materials, page 52) was added to each well and the whole plate was incubated 30 minutes on a laboratory rocking platform shaker at 4°C. Now cells were scraped with a cell scraper and transferred in labelled eppendorf tubes. The lysates were centrifuged at 16060 x g at 4°C for 20 min and the obtained supernatant was carefully transferred into a new eppendorf tube. Protein samples were stored at -20°C for gel electrophoresis or immediately used for protein quantification.

3.3.3 Bradford assay

The Bradford assay was used to determine the total protein concentration of a sample. The method is based on the binding of Coomassie Brilliant Blue to protein molecules resulting in a visible color change (from blue to brown). The method was performed with Bradford reagent (Roti®-quant solution) using bovine serum albumin (BSA) as the reference standard.

First 45 µl ddH_2O was mixed with 5 µl of protein supernatant to get a protein dilution of 1:10. These samples could be stored now at -20°C or immediately used. In total 205 µl were added to each well in a 96-well plate (see **Figure 12**). For cell lysates that means 5 µl diluted sample and 10 µl ddH_2O and for bovine serum standards (BSA) 5 µl diluted lysis buffer and 10 µl BSA standard in each well. As a last step 190 µl freshly prepared Roti®-quant reagent solution was added. All samples were prepared in triplicates and absorbance was measured in a Tecan Sunrise™ plate reader at 595 nm. The acquired values from bovine serum standards were used to generate of a standard curve.

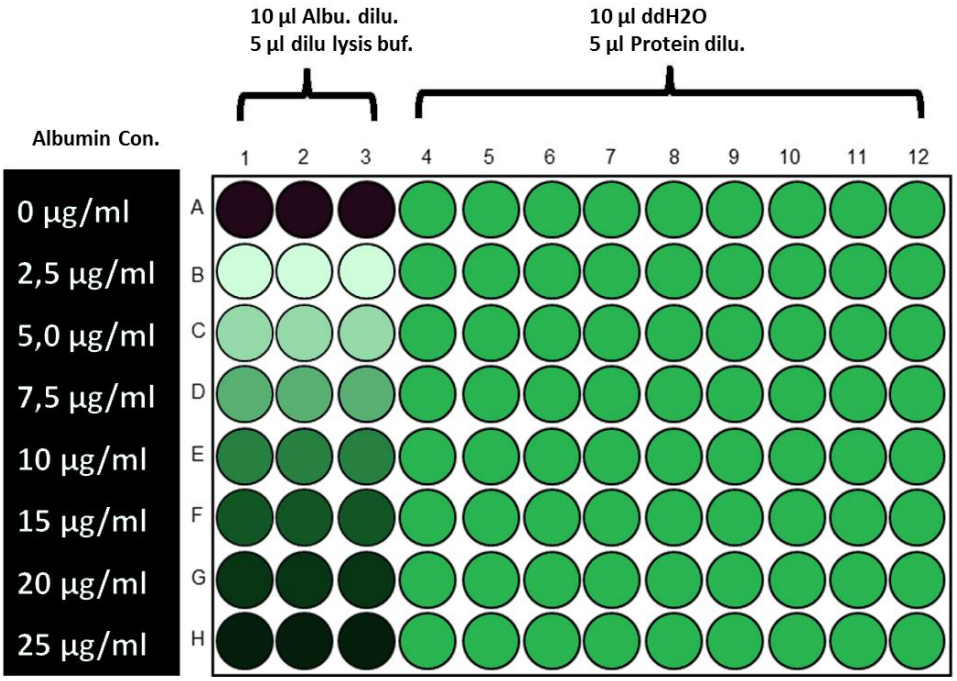

Figure 12: Schematic sketch for Bradford assay

All the samples were measured in triplicates. BSA standards were used in 7 increasing concentrations.

3.3.4 SDS-polyacrylamide gel electrophoresis

For the separation of proteins by molecular weight sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed. 60 µl of protein lysate were mixed with 20 µl sample buffer. Because of the size of the investigated proteins (42 kDa, 220 kDa) 10% gels were used. The freshly prepared gels were put in an electrophoretic chamber and filled with Epho buffer. 20 µg protein was loaded per slot. As a marker Precision Plus Protein^{TM} Standards All Blue was used. Proteins were separated for 70 minutes at 25 mA per gel or until the dye front of the samples reached the bottom of the gels. For detailed information regarding the used buffers and solutions see **Table 11**, chapter Materials, page 53.

3.3.5 Blotting

Polyvinylidenfluoride (PVDF) membranes were equilibrated in methanol and blotting buffer. The blotting sandwich was prepared as follows: sponge, filter papier, membrane, gel, filter papier and sponge again. This sandwich was then inserted into a cassette (see **Figure 13**). To avoid overheating during the blotting process a cooling box was added to the tank blot. Blotting was performed for 2 hours at 100 V. After blotting, the membranes were blocked with 5% milk in Tris-buffered saline supplemented with Tween-20 (TBS-T) at room temperature for 2 hours to saturate unspecific binding sites on the membrane. Details about the used reagents are given in **Table 12**, chapter Materials, page 54.

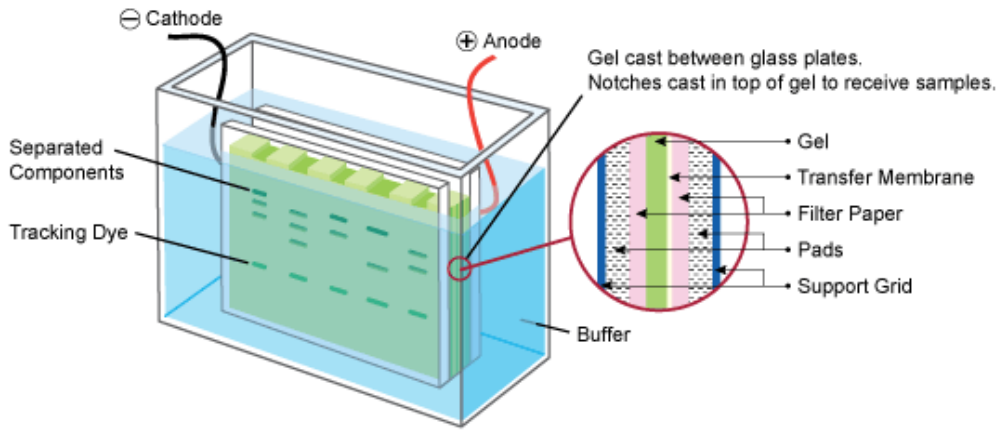

Figure 13: Western blotting schema

Figure adapted from [89]

3.3.6 Immunodetection

For chemiluminescent detection of the investigated protein the membranes were incubated with two antibodies (more details in **Table 13**, chapter Materials, page 55). The primary antibody binds directly to the target protein and is in a second reaction recognized by the secondary antibody, which is linked to horseradish peroxidase

(HRP). The chemiluminescence reaction exerts HRP conversion of luminol to light in the presence of hydrogen peroxide and p-coumaric acid.

After blocking the membranes were washed three times with TBS-T and incubated with primary antibody at 4°C overnight on a rotator. Afterwards the membranes were washed three times for 10 minutes with TBS-T and incubated with the secondary antibody for two hours at room temperature. Freshly prepared ECL solution was added and the membrane was incubated with it for 1 minute. The protein detection was performed at a LAS-3000™ Luminescent Image Analyzer and the intensity of emitted light was evaluated by the AIDA™ software. After measurement the membranes were washed with TBS-T and stripped with 0.5 M NaOH, which enables further incubation of the membrane. Subsequently the membranes were incubated with an actin antibody. The obtained ABCA1 and SR-B1 bands were then normalized to the matching actin bands (loading controls).

3.4 Statistics

The statistical analysis was performed with the GraphPad PRISM™ software by using one-way analysis of variance (ANOVA) with Bonferroni post hoc test. Each experiment was performed at least three times to receive reliable results. All values are mean ± standard deviation (mean ± SD). The statistical significance were considered at p values (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

3.5 Materials

3.5.1 Cell lines

Table 6: Media, reagents and solutions used in cell culture

Name	Components
Complete DMEM medium (Lonza Group Ltd)	500 ml DMEM without phenol red, 4.5 g/ml glucose 10% heat-inactivated foetal bovine serum (FBS) 2 mM L-glutamine 100 U/ml penicillin 100 µg/ml streptomycin
Stripped DMEM medium (Lonza Group Ltd)	500 ml DMEM without phenol red, 4.5 g/ml glucose 5% stripped foetal bovine serum (FBS) 2 mM L-glutamine 100 U/ml penicillin 100 µg/ml streptomycin
Complete RPMI-1640 medium (Lonza Group Ltd)	500 ml RPMI-1640 medium 10% heat-inactivated foetal bovine serum (FBS) 2 mM L-glutamine 100 U/ml penicillin 100 µg/ml streptomycin
Serum-free RPMI-1640 medium (Lonza Group Ltd)	500 ml RPMI-1640 medium 2 mM L-glutamine 100 U/ml penicilin 100 µg/ml streptomycin
Phosphate buffered saline (PBS) pH 7,4	36.0 g NaCl 7.4 g Na_2HPO_4 2.15 g KH_2PO_4

	ad 5000 ml ddH_2O
Trypsin/EDTA solution	0.5 g trypsin 0.2 g EDTA 1000 ml PBS

3.5.2 Luciferase reporter gene assay

Table 7: Luciferase reporter gene assay solutions and reagents

Name	Components	Stock (µg/ml)
DNA plasmids **Luciferase reporter gene assay**	3 µg EGFP 5 µg RXRE 5 µg ABCA1 5 µg PPARE 5 µg RXRα 5 µg LXRα 5 µg LXRβ 5 µg PPARα 5 µg PPARγ	243.80 521.00 743.60 764.00 419.00 526.00 624.00 221.10 680.30
DNA plasmids **Gal-4 reporter gene assay**	1 µg FXR Gal-4 5 µg tk(MH100)4Luc2 5 µg EGFP	
2x HBS	1.6 g 280 mM NaCl 74 mg 10 mM KCl 216.2 mg 12 mM Glucose 1.2 g 50 mM HEPES 26.7 mg 1.5 mM Na_2HPO_4 ad 100 ml ddH_2O	

$CaCl_2$	11 g $CaCl_2$ 50 ml ddH_2O	

Table 8: Luciferase reporter gene assay – measurement

Name	Components
ATP solution	1 g ATP 3.3 ml 1 N NaOH 13.4 ml ddH_2O
Luciferin solution	25 mg Luciferin 8.3 ml ddH_2O
ATP buffer	16.5 ml 1 M Tricine pH 7.8 35.5 ml 0,5 M $MgCl_2$ 30.5 ml 0,1 M ATP 744.3 ml ddH_2O
Luciferin buffer	82.7 ml 10x Luciferin solution 16.5 ml 1 M Tricine pH 7.8 691.6 ml ddH_2O
Lysis buffer	1.2 ml Reporter lysis buffer 5x 6 µl 270 mM CoA 6 µl 1 M DTT 4.8 ml ddH_2O

3.5.3 Western blotting

Table 9: Treatment reagents

Name	Components
PMA	200 nM PMA
Unesterified cholesterol (UC)	20 µg/ml unesterified cholesterol
Bovine serum albumin (BSA)	0.1% bovine serum albumin

Table 10: Protein extraction and quantification buffers and solutions

Name	Components
NP40 lysis buffer 1000 µl	940 µl NP40 40 µl Complete 10 µl PMSF 5 µl 1 M NaF 5 µl 100 mM Na_3VO_4
NP40 buffer (stock solution)	150 mM NaCl 50 mM HEPES pH 7.4 1% NP40
Roti®-quant solution	1 ml Roti®-quant 3.75 ml ddH_2O

Table 11: Electrophoresis buffers and solutions

Name	Components
Sample buffer	255 µl 3x SDS-Sample buffer 45 µl β-Mercaptoethanol
3 x SDS sample buffer	37.5 ml 0,5 M Tris-HCl pH 6.8 6 g SDS 30 ml Glycerol 15 mg Bromophenol blau ad 100 ml ddH_2O
Epho buffer	100 ml 10 x Epho-buffer 900 ml ddH_2O
10 x Epho buffer	30 g Tris-Base 144 g Glycine 10 g SDS Ad 1000 ml ddH_2O
Resolving gel 10%	2.5 ml 30% PAA 1.875 ml 1,5 M Tris-HCl pH 8.8 75 µl 10% SDS 3.05 ml ddH_2O 7.5 µl TEMED 37.5 µl 10% APS
Stacking gel	640 µl 30% PAA 375 µl 1,25 M Tris-HCl pH 6.8 37.5 µl 10% SDS 2.62 ml ddH_2O 7.5 µl TEMED 37.5 µl 10% APS

Table 12: Western blotting and protein detection buffers and solutions

Name	Components
Blotting buffer	100 ml 5 x Blotting-buffer 100 ml Methanol 300 ml H_2O
5 x Blotting buffer	15.169 g Tris-Base 72.9 g Glycine ad 1000 ml ddH_2O
TBS-T	100 ml 10 x TBS-T pH 8.0 900 ml H_2O
TBS-T 10 x	30 g Tris-Base 111 g NaCl 10 ml Tween 20 ad 1000 ml ddH_2O
ECL Chemiluminescence reagent	9 ml ddH_2O 1000 µl Tris Base pH 8.5 50 µl Luminol 22 µl p-coumaric acid 3 µl H_2O_2
Luminol stock	0.44 g Luminol 10 ml DMSO
Coumaric acid stock	0.15 g p-coumaric acid 10 ml DMSO

Table 13: Antibodies

Immunogen	Molecular weight	Source of antibody	Provider	Dilution
ABCA1α	220 kDa	Rabbit, polyclonal	Novus Biologicals	1:500, 3% milk in TBS-T
Rabbit IgG	-	Rabbit, polyclonal	Cell Signaling Technology	1:1000 in TBS-T
Actinα	42 kDa	Mouse	MP Biomedicals	1:10000 in TBS-T
Mouse IgG	-	Mouse	MP Biomedicals	1:1000 in TBS-T
SR-B1	82 kDa	Rabbit	Novus Biologicals	1:500 in TBS-T

3.5.4 Technical equipment

Table 14: Used technical equipment

Name	Provider
HERAcell™ 150 Incubator	Thermo Fisher Scientific Inc.
HERAeus™ Biofuge™ fresco	Thermo Fisher Scientific Inc.
HERAsafe™ KS Safety Cabinet	Thermo Fisher Scientific Inc.
HERAeus™ Multifuge™ 1S-R Centrifuge	Thermo Fisher Scientific Inc.
LAS-3000™ Luminescent Image Analyzer	Fujifilm
Light microscope Olympus CKX 31	Olympus Europe GmbH
Multi-Microplate Genie™	Thermo Fisher Scientific Inc.
PowerPac™ Basic Power supply	BIO-RAD Laboratories
Shaking Water Bath SW23	Julabo GmbH
Tecan GENios™ Pro	Tecan Group Ltd.
Tecan Sunrise™	Tecan Group Ltd.
Vi-Cell™ XR Cell Viability Analyzer	Beckmann Coulter
Vortex shaker	VWR International Inc.

3.5.5 Software

Table 15: Used software

Name	Provider
AIDA™	Raytest GmbH
GraphPad PRISM™ version 5	GraphPad Software Inc.
Microsoft® Office Excel 2007	Microsoft
Tecan GENios™ Pro, version 4.63	Tecan
Vi-Cell™ XR 2.03	Beckmann Coulter

4. RESULTS

4.1 Leoligin derivative 2780

4.1.1 Influence on the expression of the cholesterol transporter ABCA1

In order to further characterize the already established effects of the leoligin derivative 2780 on cholesterol efflux (see chapter Preliminary research, page 34) and atherosclerosis, respectively, western blot analysis was performed.

The ABCA1 expression after treatment with the leoligin derivative 2780 was investigated in human macrophage-derived foam cells. As seen in **Figure 14** compound 2780 increased ABCA1 protein levels in a concentration dependent manner resulting in enhanced cholesterol efflux. At the concentrations of 30 µM and 10 µM 2780 achieved the maximal ABCA1 expression levels, showing a 1.9 and 1.7 fold activation compared to the negative control (DMSO). Pioglitazone, a known PPARγ agonist in clinical use, was used as positive control in this experiment.

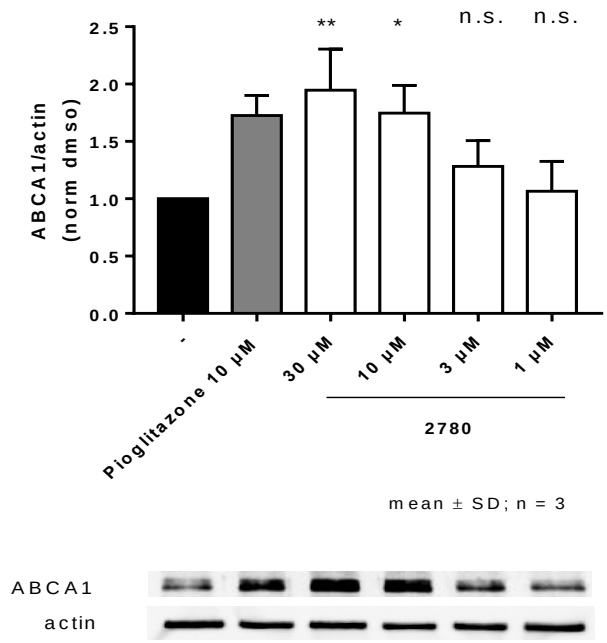

Figure 14: ABCA1 protein levels induced by leoligin derivative 2780

ABCA1 protein levels after treatment with leoligin derivative 2780, 0.1% DMSO (negative control) and 10 µM pioglitazone (Pio, positive control). The graph represents data from three independent experiments (n=3), shown as mean ± SD. The figure shows representative blots

with ABCA1 bands and corresponding actin bands that were used for normalization. The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; n.s., no significance).

4.1.2 Influence on the expression of the cholesterol transporter SR-B1

Western blotting analysis was performed to determine whether SR-B1 expression in macrophage-derived foam cells is influenced by leoligin derivative 2780. As seen in **Figure 15** the SR-B1 protein levels after treatment with the compound 2780 were not increased. None of the investigated concentrations achieved the effects on expression SR-B1 levels compared to the negative control (DMSO).

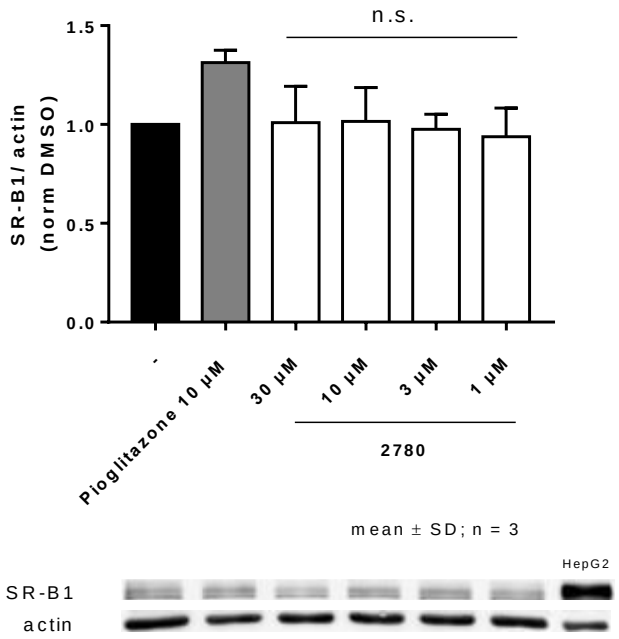

Figure 15: SR-B1 protein levels induced by leoligin derivative 2780

SR-B1 protein levels after treatment with leoligin derivative 2780, 0.1% DMSO (negative control) and HepG2 lysate (positive control). The graph represents data from three independent experiments (n=3), shown as mean ± SD. The figure shows representative blots with ABCA1 bands and corresponding actin bands that were used for normalization. The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; n.s., no significance).

4.1.3 Effect on diverse nuclear receptors

To establish an influence of the leoligin derivative 2780 on different NRs and thereby investigate the molecular mechanisms influencing cholesterol elimination, luciferase reporter gene assays were performed. All investigated NRs were identified as important regulators of cholesterol metabolism. It was established that PPARγ and LXRs cooperate in the regulation of ABCA1 expression leading to enhanced macrophage cholesterol efflux (PPARγ-LXR-ABCA1 pathway) [77].

As presented in **Table 16** none of these receptors were influenced by compound 2780 in the tested concentration range (from 0.1 to 30 µM). Therefore leoligin derivative 2780 seems to be a selective FXR agonist *in vitro*. So far FXR agonists were not established as potential enhancers of ABCA1 protein expression. Therefore these results suggest the existence of multiple molecular mechanisms in the regulation of cholesterol homeostasis by this compound.

Table 16: Effect of compound 2780 on nuclear receptors

Results from luciferase reporter gene assay in HEK-293 cells. The cells were treated with leoligin derivative 2780 in different concentrations (from 0.1 to 30 µM). The appropriate receptor agonists were used as positive controls and DMSO was used as solvent control. The obtained luminescence values were standardized to EGFP-derived fluorescence (RLU/RFU ratio) and normalized to the solvent control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments. (n=3).

Nuclear receptor	Used agonists	Activation by 2780
Retinoid X receptor α	5 µM 9-cis retinoic acid 10 µM bexarotene	×
Liver X receptor α	1 µM GW3965	×
Liver X receptor β	1 µM GW3965	×
Peroxisome prolifator-activated receptor α	50 nM GW7647	×
Peroxisome prolifator-activated receptor γ	5 and 10 µM pioglitazone	×

4.1.4 Activity on farnesoid X receptor

In order to investigate the direct effect of leoligin derivative 2780 on the transcriptional activity of FXR and thereby confirm this compound as specific FXR agonist, the Gal-4 reporter gene assay was performed. In addition to compound 2780 these experiments were also performed with the parent compound leoligin.

As shown in **Figure 16** and **17** both compounds are able to directly bind to the FXR-LBD and thereby activate Gal-4-FXR transcriptional activities. At a concentration of 10 µM leoligin derivative 2780 achieved the maximal fold activation of 14. In comparison to these data the maximal fold activation of leoligin at 20 µM was only 2. The values of both compounds were compared to the negative control (DMSO). Interestingly, the synthetic derivative 2780 elicited a significantly higher activation on the FXR-Gal4 construct compared to the leoligin.

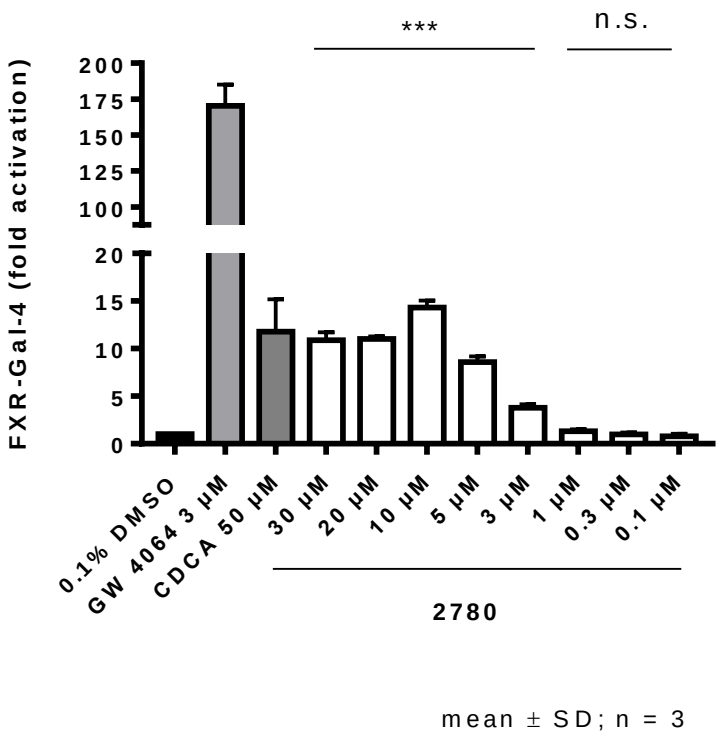

Figure 16: Dose-dependent transactivation of FXR by leoligin derivative 2780

Results from Gal-4 reporter gene assay in HEK-293 cells.

HEK-293 cells were transfected with an expression plasmid (FXR Gal-4), a reporter plasmid (tk(MH100)4Luc2) coupled to the luciferase reporter and EGFP as an internal control. Cells were stimulated with different concentrations of leoligin derivative for 18 hours.

The FXR agonists GW4064 and CDCA were used as positive controls and DMSO was used as solvent control. The luminescence values were standardized to EGFP-derived fluorescence (RLU/RFU ratio) and the results were expressed as fold activation normalized to the vehicle control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments (n=3). The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (***, $p < 0.001$; n.s., no significance).

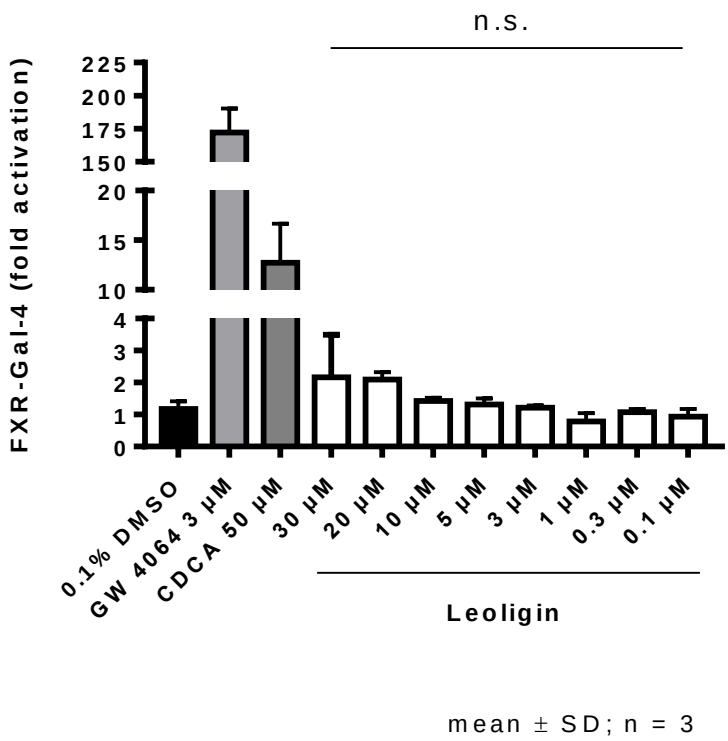

Figure 17: Dose-dependent transactivation of FXR by leoligin

Results from Gal-4 reporter gene assay on HEK-293 cells.

HEK-293 cells were transfected with an expression plasmid (FXR Gal-4), a reporter plasmid (tk(MH100)4Luc2) coupled to the luciferase reporter and EGFP as an internal control. Cells were stimulated with different concentrations of leoligin for 18 hours.

The FXR agonists GW4064 and CDCA were used as positive controls and DMSO was used as solvent control. The luminescence values were standardized to EGFP-derived fluorescence (RLU/RFU ratio) and the results were expressed as fold activation normalized to the vehicle control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments (n=3). The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (n.s., no significance).

4.2 Guggulsterone

4.2.1 Influence on the expression of the cholesterol transporter ABCA1

Next the influence of guggulsterone, a FXR modulator with hypolipidemic activities, on ABCA1 expression was investigated.

As determined by western blotting, guggulsterone is able to dose-dependently increase the expression of the ABCA1 in macrophage-derived foam cells (see **Figure 18**). The treatment with 30 µM and 10 µM guggulsterone leads to the upregulation of the ABCA1 protein levels in comparison to the negative control (2.4 fold). It has to be mentioned that this set of experiments was only performed twice, therefore no statistical analysis could be performed. Pioglitazone, a known PPARγ agonist in clinical use, was used as positive control in this experiment.

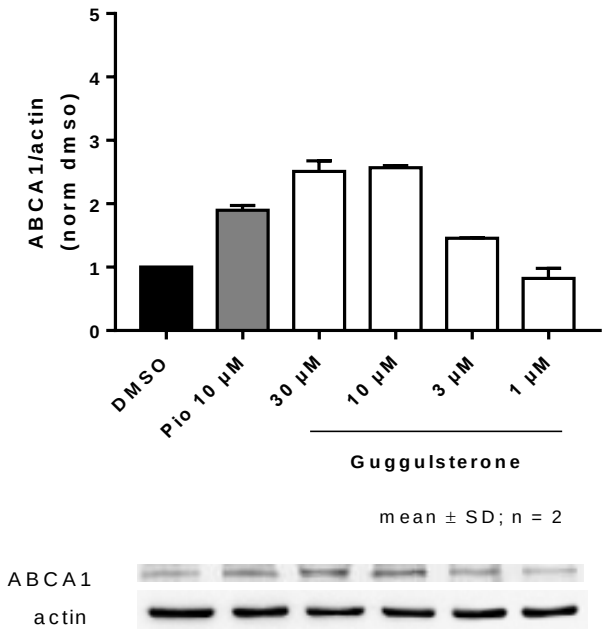

Figure 18: ABCA1 protein levels induced by guggulsterone

ABCA1 protein levels after treatment with guggulsterone, 0.1% DMSO (negative control) and 10 µM pioglitazone (Pio, positive control). The graph represents data from two independent experiments (n=2), shown as mean ± SD. The figure shows representative blots with ABCA1 bands and corresponding actin bands that were used for normalization.

4.2.2 Effect on different nuclear receptors

To investigate the role of RXRα and LXRs in the guggulsterone-mediated ABCA1 expression, luciferase reporter gene assays were performed. LXRs are able to bind to the ABCA1 promotor and upregulate its protein levels [74]. Accordingly in experiments with LXRs a reporter construct containing an ABCA1 promotor was used to determine whether the cholesterol efflux promoting effect of guggulsterone is caused by LXR activation and subsequent modulation of ABCA1 protein levels.

The RXR can regulate gene expression as homodimer or forms heterodimers with for instance FXR or LXR. [79].

In addition to the positive effects of guggulsterone on expression of ABCA1 protein levels (described in previous chapter), LXRα and LXRβ were activated by this FXR modulator without affecting cell viability. In the tested concentration range (0.1 to 30 µM) the maximal fold activation of LXRs was achieved after treatment with 10 µM guggulsterone showing 3.5 fold activation for LXRα and 2.0 fold activation for LXRβ (see **Figure 19** and **20**). These results indicate that the expression of ABCA1 could be enhanced through the activation LXRα and LXRβ. Interestingly guggulsterone did not significantly influence RXRα transactivation (data not shown). **Table 17** summarizes the effects of guggulsterone on different NRs in luciferase reporter gene assay.

Table 17: Effect of guggulsterone on nuclear receptors

Results from luciferase reporter gene assay in HEK-293 cells. The cells were treated with different concentrations of guggulsterone. The appropriate receptor agonists were used as positive controls and DMSO was used as solvent control. The luminescence values were standardized to EGFP-derived fluorescence and normalized to vehicle control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments (n=3).

Nuclear receptor	Used agonists	Activation by guggulsterone
Retinoid X receptor α	5 µM 9-cis retinoic acid	×
Liver X receptor α	1 µM GW3965	✓
Liver X receptor β	1 µM GW3965	✓

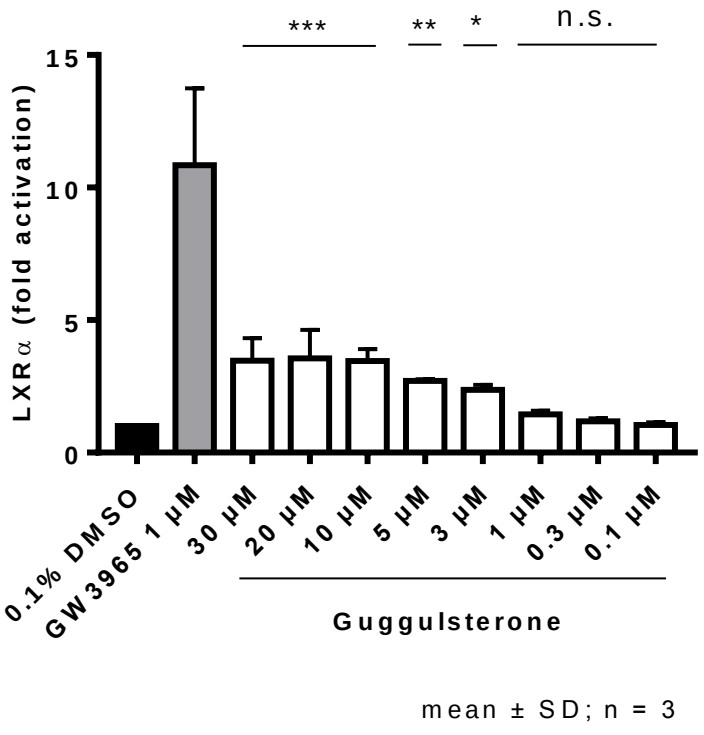

Figure 19: Effect of the guggulsterone on LXRα

Results from luciferase reporter gene assay in HEK-293 cells.

HEK-293 cells were transfected with an LXRα expression plasmid, a reporter plasmid containing ABCA1 promotor coupled to a luciferase reporter and EGFP as an internal control. Cells were stimulated with different concentrations of guggulsterone for 18 hours.

The synthetic LXR agonist GW3965 was used as positive control and DMSO was used as solvent control. The obtained luminescence values were standardized to EGFP-derived fluorescence (LFU/RFU ratio) and the results were expressed as fold activation normalized to the vehicle control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments (n=3). The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; n.s., no significance).

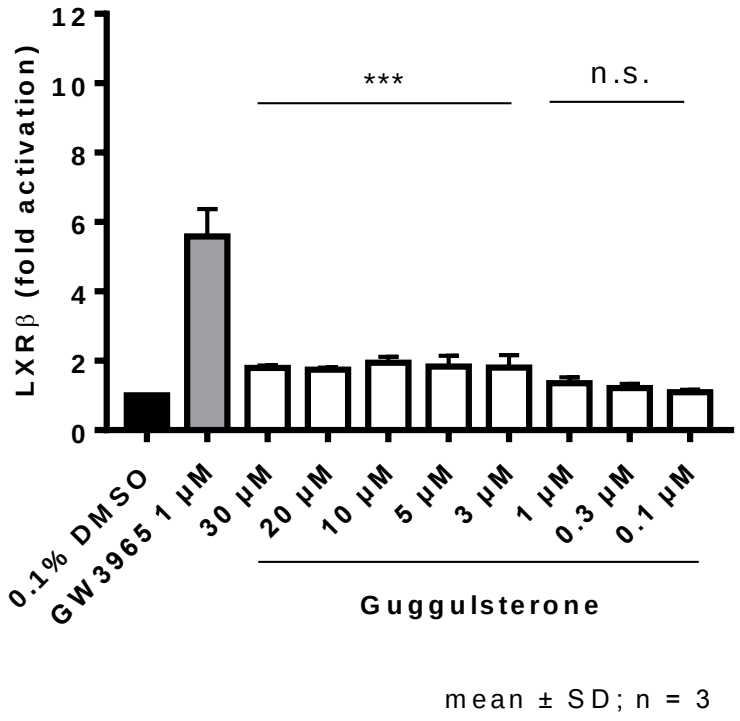

Figure 20: Effect of the guggulsterone on LXRβ

Results from luciferase reporter gene assay in HEK-293 cells.

HEK-293 cells were transfected with an LXRβ expression plasmid, a reporter plasmid containing ABCA1 promotor coupled to a luciferase reporter and EGFP as an internal control. Cells were stimulated with different concentrations of guggulsterone for 18 hours.

The synthetic LXR agonist GW3965 was used as positive control and DMSO was used as solvent control. The obtained luminescence values were standardized to EGFP-derived fluorescence (LFU/RFU ratio) and the results were expressed as fold activation normalized to the vehicle control (DMSO). Each sample was tested in quadruplicate in a total of three independent experiments (n=3). The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (***, $p < 0.001$; n.s., no significance).

4.3 Chenodeoxycholic acid

4.3.1 Influence on the expression of the cholesterol transporter ABCA1

Western blotting analysis was performed to determine whether ABCA1 expression in macrophage-derived foam cells is influenced by chenodeoxycholic acid (CDCA), the most potent endogenous agonist of human FXR. The results are given in **Figure 21** and revealed a dose-dependent increase in ABCA1 protein levels, similar to the data received from the experiment with the leoligin derivative. In comparison to the negative control 50 µM CDCA significantly ($p < 0.05$) increases ABCA1 expression. Pioglitazone, a known PPARγ agonist in clinical use, was used as positive control in this experiment.

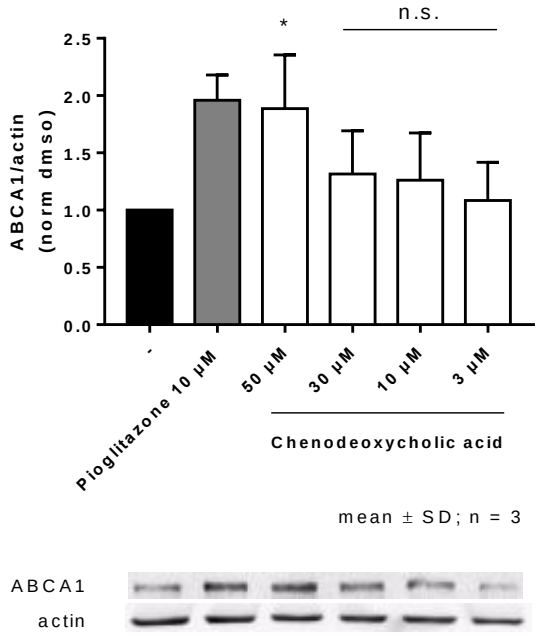

Figure 21: ABCA1 protein levels induced by chenodeoxycholic acid (CDCA)

ABCA1 protein levels after treatment with chenodeoxycholic acid, 0.1% DMSO (negative control) and 10 µM pioglitazone (Pio, positive control). The graph represents data from three independent experiments (n=3), shown as mean ± SD. The figure shows the representative blots with ABCA1 bands and corresponding actin bands that were used for normalization. The statistical significance was analyzed by one-way ANOVA (analysis of variance) with Bonferroni post hoc test (*, $p < 0.05$; n.s., no significance).

5. DISCUSSION

The main objective of this study was the characterization of the synthetic leoligin derivative 2780, which was shown to increase cholesterol efflux mediated by apo A-1 in previous experiments. The cholesterol efflux to apo A-1 in macrophages depends largely on expression of ABCA1 [40]. In this study, it was established that micromolar concentrations of the leoligin derivative 2780 significantly upregulate ABCA1 expression levels. This dose-dependent increase of ABCA1 protein clarifies the before described role of compound 2780 in cholesterol efflux. In this work human THP-1 macrophage-derived foam cells were used, which are able to mimic the *in situ* pathologies of macrophages in atherosclerosis [90]. But it is necessary to mention that cholesterol loading of THP-1 cells on its own could induce the expression of ABCA1 levels [91]. However, in our experimental setting, a false positive was avoided as our control (0.1% DMSO) was also loaded with cholesterol.

Next we focused on the investigation of other cholesterol transporters, including ABCG1 and SR-B1 transporters. The assays performed with ABCG1 transporter, which has just a minor role in faciliting efflux, were unsuccessful and therefore these data are not shown. Accordingly compound 2780 showed no significant effects on the expression of SR-B1 protein levels. The SR-B1 transporter is expressed mostly in the liver and thereby has only a negligible role in promoting cholesterol efflux from macrophages.

Nevertheless the ABCA1 transmembrane protein is a key transporter in apo A-1 mediated macrophage cholesterol efflux [50] and therefore leoligin derivative 2780 could be established as enhancer of this anti-atherogenic process.

Given the fact that compound 2780 increases ABCA1 protein levels, we investigated its underlying mechanism of action. In this study special attention was given to a possible connection between the compound-mediated increase in ABCA1 expression and transactivation of NRs. Several members of the NR family were described to play a role

in the regulation of cholesterol metabolism and in particular in the upregulation of ABCA1 [92]. To examine the possibility that compound 2780 may elicit its cholesterol efflux promoting function via NRs, luciferase reporter gene assay with different NRs relevant in lipid and cholesterol metabolism, were performed. However, none of the tested NRs (see **Table 16**, page 61) were activated by compound 2780.

Thanks to the results obtained from the FXR-Gal-4 assay, which represents the direct activation of FXR, leoligin derivative 2780 could be confirmed as specific FXR agonist with a maximal activation at 10 µM. Although several studies reported that FXR is expressed in THP-1 derived macrophages and exerts anti-inflammatory and immunoregulatory effects [60, 63], so far published results do not indicate an active role of FXR in the upregulation of cholesterol transporter or a direct influence on cholesterol efflux. Despite these statements it cannot be excluded that activation of FXR may somehow be involved in cholesterol efflux. In future studies expression of FXR in THP-1 macrophages has to be confirmed before a possible link between these two observed activities can be investigated.

Another compound that was tested in the course of this thesis was chenodeoxycholic acid (CDCA). CDCA treatment elicited enhanced levels of ABCA1 protein expression in a dose-dependent manner. Direct comparison with the leoligin derivative showed, that both compounds reached almost the same ABCA1 levels. Interestingly CDCA is the most potent endogenous agonist of FXR. Similar to the leoligin derivative the mechanism of ABCA1 upregulation after CDCA treatment is not known and further studies need to be performed to elucidate a possible connection to its FXR activity.

Finally we examined whether the FXR modulator, guggulsterone is able to influence ABCA1. Interestingly, guggulsterone was able to dose-dependently increase ABCA1 protein levels in THP-1 macrophage-derived foam cells. The following experiments suggested that this effect may be due to LXRα or LXRβ transactivation as LXR ligands are able to induce the genes involved in the macrophage cholesterol efflux, such as

ABCA1 [92]. To clarify a possible role of PPARγ in the guggulsterone elicited increase in ABCA1 levels, as suggested in the PPARγ-LXR-ABCA1 pathway promoting cholesterol efflux [77], further studies should determine a direct PPARγ activation of this compound.

In comparison these three compounds, all increase ABCA1 protein levels and modulate FXR. Future studies need to investigate a possible interaction of FXR with macrophage cholesterol efflux.

6. CONCLUSION

In this study the main aim was to test the influence of the synthetic leoligin derivative 2780 in addition to other FXR modulators on the expression of cholesterol transporters. In addition we aimed to investigate the effects of these compounds on the transactivation of NRs influencing cholesterol efflux as a possible mode of action.

This study succeeded previous research where the leoligin derivative 2780 was shown on the one hand to promote cholesterol efflux from macrophage-derived foam cells and on the other hand to transactivate FXR. Our obtained results confirmed the role of compound 2780 in mediating cholesterol efflux as it was able to induce the expression of ABCA1 protein. To connect this effect with a possible transcriptional effect NRs, including LXRα, LXRβ, PPARα, PPARγ, RXRα, were investigated. However none of the mentioned receptors were activated by this compound.

To investigate the effect of other known FXR modulators on ABCA1 protein expression western blot analysis with chenodeoxycholic acid and guggulsterone were performed. CDCA elicited increased ABCA1 protein levels with very similar maximal fold activation as compound 2780 (around 1.9). In addition the FXR modulator guggulsterone also increased ABCA1 protein levels, but in contrast to the other tested compounds was able to activate LXRα and β.

To conclude, all three investigated compounds were able to increase ABCA1 protein levels in a dose-dependent manner that makes them interesting tools for further investigations. Further experiments are required to elucidate their particular cholesterol efflux promoting mechanism of action.

7. ABBREVIATIONS

ABC	ATP-binding cassette
ACAT	acyl-coenzyme A:cholesterol acyltransferase 1
Apo A-1	apolipoprotein A-1
ApoB	apolipoprotein B
ATP	adenosine triphosphate
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CE	cholesterol ester
CEPT	cholesterol ester transfer protein
CD 36	cluster of differentiation 36
CDCA	chenodeoxycholic acid
CSF	macrophage colony-stimulating factor
CVD	cardiovascular disease
DMSO	dimethylsulfoxide
EDTA	ethylene diamine tetraacetic acid
FC	free cholesterol
FXR	farnesoid X receptor
GM-CSF	granulocyte-macrophage colony-stimulating factor
HDL	high-density lipoprotein
HRP	horseradish peroxidase
ICAM-1	intracellular adhesion molecule
IDL	intermediate-density lipoprotein
IL-1, 6, 10, 12, 23, 34	interleukin-1, 6, 10, 12, 23, 34
INF-γ	interferon-γ
LBD	ligand binding domain
LCA	lithocholic acid
LCAT	lecithin:cholesterol acyltransferase
LDL	low-density lipoprotein
LXR	liver X receptor
MCP-1	monocyte chemoattractant protein-1

NBD	nucleotide binding domain
NF-κB	nuclear factor-kappa B
NO	nitric oxide
NR	nuclear receptor
PAMP	pathogen-associated molecular patterns
PBS	phosphate buffered saline
PVDF	polyvinylidenfluoride
PPAR	peroxisome proliferator-activated receptor
RE	response elements
RCT	reverse cholesterol transport
ROS	reactive oxygen species
RXR	retinoid X receptor
SR-A1	scavenger receptor class A type 1
SR-B1	scavenger receptor class B type 1
SDS-PAGE	sodium dodecyl sulfate- polyacrylamide gel electrophoresis
STAT-1	signal transducer and activator of transcription 1
TBS-T	tris-buffered saline supplemented with Tween-20
TGF-β	transforming growth factor β
TLR	toll-like receptor
TNF-α	tumor necrosis factor α
UC	unesterified cholesterol
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low-density lipoprotein

8. LIST OF TABLES

9. LIST OF FIGURES

10. REFERENCES

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