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„The influence of selected natural products on nuclear receptors controlling cholesterol homeostasis in intestinal cells and macrophages“

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Mag.pharm. Verena Hiebl

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To my family

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Abstract

Atherosclerosis is a chronic disease affecting the arterial walls, characterized by the development of plaques that contain cholesterol crystals and lipid-laden foam cells. Its complications, stroke and myocardial infarction, are major causes of death in industrialized countries. Therefore, modulating cholesterol absorption and excretion pathways is of particular interest.

As the intestine is an important interface in cholesterol uptake and efflux, it constitutes a pharmacologically interesting target tissue. In addition, macrophages play a crucial role in the development of atherosclerosis, as their uptake of lipids results in the formation of foam cells that drive the progression of atherosclerosis. The nuclear receptors LXR, FXR, PPAR and their heterodimeric partner RXR are crucial regulators of metabolic processes. Their target genes include major cholesterol transporters in the intestine and in macrophages, e.g. ABCA1, ABCG1, ABCG5/G8 and NPC1L1, highlighting their great potential as drug targets.

The aims of this study were to set up protocols for assessing intestinal cholesterol transport processes *in vitro* and to test selected natural products for their influence on cholesterol uptake and efflux in macrophages and intestinal cells.

To assess the effect of a certain natural compound on intestinal cholesterol transport, the human intestinal Caco-2 cell line was established in our laboratory. Many publications using the Caco-2 cell line exist, however, the used methodology differs between laboratories, or descriptions are undetailed, therefore not allowing reproduction. Comprehensive and quality-controlled protocols for the cultivation of the Caco-2 cell line and for viability assessment were established. A cholesterol uptake and efflux assay were set up, as well as a luciferase reporter gene assay for the purpose of determining nuclear receptor activation. Since measurement of protein and mRNA levels of cholesterol transporters in differentiated cells turned out very challenging, critical steps and alternative approaches were evaluated.

Natural products represent an interesting pool of potential nuclear receptor modulators and a valuable source for drug discovery. Soraphen A, a polyketide natural product, was shown to be an inhibitor of acetyl-CoA carboxylases, thereby inhibiting *de novo* fatty acid synthesis and increasing fatty acid β-oxidation. In this study, we could show that soraphen A increases cholesterol efflux from macrophages *via* ABCA1. Soraphen A induced ABCA1

expression by increasing the transcriptional activity of LXR. The increase in LXR activity turned out to be a consequence of increased free cholesterol levels, most likely due to soraphen A-related inhibition of fatty acid synthesis.

Piperine, the major pungent alkaloid of *Piper nigrum* L., was shown to have cardioprotective effects *in vivo*. A series of piperine derivatives was synthesized and investigated in regard to ABCA1 protein expression in macrophages, identifying LAU398 as the most potent piperine derivative. LAU398 increased cholesterol efflux from macrophages in an ABCA1-dependent manner, while it reduced intestinal cholesterol uptake. This compound did not activate nuclear receptors, but it increased the protein expression of LXRα. It was shown in previous studies that LAU398 increases capsaicin-induced currents through TRPV1 channels. Co-administration of either TRPV1 or LXR antagonists could not reverse the effect of LAU398 on cholesterol uptake. The exact mechanism of action of LAU398 on cholesterol transport still remains to be elucidated.

In summary, this work describes the establishment of protocols based on the use of the Caco-2 cell line for assessing intestinal cholesterol transport. It shows the impact of the natural product soraphen A and of the natural product derivative LAU398 on cholesterol homeostasis and analyses the underlying molecular mechanisms.

Zusammenfassung

Atherosklerose ist eine chronische Erkrankung der Arterien, gekennzeichnet durch die Entstehung von Plaques, die Cholesterinkristalle sowie lipidreiche Schaumzellen enthalten. Die aus Atherosklerose resultierenden Komplikationen, wie Schlaganfall und Herzinfarkt, gehören zu den häufigsten Todesursachen in Industrieländern. Daher ist die Modulierung der Cholesterinaufnahme und -ausscheidung von großem Interesse.

Da der Darm eine wichtige Schnittstelle in Cholesterinaufnahme und -ausscheidung ist, stellt er ein interessantes pharmakologisches Ziel dar. Darüber hinaus spielen Makrophagen eine entscheidende Rolle in der Entstehung von Atherosklerose, da ihre Aufnahme von Lipiden in der Entstehung von Schaumzellen resultiert, welche das Fortschreiten von Atherosklerose begünstigen. Die nukleären Rezeptoren LXR, FXR, PPAR und ihr Heterodimer-Partner RXR, sind wichtige Regulatoren von metabolischen Vorgängen. Ihre Zielgene umfassen bedeutende Cholesterintransporter sowohl im Darm als auch in Makrophagen, wie zum Beispiel ABCA1, ABCG1, ABCG5/G8 und NPC1L1, was ihr großes Potenzial als Wirkstoffziele verdeutlicht.

Das Ziel dieser Arbeit war, Protokolle für die *in vitro* Untersuchung intestinaler Cholesterintransport-Prozesse aufzusetzen und ausgewählte Naturstoffe hinsichtlich ihres Einflusses auf die Cholesterinaufnahme sowie -ausscheidung in Makrophagen und intestinalen Zellen zu testen.

Um den Effekt eines bestimmten Naturstoffes auf den intestinalen Cholesterintransport untersuchen zu können, wurde die humane intestinale Caco-2 Zelllinie in unserem Labor etabliert. Viele Publikationen existieren bereits, welche die Caco-2 Zelllinie verwenden, jedoch unterscheidet sich die verwendete Methodik zwischen den verschiedenen Laboratorien, oder sie wird nur skizzenhaft beschrieben, wodurch die Reproduktion erschwert wird. Umfangreiche Protokolle für die Kultivierung der Caco-2 Zelllinie und die Viabilitätsbestimmung wurden erstellt und einer Qualitätskontrolle unterzogen. Ein Cholesterinaufnahme- und ein Cholesterinefflux-Assay wurden aufgesetzt, ebenso wie ein Luziferase-Reportergen-Assay, welcher eine potentielle Aktivierung von nukleären Rezeptoren zeigen soll. Die Messung der Protein- und mRNA-Level von Cholesterintransportern stellte eine große Herausforderung dar, weshalb kritische Schritte und alternative Herangehensweisen evaluiert wurden.

Naturstoffe sind ein interessanter Pool an potentiellen Liganden für nukleäre Rezeptoren und eine wichtige Ressource für die Wirkstoffentwicklung. Soraphen A, ein Naturstoff aus der Gruppe der Polyketide, ist ein Inhibitor von Acetyl-CoA Carboxylasen, wodurch es die *de novo* Fettsäuresynthese hemmt und die Fettsäure β-Oxidation erhöht. In dieser Studie konnten wir zeigen, dass Soraphen A den Cholesterinefflux aus Makrophagen über den ABCA1 Transporter erhöht. Soraphen A induzierte die Expression von ABCA1 über eine Erhöhung der transkriptionellen Aktivität von LXR. Es zeigte sich, dass die Aktivierung von LXR die Konsequenz einer Erhöhung freier Cholesterin-Level ist, welche höchstwahrscheinlich auf die Inhibierung der Fettsäuresynthese zurückzuführen ist.

Piperin, ein in *Piper nigrum* L. vorkommender Scharfstoff aus der Gruppe der Alkaloide, besitzt kardioprotektive Eigenschaften *in vivo*. Eine Reihe von Piperin-Derivaten wurde synthetisiert und in Bezug auf die ABCA1 Protein-Expression untersucht, wobei LAU398 als potentestes Piperin-Derivat identifiziert wurde. LAU398 erhöhte den Cholesterinefflux aus Makrophagen über ABCA1, während es die intestinale Aufnahme von Cholesterin verringerte. Die Verbindung aktivierte keine nukleären Rezeptoren, erhöhte aber die Protein-Expression von LXRα. In früheren Studien wurde gezeigt, dass LAU398 Capsaicin-induzierte Ströme durch TRPV1-Kanäle verstärkt. Nichtsdestotrotz konnte weder die Co-Applizierung mit einem TRPV1-, noch mit einem LXR-Antagonisten die Effekte auf die Cholesterinaufnahme aufheben. Der exakte Wirkmechanismus von LAU398 bleibt noch zu klären.

Kurz zusammengefasst beschreibt diese Arbeit die Etablierung von Protokollen für die Untersuchung des intestinalen Cholesterintransports basierend auf der Verwendung der Caco-2 Zelllinie. Sie zeigt den Einfluss des Naturstoffes Soraphen A sowie des Naturstoff-Derivates LAU398 auf die Cholesterinhomöostase und analysiert die zugrundeliegenden molekularen Wirkmechanismen.

Contents

1 Introduction

1.1 Atherosclerosis

Atherosclerosis and its subsequent complications, like stroke and myocardial infarction, are major causes of death in industrialized countries [1]. Atherosclerosis is a chronic disease affecting the arterial walls, characterized by the development of atherosclerotic plaques.

The normal arterial wall consists of three layers, which are, from the interior to the exterior, the tunica intima, the tunica media and the tunica adventitia (Figure 1.1). The tunica intima consists of a basement membrane that is covered by a layer of endothelial cells, which is in contact with the blood. Smooth muscle cells (SMCs) are residing in the human tunica intima, which is not the case in many other animal species. The tunica media of larger vessels consists of extracellular matrix components like elastin and collagen and, like the tunica intima, contains SMCs. The outermost layer, the adventitia, also contains extracellular matrix components, as well as fibroblasts, mast cells, nerve endings and small arteries for nutrient supply [2, 3].

Atherosclerosis is initiated at predisposed sites with slow or so-called disturbed flow, partly by the activation of endothelial cells. Accumulating SMCs then cause a thickening of the intima at these predisposed areas. Current knowledge suggests that platelet-derived growth factor (PDGF) that is secreted by the activated endothelium is driving the accumulation of SMCs. Activated endothelial cells further secrete chemoattractants and express adhesion molecules, like e.g. vascular cell adhesion molecule-1 (VCAM-1), leading to the adhesion of leukocytes, including monocytes, to endothelial cells. Due to changes in the permeability of the endothelium, the accumulation of apolipoprotein B (apoB)-containing particles like low-density lipoprotein (LDL) is promoted, and these are retained and can be modified within the subendothelium. Bound leukocytes migrate into the tunica intima, followed by the differentiation of monocytes to macrophages and their uptake of lipoprotein particles, yielding foam cells (Figure 1.1) [2, 4–7].

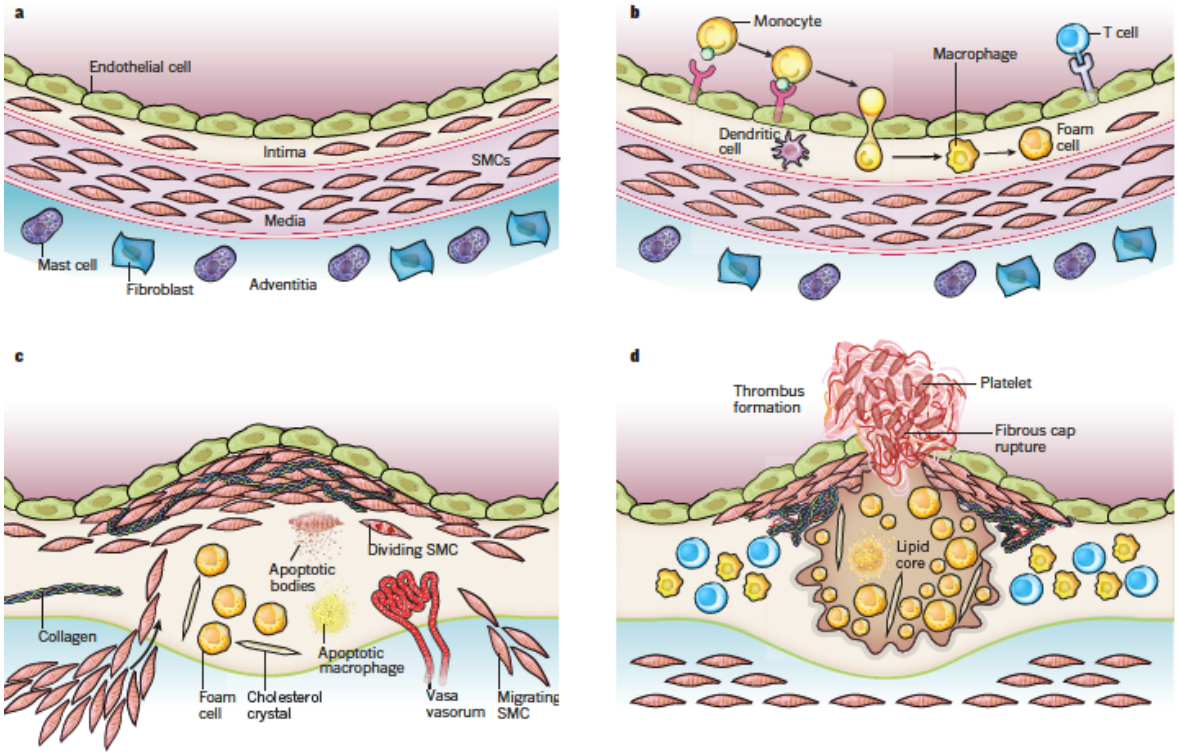

Figure 1.1: Development of atherosclerosis. **(a)** Normal arterial wall consisting of three layers – the tunica intima, the tunica media and the tunica adventitia. The tunica intima is covered by a layer of endothelial cells. **(b)** Initial steps of atherosclerosis showing the adhesion of leukocytes, their migration into the tunica intima, the differentiation of monocytes into macrophages and their uptake of lipids, resulting in foam cell formation. **(c)** Progression of the lesion with augmented migration of SMCs into the intima and subsequent production of macromolecules, like collagen. Release of lipids by dying macrophages and SMCs. In advanced stages, cholesterol crystals and microvessels can be found. **(d)** Thrombus formation upon rupture of the fibrous cap of the plaque. Reprinted by permission from Springer Nature, Nature, Libby et al. (2011) [2], Copyright 2020.

Notably, inflammation also plays an important role in atherosclerosis. Macrophages in the atherosclerotic plaque can show pro-inflammatory functions, characteristic of M1 macrophages. These macrophages, but also activated endothelial cells, secrete cytokines like interleukin-1β (IL-1β), tumor necrosis factor (TNF) and matrix metalloproteinases. Besides macrophages, various other leukocytes like mast cells and lymphocytes also accumulate in atherosclerotic lesions. Further steps in atherosclerosis development include the production of macromolecules like elastin and collagen by SMCs, leading to the formation of a fibrous cap that covers the plaque. In the developing atherosclerotic plaque, SMCs and macrophages can die by e.g. apoptosis and thereby release lipids. Efferocytosis – a process of inefficient clearance of dead cells – promotes the accumulation of both extracellular lipids and cell debris, leading to the formation of a so-called necrotic core

rich in lipids in the center of the plaque. Besides, cholesterol crystals and microvessels can also be found in atherosclerotic plaques [2, 4–7].

Atherosclerotic plaques can limit blood flow by causing stenoses, thereby leading to tissue ischaemia. Rupture of a plaque can result in a local thrombus that blocks blood flow or, upon embolization and movement to distal arteries, it can even lead to stroke or myocardial infarction [2, 7].

Atherosclerotic plaques prone to rupture are most often those having a thin fibrous cap, which is poor in collagen, and containing a low number of SMCs but a multitude of macrophages. Inflammatory cells are held responsible for the death of collagen-producing SMCs by the production of certain mediators, as well as for the degradation of collagen by the secretion of collagenolytic enzymes [2]. This can lead to physical disruption of the plaque and can expose the pro-coagulant material of the core to coagulation proteins in the blood, finally leading to the formation of a thrombus, as mentioned above and shown in Figure 1.1 [2].

1.1.1 Risk factors for atherosclerosis

The exact causes underlying atherosclerosis are not known, however, some circumstances and a certain lifestyle can increase the chance of developing atherosclerosis. Major risk factors for atherosclerosis include hypertension, diabetes, inflammation, smoking, obesity, older age and lack of physical activity [8]. In addition, lipids are known to play a significant role in the formation of atherosclerotic plaques, and data suggest a strong association between lipid levels in plasma and the occurrence of cardiovascular disease [9].

It has been reported that high levels of LDL increase the risk of cardiovascular events and the likelihood of an individual to develop atherosclerosis. Lowering LDL levels has been associated with a reduction in atherosclerotic events [10]. In contrast to LDL, high density lipoprotein (HDL) levels are inversely correlated with the risk for cardiovascular events [11]. Great effort has been made to find lipid-lowering strategies, yielding the very important drug classes of fibrates and statins. Fibrates act as agonists of the nuclear receptor peroxisome proliferator activated receptor α (PPARα) and reduce levels of fatty acids and triglycerides by inducing peroxisomal β-oxidation [12, 13]. Statins lower cholesterol levels by inhibiting 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMG-CoA reductase), a rate-limiting enzyme in cholesterol biosynthesis [14, 15]. However, a residual risk of cardiovascular events remains even in patients treated with statins [16]. Therefore, new lipid-lowering strategies or other effective treatments targeting e.g. inflammation are still needed, and research is focusing on several new biological targets [2, 17].

1.2 Cholesterol and its physiological functions

Cholesterol (3-hydroxy-5,6-cholestene) is a steroid (Figure 1.2), which contains a cyclopentanoperhydro-phenanthrene nucleus, that is present in all animal steroids [18]. In addition to this hydrophobic nucleus, it also contains a hydrocarbon tail and a polar hydroxyl group. Cholesterol is therefore amphipathic, containing both a hydrophobic and a small hydrophilic part [19, 20].

In adults, about 1 g of cholesterol is synthesized and 0.3 g consumed daily [20]. Almost every cell is able to synthesize cholesterol, highlighting its importance in physiological functions. Cholesterol mainly localizes to cell membranes, where it regulates their rigidity and permeability [21, 22]. Cholesterol-rich membrane domains, so-called lipid rafts, have an important role in signal transduction [23]. In addition to its role in membrane function and structure, cholesterol can be metabolized to oxysterols and bile acids [22, 24, 25], which are important for nutrient uptake. Moreover, cholesterol is the precursor for pregnenolone, which in turn is the precursor for all other steroid hormones. Furthermore, cholesterol was shown to be important for embryonic development [22, 26, 27].

For physiological homeostasis, it is necessary to stringently control systemic and cellular cholesterol levels. Despite being essential for mammalian cells, excess cholesterol is toxic to cells and drives the emergence of cardiovascular disease, as highlighted above [28–30].

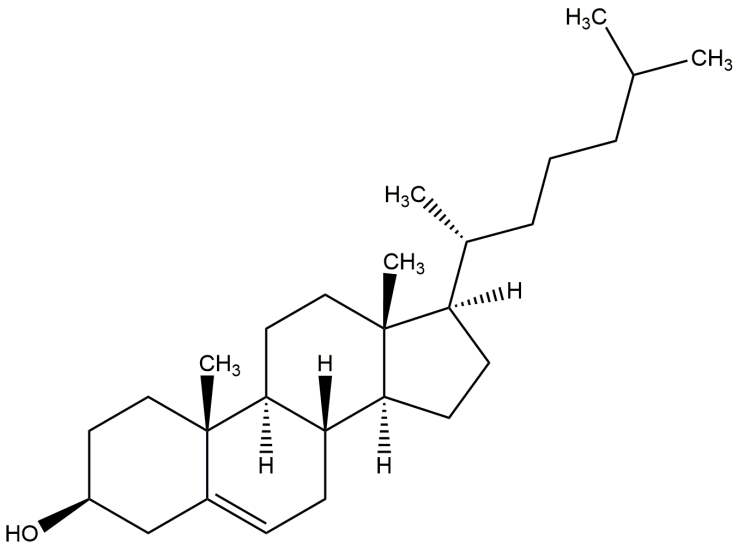

Figure 1.2: Structure of cholesterol.

1.3 Cholesterol homeostasis

As lipids, including cholesterol, play a pivotal role in atherosclerotic plaque formation, cholesterol homeostasis is of major interest. Cholesterol homeostasis is a very complex network involving the processes of cholesterol biosynthesis, uptake, metabolism, storage, transport and excretion [22]. All these processes cannot be described in full detail, but a brief overview is provided below and cholesterol excretion pathways are described in more detail in the next subchapter.

Although nearly all cells are capable of synthesizing cholesterol, 50% of cholesterol synthesis in humans takes place in the liver [31]. It ensues from acetyl-CoA and involves more than 20 different enzymes, which are mostly localized in the membrane of the endoplasmic reticulum (ER) [22]. Besides being synthesized, cholesterol can also be taken up from the diet by intestinal enterocytes [32]. Cholesterol can be secreted from enterocytes as chylomicrons and the cholesterol contents can then be taken up by the liver. The liver secretes cholesterol – from dietary source and endogenously synthesized – in the form of very low density lipoproteins (VLDLs) into the blood [22]. VLDLs are processed, generating LDLs, that can be taken up by peripheral cells [10]. Inside the cell, vesicular and non-vesicular mechanisms transport cholesterol so that it can accomplish its functions [33]. Apolipoprotein A1 (apoA1) that is produced by the liver, intestine and pancreas, can serve as acceptor for excess cholesterol and thereby generate HDLs [34]. Cholesterol can also be esterified to cholesteryl esters by acyl CoA:cholesterol acyltransferase (ACAT) [35]. Cholesteryl esters can either be stored in the cytoplasm in the form of lipid droplets, or they can be released as a component of plasma lipoproteins (chylomicrons, VLDLs, LDLs, HDLs). HDL can be transported to the liver and the intestine, where cholesterol is taken up and then either recycled or excreted [22].

The processes involved in cholesterol homeostasis are tightly controlled by a complex transcriptional and post-translational regulatory system, which is responsive to a variation in lipid levels [22].

1.3.1 Cholesterol excretion

There are two major pathways for cholesterol excretion: the biliary reverse cholesterol transport (RCT) and the non-biliary RCT, also termed transintestinal cholesterol efflux (TICE) [36–39]. Figure 1.3 shows a simplified overview of RCT and TICE in humans.

A very important lipoprotein in the process of RCT is HDL, which is produced mainly by liver hepatocytes and intestinal enterocytes [40]. One of the first steps in RCT is the efflux of cholesterol from peripheral tissues, like macrophages in the arterial wall, to apoA1

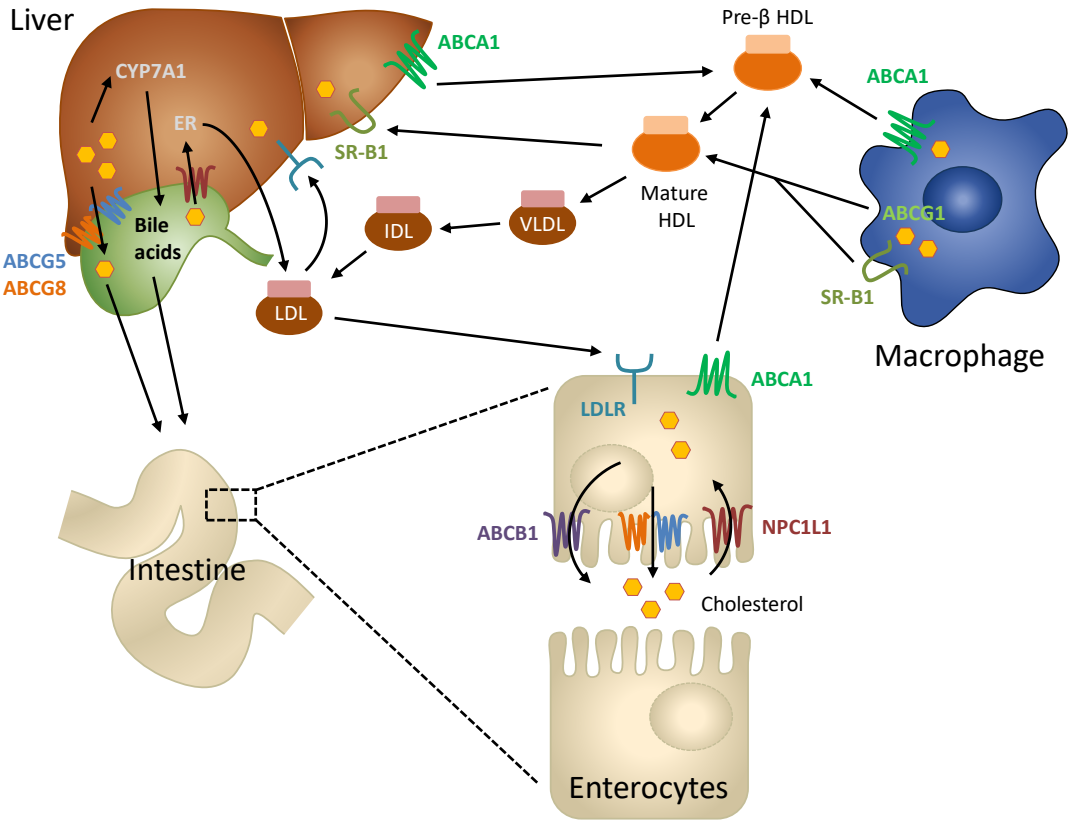

Figure 1.3: Overview of RCT and TICE pathways in humans. For details, see text.

or HDL by the ATP-binding cassette transporters A1 (ABCA1) and ABCG1, or by the scavenger receptor class B type 1 (SR-B1) [41–43]. Free cholesterol in HDL particles can be esterified and then transferred to apoB-containing lipoproteins, like LDL or VLDL, accomplished by cholesteryl ester transfer protein (CETP) [44]. VLDL and LDL can be taken up by the liver *via* LDL receptor (LDLR) and VLDLR, whereas cholesteryl esters from HDL can be taken up primarily by SR-B1 [45, 46]. Following uptake in the liver, cholesterol can be secreted into the bile, which is accomplished mainly by the transporter heterodimer ABCG5/G8 [47]. Moreover, cholesterol can be processed to bile acids, by the action of the rate limiting enzyme cholesterol 7α-hydroxylase (CYP7A1), and bile acids are also secreted into the bile [25]. Biliary cholesterol can finally be transferred into the intestinal lumen, where it can either be reabsorbed by the transporter Niemann-Pick C1-like protein 1 (NPC1L1) or leave the body *via* the faeces [48].

The hepatobiliary RCT was considered as the exclusive route for cholesterol elimination from the body, until it was noticed that a great part of cholesterol found in faeces does not stem from bile or diet. The secretion of this part of cholesterol found in faeces is accomplished by the small intestine and this process is termed TICE, representing a non-biliary pathway for RCT [38, 49, 50]. TICE is less well-understood compared to the

biliary RCT. Current knowledge suggests that this pathway can be initialized amongst others by the reuptake of cholesterol from bile by the liver *via* the transporter NPC1L1. In liver hepatocytes, cholesterol is then shuttled to the endoplasmic reticulum, where it is transferred to apoB-containing lipoproteins. These lipoproteins are secreted into the blood and taken up by the small intestine *via* LDL receptors and perhaps other processes [36, 51–53]. Cholesterol can then be secreted into the intestinal lumen by apically located ABCG5/G8 [54, 55], ABCB1 [53] and probably other mechanisms. In contrast to the biliary RCT, indications that SR-B1 is involved in TICE are controversial [56].

1.3.2 Cholesterol and the intestine

The intestine has a crucial role in cholesterol homeostasis, as it is a major player in TICE (as described above) and therefore in cholesterol excretion. In addition to its relevant role in TICE, the intestine is also an interface in cholesterol uptake and metabolism.

In the intestine, four main cell types can be found, one of them being the absorptive enterocytes. Enterocytes are polarized cells, meaning that they differentiate into cells with an apical and a basolateral side [57, 58]. The apical side is directed to the intestinal lumen, whereas the basolateral side is in contact with lymphatic vessels and thereby with the circulation [59].

Lipids in the intestinal lumen (predominantly triglycerides, phospholipids and cholesteryl esters), stemming from diet or bile, are solubilized in micelles with the help of bile acids. Triglycerides are hydrolyzed by lipases, yielding free fatty acids and monoacylglycerides, and cholesteryl esters are hydrolyzed to fatty acids and free cholesterol. They are then taken up on the apical side of the enterocyte by diffusion and by protein-mediated transport. Transporters implicated in the uptake of lipids are the scavenger receptor class B type 3 (CD36), several fatty acid binding and transport proteins, and NPC1L1, which mediates the uptake of free cholesterol [17, 29]. Of note, the presence of bile acids in the intestinal lumen is inevitably required for the absorption of cholesterol [60].

Once free cholesterol is inside the enterocyte, it can be shuttled to the endoplasmic reticulum, where it is esterified by the enzyme ACAT2. Absorbed free fatty acids and monoacylglycerides are used for the synthesis of cholesteryl esters and triglycerides. Cholesteryl esters are assembled into chylomicrons, along with triglycerides, phospholipids and proteins, with the help of microsomal transfer protein (MTP). Chylomicrons are then secreted into the lymph at the basolateral side of the enterocyte. Alternatively, cholesteryl esters can also be stored in the form of lipid droplets in the cytoplasm [17, 29].

Free cholesterol in the enterocyte can also be transported back into the intestinal lumen by the transporters ABCG5/G8 or ABCB1 in the apical membrane of the enterocyte.

Alternatively, free cholesterol can be transferred to apoA1 *via* basolaterally located ABCA1, resulting in the formation of HDL [17, 29].

1.3.3 Cholesterol and macrophages

As macrophage foam cells contribute to the development and progression of atherosclerosis, cholesterol homeostasis, in particular cholesterol efflux from these cells is of special interest.

In macrophages, lipid uptake is mainly mediated by LDLR, CD36 and scavenger receptor-A (SR-A). The LDLR recognizes native LDL and facilitates its endocytosis, whereas CD36 and SR-A mediate the endocytosis of modified LDL. Endocytosed LDL is then transported to the lysosome, where cholesteryl esters are hydrolyzed [17, 61, 62].

Free cholesterol is then shuttled to the endoplasmic reticulum, where it is esterified by ACAT1. Cholesteryl esters, along with triglycerides and phospholipids, are then stored in the form of cytoplasmic lipid droplets, that are a characteristic feature of foam cells [17, 61, 62]. Cholesteryl esters from these lipid droplets can again be hydrolyzed, enabling the transport of free cholesterol to the plasma membrane, where it can be effluxed to lipid-poor apolipoproteins (apoA1 and apoE) or to HDL, which helps to reduce foam cell formation [17, 63–65]. Three transporters have a crucial role in the efflux of free cholesterol, namely ABCA1, ABCG1 and SR-B1. ABCA1 mediates the efflux of free cholesterol to apoA1 and apoE, thereby generating nascent HDL particles. ABCG1 and SR-B1 stimulate the efflux of free cholesterol to nascent HDL particles or mature HDL [17, 34, 66–68].

1.4 Major cholesterol transporters in the intestine and in macrophages

1.4.1 ABCA1

The gene encoding ABCA1, which was originally called ABC1, was identified and first cloned in 1994 [69]. In humans, it encodes a membrane-bound transport protein that is ATP-dependent. It consists of 2261 amino acids and has a molecular weight of 254 kDa [34, 70]. Following the general architecture of ABC transporters, ABCA1 possesses four core domains, which are constituted of two transmembrane domains (TMDs) and two nucleotide binding domains (NBDs). ABCA1 is predicted to have 12 transmembrane helices and two extracellular domains (ECDs): one between helices one and two, and another between helices seven and eight. Glycosylation sites as well as disulfide bond sites can be found in these extracellular domains [71–75]. Mutations in the two ECDs cause

Tangier disease, a disorder with very low plasma HDL levels and often a premature onset of atherosclerosis [71, 76].

ABCA1 does not only mediate cellular cholesterol efflux, but also enables the efflux of phospholipids [34, 70] and has a central role in the biosynthesis of HDL by lipidation of apoA1 [77]. It is also implicated in the transport of other substances, e.g. apoE [78], and in phospholipid translocation [79]. Some indications point towards ABCA1 transporting small molecule drugs [80], which, however, needs further confirmation.

ABCA1 is ubiquitously expressed in tissues, with the liver and the intestine being the primary sources for plasma HDL generated *via* ABCA1 activity [77, 81]. In intestinal enterocytes, ABCA1 is located in the basolateral membrane [82, 83]. Various subspecies of HDL with different sizes are generated that induce the efflux of cholesterol from cells in peripheral tissues, e.g. cholesterol-loaded macrophage foam cells. In macrophages, ABCA1 and another cholesterol transporter, ABCG1, synergize in promoting cholesterol efflux. ABCA1 creates nascent HDL particles that then further induce cholesterol efflux *via* ABCG1 [84–88]. The activity of ABCA1 is therefore considered to be anti-atherogenic.

The expression of ABCA1 can be regulated at the transcriptional, post-transcriptional and post-translational level.

In regard to transcriptional regulation, nuclear receptors like the liver X receptor (LXR), the retinoid X receptor (RXR), the retinoic acid receptor (RAR) and the peroxisome proliferator activated receptor (PPAR) play an important role. More details on nuclear receptors can be found in chapter 1.5. LXR leads to an upregulation of ABCA1 by direct binding to the ABCA1 promoter at direct repeat 4 elements (DR-4, response element consisting of a direct repeat with 4 spacing nucleotides) [89, 90]. LXR acts as a heterodimer with its permissive partner RXR to increase the expression of ABCA1 [91]. Likewise, RAR also upregulates ABCA1 expression *via* its promoter together with RXR [92, 93]. PPARα and PPARγ were shown to upregulate LXRα expression which then further causes an increase in ABCA1 transcription and expression [94–96]. Rather inconsistent effects on ABCA1 gene expression were shown for cytokines, like interleukin, interferons and tumor necrosis factor [97].

Concerning the regulation of ABCA1 expression at the post-transcriptional level, microRNAs (miRNAs) were shown to cause a destabilization of the RNA or to repress translation by binding to the 3'-untranslated regions (3'-UTR) of mRNA transcripts of ABCA1 [98, 99]. A multitude of miRNAs were reported to target the 3'UTR of ABCA1 mRNA, like e.g. miRNA-33a and b [100], miRNA-106b [101] and miRNA-148a [102]. Besides miRNAs, RNA-binding proteins (RBPs), including human antigen R (HuR) [103], may also target the 3'UTR of the ABCA1 mRNA transcript and thereby regulate its

expression. In addition, long noncoding RNAs (lncRNAs), among them lnc-HC [104] and MeXis [105], can also regulate ABCA1 expression at both the transcriptional and post-transcriptional level.

Finally, ABCA1 expression is also regulated post-translationally. Major systems involved in the degradation of the ABCA1 protein are the proteasomal [106, 107], the lysosomal [108, 109] and the calpain systems [110]. The so-called PEST motif, a sequence rich in proline, glutamic acid, serine, and threonine located in the first intracellular loop of ABCA1, is very important for enabling proteolysis by calpain [111]. In contrast, the PDZ binding motif in the ABCA1 protein is recognized by the PDZ proteins α1-syntrophin and β1-syntrophin that inhibit the degradation of ABCA1 [112, 113].

1.4.2 ABCG5/G8

ABCG5 and ABCG8 are membrane transporters of the ABC family that are so-called half-transporters, meaning that they possess only one TMD and one NBD and therefore work as an obligate heterodimer [114, 115]. The ABCG5 and ABCG8 genes are oriented in a head-to-head manner on opposite strands, and are only separated by 374 base pairs. It was suggested that these genes share a bidirectional promoter and regulatory elements, which is supported by their proximity and opposite orientation [116–118]. ABCG5 consists of 651 amino acids, whereas ABCG8 is made up by 673 amino acids [119]. Both ABCG5 and ABCG8 are predicted to have six transmembrane helices and one ECD between helix five and six [114, 115]. ABCG5 and ABCG8 are both glycoproteins, existing in different glycosylated and non-glycosylated isoforms during the process of maturation [120].

When ABCG5 and ABCG8 were identified to be mutated in a rare disease, sitosterolemia, the understanding of how sterols, especially cholesterol, traffic and are being eliminated from the body took a leap [121, 122]. ABCG5 and ABCG8 are responsible for sterol export, whereby xenosterols (plant sterols and others) are actually preferred over cholesterol [122]. The only cells expressing ABCG5 and ABCG8 are hepatocytes, enterocytes and cells of the gallbladder epithelium [47, 117, 118, 122, 123]. Being expressed in hepatocytes as well as in the apical membrane of intestinal enterocytes, ABCG5/G8 enhances biliary and fecal sterol/cholesterol excretion, respectively [123]. ABCG5/G8 thereby completes reverse cholesterol transport, as this heterodimer promotes the excretion of cholesterol *via* liver and intestine. Consequently, transgenic mice overexpressing ABCG5/G8 exhibited lower plasma cholesterol levels and were protected from atherosclerosis [118, 124, 125].

Major regulation of ABCG5/G8 expression appears to occur at the transcriptional level. LXR was reported to play an important role and to increase ABCG5/G8 expression [126]. LXR response elements regulating ABCG5/G8 expression were found in two evolutionary

conserved regions distal of the intergenic promoter [127]. Within the intergenic promoter, regulatory elements for liver receptor homolog-1 (LRH-1), hepatocyte nuclear factor 4α (HNF4α), nuclear factor kappa B (NFκB) and Forkhead box protein O1 (FoxO1) have been found [122, 128–131]. Agonists for farnesoid X receptor (FXR) have also been shown to regulate the expression of ABCG5/G8, however, response elements have not yet been found [132, 133]. *In vivo* studies indicate that bile acids can modulate ABCG5/G8 mRNA and protein expression in both the intestine and the liver, possibly *via* FXR [133–136]. Moreover, it was reported that bile acids could induce ATP hydrolysis and could thereby regulate the activity of ABCG5/G8 [137].

In regard to the tissue-specific expression of ABCG5/G8, epigenetic mechanisms, like e.g. differential histone acetylation, appear to be implicated [138].

Indications for post-translational regulation of subcellular localization stem from experiments in canine gall bladder epithelial cells, where both LXR activation and exposure to model bile stimulated translocation of ABCG5 and ABCG8 from intracellular compartments to the apical surface, but did not alter their RNA expression [139].

Several other factors have also been shown to regulate expression of ABCG5/G8 in the liver, the intestine or the gallbladder, including thyroid hormone signaling, dietary calcium, iron depletion and constitutive androstane receptor (CAR) agonists, amongst others. The underlying molecular mechanisms of the regulation by these factors have, however, not yet been identified [140–143].

1.4.3 NPC1L1

NPC1L1 is a protein that has an essential role in intestinal and hepatic cholesterol uptake [32, 51, 144]. The NPC1L1 gene codes for the predominant protein consisting of 1332 amino acids, however, two alternatively spliced variants exist, whose biological significance still remains unknown [145]. The NPC1L1 protein is predicted to be constituted of 13 transmembrane domains, five out of these making up a sterol sensing domain (SSD) [32, 145, 146]. The function of this SSD is still unknown, but expected to be involved in the regulation of NPC1L1 protein trafficking by cholesterol [147]. The NPC1L1 protein is a homolog of the Niemann-Pick C1 protein (NPC), sharing 42% amino acid identity. Mutations in NPC cause the lipid storage disorder Niemann-Pick disease type C1 [145, 148, 149]. Besides the transmembrane domains, the human NPC1L1 protein is predicted to have a typical signal peptide, a conserved amino-terminal NPC1 domain and many N-glycosylation sites, resulting in high glycosylation [32, 145].

In humans, NPC1L1 is highly expressed in the small intestine. It is localized in the apical membrane of absorptive enterocytes and has a central role in cholesterol uptake from

the intestinal lumen. Several studies support the assumption that NPC1L1 is the target of the drug ezetimibe (e.g. Zetia®, Ezetrol®), which is a potent inhibitor of cholesterol absorption and which was shown to efficiently lower plasma total and LDL cholesterol levels [32, 145]. In addition to the intestine, NPC1L1 is also expressed in the human liver, where it is situated in the canalicular (apical) membrane of hepatocytes. It is suggested to mediate the transport of cholesterol from bile back into hepatocytes and to thereby regulate biliary cholesterol excretion. It is within the bounds of possibility that ezetimibe also interacts with hepatic NPC1L1, since a study in liver-specific NPC1L1 transgenic mice found that impaired biliary cholesterol excretion was restored and plasma cholesterol levels were normalized by treatment with this compound [51].

Experiments in cultured cells have shown that NPC1L1 can not only localize to the plasma membrane, but cycles between plasma membrane and intracellular compartments. This trafficking is suggested to be cholesterol-dependent, since loading with cholesterol results in prevalent NPC1L1 localization in the endocytic recycling compartment, whereas depletion of cholesterol leads to movement of NPC1L1 to the plasma membrane. As mentioned above, this trafficking could be regulated *via* the SSD [150].

The transcriptional regulation of NPC1L1 is still not completely clear. Several nuclear receptors have been shown to regulate mRNA and protein expression of NPC1L1, namely LXR, RXR, PPARα and PPARβ/δ. If this regulation occurs directly or is an indirect effect is still not fully understood [151–155]. It has, however, been shown that the membrane-bound transcription factor sterol regulatory element binding protein-2 (SREBP-2) can bind to two putative sterol regulatory elements (SREs) in the promoter of human NPC1L1 and that it can regulate NPC1L1 promoter activity and mRNA expression in the Caco-2 cell line [156]. Several studies suggest that cellular cholesterol levels could possibly regulate NPC1L1 expression *via* SREBP-2 [157, 158]. Animal studies reported increased NPC1L1 mRNA levels upon cholesterol deprivation and reduced expression of NPC1L1 when feeding a high cholesterol and bile acid diet [159–161]. Nonetheless, the results from other studies are somewhat contradictory. It was reported that feeding a high cholesterol diet without addition of bile acids cannot significantly reduce NPC1L1 expression and that ezetimibe treatment, which reduces cholesterol content in enterocytes, does not increase NPC1L1 mRNA [153, 162]. Lee et al. identified LRH-1 response elements in the NPC1L1 gene *via* analysis of evolutionary conserved regions, and could show that LRH-1 regulates NPC1L1 transcription. Moreover, transcriptional activation was synergistic when LRH-1 was co-expressed with SREBP-2 [163].

Epigenetic modulation by DNA methylation has also been proposed to influence NPC1L1 expression and seems to account for the absence of NPC1L1 expression in the colon [164].

Regarding post-translational regulation of NPC1L1 expression in intestinal cells, the proteasomal and lysosomal systems seem to be implicated in the degradation of this protein [165].

1.4.4 ABCB1

ABCB1, also known as P-glycoprotein (P-gp) or MDR-1, was the first ABC transporter cloned and is probably the best characterized protein of this superfamily. ABCB1 is constituted of 1280 amino acids and is a membrane-bound glycoprotein with an approximate molecular weight of 170 kDa. Following the general structure of ABC transporters, it possesses two NBDs and two TMDs that consist of six transmembrane α-helices each [166–169]. The N-terminus, the C-terminus, and the NBDs are intracellularly located, as is the highly charged linker region that can be phosphorylated by protein kinases [166, 170].

ABCB1 is expressed in normal human cells, but also in cancer cells. It uses ATP hydrolysis to mediate the efflux of various substrates across cellular membranes [166, 171]. ABCB1 is associated with multidrug resistance (MDR), as it has a significant role in providing cancer cells with resistance to chemotherapeutic drugs [172–175]. The ABCB1 protein is broadly expressed in the plasma membrane and the Golgi membrane of tissues like the liver, intestine, kidney, placenta, macrophages and endothelial cells at the blood-brain, blood-placenta, and blood-testis barriers. ABCB1 provides a mean for the transport and efflux of different substrates and thereby protects these tissues from cytotoxic compounds, xenobiotics and physiologically active substances [166, 175–177]. Among the substrates of this transporter are small molecules like organic cations, carbohydrates, amino acids, lipids like cholesterol and phospholipids [53, 178], and macromolecules like polysaccharides and proteins [166, 179]. Moreover, drug compounds like antibiotics, statins, digoxin and a variety of anticancer drugs, like taxanes and vinca alkaloids are also substrates of ABCB1 [175, 180–182].

ABCB1 is present in the apical membrane of enterocytes and can act as a cholesterol floppase, thereby relocating cholesterol from the cytosolic leaflet of the plasma membrane to the exoplasmic leaflet. Studies in mice could show that ABCB1 contributes to TICE and that knockout results in decreased fecal sterol excretion [53, 178]. ABCB1 expression was reported to be induced by the process of differentiation of monocytes to macrophages [183, 184]. Moreover, ABCB1 mRNA levels were found to be increased in atherosclerotic specimens. However, the exact role of ABCB1 in macrophage lipid homeostasis remains to be elucidated [184, 185].

The expression of ABCB1 can be regulated at the transcriptional, the post-transcriptional and post-translational level.

In regard to transcriptional control, nuclear receptors play an important role. The pregnane X receptor (PXR) and the CAR that form permissive heterodimers with RXR were shown to increase the expression of ABCB1 *via* response elements in the promoter region [186, 187]. In macrophages, LXR was shown to increase ABCB1 levels [183], which, however, does not hold true for other cell types [188]. Several other transcription factors were found to regulate ABCB1 expression by binding to sequences in the promoter region of the ABCB1 gene, e.g. activator protein-1 (AP-1), NFκB, FoxO1, FoxO3a, T-cell factor/lymphoid enhancer factor (TCF/LEF), Sp1 and Y-box binding protein-1 (YB-1) [166, 175].

Concerning post-transcriptional regulation of ABCB1 expression, several miRNAs have been shown to either suppress or induce the expression of this transport protein. Among the miRNAs directly binding to the 3'UTR of ABCB1 mRNA transcripts and thereby suppressing ABCB1 expression are miR-451, miR-298 and miR-145 [189–191]. In contrast, the miRNAs miR-19a/b and miR-130a seem to increase the expression of ABCB1 [192, 193].

Several factors were found to regulate ABCB1 expression post-translationally. The proteasomal system was reported to regulate the degradation of ABCB1, leading to rapid proteolysis [194]. Moreover, trafficking and recycling of ABCB1 were shown to be controlled by the small GTPases Rab4 and Rab5, amongst others [195]. The serine/threonine protein kinase Pim-1L can mediate phosphorylation of ABCB1 and thereby seems to protect it from proteasomal degradation, further allowing glycosylation of ABCB1 and plasma membrane expression [196]. Both protein kinase C (PKC) and PKA were reported to phosphorylate ABCB1 at several residues in the linker region. Some research groups found that these phosphorylations were important for the translocation and function of ABCB1, whereas others could not confirm these findings [175, 197, 198].

1.4.5 ABCG1

ABCG1 is an ABC half-transporter, consisting of only one TMD and one NBD. To build a functional transporter, it therefore has to dimerize, with either another ABCG1 or with ABCG4. The TMD is made up by six putative transmembrane α-helices, with an extracellular loop located between transmembrane helices five and six [22, 177, 199, 200]. First cloning of ABCG1 cDNA was performed by two independent groups, namely Chen et al. [199] and Croop et al. [201]. They found that the cDNA encodes for a protein consisting of 674 and of 638 amino acids, respectively. Further studies showed that other variants exist due to alternative splicing [202–204]. Moreover, different translational start

sites can lead to further N-terminal variants [205]. In human macrophages, two different molecular masses have been reported, namely 60 kDa and 110 kDa [206, 207].

ABCG1, like ABCA1, is expressed in macrophages and in cells of many other tissues, with the highest expression levels found in adrenal glands, lung, heart and spleen. However, expression in hepatocytes is low and ABCG1 is not expressed in enterocytes [22, 41, 206]. ABCG1 mediates the efflux of cholesterol to different extracellular acceptors, like mature HDL, but also to LDL, albumin and liposomes, amongst others. In contrast to ABCA1, it does not mediate the efflux of cholesterol to lipid-free apolipoproteins [22, 41, 42, 87, 208]. Other substrates for ABCG1 include oxysterols and choline phospholipids like sphingomyelin [208, 209]. The subcellular localization of ABCG1 still remains inconclusive, since research groups have reported localization in the plasma membrane [210, 211], and along both secretory and endocytic pathways [212], whereas others found ABCG1 located in endosomal vesicles only [213, 214]. Likewise conflicting are the reports in regard to its role in atherosclerosis development [22, 215]. Nonetheless, it has been shown that combined knockout of ABCA1 and ABCG1 leads to massive lipid accumulation in tissues rich in macrophages [216]. Moreover, in LDLR-knockout mice receiving chow diet, ABCA1 and ABCG1 deficiency in macrophages was reported to increase atherosclerosis [217]. These results indicate that cholesterol efflux from macrophages mediated by ABCA1 and ABCG1 is anti-atherosclerotic.

The expression of ABCG1 can be controlled at the transcriptional, post-transcriptional and post-translational level.

At the transcriptional level, the LXR/RXR heterodimer was shown to upregulate ABCG1 expression *via* multiple response elements present in the ABCG1 gene [22, 202]. Besides, PPARγ leads to an increased expression of ABCG1 in macrophages [218, 219], and a putative PPAR binding motif was found in the promoter of ABCG1 [203, 220]. Furthermore, the promoter of the ABCG1 gene carries binding sites for transcription factors including Sp1, AP-2 and NFκB, and a sterol regulatory element which can be regulated by SREBP [221].

Several miRNAs were found to regulate ABCG1 expression post-transcriptionally by direct binding to its 3'UTR, including miR-33, miR-10b and miR-128-2 [100, 222–224]. Another post-transcriptional regulatory mechanism was identified *via* AMP-activated protein kinase (AMPK), which can activate the extracellular signal-regulated protein kinase (ERK) pathway and thereby stabilizes ABCG1 mRNA [22, 225].

ABCG1 expression is also extensively regulated at the post-translational level, and the fact that this protein exists in different isoforms in human cells adds another level of complexity. Eicosanoids, produced by 12/15-lipoxygenase (12/15-LO) activity from

fatty acids, can induce serine phosphorylation of ABCG1, which was shown to enhance its degradation in macrophages, thereby reducing its expression [226]. It was further shown that this induction of degradation is mediated *via* p38- and JNK2-dependent pathways [227]. Concerning the degradation of ABCG1, the proteasomal and the calpain systems have been shown to be implicated [228, 229]. In regard to lysosomal degradation of ABCG1, contradictory results exist, which might be caused by different cell types used [106, 228]. Phosphorylation of one isoform of ABCG1 by PKA was shown to destabilize this protein, whereas phosphorylation mediated by PKC stabilizes and thereby increases ABCG1 expression [205, 230].

1.4.6 SR-B1

SR-B1 is a member of the class B scavenger receptor family that is encoded by the SCARB1 gene. Predictions using UniProt suggest that five protein variants could be produced by alternative splicing, where isoform 3 is the longest variant. Isoform 1, however, is the major gene product consisting of 509 amino acids [231, 232]. SR-B1 has two transmembrane domains that are separated by an extracellular domain that is extensively N-glycosylated, and both the N- and the C-terminus are located intracellularly [233]. It has at least one phosphorylation site, and a transmembrane glycine dimerization motif at the N-terminus that is essential for receptor oligomerization and lipid transport [231, 234, 235]. Four cysteine residues in the extracellular domain were reported to be necessary for HDL binding activity, cholesteryl ester uptake and trafficking to the plasma membrane [236].

SR-B1 is a membrane receptor protein that is expressed in various tissues and cell types, e.g. intestine, liver, macrophages, endothelial cells, smooth muscle cells, adipocytes and steroidogenic tissues (e.g. adrenal glands, ovary, testis) [231, 237]. It was first identified to bind both modified and native LDL [238], and was later found to also bind VLDL, chylomicrons and HDL [224]. Subsequent studies showed that it serves as a very important HDL receptor that mediates the uptake of cholesteryl esters from HDL [45]. SR-B1 can also transfer other lipids from HDL to cells, like triglycerides, free cholesterol and phospholipids [239]. Furthermore, it facilitates the efflux of cholesterol from peripheral tissues like macrophages to mature HDL *via* passive diffusion [34, 231]. It was shown that SR-B1 can bind and transport lipid-soluble vitamins, like vitamin E and carotenoids [240, 241], and silica [242].

Several studies were investigating the role of SR-B1 in macrophages. The ones that were performed in mouse macrophages are rather inconclusive, whereas the use of human macrophages clearly indicates that SR-B1 plays a role in cholesterol efflux together with ABCA1 [243–247]. SR-B1 was shown to be atheroprotective, since its partial or total loss

increased atherosclerosis in mouse models, whereas overexpression in the liver reduced atherosclerosis [231, 248–251]. Uptake of cholesteryl esters from HDL by the liver through SR-B1 enables HDL to accept more cholesteryl esters from peripheral tissues, suggesting that SR-B1 promotes RCT [252].

Several levels of regulation have been identified for SR-B1 expression. Various transcription factors have been shown to exert transcriptional control of SR-B1 expression. SREBP-1a can regulate SR-B1 expression *via* binding sites in the promoter [253]. Furthermore, the nuclear receptors LXRα, LXRβ, FXR, PPARα and PPARγ were found to upregulate SR-B1 expression due to response elements in the promoter region [224]. Likewise, LRH-1 and the estrogen receptors α and β can induce the expression of SR-B1 [254, 255]. Nuclear receptors that repress promoter activity include PXR and NR0B1 (DAX-1) [256, 257]. SR-B1 expression is also regulated by steroidogenic factor-1 (SF-1). It was shown that SR-B1 expression is sensitive to trophic hormones, like adrenocorticotropin (ACTH), in a tissue-specific manner and that this hormone stimulation is mediated *via* cAMP and SF-1 [231, 258].

The expression of SR-B1 was reported to be diminished post-transcriptionally by several miRNAs in the liver and in macrophages, namely miR-185, miR-96 and miR-223 [259]. In steroidogenic tissues, miR-125a and miR-455 were demonstrated to negatively regulate SR-B1 expression by binding to the 3'UTR of SR-B1 mRNA transcripts [231, 260].

Finally, the expression of SR-B1 can also be regulated at the post-translational level *via* several different mechanisms, and at least a few shall be mentioned. In the liver, PDZ-containing kidney protein 1 (PDZK1 or NHERF3) was reported to control SR-B1 protein stability, since its knockout in mice reduced hepatic SR-B1 protein levels by more than 95%, but not its mRNA levels [261]. In steroidogenic cells, two other members of the NHERF family, NHERF1 and NHERF2, were shown to interact with SR-B1 and thereby decrease its protein expression [262]. SR-B1 protein levels were further shown to be influenced by the Ras/MEK/ERK signaling pathway, and it was demonstrated that this effect occurs *via* degradation pathways that can be induced by PPARα. PPARα agonists induced a degradation that was independent of the proteasomal, lysosomal and calpain systems, in a post-endoplasmatic reticulum compartment [224, 263, 264].

1.5 Nuclear receptors

Nuclear receptors (NR) are a superfamily of ligand-dependent transcription factors, meaning that they modulate gene transcription upon activation by various (endogenous) ligands

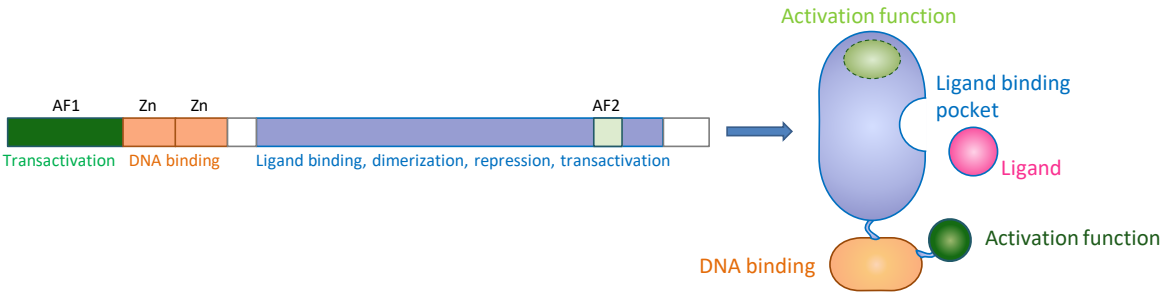

Figure 1.4: Structural features of nuclear receptors. See text for details. Adopted from Hiebl et al. (2018) [277].

and are thereby involved in a plethora of physiological pathways, including metabolic processes [265, 266]. In humans, this superfamily consists of 48 family members [267].

Nuclear receptors possess common structural features (Figure 1.4): an N-terminal region with a ligand-independent activation domain (AF-1), a central zinc-finger DNA-binding domain, a hinge region and a C-terminal ligand-binding domain containing a ligand-dependent activation function (AF-2) [268]. The DNA-binding domain (DBD) binds to a specific DNA sequence termed response element (RE) [269]. The ligand-binding domain (LBD) is not only responsible for the binding of the ligand, but also for receptor dimerization and cofactor interaction [268].

Major nuclear receptors regulating metabolism belong to the class of type 2 nuclear receptors. Type 2 nuclear receptors are localized in the nucleus, even in their inactivated state, where they are already bound to the RE as a homo- or heterodimer, but the transcription of target genes is repressed by the binding to a corepressor complex. Upon ligand binding, the three-dimensional conformation of the AF-2 changes, whereupon corepressors are released and coactivators are recruited, resulting in target gene transcription (Figure 1.5) [265, 270].

Most type 2 nuclear receptors act as heterodimers with the RXRs [91], whereas RXRs form also homodimers or homotetramers [271, 272]. Type 2 nuclear receptors that are important regulators of metabolic pathways are the liver X receptors (LXRα (NR1H3) and LXRβ (NR1H2)), the farnesoid X receptor (FXR (NR1H4)), the peroxisome proliferator activated receptors (PPARα (NR1C1), PPARβ/δ (NR1C2) and PPARγ (NR1C3)) and the retinoid X receptors (RXRα (NR2B1), RXRβ (NR2B2) and RXRγ (NR2B3)). Ligands activating these nuclear receptors include retinoids, oxysterols, bile acids and fatty acids, highlighting their importance in regulating metabolic processes [273–276]. As disturbances in lipid metabolism contribute highly to the development of atherosclerosis, regulating these nuclear receptors is of great interest in drug discovery and development.

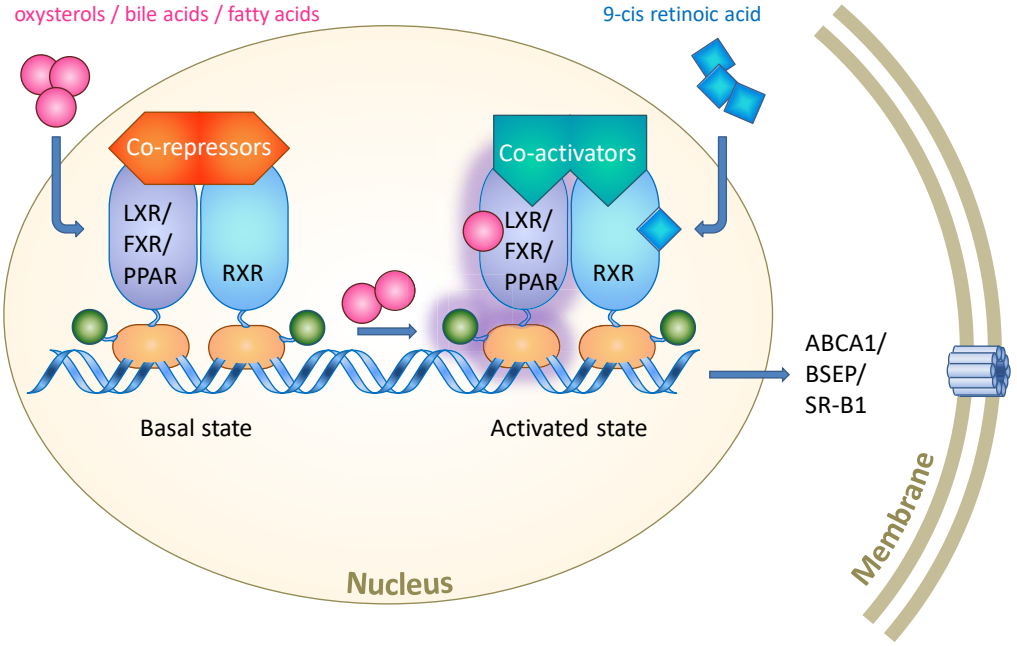

Figure 1.5: Mechanism of nuclear receptor activation. LXR, FXR and PPAR form heterodimers with RXR, which are bound to response elements on the DNA. Corepressor binding inhibits target gene transcription in the basal state. Upon ligand binding, conformational changes lead to the substitution of corepressors for coactivators, thereby triggering the transcription of target genes. Adopted from Hiebl et al. (2018) [277].

The nuclear receptors described herein regulate a plethora of target genes and as it would go beyond the constraints of this work, only the most important ones in regard to lipid homeostasis will be outlined.

1.5.1 Nuclear receptors with a special relevance in cholesterol homeostasis

1.5.1.1 LXR (Liver X Receptor)

The liver X receptor exists as two different isotypes, LXRα and LXRβ. LXRβ is ubiquitously expressed, whereas LXRα can be found mainly in tissues with high metabolic rates, like kidney, liver, macrophages and intestine [31, 278, 279]. Endogenous ligands for both LXR isotypes are, for instance, oxysterols, which are oxygenated derivatives of cholesterol. Most oxysterols, including 22*(R)*-hydroxycholesterol, 24*(S)*-hydroxycholesterol and 24*(S)*,25-epoxycholesterol, are agonists for both isotypes [274, 280–282]. Of note, endogenous antagonists for LXR also exist. A competitive antagonist of LXR is arachidonic acid, a polyunsaturated fatty acid (PUFA) [283, 284]. Another LXR ligand identified to be found both endogenously and in processed foods is 5α,6α-epoxycholesterol [285, 286].

It was shown that this compound can function either as an agonist, antagonist or inverse agonist, depending on the target gene and the cell type [286]. A very prominent synthetic agonist of both LXRα and LXRβ is GW3965 [287].

LXRs form heterodimers with RXRs, which are so-called permissive heterodimers, meaning that activation of the receptor dimer can be achieved by ligands for LXR or for RXR, or by both synergistically [279]. LXREs bound by the LXR/RXR heterodimer contain direct repeats of the consensus sequence AGGTCA separated by four nucleotides (DR-4) [273]. An inverted repeat of this consensus sequence without spacing nucleotide (IR-0) as well as an inverted repeat with one spacing nucleotide (IR-1) have also been reported to be bound by LXR [288–290]. Activation of LXR by a ligand leads to the release of corepressors (e.g. nuclear receptor corepressor (NCoR)) and to specific coactivator (e.g. steroid receptor coactivator-1 (SRC-1)) recruitment. This coactivator recruitment may e.g. change local chromatin architecture by histone acetylation, which finally results in target gene transcription [277, 291–293].

LXRs cannot only activate target gene transcription, but they can also repress expression of certain genes. However, this transrepression is mediated in a different manner compared to transactivation. Current knowledge suggests that LXR is activated by a ligand and hereafter SUMOylated by conjugation to a small ubiquitin-like modifier protein (SUMO). SUMOylated LXR monomers form a complex with and stabilize transcriptional corepressors on promoters of (especially proinflammatory) target genes [294–296].

LXRs are key regulators of cholesterol and lipid homeostasis. Their target genes are implicated in cholesterol absorption, biosynthesis, cellular uptake, excretion, RCT and TICE, revealing the unique role of LXRs as master regulators of these processes [293]. A bona fide LXR target that was one of the first identified as such is ABCA1 [297, 298]. It has been shown that activation of LXR increases ABCA1 expression substantially in the intestine, in macrophages and in the liver [299–301]. Likewise, ABCG1 is an LXR target gene that is induced upon its activation [202]. In the intestine as well as in the liver, ABCG5/G8 is upregulated upon LXR activation, leading to increased fecal and biliary cholesterol excretion [47, 123, 126, 302]. Activating LXR was reported to downregulate NPC1L1, thereby limiting intestinal cholesterol absorption [154]. Several proteins that are involved in lipid remodeling were found to be regulated by LXR, including CETP, phospholipid transfer protein (PLTP) and lipoprotein lipase (LPL) [303–305]. The gene cluster of apolipoproteins E, C1, C2 and C4 (apoE, apoC1, apoC2 and apoE) has also been shown to be induced by LXR [306, 307].

Besides cholesterol homeostasis, triglyceride and fatty acid homeostasis are also under regulation of LXR. LXR upregulates SREBP-1c, which in turn regulates *de novo* fatty acid synthesis [308]. LXRs can thereby increase the expression of several lipogenic enzymes,

including stearoyl-CoA desaturase-1 (SCD-1), acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS), which are target genes of SREBP-1c [309–312]. FAS and SCD-1 can also be directly upregulated by LXR *via* response elements in their promoters [313, 314]. Moreover, LXR increases the expression of inducible degrader of the LDLR (IDOL), which induces, for instance, the ubiquitination and degradation of LDLR and VLDLR [315–317].

Several studies have indicated that the activation of LXR has anti-atherosclerotic effects. In LDLR$^{-/-}$ and apoE$^{-/-}$ mouse models, the ablation of LXRs accelerated the development of atherosclerosis, while administration of LXR agonists reduced the development of atherosclerotic lesions [318–321]. Applying an LXR agonist has been shown to modify the cellular composition of atherosclerotic plaques, reducing inflammation and adhesion molecules [319]. Transfer of LXR-deficient bone marrow into LDLR$^{-/-}$ mice was reported to drive atherosclerosis, whereas overexpression of LXRα in macrophages reduced atherosclerosis, revealing the importance of macrophage LXR expression [293, 318, 322, 323]. However, other tissues than the bone marrow seem to be implicated in the anti-atherogenic effects of LXR, since LXR$\alpha^{-/-}$ in the bone marrow did not entirely reproduce the phenotype of complete LXRα knockout [324].

It was shown that in primates and humans, LXR agonists elevate plasma LDL levels and reduce LDLR expression in the liver, possibly *via* the IDOL pathway mentioned above [325, 326]. Moreover, due to its effects on *de novo* lipogenesis, LXR activation leads to pronounced liver steatosis [314, 327]. This undesirable feature has hampered the use of synthetic LXR agonists, like GW3965 and T0901317, in clinical trials. A possible strategy to avoid the formation of hepatic steatosis would be the development of LXRβ-selective agonists. The rationale behind is that LXRα is the predominant isotype expressed in the liver, and thought to be the major inducer of SREBP-1c expression [323]. Although the development of LXRβ-selective agonists is tricky due to the high sequence similarity in the LBDs of the two LXR isotypes, some compounds with partial selectivity have already been developed [292, 328]. A different strategy to overcome some side effects of LXR agonists would be the design of tissue-selective agonists. GW6340 is an intestine-specific LXR agonist that was found to induce LXR target genes in the intestine, but not in the liver, and promotes macrophage reverse cholesterol transport [329].

The search for potential LXR agonists for therapeutic use is still ongoing. Natural products with a tissue- or isotype-specific effect or with a lower potency could also be suitable candidates. These features would help to avoid undesired side effects by regulating only a subset of target genes [277].

Not only the activity of LXR is important for target gene expression, but also the expression level of LXR itself. It was shown that an auto-regulatory mechanism exists

for LXRα expression. The promoter of human LXRα contains three LXREs, and both LXRα and LXRβ were found to strongly activate one of these, whereas the other two are only bound by LXRα [330, 331]. Moreover, the LXRα gene contains PPREs and PPARγ agonists were shown to increase LXRα mRNA and protein levels. Other studies reported that also PPARα agonists can induce the expression of LXRα, however, contradictory results were reported in this regard, which might depend on the different cell lines used [94, 96, 331, 332].

Several post-translational mechanisms can also regulate the activity and/or the expression of LXR. Deacetylation of lysine residues by the sirtuin 1 (SIRT1) deacetylase was found to lead to the ubiquitination and subsequent degradation of LXR, whereas at the same time it induces the transcriptional activity of LXR. The model proposed by Li et al. is that the degradation leads to the clearance of LXR from promoters, which then enables the next round of transcription and increased target gene expression [333]. Furthermore, phosphorylation by several kinases, including protein kinase A, was reported to modulate LXRα activity, in some cases in a gene-specific manner [334, 335].

1.5.1.2 FXR (Farnesoid X Receptor)

Two different FXR genes are known, FXRα (NR1H4) and FXRβ (NR1H5), which share great homology. Both encode for functional proteins in several species, including mice, rats, rabbits and dogs. In humans and primates, however, FXRβ is a pseudogene and only FXRα yields a functional protein. In this work, FXRα will be simplified to FXR, since FXRβ is not of relevance in humans [336]. Four different isoforms of FXR exist due to distinct promoters and alternative splicing [337].

FXR can be found mainly in the liver, kidney, intestine and adrenal glands. Its name stems from the first identified ligand farnesol, which is an intermediate in the mevalonate biosynthetic pathway and a very weak agonist at supraphysiological levels [282]. The endogenous ligands for FXR were later identified to be bile acids, rendering FXR a nuclear bile acid receptor. The most potent bile acid in regard to activation of FXR is chenodeoxycholic acid (CDCA), followed by deoxycholic acid (DCA), lithocholic acid and cholic acid (CA) [338]. A non-steroidal synthetic agonist of FXR is GW4064 [339].

FXR can either act as a monomer, a homodimer or as a heterodimer with RXR on its target genes [340]. FXR/RXR heterodimers are permissive, meaning that they can be activated by ligands for either partner in the heterodimer [341]. FXREs recognized by FXR consist of an inverted repeat of the consensus sequence separated by one nucleotide (IR-1), direct repeats with one spacing nucleotide (DR-1) or everted repeats with eight spacing nucleotides (ER-8) [342].

FXR can either directly stimulate target gene expression or indirectly repress it *via* regulating expression of small heterodimer partner (SHP) [343, 344]. Several coactivators have been identified for FXR, including peroxisome proliferator activated receptor-gamma coactivator-1α (PGC-1α) and vitamin D receptor-interacting protein 205 (DRIP205) [345, 346].

FXR controls a variety of target genes that are involved in lipid and bile acid metabolism. It has been reported that FXR contributes to lower triglyceride levels, as it induces apoC2 expression *via* its promoter. ApoC2 enables the interaction of VLDLs and chylomicrons with LPLs, leading to the release and the hydrolysis of triglycerides [323, 347]. Moreover, apoC3 that acts in an opposite manner is suppressed upon FXR activation [348]. Other genes with importance in lipid metabolism that are regulated by FXR include apoE, VLDLR, PLTP and FAS [290, 349, 350]. Activation of FXR induces the expression of PPARα, whereas it suppresses the expression of SREBP-1c *via* SHP [351, 352].

FXR activation further leads to the repression of CYP7A1 transcription, which is a key enzyme in the conversion of cholesterol to bile acids. The transcription of an enzyme important for the synthesis of cholic acid, cytochrome P450 8B1 (CYP8B1), is also suppressed by FXR. In the liver, FXR activation induces the expression of SHP, which interacts with LRH-1 and thereby suppresses CYP7A1. Repression of HNF4α by SHP further inhibits CYP8B1 transcription [343, 344, 353–355].

Activation of FXR in the intestine leads to the expression of fibroblast growth factor 19 (FGF19, FGF15 in rodents). FGF19 is released into the circulation and acts as a hormone on the liver, by binding to the fibroblast growth factor receptor 4 (FGFR4). *Via* FGFR4, ERK1/2 and JNK1/2, it represses the expression of CYP7A1 and CYP8B1 [356–358].

Furthermore, FXR regulates the expression of genes involved in the conjugation and in the detoxification of bile acids [359, 360]. In both the liver and the intestine, it regulates genes for bile acid import and export, as well as for the shuttling of bile acids from the apical to the basolateral side of the enterocyte [323, 338, 361, 362].

Some interesting connections between FXR and atherosclerosis have been found. Studies in mice have revealed that FXR knockout leads to hypercholesterolemia, hypertriglyceridemia and increased cholesterol absorption in the intestine, however, these mice do not develop atherosclerosis on a Western diet [363, 364]. Studies in apoE$^{-/-}$, LDLR$^{-/-}$ and FXR-deficient mice yielded mixed results and indicated that the effects of FXR are gender-specific. Male FXR- and LDLR-deficient mice had reduced atherosclerotic lesion area, as well as reduced plasma levels of LDL and HDL [364]. In contrast, female FXR- and LDLR-deficient mice showed no reduction in lesion area and had elevated plasma cholesterol levels. FXR- and apoE-deficient mice on a Western diet were reported to have a marked increase in lesion

area as well as in plasma cholesterol levels [365]. A different study, however, showed that female FXR- and apoE-deficient mice have a reduction in lesion area, despite elevated plasma total cholesterol [366]. The outcome of a specific study might also depend on the genetic background of the mice used.

Activation of FXR by different agonists was shown to yield more consistent results, indicating an anti-atherogenic effect [367–369]. The FXR agonist obeticholic acid (INT-747) reduced aortic plaque formation in apoE$^{-/-}$ mice [368]. FXR activation with the agonist WAY-362450 in LDLR- or apoE-knockout mice reduced the levels of non-HDL cholesterol and the formation of aortic plaques in both male and female mice. To investigate the contribution of SHP, the authors applied WAY-362450 in LDLR- and SHP-deficient, as well as in apoE- and SHP-deficient mice. Notably, the reduction in non-HDL cholesterol and lesion area was then only observed in male mice, but not in female [367, 369].

Regulating the expression of FXR could also influence the expression of FXR target genes. It has been shown that FXR expression can be regulated by the transcription factors HNF1α and *caudal*-related Homeobox 2 (CDX2) [370, 371]. Moreover, FXR expression and/or activity can be regulated at the post-translational level. It was shown that FXR can be acetylated by p300, which increases protein stability but reduces heterodimerization with RXRα and DNA binding. Like LXR, FXR can also be deacetylated by SIRT1 [372]. Phosphorylation of FXR at two serine residues by PKC was reported to increase its transcriptional activity [373]. In contrast, phosphorylation by AMPK leads to reduced transcriptional activity [335, 374].

1.5.1.3 PPAR (Peroxisome Proliferator Activated Receptor)

Three different isotypes of the peroxisome proliferator activated receptor exist – PPARα, PPARβ/δ and PPARγ. Two isoforms of PPARγ have been reported, PPARγ1 and PPARγ2, derived by the usage of two distinct promoters or alternative splicing. PPARα is expressed in tissues like liver, skeletal muscle, intestine, macrophages and brown fat. PPARβ/δ is ubiquitously expressed, whereas PPARγ can be found mainly in adipose tissue and cells belonging to the immune system, also in macrophages [375, 376].

Endogenous ligands for PPARs include PUFAs, conversion products of polyunsaturated essential fatty acids like arachidonic acid, eicosanoids and components of oxLDL like 9- and 13-hydroxyoctadenoic acid [377, 378]. Moreover, several synthetic ligands of the different PPARs were generated. Ligands for PPARα are the hypolipidemic drugs fenofibrate and clofibrate [12, 13], while thiazolidinedione drugs like rosiglitazone and pioglitazone are agonists of PPARγ [379], some of them still in use as antidiabetics. GW501516 and GW0742 are ligands of PPARβ/δ [380].

PPARs act as heterodimers together with RXRs and bind PPREs that consist of a direct repeat of the consensus sequence separated by one nucleotide (DR-1). PPAR/RXR heterodimers are permissive and can therefore also be activated by RXR ligands [381–383]. PPARs can either activate gene expression or lead to its repression. Several models of how this repression could occur have been proposed for PPARγ, but for PPARα and PPARβ/δ the underlying mechanisms are less well understood [382]. Important coactivators of PPARs include PGC-1α, thyroid hormone receptor-associated protein 220 (TRAP220), PPAR-binding protein (PBP) and mediator complex subunit 1 (MED1) [384].

The target genes of PPARs play an important role in lipid and glucose metabolism and are important inhibitors of inflammatory gene expression. PPARα activates mitochondrial and peroxisomal fatty acid β-oxidation by inducing, for instance, the target genes carnitine palmitoyltransferase 1B and acyl-coenzyme A oxidase [385]. PPARα also regulates systemic lipid metabolism by e.g. reducing the expression of apoB48 and inducing LPL [386–388]. Furthermore, activation of PPARα upregulates NPC1 and 2, as well as SR-B1, while it decreases cholesterol esterification by decreasing the availability of substrates for ACAT1 [389–391]. PPARβ/δ is implicated in the regulation of mitochondrial respiration, fatty acid metabolism, thermogenesis and the programming of muscle fiber type [382]. Upon activation, it was shown to induce the expression of scavenger receptors CD36 and SR-A [392]. Moreover, it increases the expression of ABCA1, while it reduces apoE expression [392, 393]. Like PPARα, it also induces carnitine palmitoyltransferase 1B and acyl-coenzyme A oxidase expression [394]. PPARγ activates fatty acid synthesis and storage and has a crucial role in adipocyte differentiation [383, 395, 396]. Some of the target genes of PPARγ are LPL, adipocyte fatty acid binding protein, phosphoenolpyruvate carboxykinase, CD36 and LXRα [378, 397]. Activation of PPARγ upregulates the expression of ABCG1 and SR-B1, while it downregulates apoB48 [218, 387, 391].

Like LXR, PPARα and PPARβ/δ are able to stimulate RCT and TICE upon activation [398, 399]. PPARα activation in the intestine leads to a reduction in cholesterol esterification, an upregulation of ABCA1 expression and a rise in apoA1 secretion, thus also increasing HDL cholesterol levels [398]. Activation of PPARβ/δ results in an increase of HDL levels, as well as in a downregulation of NPC1L1, thereby reducing cholesterol absorption [151, 400]. In contrast to PPARα and β/δ, little is known about the role of PPARγ in regard to cholesterol homeostasis in the intestine.

The roles of the different PPARs in atherosclerosis development and the effect of their activation have been extensively studied in various mouse models. In regard to PPARα, results are somewhat contradictory. Results from apoE-deficient and PPARα-deficient mice show that PPARα-deficient mice have less atherosclerotic lesions compared to control

animals [401]. In contrast, LDLR-deficient mice on a Western diet receiving bone marrow from PPAR$\alpha^{-/-}$ mice had increased atherosclerotic lesions compared to those receiving bone marrow from PPAR$\alpha^{+/+}$ mice [402]. The use of PPARα agonists, however, supports a protective role of this nuclear receptor in atherosclerosis. For instance, fenofibrate treatment of dyslipidemic mice reduced the accumulation of lipids in the aortic sinus and improved lipoprotein metabolism [403]. In mice transgenic for human apoA1, the PPARα agonist GW7647 promoted reverse cholesterol transport while it did not in WT animals [383, 404]. LDLR knockout mice receiving bone marrow from PPARβ/δ-deficient mice had less atherosclerotic lesion areas compared to those receiving bone marrow from control mice. However, PPARβ/δ was suggested to control the inflammatory status of macrophages in atherosclerotic lesions, and its modulation by a ligand could help to protect from atherosclerosis progression [405]. Supporting this suggestion, the PPARβ/δ agonist GW0742 suppressed the expression of inflammatory and atherogenic genes in the artery in a mouse model of AngII-accelerated atherosclerosis [406]. A different PPARβ/δ agonist, GW501516, reduced atherosclerosis in apoE-deficient mice [407]. Numerous studies have reported an anti-atherosclerotic effect of PPARγ by using both PPARγ-deficiency and PPARγ agonists. Increased lesion formation was observed when LDLR-knockout mice were transplanted with PPARγ-deficient bone marrow [94]. In apoE$^{-/-}$ mice, a PPARγ agonist reduced lesion formation [408]. This was also the case in LDLR$^{-/-}$ mice under PPARγ agonist treatment, however, only in male animals [409, 410].

The expression of PPARs can be regulated at various levels. The expression of PPARγ can be induced by transcription factors *via* binding sites in the promoter, like CCAAT/enhancer-binding proteins (C/EBPs) and SREBP-1 [411, 412]. An excellent recent review highlights extensive post-translational modifications that can influence the expression and/or activity of PPARs [413]. It has been shown that all three proteins can undergo SUMOylation. PPARα and PPARγ were further reported to be extensively phosphorylated by different kinases and acetylation of several lysine residues was shown for PPARγ [413].

1.5.1.4 RXR (Retinoid X Receptor)

In mammals, RXR is expressed as three different isotypes, namely RXRα, RXRβ and RXRγ. These three isotypes are differentially expressed in distinct tissues, however, at least one isotype is expressed in each cell. In regard to their function, the three RXR isotypes are mostly interchangeable [414–416]. RXRα is expressed e.g. in the liver and the intestine, RXRβ is found in virtually every human tissue, and RXRγ is mainly expressed in the pituitary gland, brain and muscles [415, 417].

Endogenous ligands for RXR are derived from the retinol (vitamin A) metabolism. It was suggested that 9-*cis* retinoic acid is an endogenous ligand of RXR, however, this is still under debate since it is not detectable in serum under normal conditions. Nonetheless, one study could show the presence of 9-*cis* retinoic acid in the pancreas, regulating insulin secretion [418–420]. In mice, 9-*cis*-13,14-dihydroretinoic acid was found to be an endogenous retinoid and RXR ligand with physiological relevance [421]. Furthermore, unsaturated fatty acids, stemming from diet, are ligands of RXR. Among these fatty acids are docosahexaenoic acid (22:6), arachidonic acid (20:4) and oleic acid (18:1) [422, 423]. A very important synthetic and high-affinity agonist of all three RXR isotypes is bexarotene, which is frequently used in experimental studies, but is also marketed as Targretin™ for advanced stage cutaneous T-cell lymphoma [424, 425]. Synthetic compounds that bind and activate RXRs are also called rexinoids [423].

RXR does not only function as a heterodimer partner for other nuclear receptors, but can also form homodimers and homotetramers [271, 272]. In regard to the heterodimers, we distinguish "permissive" and "non-permissive" for RXR activity. As mentioned above, LXR, FXR and PPAR belong to the group of permissive heterodimers. The vitamin D receptor and the thyroid hormone receptors are non-permissive for RXR, meaning that they cannot be activated by RXR ligands and RXR remains a silent partner [265, 426, 427]. RXREs consist either of a direct repeat of the consensus sequence separated by one nucleotide (DR-1) or of an inverted repeat without spacing nucleotide (IR-0), and are bound by RXR homodimers. In a heterodimer, the nuclear receptor partner of RXR defines the response element to which the RXR/NR heterodimer binds to [265, 428, 429].

Several different coactivators were shown to interact with RXR, like SRC-1 and nuclear receptor coactivator 2 (NCoA2) [430].

As RXR is the obligate heterodimer partner of the metabolic sensors LXR, FXR and PPAR, it has a unique role in integrating the action of these receptors, and ligands for RXR can exert a variety of biological functions by modulating these permissive receptor heterodimers [416, 429]. *Via* modulation of PPARs and LXRs, in particular, RXR ligands control the uptake, efflux and storage of cholesterol [383, 431, 432]. In macrophages, lipid uptake is mainly mediated by scavenger receptors, particularly CD36 and SR-A [433]. RXR agonists were shown to mediate moderate oxLDL uptake in macrophages by an upregulation of the PPARγ-target gene CD36 and a reduction in SR-A activity [434, 435]. RXR activation by different agonists was further reported to increase ABCA1 and ABCG1 expression in different monocyte/macrophage cell systems, thus promoting cholesterol efflux, most likely mediated *via* PPARγ and LXR. Thus, the net effect of activating this nuclear receptor in macrophages is a reduction in cholesteryl ester accumulation [434–436].

It has to be mentioned that some RXR agonists were shown to cause elevated plasma triglyceride levels by inducing the expression of LXR/RXR-target genes responsible for lipogenesis in the liver, e.g. SREBP-1c and FAS [436–438]. Nonetheless, there seem to be substantial differences between specific RXR agonists regarding the induction of lipogenic genes [439, 440]. The final expression pattern induced by RXR ligands can be influenced by several issues, including the differential protein abundance of RXR, its heterodimer partners, and coregulators in different tissues. Moreover, the allosteric changes induced by RXR ligands and consequently the interaction with the heterodimer partner and coregulators, as well as the promoter context may also cause a variation in the gene expression pattern [272, 277].

The RXR agonist bexarotene was reported to inhibit atherosclerotic lesion formation and intestinal cholesterol absorption in apoE2 knockin mice. In the same mouse model, bexarotene treatment improved macrophage lipid homeostasis [436]. Another mouse model of dyslipidemia ($apoE^{-/-}$) also showed that RXR activation by a rexinoid reduced atherogenesis and increased cholesterol efflux from macrophages from WT but not from LXRα/β double knockout mice. Thus, the RXR/LXR heterodimer likely mediated the effects of the rexinoid [441]. Anti-atherogenic effects have been reported for LXR, PPARβ/δ and PPARγ agonists, further supporting the hypothesis that RXR agonists exert their protective effects *via* heterodimers with these partners [431, 432].

RXR expression could also have an impact on the expression of its target genes. In the human RXRα gene, putative binding sites for several transcription factors, including Sp1, AP-1 and GATA-1/2 were found [442]. Moreover, post-translational modifications were also shown to modulate RXR expression and/or activity. RXRα and RXRγ were shown to be targeted by the p300 acetyltransferase. Acetylation of RXRα was reported to increase its transcriptional activity [443]. RXR can also be phosphorylated by different kinases at different residues. Phosphorylation of RXRα at serine 260 was found to confer resistance to proteasomal degradation and to lead to loss of heterodimeric activity [444–446]. Several other phosphorylations were reported that can inhibit transcriptional activity [335, 447].

1.5.2 Natural products as modulators of nuclear receptors

Natural products constitute an interesting and multifaceted source for drug discovery. In regard to their chemical structures, natural products are very diverse and they differ from synthetic compounds in several aspects. Generally speaking, they are sterically more complex compared to synthetics, incorporate more oxygen atoms, but less nitrogen, halogen and sulphur atoms, and possess a higher molecular weight [448–451]. Besides their chemical diversity, natural products show profuse functionality, interacting with a

multitude of biological targets. Their production in living systems and refinement under evolutionary pressure could account for the often observed favorable pharmacokinetic properties [277, 448–450, 452].

The search for potential nuclear receptor modulators for therapeutic use is still ongoing. As metabolic nuclear receptors regulate a plethora of target genes involved in lipid homeostasis, the modulation of these receptors enables the manipulation of a multitude of transporters and enzymes, for instance. However, this diverseness can also be a disadvantage in regard to potential side effects. Thus, compounds with tissue- or target gene-specific effects, or acting as partial agonists instead of being full agonists could be more favorable [277]. Natural products represent an interesting pool of potential ligands and a valuable source for the discovery of new drugs [277, 453]. Natural products with a presumably lower potency compared to synthetic agonists or with a tissue- or isotype-specific effect could be suitable candidates for nuclear receptor modulation [277].

Several natural products have already been identified as modulators of nuclear receptors. For instance, taurine, which is mainly taken up with diet, has been shown to activate LXRα, but not LXRβ, without leading to hepatic lipogenesis [454, 455]. Honokiol, a natural compound present in *Magnolia* species, was reported to be a partial agonist of RXRα and to increase cholesterol efflux from murine macrophages by inducing ABCA1 and ABCG1 mRNA expression [456]. This reinforces that natural compounds can have valuable activities, and even if a natural product itself cannot be used as a drug, it can serve as a lead for synthetic mimetics [449].

1.6 Caco-2 cells as an intestinal cell model

The Caco-2 cell line is a human intestinal cell line, derived from a colon adenocarcinoma. Caco-2 cells are commonly used as an *in vitro* model of the intestinal epithelial barrier. When grown to confluence and kept in post-confluent cultures, these cells spontaneously differentiate and develop the biochemical and morphological features of polarized small intestinal enterocytes. Cultivation of Caco-2 cells on filter inserts improves their functional and morphological differentiation, underpinning this form of cultivation [457–459]. Moreover, it gives access to both the apical and the basolateral side of the cells, enabling transport experiments. Caco-2 cells are widely used to determine drug bioavailability [460], but they are also a suitable model for studying lipid and cholesterol homeostasis [461–463], like cholesterol uptake and efflux.

1.7 Natural products and derivatives investigated in this study

1.7.1 Soraphen A

Soraphen A (Figure 1.6) is a polyketide natural product, belonging to the group of soraphens. It was discovered around 1993, by the group of Hans Reichenbach and Gerhard Hoefle. It is produced and secreted into the culture broth by the myxobacterium *Sorangium cellulosum* strain So ce26 [464–466].

Soraphen A possesses a strong broad-spectrum antifungal activity [466]. Later on, it was demonstrated that soraphen A inhibits the biotin carboxylase domain of acetyl coenzyme A carboxylase 1 and 2 (ACC1/2) of human and other eukaryotic species [467–470]. ACC1 is mainly expressed in the liver and in adipose tissue and is located in the cytoplasm. ACC2 is primarily found in oxidative tissue, like skeletal muscle, heart and liver, and is associated with the mitochondrial membrane. Both ACCs catalyze the conversion of acetyl-CoA to malonyl-CoA, which is the first and rate limiting step in *de novo* fatty acid synthesis. Malonyl-CoA formed by ACC1 in the cytoplasm is primarily used for fatty acid synthesis and elongation, whereas the formed malonyl-CoA by ACC2 regulates mitochondrial fatty acid oxidation *via* inhibition of carnitine palmitoyltransferase 1, which conjugates free fatty acyls to carnitine to enable transport across the mitochondrial membrane [471, 472].

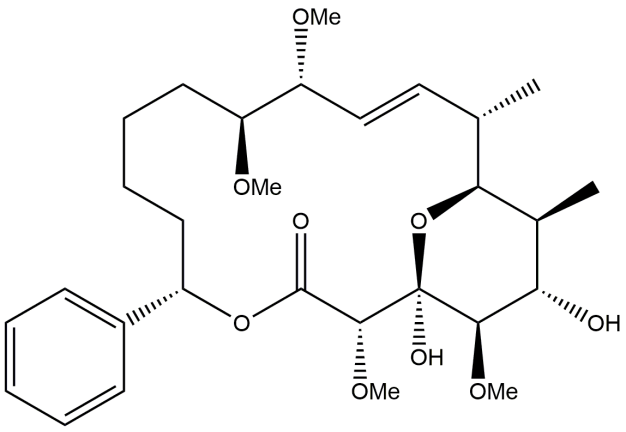

Figure 1.6: Structure of the polyketide soraphen A.

Inhibition of ACCs by soraphen A was reported to lower malonyl-CoA levels, thereby inhibiting *de novo* fatty acid synthesis and increasing fatty acid β-oxidation [471, 473]. Moreover, soraphen A was shown to attenuate the formation of very long chain saturated, mono- and polyunsaturated fatty acids [473]. In murine 3T3-L1 preadipocytes, soraphen

A inhibited lipid accumulation [474]. These studies indicate that inhibition of ACCs by soraphen A can have a great influence on cellular fatty acid metabolism.

1.7.2 The piperine derivative LAU398

Piperine (Figure 1.7) is the major pungent alkaloid present in the fruits of the plant black pepper (*Piper nigrum* L.). Green pepper, black pepper and red pepper, spices used worldwide, represent the different harvesting stages of the fruits of *Piper nigrum* and all contain piperine [475–477]. Pepper fruits are also used in traditional Chinese and Indian medicine for treating abdominal pain, diarrhea, epilepsy with much phlegm or diabetes, for instance [478, 479].

Several *in vivo* studies have suggested a cardioprotective effect of piperine. Rats on a high fat diet that received a piperine-supplemented food had decreased plasma total and LDL cholesterol levels, but increased HDL cholesterol levels [480, 481] A different study could show that piperine-supplemented food normalized blood pressure and ameliorated aortic reactivity in rats on a high carbohydrate, high fat diet [482]. Results from *in vitro* studies indicate that piperine increases ABCA1 expression by inhibiting its degradation in THP-1-derived macrophages, thereby promoting cholesterol efflux [479]. In regard to the metabolic nuclear receptors described above, it was shown that piperine decreases the mRNA expression of LXRα and further inhibits its transcriptional activity [483]. Moreover, studies also found that piperine influences PPARγ, however, the results are conflicting, which might be attributable to the different model systems used. In 3T3-L1 cells, it reduced the expression and the transcriptional activity of PPARγ [484]. In contrast, it activated PPARγ in a mouse model of cardiac fibroses and in mice receiving doxorubicin treatment [485]. In addition, similar to other pungent compounds like capsaicin, piperine is an agonist of transient receptor potential cation channel subfamily V member 1 (TRPV1, also known as vanilloid receptor 1 (VR1)) [486].

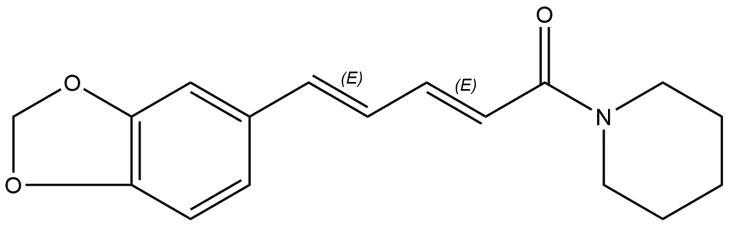

Figure 1.7: Structure of the alkaloid piperine.

The biological activities exhibited by piperine make it an interesting lead compound for the development of derivatives with either higher potency or selectivity for a certain target. Therefore, a series of piperine derivatives was synthesized by the Vienna University

of Technology (Univ.-Prof. Dr. Marko D. Mihovilovic, Institute for Applied Synthetic Chemistry, Austria). The 31 derivatives synthesized were subjected to a screening in regard to ABCA1 protein expression in THP-1-derived macrophages by a former PhD student in our group, Dr. Limei Wang. The most potent derivative identified *via* this screening was the piperine derivative LAU398 (Figure 1.8). In 2015, it was already shown by a different group at the University of Vienna that LAU398 enhances capsaicin-induced currents through TRPV1 channels [487].

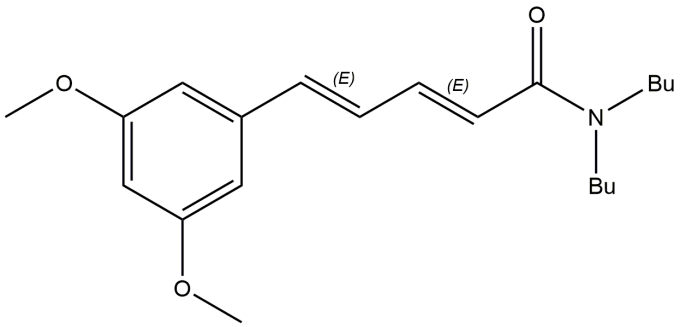

Figure 1.8: Structure of the piperine derivative LAU398 ((*2E,4E*)-*N,N*-dibutyl-5-(3,5-dimethoxyphenyl)penta-2,4-dienamide).

2 Aims of the study

As described in the introduction, the intestine plays a major role in cholesterol turnover, as it regulates both cholesterol uptake, and excretion *via* TICE. Agonists of the metabolic nuclear receptors LXR, FXR, PPAR and RXR could regulate the expression of major cholesterol transporters in the intestine, amongst others, and could thereby positively influence cholesterol homeostasis. In order to be able to study different natural products regarding their potential effects on nuclear receptors in the intestine and regarding their influence on cholesterol transport in this tissue, we first had to establish the Caco-2 cell model in our laboratory. Many publications exist that make use of the Caco-2 cell line. The descriptions of the used methodology, however, are either undetailed, making reproduction impossible, or not consistent among different laboratories [457, 460, 488–490]. The first aim of this work was therefore to consider the different approaches of the different laboratories and to evaluate and modify the assay conditions to achieve an optimal outcome.

Several published studies indicated that inhibition of ACCs by soraphen A can have a major influence on fatty acid metabolism [473, 474]. As described in the introduction, fatty acid metabolism is also important for lipid homeostasis in macrophages, since excessive accumulation of cholesteryl esters can drive the formation of foam cells. However, to the best of our knowledge, no studies have been published so far examining the effect of soraphen A on cholesterol efflux from macrophages. The second goal of this work was therefore to investigate the influence of soraphen A on macrophage cholesterol homeostasis and to uncover a potential relationship to its effects on fatty acid metabolism. This work was carried out together with a former member of our group, Dr. Dongdong Wang. Soraphen A was provided by our collaboration partner Univ.-Prof. Dr. Rolf Mueller from the Helmholtz Institute for Pharmaceutical Research Saarland (Saarland University, Germany).

A series of 31 piperine derivatives was synthesized by the Vienna University of Technology (Univ.-Prof. Dr. Marko D. Mihovilovic, Institute of Applied Synthetic Chemistry, Austria) and screened regarding ABCA1 expression by Dr. Limei Wang. She identified the piperine derivative LAU398 as the most potent one. The third aim of my work was to further characterize LAU398 in regard to cholesterol homeostasis in intestinal cells and

macrophages, and to elucidate the underlying mechanisms leading to the induction of ABCA1.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (Vienna, Austria) or Carl Roth (Karlsruhe, Germany), unless otherwise stated.

3.1.2 Cell culture

Table 3.1: Cell culture - Materials

Material	Provider
HEK293 cells	ATCC (Manassas, VA, USA)
Caco-2 cells	DSMZ (Braunschweig, Germany)
THP-1 cells	ATCC (Manassas, VA, USA)
DMEM (Dulbecco's Modified Eagle Medium) with 4.5 g/l glucose without phenol red	Lonza Group Ltd. (Basel, Switzerland)
EMEM (Eagle's Minimum Essential Medium) without glutamine	Lonza Group Ltd. (Basel, Switzerland)
RPMI-1640 (Roswell Park Memorial Institute)	Lonza Group Ltd. (Basel, Switzerland)
L-glutamine	Lonza Group Ltd. (Basel, Switzerland)
Penicillin/streptomycin mixture (potassium penicillin/streptomycin sulfate)	Lonza Group Ltd. (Basel, Switzerland)
Trypsin	Gibco *via* Invitrogen (Lofer, Austria)
FBS (fetal bovine serum)	Gibco *via* Invitrogen (Lofer, Austria)
100x NEAA (non-essential amino acids)	Lonza Group Ltd. (Basel, Switzerland)

Continued on the next page

Table 3.1: Cell culture - Materials: continued

Material	Provider
PET translucent/transparent filter inserts (0.4 μm pore size)	Sarstedt (Nuembrecht, Germany)
Companion 12-well plates for filter inserts	Sarstedt (Nuembrecht, Germany)

Table 3.2: Cell culture - Media and reagents

Name	Content
HEK293 complete growth medium	DMEM 10% heat-inactivated FBS 2 mM L-glutamine 100 U ml^{-1} penicillin 100 μg ml^{-1} streptomycin
HEK293 stripped medium	DMEM 5% charcoal stripped FBS 2 mM L-glutamine 100 U ml^{-1} penicillin 100 μg ml^{-1} streptomycin
THP-1 complete growth medium	RPMI 10% heat-inactivated FBS 2 mM L-glutamine 100 U ml^{-1} penicillin 100 μg ml^{-1} streptomycin
THP-1 2.5% FBS medium	RPMI 2.5% heat-inactivated FBS 2 mM L-glutamine 100 U ml^{-1} penicillin 100 μg ml^{-1} streptomycin
THP-1 medium containing 0.1 % BSA	RPMI 2 mM L-glutamine
	Continued on the next page

Table 3.2: Cell culture - Media and reagents: continued

Name	Content
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
	0.1% BSA
Caco-2 complete growth medium	EMEM
	20% heat-inactivated FBS
	2 mM L-glutamine
	1x NEAA
Caco-2 apical medium	EMEM
	2 mM L-glutamine
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
Caco-2 basolateral medium	EMEM
	20% heat-inactivated FBS
	2 mM L-glutamine
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
Caco-2 DMEM comp. growth medium	DMEM
	20% heat-inactivated FBS
	2 mM L-glutamine
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
Caco-2 DMEM stripped medium	DMEM
	5% charcoal stripped FBS
	2 mM L-glutamine
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
Caco-2 medium containing 0.5% BSA	DMEM
	2 mM L-glutamine

Continued on the next page

Table 3.2: Cell culture - Media and reagents: continued

Name	Content
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
	0.5% BSA
Caco-2 serum-free medium	DMEM
	2 mM L-glutamine
	1x NEAA
	100 U ml^{-1} penicillin
	100 μg ml^{-1} streptomycin
PBS (phosphate buffered saline, pH 7.4)	123 mM NaCl
	10.4 mM Na_2HPO_4
	3.16 mM KH_2PO_4
Trypsin/EDTA	0.05% trypsin
	0.02% Na_2EDTA

3.1.3 Plasmid DNA preparation

Table 3.3: Plasmid DNA preparation - Materials

Material	Provider
Competent *E.coli* cells (JM109)	Sigma-Aldrich (Vienna, Austria)
peqGOLD Plasmid Miniprep Kit I	PeqLab (Linz, Austria)
PureYield™ Plasmid Midiprep System	Promega (Mannheim, Germany)

3.1.4 Transient transfection and luciferase assay

Table 3.4: Transient transfection and luciferase assay - Materials

Material	Provider
Lipofectamine™ LTX with PLUS™ reagent	Fisher Scientific (Vienna, Austria)
FuGENE® HD transfection reagent	Promega (Mannheim, Germany)
LPEI (linear polyethylenimine)	Univ.-Prof. Dr. Manfred Ogris (Department of Pharmaceutical Chemistry, University of Vienna, Vienna, Austria)
Luciferin	Synchem (Flesburg, Germany)
Reporter lysis 5x buffer	Promega (Mannheim, Germany)
OptiMEM	Thermo Fisher Scientific (Vienna, Austria)
Expression plasmids	
EGFP (enhanced green fluorescent protein, pEGFP-N1)	Clontech Laboratories (Mountain View, CA, USA)
hLXRα (human LXRα, full length, pCMV)	Missouri S&T cDNA Resource Center (Rolla, MO, USA)
hLXRβ (human LXRβ, full length, pcDNA3.1+)	Missouri S&T cDNA Resource Center (Rolla, MO, USA)
hRXRα (human RXRα, full length, pcDNA3.1+)	Missouri S&T cDNA Resource Center (Rolla, MO, USA)
hPPARβ/δ (human PPARβ/δ, full length, pSG5)	Prof. Walter Wahli (Université de Lausanne, Lausanne, Switzerland)
hPPARγ1 (human PPARγ1, full length, pSG5)	Prof. Walter Wahli and Prof. Beatrice Desvergne (Université de Lausanne, Lausanne, Switzerland)
hLXRαGal4 (chimeric expression of LXRαLBD/Gal4DBD, pCMX)	Prof. Makoto Makishima (Nihon University School of Medicine, Tokyo, Japan)
hLXRβGal4 (chimeric expression of LXRβLBD/Gal4DBD, pCMX)	Prof. Makoto Makishima (Nihon University School of Medicine, Tokyo, Japan)

Continued on the next page

Table 3.4: Transient transfection and luciferase assay - Materials: continued

Material	Provider
hRXRαGal4 (chimeric expression of RXRαLBD/Gal4DBD, pCMX)	Prof. Ronald M. Evans (Howard Hughes Medical Institute, Chevy Chase, MD, USA)
hRXRβGal4 (chimeric expression of RXRβLBD/Gal4DBD, pCMX)	Dr. Hinrich Gronemeyer (Université de Strasbourg, Strasbourg, France)
hPPARγGal4 (chimeric expression of PPARγLBD/Gal4DBD, pCMX)	Prof. Ronald M. Evans (Howard Hughes Medical Institute, Chevy Chase, MD, USA)
hFXRGal4 (chimeric expression of FXRLBD/Gal4DBD, pFA-CMV)	Dr. Daniel Merk (ETH Zuerich, Zuerich, Switzerland)
Reporter plasmids	
ABCA1_Luc (luciferase reporter vector driven by the ABCA1 promoter, pGL4.14)	Prof. Ira G. Schulman (University of Virginia Health System, Charlottesville, VA, USA) [491]
RXRE_Luc (luciferase reporter vector driven by RXR response elements, pTL-Luc)	Panomics Srl (Milan, Italy)
PPRE_Luc (luciferase reporter vector driven by PPAR response elements, tk-PPREx3-luc)	Prof. Ronald M. Evans (Howard Hughes Medical Institute, Chevy Chase, MD, USA) [381]
UAS_Luc (Gal4 responsive luciferase reporter vector, pTK-MH100x4-Luc)	Prof. Makoto Makishima (Nihon University School of Medicine, Tokyo, Japan)

Table 3.5: Transient transfection and luciferase assay - Buffers and solutions

Solution	Content
Transfection mix for the calcium phosphate precipitation method	Plasmids in the required concentration 750 μl 2x HBS
	Continued on the next page

Table 3.5: Transient transfection and luciferase assay - Buffers and solutions: continued

Solution	Content
	94 μl $CaCl_2$ 2 M ddH_2O ad 1.5 ml
2x HBS (HEPES-buffered saline)	280 mM NaCl 10 mM KCl 1.5 mM $Na_2HPO_4 \cdot 2\ H_2O$ 12 mM dextrose 50 mM HEPES pH adjusted to 7.05
Ready-to-use lysis buffer	4.8 ml ddH_2O 1.2 ml reporter lysis 5x buffer 6 μl CoA (coenzyme A trilithium salt, 270 mM) 6 μl DTT (dithiothreitol, 1 M)
ATP solution	20 mM tricine pH 7.8 21.5 mM $MgCl_2$ 3.8 mM adenosin-5'-triphosphate disodium salt
Luciferin solution	32 mg/ml luciferin 21 mM tricine pH 7.8

3.1.5 Oil Red O staining

Table 3.6: Oil Red O staining - Materials

Material	Provider
Oil Red O	Fluka Analytical/Sigma-Aldrich (Vienna, Austria)

Table 3.7: Oil Red O staining - Solutions

Solution	Content
Oil Red O working solution	0.21% Oil Red O in 60% isopropanol

3.1.6 Amplex™ Red assay

Table 3.8: Amplex™ Red assay - Materials

Material	Provider
Amplex™ Red Cholesterol Assay Kit	Invitrogen/Thermo Fisher Scientific (Warsaw, Poland)

3.1.7 Cholesterol efflux assay

Table 3.9: Cholesterol efflux assay - Materials

Material	Provider
Human plasma	Healthy, young volunteers
[1,2-^{3}H(N)]-cholesterol	Perkin Elmer (Traiskirchen, Austria)
Ultima Gold™ liquid scintillation cocktail	Perkin Elmer (Traiskirchen, Austria)
Liquid scintillation vials (MidiVials)	Perkin Elmer (Traiskirchen, Austria)
2-Oleoylglycerol	Cayman Chemical *via* Sanova (Vienna, Austria)

Table 3.10: Cholesterol efflux assay - Buffers

Buffer	Content
NP40 lysis buffer (pH 7.4)	150 mM NaCl 50 mM HEPES 1% NP40 ddH_2O

3.1.8 Western Blot

Table 3.11: Western Blot - Materials

Material	Provider
cOmplete™ protease inhibitor cocktail	Roche Diagnostics (Vienna, Austria)
Immun-Blot® PVDF (polyvinylidene difluoride) membrane	BIO-RAD Laboratories (Hercules, CA, USA)

Table 3.12: Western Blot - Primary antibodies

Target (source)	Dilution	Provider
ABCA1 (rabbit, pAb)	1:500 in TBST	Novus Biologicals (Vienna, Austria)
LXRα (mouse, mAb)	1:1000 in TBST with 5% nonfat dry milk	Perseus Proteomics *via* R&D systems (Abingdon, United Kingdom)
Actin (mouse, mAb)	1:10000 in TBST	MP Biomedicals *via* Fisher Scientific (Vienna, Austria)

Table 3.13: Western Blot - Secondary antibodies

Target	Dilution	Provider
Anti-mouse IgG, HRP-linked	1:1000 in TBST with 5% nonfat dry milk	Cell Signaling *via* New England Biolabs (Frankfurt am Main, Germany)
Anti-rabbit IgG, HRP linked	1:1000 in TBST with 5% nonfat dry milk	Cell Signaling *via* New England Biolabs (Frankfurt am Main, Germany)

Table 3.14: Western Blot - Buffers and solutions

Buffer/Solution	Content
NP40 lysis buffer (pH 7.4)	150 mM NaCl 50 mM HEPES 1% NP40 ddH_2O
25x stock cOmplete™	1 tablet 2 ml ddH_2O
NP40 ready-to-use lysis buffer	NP40 lysis buffer 40:1000 cOmplete™ (25x stock) 10:1000 PMSF (100 mM in isopropanol) 5:1000 NaF (200 mM) 5:1000 Na_3VO_4 (200 mM)
3x sample buffer	187.5 mM TRIS-HCl (pH 6.8) 0.2 M SDS 30% glycerol 0.2 mM bromophenol blue
3x sample buffer containing β-mercaptoethanol	85% 3x sample buffer 15% β-mercaptoethanol

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Table 3.14: Western Blot - Buffers and solutions: continued

Buffer/Solution	Content
Diluted Bradford reagent	1 ml Roti®-Quant 3.75 ml ddH_2O
Resolving gel	7.5% acrylamide (Rotiphorese® Gel30) 375 mM TRIS-HCl pH 8.8 0.1% SDS 0.1% TEMED 0.05% APS ddH_2O
Stacking gel	5% acrylamide (Rotiphorese® Gel30) 125 mM TRIS-HCl pH 6.8 0.1% SDS 0.1% TEMED 0.05% APS ddH_2O
Electrophoresis buffer 10x	250 mM TRIS-base 1.92 M glycine 35 mM SDS
Electrophoresis buffer 1x	100 ml electrophoresis buffer 10x ddH_2O ad 1000 ml
Blotting buffer 5x	125 mM TRIS-base 970 mM glycine
Blotting buffer 1x	200 ml Blotting buffer 5x 200 ml methanol ddH_2O ad 1000 ml
TBST (TRIS-buffered saline with Tween-20, pH 8.0)	25 mM TRIS-base 190 mM NaCl 0.1% Tween-20 ddH_2O
ECL reagent	100 mM TRIS-base (pH 8.5)

Continued on the next page

Table 3.14: Western Blot - Buffers and solutions: continued

Buffer/Solution	Content
	1.24 mM luminol 0.2 mM p-coumaric acid 0.009% H_2O_2 ddH_2O

Table 3.15: Serial dilutions of BSA

BSA endconcentration (μg/ml)	Preparation (x μl BSA (1 mg/ml) + x μl ddH_2O)
2.5	50 + 950
5.0	100 + 900
7.5	150 + 850
10	200 + 800
15	300 + 700
20	400 + 600
25	500 + 500

3.1.9 Permeability assays

Table 3.16: Permeability assays - Materials

Material	Provider
D-[2-^{14}C]-mannitol	Perkin Elmer (Traiskirchen, Austria)
Dextran Blue (from *Leuconostoc spp.*)	Sigma-Aldrich (Vienna, Austria)
Human plasma	Healthy, young volunteers

Table 3.17: Permeability assays - Solutions

Solution	Content
Dextran Blue stock solution	20 mg/ml Dextran Blue in serum-free medium kept at 70 ℃ and 1400 rpm until it dissolves
[^{14}C]-mannitol stock solution	0.5 μCi/ml [^{14}C]-mannitol in serum-free medium

3.1.10 Confocal microscopy

Table 3.18: Confocal microscopy - Materials

Material	Provider
Fluoromount™ aqueous mounting medium	Sigma-Aldrich (Vienna, Austria)
Precision cover slips (Ø 18 mm, borosilicate glass, 0.17 ± 0.005 mm)	Carl Roth (Karlsruhe, Germany)
Microscope slides	Carl Roth (Karlsruhe, Germany)
Leica Type F Immersion liquid	Leica Microsystems (Wetzlar, Germany)

Table 3.19: Confocal microscopy - Primary antibodies

Target (source)	Provider
Alexa Fluor 594 conjugated anti-ZO-1 (zonula occludens-1) antibody (mouse, mAb)	Thermo Fisher Scientific (Vienna, Austria)
NPC1L1 (rabbit, pAb)	Thermo Fisher Scientific (Vienna, Austria)

Continued on the next page

Table 3.19: Confocal microscopy - Primary antibodies: continued

Target (source)	Provider
ABCG5 (rabbit, pAb)	Thermo Fisher Scientific (Vienna, Austria)
ABCB1 (rabbit, pAb)	Thermo Fisher Scientific (Vienna, Austria)

Table 3.20: Confocal microscopy - Secondary antibodies and other fluorophores

Material	Provider
Alexa Fluor 488 conjugated goat anti-rabbit secondary antibody	Thermo Fisher Scientific (Vienna, Austria)
DAPI (4',6-diamidine-2'-phenylindole dihydrochloride)	Sigma-Aldrich (Vienna, Austria)

3.1.11 RT-qPCR

Table 3.21: RT-qPCR - Materials

Material	Provider
TRIzol	Invitrogen/Thermo Fisher Scientific *via* Life Tech Austria (Vienna, Austria)
RNase-free glycogen	Invitrogen/Thermo Fisher Scientific *via* Life Tech Austria (Vienna, Austria)
Phasemaker™ tubes	Invitrogen/Thermo Fisher Scientific *via* Life Tech Austria (Vienna, Austria)
High Capacity cDNA Reverse Transcription Kit	Invitrogen/Thermo Fisher Scientific *via* Life Tech Austria (Vienna, Austria)
RNase inhibitor	Invitrogen/Thermo Fisher Scientific *via* Life Tech Austria (Vienna, Austria)

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Table 3.21: RT-qPCR - Materials: continued

Material	Provider
PeqGOLD total RNA kit	PeqLab (Linz, Austria)
SYBR® Green I Master	Roche Diagnostics (Vienna, Austria)

Table 3.22: RT-qPCR - Primers

Primer	Provider
ABCA1 (Hs_ABCA1_1_SG QuantiTect Primer Assay)	Qiagen (Vienna, Austria)
ABCG5 (Hs_ABCG5_1_SG QuantiTect Primer Assay)	Qiagen (Vienna, Austria)
NPC1L1 (Hs_NPC1L1_1_SG QuantiTect Primer Assay	Qiagen (Vienna, Austria)
ABCB1 (Hs_ABCB1_1_SG QuantiTect Primer Assay)	Qiagen (Vienna, Austria)
GAPDH (glyceraldehyde-3-phosphate dehydrogenase; Hs_GAPDH_1_SG QuantiTect Primer Assay)	Qiagen (Vienna, Austria)

3.1.12 Cholesterol uptake assay

Table 3.23: Cholesterol uptake assay - Materials

Material	Provider
Human plasma	Healthy, young volunteers
[1,2-^{3}H(N)]-cholesterol	Perkin Elmer (Traiskirchen, Austria)
Ultima Gold™ liquid scintillation cocktail	Perkin Elmer (Traiskirchen, Austria)

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Table 3.23: Cholesterol uptake assay - Materials: continued

Material	Provider
Liquid scintillation vials (MidiVials)	Perkin Elmer (Traiskirchen, Austria)
2-Oleoylglycerol	Cayman Chemical *via* Sanova (Vienna, Austria)

Table 3.24: Cholesterol uptake assay - Buffers and solutions

Buffer/solution	Provider
NP40 lysis buffer (pH 7.4)	150 mM NaCl 50 mM HEPES 1% NP40 ddH_2O
Diluted Bradford reagent	1 ml Roti®-Quant 3.75 ml ddH_2O

Serial dilutions of BSA see Table 3.15.

3.1.13 Resazurin conversion assay and crystal violet staining

Table 3.25: Resazurin conversion assay & crystal violet staining - Materials

Material	Provider
Resazurin sodium salt	Sigma-Aldrich (Vienna, Austria)
Crystal violet	Sigma-Aldrich (Vienna, Austria)

Table 3.26: Resazurin conversion assay & crystal violet staining - Solutions

Solution	Content
Crystal violet solution	1% crystal violet in 20% methanol filtered before use
Sodium citrate:ethanol solution	0.05 M sodium citrate in 50% ethanol

3.1.14 Compounds used in this study

Table 3.27: Compounds used for treatment

Compound	Provider
DMSO (dimethylsulfoxide) as vehicle control	Sigma-Aldrich (Vienna, Austria)
GW3965	Sigma-Aldrich (Vienna, Austria)
T0901317	Sigma-Aldrich (Vienna, Austria)
Bexarotene	Sigma-Aldrich (Vienna, Austria)
GW4064	Sigma-Aldrich (Vienna, Austria)
CDCA (chenodeoxycholic acid)	Sigma-Aldrich (Vienna, Austria)
Pioglitazone	Molekula (Shaftesbury, United Kingdom)
GW0742	Cayman Chemical *via* Sanova (Vienna, Austria)
DLPC (dilauroyl phosphatidylcholine)	Tocris *via* biotechne (Abingdon, United Kingdom)
Valproic acid sodium salt	Sigma-Aldrich (Vienna, Austria)
Piperine	Sigma-Aldrich (Vienna, Austria)
Capsazepine	Sigma-Aldrich (Vienna, Austria)
GSK2033	Biomedica Medizinprodukte (Vienna, Austria)

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Table 3.27: Compounds used for treatment: continued

Compound	Provider
Probucol	Sigma-Aldrich (Vienna, Austria)
ND-630	Cayman Chemical *via* Sanova (Vienna, Austria)
Arachidonic acid	Sigma-Aldrich (Vienna, Austria)
Eicosapentaenoic acid	Sigma-Aldrich (Vienna, Austria)
Oleic acid	Sigma-Aldrich (Vienna, Austria)
Digitonin	Sigma-Aldrich (Vienna, Austria)
Soraphen A	Univ.-Prof. Dr. Rolf Mueller (Helmholtz Institute for Pharmaceutical Research Saarland, Saarland University, Saarbruecken, Germany)
Piperine derivative LAU398	Univ.-Prof. Dr. Marko D. Mihovilovic (Institute of Applied Synthetic Chemistry, Vienna University of Technology, Vienna, Austria)

3.1.15 Technical equipment and scientific software

Table 3.28: Technical equipment

Name	Manufacturer
EVOM2 epithelial voltohmmeter	World Precision Instruments Germany (Berlin, Germany)
ViCell™ XR cell viability analyzer	Beckman Coulter (Brea, CA, USA)
Tecan Sunrise™ plate reader	Tecan (Mannedorf, Switzerland)
Tecan GENios™ Pro plate reader	Tecan (Mannedorf, Switzerland)
Tecan Spark™ plate reader	Tecan (Mannedorf, Switzerland)
Leica DMi8 Confocal Laser Scanning Microscope	Leica Microsystems (Wetzlar, Germany)
LightCycler® 480	Roche (Basel, Switzerland)

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Table 3.28: Technical equipment: continued

Name	Manufacturer
NanoDrop 2000c	Thermo Fisher Scientific (Waltham, MA, USA)
Tri-carb® 2910TR liquid scintillation counter	Perkin Elmer (Waltham, MA, USA)
LAS-3000™ Luminescent Image Analyzer	Fujifilm (Tokyo, Japan)
Mini-PROTEAN™ 3 Cell System	BIO-RAD Laboratories (Hercules, CA, USA)
Mini Trans-Blot™ Electrophoretic Transfer Cell System	BIO-RAD Laboratories (Hercules, CA, USA)
Power supply PowerPac™ HC	BIO-RAD Laboratories (Hercules, CA, USA)
Heraeus™ Multifuge 1 S-R	Kendro Laboratory Products (Langenselbold, Germany)
Heracell™ 150 Incubator (37 °C and 5% CO_2)	Thermo Electron Corporation (Waltham, MA, USA)
Heraeus™ Biofuge fresco	Kendro Laboratory Products (Langenselbold, Germany)
Eppendorf® Thermomixer compact	Eppendorf AG (Hamburg, Germany)
C1000™ Thermal Cycler	BIO-RAD Laboratories (Hercules, CA, USA)
Swip SM25 DIGI orbital shaker	Edmund Buehler GmbH (Bodelshausen, Germany)
Thermo Scientific™ CO_2 resistant orbital shaker (#88881102)	Thermo Fisher Scientific (Waltham, MA, USA)
Light Microscope Olympus CKX31	Olympus Europa GmbH (Hamburg, Germany)
Olympus Live View Digital SLR Camera E-330	Olympus Europa GmbH (Hamburg, Germany)

Table 3.29: Scientific software

Name	Manufacturer
ViCell™ XR 2.03	Beckman Coulter (Brea, CA, USA)
Tecan XFLUOR4 V4.51	Tecan (Mannedorf, Switzerland)
QuantaSmart™ V4.00	Perkin Elmer (Waltham, MA, USA)
Tecan SparkControl™ V2.1	Tecan (Mannedorf, Switzerland)
Image Reader LAS-3000™ V2.1	Fujifilm (Tokyo, Japan)
MultiGauge V3.0 Science Lab 2005	Fujifilm (Tokyo, Japan)
Leica LASX	Leica Microsystems (Wetzlar, Germany)
NanoDrop 2000 software V1.4.2	Thermo Fisher Scientific (Waltham, MA, USA)
LightCycler® 480 software V1.5.1.62	Roche (Basel, Switzerland)
GraphPad Prism™ 6.01	GraphPad Software (San Diego, CA, USA)
Microsoft Excel 2016	Microsoft (Redmond, WA, USA)
ChemDraw 16.0	Perkin Elmer (Waltham, MA, USA)
IrfanView 4.53	Irfan Skiljan (Wiener Neustadt, Austria)

3.2 Methods

3.2.1 General maintenance of different cell lines

3.2.1.1 HEK293 cells

Human embryonic kidney 293 cells (HEK293 cells) were purchased from ATCC. They were grown in 175 cm^2 flasks in complete growth medium, in an incubator with a humidified atmosphere at 37 ℃ and 5% CO_2. They were passaged every other day (three times per week). To do so, they were first washed with PBS and then incubated with trypsin/EDTA for 2 minutes. The trypsin/EDTA solution was then neutralized by the addition of complete growth medium. A small aliquot of the cell suspension was removed for viability measurement and cell counting on a ViCell™ XR cell viability analyzer. A specific number of cells was then seeded into a new 175 cm^2 flask with fresh complete growth medium. HEK293 cells were used for experiments between passages 15 and 40. Only cells with a viability of at least 95% were used for experiments.

3.2.1.2 THP-1 cells

The human THP-1 monocytic cell line was purchased from ATCC. THP-1 cells were grown in 175 cm^2 flasks in complete growth medium. They were kept in an incubator with a humidified atmosphere set at 37 ℃ and 5% CO_2. They were passaged every other day (three times per week) as described hereafter. The cell suspension was transferred into a conical centrifuge tube and centrifuged at 150 x g for 4 minutes. Medium was then poured off and the cell pellet was resuspended in complete growth medium by gently pipetting up and down. An aliquot of the cell suspension was removed for cell counting and viability assessment. A certain number of cells was then seeded into a new 175 cm^2 cell culture flask with fresh complete growth medium. THP-1 cells were used for experiments within three months from the date of thaw. Cells with a viability of at least 95% were used for experiments.

3.2.1.3 Caco-2 cells

The human colon adenocarcinoma cell line Caco-2 was purchased from DSMZ. They were grown in 75 cm^2 flasks in complete growth medium, in a humidified incubator at 37 ℃ and 5% CO_2. They were passaged twice a week, on Mondays and Thursdays. Old medium was poured off and cells were washed with PBS. They were then incubated with 3 ml trypsin/EDTA for 5 minutes in the incubator. The flask was then hit on its sides to detach still adherent cells and the trypsin/EDTA solution was next neutralized by the

addition of complete growth medium. An aliquot of the cell suspension was removed for cell counting and viability assessment. A specific number of cells was then seeded into a new 75 cm^2 flask containing fresh complete growth medium. Although subculturing was only performed twice a week, medium was renewed every other day (Wednesday and Friday). Caco-2 cells were used for experiments between passages 5 and 20 and only when the viability was at least 95%.

3.2.2 Plasmid DNA preparation

Plasmid DNA was obtained commercially or kindly provided by other research groups. Competent *E.coli* cells were transformed in our laboratory with the respective plasmid DNA and then stored as a glycerol stock at -80 °C. First, an amplification and isolation of the plasmid DNA in a small scale was performed, using a Miniprep system. Obtained plasmids were verified by digestion with suitable restriction enzymes and subsequent analysis of the obtained fragments by gel electrophoresis. After verification, amplification and isolation of the plasmids was carried out in a larger scale using the PureYield™ Plasmid Midiprep System. Both Miniprep and Midiprep were performed according to the manufacturer's instructions.

3.2.3 Transient transfection and luciferase reporter gene assay

The luciferase reporter gene assay is a very useful method to determine the activation of a nuclear receptor by a test compound. Transient transfection with an expression plasmid and a reporter plasmid is required for this assay. The expression plasmid codes for the nuclear receptor of interest, and the reporter plasmid carries the promoter of its target gene or a repetition of response elements, coupled to the luciferase gene. Activation of the nuclear receptor under investigation causes binding to the promoter or response elements, leading to the transcription of the luciferase gene. Luciferase activity can then be measured by the luminescence emerging upon the addition of luciferin, ATP and Mg^{2+} [492, 493]. From the resulting luminescence one can conclude if a nuclear receptor was activated by a certain compound and to which extent in comparison to the vehicle control.

3.2.3.1 HEK293 cells - Transient transfection

HEK293 cells were transiently transfected with plasmid DNA using the calcium phosphate precipitation method [494]. For that purpose, HEK293 cells were seeded at 6 x 10^6 cells/dish into 15 cm diameter dishes containing 20 ml complete growth medium. A control

was prepared in a 10 cm diameter dish, consisting of 4 x 10^6 cells that were not transfected. Cells were grown overnight in a humidified incubator at 37 ℃ and 5% CO_2.

A transfection mix of 1.5 ml was prepared for every dish that needed to be transfected. The transfection mix contained the appropriate amounts of chemicals (ddH_2O, HBS buffer and calcium chloride) and plasmids. For the luciferase assays using human full-length receptors, the respective human full-length receptor expression plasmid, the respective luciferase reporter plasmid and the EGFP expression plasmid were used for transfection in the amounts listed in Table 3.30. For the luciferase assays using the Gal4-UAS system, 10 μg of an expression plasmid encoding a chimeric protein consisting of the yeast Gal4 DNA binding domain (DBD) fused to the respective nuclear receptor ligand binding domain (LBD), 2 μg of the luciferase reporter plasmid containing specific upstream activating sequences (UAS) and 2 μg of the EGFP expression plasmid were used per dish to be transfected. Transfection was carried out for 6 hours. After transfection, cells were re-seeded as described in the following section.

Table 3.30: Plasmids used for the luciferase reporter gene assay in HEK293 cells

Plasmids	**μg/dish**
LXRα : ABCA1_Luc : EGFP	6 : 6 : 3
LXRβ : ABCA1_Luc : EGFP	6 : 6 : 3
RXRα : RXRE_Luc : EGFP	6 : 6 : 3
PPARβ/δ : PPRE_Luc : EGFP	6 : 6 : 3
PPARγ : PPRE_Luc : EGFP	6 : 6 : 3

3.2.3.2 HEK293 cells - Luciferase reporter gene assay

Prior to re-seeding, compound dilutions were prepared in stripped medium and brought in first into a 96-well plate. Each condition was measured in quadruplicate (4 technical replicates).

For the luciferase assays with fatty acid treatments, fatty acids needed to be conjugated to BSA first, in order not to be toxic to the cells. For that reason, a solution of 4% fatty acid-free BSA in serum-free medium was prepared. Stock solutions of arachidonic acid, eicosapentaenoic acid and oleic acid were prepared in DMSO purged with nitrogen gas. Stock solutions of the fatty acids were diluted in BSA-solution to achieve 4-fold of the final concentration. These dilutions were then subjected to sonication in a water bath for

15 minutes, followed by incubation on an Eppendorf® thermomixer compact shaker (set at 400 rpm and 37 ℃) for 1 hour. The 4-fold dilutions were then pipetted into a 96-well plate and diluted to onefold by re-seeding of transfected cells, as described below. The final concentration of BSA was thus 1%.

After 6 hours of transfection, cells were harvested and re-seeded into the 96-well plate containing the compound dilutions. Cells were re-seeded in stripped medium at 0.05 x 10^6 cells/well. After 18 hours of treatment, medium was aspirated and the plate was frozen at -80 ℃ for at least one hour and up to one week. Hereafter, the plate was thawed and 50 μl of lysis buffer were added per well, followed by shaking for 10 minutes on a plate shaker. 40 μl of the lysates were transferred into a black 96-well plate before fluorescence and luminescence were measured on a Tecan GENios™ Pro or a Tecan Spark™ plate reader. Fluorescence was measured first, and luminescence was measured after the injection of 50 μl of an ATP solution and a luciferin solution, respectively. The background fluorescence and luminescence of untransfected cells was subtracted from the corresponding values of transfected cells. The luminescence was then normalized to the EGFP-derived fluorescence and results were expressed as fold induction compared to the vehicle control (DMSO 0.1%).

3.2.3.3 Caco-2 cells - Transient transfection

Caco-2 cells were transiently transfected using the commercially available Lipofectamine™ LTX.

Caco-2 cells were seeded at a density of 0.02 x 10^6 cells/well in 100 μl DMEM complete growth medium in a 96-well plate. They were grown overnight in an incubator with a humidified atmosphere at 37 ℃ and 5% CO_2.

One hour prior to transfection, medium was aspirated and fresh OptiMEM reduced serum medium (100 μl) was added to the wells. The transfection mix was prepared in OptiMEM reduced serum medium, according to the manufacturer's instructions. The plasmid DNA/PLUS solution and the Lipofectamine™ LTX solution were prepared separately in a ratio of 1:1, before being combined. The volume of PLUS™ solution used was 0.1 μl/well and the volume of Lipofectamine™ LTX used was 0.5 μl/well. The total amount of plasmid DNA was 0.1 μg/well, and the ratios of the plasmids used are given in Table 3.31. After an incubation of 5 minutes at room temperature, 10 μl of the plasmid DNA/Lipofectamine solution were added to each well. Transfection was carried out for 24 hours. After transfection, cells were treated as described in the section below.

Table 3.31: Plasmids used for the luciferase reporter gene assay in Caco-2 cells

Plasmids	Ratios
LXRα : ABCA1_Luc : EGFP	1 : 3 : 1
LXRβ : ABCA1_Luc : EGFP	1 : 3 : 1
RXRα : RXRE_Luc : EGFP	1 : 3 : 1
LXRαGal4 : UAS_Luc : EGFP	5 : 3 : 1
LXRβGal4 : UAS_Luc : EGFP	5 : 3 : 1

3.2.3.4 Caco-2 cells - Luciferase reporter gene assay

Compound dilutions were prepared in DMEM stripped medium. Each condition was measured in quadruplicate (4 technical replicates). After 24 hours of transfection, medium was aspirated from the plate and DMEM stripped medium was added. The compound dilutions were then added to the corresponding wells of the plate and cells were incubated with the compounds for 24 hours. Medium was then aspirated and 50 μl of lysis buffer were added per well. The plate was then frozen at -80 ℃ for at least one hour and up to one week. For measurement of luciferase activity, the plate was thawed and then put on a shaker for 3 minutes. Hereafter, 40 μl of the lysates were transferred into a black 96-well plate before fluorescence and luminescence were measured on a Tecan Spark™ plate reader. Fluorescence was measured first, and luminescence was measured after the injection of 50 μl of an ATP solution and a luciferin solution, respectively. The background fluorescence and luminescence of untransfected cells was subtracted from the corresponding values of transfected cells. The luminescence was then normalized to the EGFP-derived fluorescence and results were expressed as fold induction compared to the vehicle control (DMSO 0.1%).

3.2.4 Oil Red O staining with THP-1 macrophages

Oil Red O is a lipid soluble dye, which can be used to stain all neutral lipids inside the cell [495]. The staining can be visually observed under a microscope, and the dye can also be extracted with the help of isopropanol and absorbance can be measured for quantification.

THP-1 cells were seeded into 12-well plates at a density of 0.12 x 10^6 cells/ml in 2 ml of complete growth medium per well. The cell seeding suspension contained 200 nM phorbol-12-myristate-13-acetate (PMA) in order to differentiate monocytes into macrophages. Cells were incubated for 72 h in an incubator with a humidified atmosphere at 37 ℃ and 5%

CO_2. After differentiation, cells were washed once with PBS and loaded with cholesterol (water-soluble, 20 μg/ml, 1 ml per well) for 24 hours in 2.5% FBS medium. Cells were then washed once with PBS and treated for 24 hours with the different compounds or with the solvent control (DMSO 0.1%) in medium containing 0.1% BSA. Cells were again washed once with PBS and then fixed for 5 minutes with 10% formaldehyde in PBS at room temperature. Formalin was then aspirated and fresh formalin was added for at least 1 hour and up to one day. Hereafter, cells were washed with 60% isopropanol and afterwards air dried. Cells were then incubated with Oil Red O working solution for 10 minutes at room temperature, followed by several washing steps with deionized water to remove unincorporated stain. Cells were inspected under a light microscope at 100x magnification and pictures were taken. Residual water was then decanted and the plate dried overnight. Oil Red O was eluted by incubation with 100% isopropanol for 15 minutes at room temperature. A small aliquot was transferred into a 96-well plate and OD was measured at 492 nm with a Tecan Sunrise™ plate reader. Obtained results were normalized to the solvent control.

3.2.5 Amplex™ Red assay with THP-1 macrophages

The Amplex™ Red assay is a fluorometric assay for the quantification of cholesterol. Cholesteryl esters are hydrolyzed to cholesterol with the help of cholesterol esterase, and cholesterol is then oxidized by cholesterol oxidase, yielding a ketone and H_2O_2. The formed H_2O_2 reacts with Amplex™ Red in the presence of HRP (horseradish peroxidase), yielding resorufin which is highly fluorescent and can be measured on a plate reader [496].

For the Amplex™ Red assay, THP-1 cells were seeded into 6-well plates at a density of 0.15 x 10^6 cells/ml in 4 ml complete growth medium per well, containing 200 nM PMA for differentiation. After 72 hours, cells were washed once with PBS and loaded with cholesterol (water-soluble, 20 μg/ml, 2 ml per well) for 24 hours in 2.5% FBS medium. Cells were then washed once with PBS and treated for 24 hours with the different compounds or with the solvent control (DMSO 0.1%) in medium containing 0.1% BSA. Cells were again washed once with PBS and then lipids were extracted with hexane:isopropanol (3:2) for 30 minutes at 4 °C. Lipid extracts were transferred into brown glass tubes with a screw cap and evaporated to dryness under a gentle flow of nitrogen. Each sample was then reconstituted in 800 μl of 1x reaction buffer provided with the kit. Samples were vortexed vigorously to entirely dissolve them. Levels of both free and total cholesterol were then determined as described by the manufacturer.

The Amplex™ Red reagent mixture can be used with or without the addition of cholesterol esterase. When no cholesterol esterase is added to the reagent mixture, levels

of free cholesterol can be determined, while the reagent mixture containing cholesterol esterase is used to detect levels of total cholesterol. Fluorescence was measured on a Tecan Spark™ plate reader and results were then expressed as the ratio of free to total cholesterol.

3.2.6 Cholesterol efflux assay with THP-1 macrophages

To assess the influence of a compound on cholesterol efflux from macrophages, the cholesterol efflux assay using radioactively labelled cholesterol was performed.

THP-1 cells were seeded into 24-well plates at a density of 0.2 x 10^6 cells/ml in 1 ml complete growth medium per well. They were differentiated into macrophages by adding 200 nM PMA for 72 hours. Cells were washed once with PBS and loaded with cholesterol (water-soluble, 20 μg/ml) plus cholesterol tracer [^{3}H]-cholesterol (0.3 μCi/ml) in 0.5 ml 2.5% FBS medium for 24 hours. Cells were then washed once with PBS and treated for 24 hours with the different compounds or with the solvent control (DMSO 0.1%) in medium containing 0.1% BSA. In some cases, compounds were co-applied with probucol, an ABCA1 inhibitor [497], to investigate if an increase in cholesterol efflux provided by a compound is mediated *via* ABCA1. In all conditions, the final DMSO concentration did not exceed 0.1%. Cells were then washed once with PBS, followed by a 6-hour incubation with medium containing 0.1% BSA as well as human plasma (1%, v/v) and the corresponding compounds. Cells were lysed with the help of 0.1 N NaOH. Both the supernatant (effluxed cholesterol) and the cell lysate (intracellular cholesterol) were measured for [^{3}H]-cholesterol by liquid scintillation counting on a Tri-carb® 2910TR liquid scintillation counter. The percentage of cholesterol effluxed was calculated as the ratio of radioactively labelled cholesterol in the supernatant to that in both supernatant and cell lysate.

3.2.7 Resazurin conversion assay with THP-1 macrophages

To assess the viability of THP-1 macrophages after compound treatment, the resazurin conversion assay was performed. Resazurin is a non-fluorescent compound that can be reduced by metabolically active cells to resorufin, which is highly fluorescent. The fluorescence of resorufin can be measured and gives indication on the viability of the cells [498, 499].

THP-1 cells were seeded in 150 μl complete growth medium at a density of 0.03 x 10^6/well into 96-well plates and differentiated for 72 hours with the help of 200 nM PMA. They were then washed once with PBS and loaded with 20 μg/ml cholesterol (water-soluble) for 24 hours in 2.5% FBS medium. They were then treated with the different compounds or with the solvent control (DMSO 0.1%) in 100 μl of medium containing 0.1% BSA for

24 hours. Treatment with the positive control digitonin (50 μg/ml) was carried out for 4 hours. Hereafter, cells were incubated with resazurin in PBS (10 μg/ml) for 4 hours. Fluorescence (excitation 535 nm, emission 590 nm) was then measured on a Tecan Spark™ plate reader.

3.2.8 Cultivation of Caco-2 cells on filter inserts

Seeding of Caco-2 cells was done on Thursdays, right after passaging. Translucent PET filter inserts (Sarstedt) were used, except for confocal laser scanning microscopy and resazurin assays with subsequent crystal violet staining, where transparent PET filter inserts were required. The filter inserts used had a growth area of 1.1 cm^2 and were placed into 12-well companion plates. The volumes used were 0.5 ml apical (insert) and 1.6 ml basolateral (well). Before seeding, filter inserts were pre-wetted with complete growth medium for at least 2 minutes. Cells were passaged as usually done and then cells were seeded at a density of 0.06 x 10^6 cells/cm^2 in 0.5 ml complete growth medium per insert. One insert was cultivated without cells, as this is required for the measurement of the transepithelial electrical resistance (TEER) blank value. Medium was changed 3 hours after seeding to basolateral medium, as this helps to avoid the formation of multilayers and adds antibiotics to the culture to avoid bacterial contamination. Medium was changed again 24 hours after seeding and hereafter every other day until the end of the cultivation period, which is 19 to 21 days after seeding. Caco-2 cells were grown under symmetric conditions (i.e. basolateral medium in both the filter inserts and the wells) for 7 days. On day 7, conditions were changed to asymmetric (i.e. apical medium in the filter inserts, basolateral medium in the wells), which was then kept till the end of cultivation. Plates with inserts were kept in an incubator with a humidified atmosphere set at 37 °C and 5% CO_2.

3.2.9 Transepithelial electrical resistance (TEER) measurement

The formation of a cell monolayer as well as the integrity of the cell monolayer at the end of the cultivation period can be monitored by TEER measurement. To do so, a pair of electrodes connected to a voltohmmeter is used. The shorter electrode is placed in the insert, whereas the longer electrode is placed in the well. A constant electrical current is applied and the resistance of the barrier (i.e. the cells on the filter insert) can be observed by measuring the voltage [500].

TEER measurement followed in basic the manufacturer's instructions. The TEER voltohmmeter was charged overnight before making any resistance measurements. It was then disconnected from the charger and both voltohmmeter and TEER electrode were

brought into the laminar airflow. The TEER electrode was disinfected in 70% ethanol for 20 minutes. In the meantime, the voltohmmeter was switched on to allow it to warm up. After 20 minutes of disinfection, the TEER electrode was air dried for a few seconds, then washed twice with sterile ddH_2O and finally placed into apical medium for app. 10 minutes. During this time, the plug at the end of the electrode cable was already inserted into the input port on the voltohmmeter. 300 μl of apical medium were then added to the inserts of the plate, in order to fully cover the electrodes during the upcoming measurement. For resistance measurements, the function switch on the voltohmmeter was set to ohms. The longer electrode was placed into the well and touched the bottom of the well, while the shorter electrode was placed into the insert without touching the cell monolayer. First, the blank resistance was measured (insert without cells), followed by measurement of the resistance of the inserts with cells. The blank resistance was subtracted and the resulting value multiplied by the growth area of the inserts (i.e. 1.1 cm^2). This yielded the results in Ω cm^2.

3.2.10 Permeability assays with Caco-2 cells: Paracellular permeability of Dextran Blue and [^{14}C]-mannitol

Another way to observe the integrity of the formed monolayer is to measure the paracellular permeability of the markers Dextran Blue and [^{14}C]-mannitol. These markers have a low permeability across Caco-2 cell monolayers. An increased permeability is thus an indicator for a damaged or destroyed monolayer [460, 501].

Cells were cultivated and fully differentiated on translucent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then incubated for 48 hours in serum-free medium. A stock solution of Dextran Blue or [^{14}C]-mannitol was then prepared in serum-free medium. The Dextran Blue stock solution as well as the [^{14}C]-mannitol stock solution were diluted 1:1 with serum-free medium directly in the insert. Cells were incubated with Dextran Blue on the apical side and serum-free medium containing 2.5% human plasma on the basolateral side for 6 hours, or with [^{14}C]-mannitol on the apical side and serum-free medium on the basolateral side for 2 hours on a shaker (200 rpm, Thermo Scientific™ CO_2 resistant orbital shaker) in an incubator with a humidified atmosphere at 37 °C and 5% CO_2.

In regard to Dextran Blue, 100 μl samples were taken from the basolateral side every hour, and 100 μl of serum-free medium containing 2.5% human plasma were added to keep

the total volume. The different samples were pipetted into a 96-well plate and absorbance was measured at 620 nm on a Tecan Sunrise™ plate reader. A Dextran Blue calibration curve was included on the same plate and the amount of Dextran Blue in percent that was found on the basolateral side at each time point was calculated.

Concerning [^{14}C]-mannitol, 100 μl samples were taken from the basolateral side every 20 minutes, and 100 μl of serum-free medium were added to keep the total volume. Each sample was then measured in a liquid scintillation counter (in dpm). The counts in 100 μl stock solution were also measured. Finally, the apparent permeability of [^{14}C]-mannitol (P_{app}) was calculated, according to the equation below.

$$P_{app} = (\text{steady state flux})(1/(A\ C_{donor})) = (\text{dpm/min})(1/(\text{cm}^2\ \text{dpm/ml}))$$

The steady state flux (in dpm/min) is normalized to the surface area of the filter insert (A, i.e. 1.1 cm^2). C_{donor} is the initial concentration of [^{14}C]-mannitol on the apical side (in dpm/ml).

3.2.11 Western blot

3.2.11.1 Caco-2 cells - Preparation of whole cell lysates

Cells were cultivated and fully differentiated on translucent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then treated for 48 hours with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium. After treatment, cells were washed once with cold PBS and then incubated for 30 minutes at 4 °C with 150 μl NP40 ready-to-use lysis buffer under gentle shaking. Cell lysates were then transferred into centrifuge tubes and centrifuged at 13000 rpm (16060 x g) and 4 °C for 20 minutes. Supernatants were transferred into new centrifuge tubes. A small aliquot was used for total protein quantification *via* the Bradford assay as described in the corresponding section. Protein samples were then diluted with 3x sample buffer containing β-mercaptoethanol (2+1, v/v) and stored at -80 °C until further use.

3.2.11.2 THP-1 cells - Preparation of whole cell lysates

Cells were seeded at a density of 0.15 x 10^6/ml in a volume of 4 ml complete growth medium per well into 6-well plates, containing 200 nM PMA for differentiation. After 72 hours, cells were washed once with PBS and loaded with cholesterol (water-soluble, 20 μg/ml,

2 ml per well) for 24 hours in 2.5% FBS medium. Cells were then washed once with PBS and treated for 24 hours with the different compounds or with the solvent control (DMSO 0.1%) in medium containing 0.1% BSA. Hereafter, cells were washed once with cold PBS and then incubated for 30 minutes at 4 ℃ with 280 μl NP40 ready-to-use lysis buffer under gentle shaking. Cell lysates were then transferred into centrifuge tubes and centrifuged at 13000 rpm (16060 x g) and 4 ℃ for 15 minutes. Supernatants were transferred into new centrifuge tubes. A small aliquot was used for total protein quantification *via* the Bradford assay as described in the next section. Protein samples were then diluted with 3x sample buffer containing β-mercaptoethanol (2+1, v/v) and stored at -80 ℃ until further use.

3.2.11.3 Protein quantification *via* the Bradford assay

Total protein content in the cell lysates was quantified *via* the Bradford method [502]. The principle of this method is that an acidic solution of Coomassie Brilliant Blue G-250 has an absorption maximum at 479 nm, which is shifted to 595 nm upon the reaction with basic and aromatic amino acids of proteins.

A small aliquot of the whole cell lysates was diluted 1:10 with distilled water. The diluted protein samples were then mixed with the diluted Bradford reagent 1:42 in a 96-well plate, in triplicate. Absorption was measured at 595 nm on a Tecan Sunrise™ plate reader. Serial dilutions of BSA were prepared in order to obtain a calibration curve and were also diluted with Bradford reagent and measured in triplicate on the same plate. The calibration curve was then used for the calculation of the protein content.

3.2.11.4 Protein separation *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis (SDS-PAGE) [503], using standard gel electrophoretic techniques. The anionic detergent SDS and the reducing β-mercaptoethanol remove hydrogen bonds and disulfide bonds, respectively, thereby denaturing the proteins. Moreover, proteins are negatively charged due to SDS, in a uniform ratio of mass to charge, enabling a separation of proteins based on their molecular weight.

A 7.5% or 10% polyacrylamide gel was used for the separation of proteins and 20 μg protein were loaded per lane. Electrophoresis was carried out at constant ampere (25 mA/gel) in 1x electrophoresis buffer, using a Mini-PROTEAN™ 3 Cell System connected to a power supply.

3.2.11.5 Western blot and protein detection

Via Western blotting, proteins are transferred from the gel to a membrane, which can then be incubated with antibodies. The membrane is incubated first with a primary antibody, directed against the protein of interest. A secondary antibody directed against the primary antibody is then used to detect the interaction of the primary antibody with the protein. The secondary antibody is conjugated to horseradish peroxidase, which can, in a certain buffer, oxidize luminol, thereby producing luminescence which can be measured and related to the amount of protein on the membrane.

Western blotting was carried out using the tank blotting technique (wet transfer), in 1x blotting buffer. Proteins were transferred to a PVDF membrane at constant volt (100 V) for 90 to 110 minutes with a Mini Trans-Blot™ Electrophoretic Transfer Cell System. Membranes were then blocked for 1.5 hours with TBST containing 5% nonfat dry milk to prevent unspecific binding of the antibody. Hereafter, they were washed 3 times in TBST and incubated overnight at 4 °C with the primary antibody. Membranes were then washed in TBST and incubated with a horseradish peroxidase conjugated secondary antibody at room temperature for 1.5 hours. Membranes were again washed in TBST and bands were visualized with an ECL reagent and a LAS-3000™ Luminescent Image Analyzer. Semi-quantitative analysis of the bands was carried out by using the software MultiGauge V3.0.

3.2.12 Confocal laser scanning microscopy (CLSM) with Caco-2 cells for ZO-1, ABCG5, NPC1L1 and ABCB1

Cells were cultivated and fully differentiated on transparent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then treated for 48 hours with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium. After treatment, cells were washed twice with cold PBS and then fixed with 3.7% formaldehyde in PBS for 15 minutes at room temperature. After washing with PBS, cells were permeabilized with 0.2% Triton X in PBS for 10 minutes. Cells were again washed with PBS and a blocking solution of 5% BSA in PBS was added for 6 hours during which the plate was placed at 4 °C.

For quality control assays, cells were then incubated with an Alexa Fluor 594 conjugated anti-ZO-1 antibody (1 μg/ml in PBS with 1% BSA) overnight at 4 ℃, followed by incubation with DAPI (1 μg/ml in PBS with 0.2% BSA) for 15 minutes in the dark.

For the detection of cholesterol transport proteins, cells were incubated with the respective primary antibody (ABCG5: 2 μg/ml, NPC1L1: 10 μg/ml, ABCB1: 2 μg/ml; in PBS with 1% BSA) overnight at 4 ℃, followed by incubation with the secondary antibody (Alexa Fluor 488 conjugated goat anti-rabbit antibody: 4 μg/ml; in PBS with 1% BSA) for 3 hours at 4 ℃ in the dark. Hereafter, cells were incubated with DAPI (1 μg/ml in PBS with 0.2% BSA) for 15 minutes in the dark.

Filter membranes were cut from the plastic insert with a scalpel and placed into mounting medium on a microscope slide. Samples were then inspected by confocal laser scanning microscopy using excitation wavelengths of 405 nm (DAPI, cell nuclei), 488 nm (ABCG5, NPC1L1, and ABCB1 antibody) and 561 nm (ZO-1 antibody). CLSM was performed on a Leica DMi8 microscope and images were obtained with the help of the Leica LASX software.

3.2.13 RT-qPCR with Caco-2 cells

3.2.13.1 RNA extraction

Cells were cultivated and fully differentiated on translucent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then incubated with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium for the indicated time points. After treatment, cells were washed once with cold PBS and then incubated for 10 minutes with TRIzol reagent. The lysates were subjected to three freeze/thaw cycles and were then transferred to phasemaker™ tubes. They were incubated with chloroform for 15 minutes, followed by centrifugation at 4 ℃ and 13000 rpm (16060 x g) for 10 minutes. The aqueous phase was then transferred into fresh tubes, RNase-free glycogen and isopropanol were added, samples were vortexed and then incubated for 10 minutes. After 30 minutes of centrifugation at 13000 rpm (16060 x g) and 4 ℃, the supernatant was removed and the cell pellet resuspended in 75% ethanol. Samples were centrifuged again at 13000 rpm (16060 x g) and 4 ℃ for 5 to 10 minutes and this washing step was then repeated once. The supernatant was carefully removed and the pellet dried at 37 ℃ for 10 minutes. The cell pellet was resuspended in RNase-free water and incubated at 56 ℃ on a heat block for 12 minutes. The RNA concentration

as well as the quality of the extracted RNA was assessed with the help of a NanoDrop 2000c UV-Vis Spectrophotometer. A ratio of the absorbance at 260 nm and 280 nm wavelength of around 2 is considered as completely pure, however, was a little bit lower for this extraction method with around 1.8. The RNA was stored at -80 ℃ until further use.

3.2.13.2 cDNA synthesis (Reverse Transcription, RT-PCR)

After total RNA extraction, mRNA, which is single stranded, has to be reverse transcribed into cDNA (complementary DNA). This is done with the help of the enzyme reverse transcriptase. The obtained cDNA can then be used for real time quantitative PCR.

The cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription Kit including an RNase inhibitor according to the manufacturer's instructions, ensuing from 0.7 μg RNA in a reaction volume of 20 μl. The thermocycler conditions used are summarized in Table 3.32.

After the RT-PCR, 30 μl of nuclease-free water were added per sample, yielding a final volume of 50 μl containing 14 ng/μl cDNA, assuming that the efficiency of the RT-PCR was 100%. The cDNA was stored at -80 ℃ until further use.

Table 3.32: Thermocycler conditions for cDNA synthesis

	Step 1	**Step 2**	**Step 3**	**Step 4**
Temperature	25 ℃	37 ℃	85 ℃	4 ℃
Time	10 min	120 min	5 min	forever

3.2.13.3 Real time quantitative PCR (qPCR)

The PCR reaction starts with a small amount of DNA, which is denatured at 95 ℃. Primers complementary to the target gene are then annealed at a lower temperature and then a DNA polymerase leads to the elongation of the DNA. These steps are carried out in repeated cycles, each doubling the amount of the DNA present, leading to an exponential and specific amplification of the target sequence of the DNA. Quantitative PCR enables the quantification of the target sequence in real time by the use of fluorescent dyes which interact with double stranded DNA. The obtained fluorescence curves enable the determination of the original amount of mRNA present in the sample. As an internal control, mRNA levels of GAPDH were measured and mRNA levels were normalized to this housekeeping gene.

The real time quantitative PCR was performed on a LightCycler® 480, using the SYBR® Green I Master Mix. The reaction volume of 15 μl contained 2 μl cDNA (i.e. 28 ng cDNA), 7.5 μl SYBR® Green I Master Mix, 4 μl of nuclease-free water and 1.5 μl of the respective primer. The real time quantitative PCR performed consisted of a HotStarTaq Polymerase activation of 5 minutes at 95 ℃, followed by 45 amplification cycles. The amplification cycles consisted of a denaturation at 95 ℃ for 10 seconds, followed by annealing at 55 ℃ for 20 seconds and elongation at 72 ℃ for 30 seconds. After the 45 amplification cycles, melting curves were generated to assure the amplification of only one PCR product. The qPCR data were analyzed using the LightCycler® 480 software and the final analysis was carried out *via* the comparative C(t) method [504].

3.2.14 Cholesterol uptake assay with Caco-2 cells

To assess the influence of a compound on cholesterol uptake into intestinal cells, the cholesterol uptake assay using radioactively labelled cholesterol was performed.

Cells were cultivated and fully differentiated on translucent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then treated for 48 hours with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium.

Approximately 2 hours before the treatment period was over, preparation of micelles was started. As incubation with micelles and treatment was carried out in parallel, micelles were prepared in 2-fold concentration and then diluted directly in the insert by the addition of fresh treatment. First, stock solutions of the substances were prepared in chloroform:methanol (2:1, v/v), except for the sodium taurocholate stock solution, which was prepared in serum-free medium. Concentrations of the stock solutions, as well as onefold concentration of the substances contained in the micelles can be found in Table 3.33. Required amounts of stock solutions were combined in a sterile glass vial with a screw cap, except for the sodium taurocholate stock solution. Hereafter, [^{3}H]-cholesterol was added (final onefold activity: 1 μCi/ml). This mixture was then evaporated to dryness under a gentle stream of nitrogen. Afterwards, the required amount of sodium taurocholate stock solution was added and the volume completed with serum-free medium to achieve the desired 2-fold concentration. The mixture was vortexed vigorously for 5 minutes and placed on a shaker (100 rpm, Swip SM25 DIGI orbital shaker) at 37 ℃ for 1 hour. The micelles were filter sterilized right before addition to the cells. Cells were then incubated with micelles and fresh treatment on the apical side, and serum-free medium containing 1%

human plasma on the basolateral side for 2 hours on a shaker (200 rpm, Thermo Scientific™ CO_2 resistant orbital shaker) in an incubator with a humidified atmosphere at 37 ℃ and 5% CO_2. Cells were washed with PBS and then incubated for 30 minutes with 150 μl NP40 lysis buffer. Cell lysates were then transferred into centrifuge tubes and centrifuged at 13000 rpm (16060 x g) and 4 ℃ for 20 minutes. Supernatants were transferred into new centrifuge tubes. A small aliquot was used for total protein quantification *via* the Bradford assay as described in the corresponding section. Another small aliquot was used for liquid scintillation counting on a Tri-carb® 2910TR liquid scintillation counter. Results were then expressed as nmol cholesterol/mg protein, before being normalized for interexperimental variance.

Table 3.33: Concentration of stock solutions and onefold concentration of the micelle components

Substance	Onefold concentration	Stock solution
Cholesterol	0.05 mM	25 mM
Sodium taurocholate	2 mM	24 mM
L-α-lysophosphatidylcholine	0.2 mM	100 mM
Oleic acid	0.6 mM	100 mM
2-Oleoylglycerol	0.2 mM	100 mM

3.2.15 Cholesterol efflux assay with Caco-2 cells

To determine the effect of a compound on cholesterol efflux from intestinal cells, the cholesterol efflux assay using radioactively labelled cholesterol was performed.

Cells were cultivated and fully differentiated on translucent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Two hours before this incubation period was over, preparation of micelles was started. Micelles were prepared as described for the cholesterol uptake assay, however, they were not co-applied with treatment, but were diluted to onefold by the addition of serum-free medium. Cells were incubated with micelles on the apical side and serum-free medium on the basolateral side for 24 hours on a shaker (200 rpm, Thermo Scientific™

CO_2 resistant orbital shaker) in an incubator with a humidified atmosphere at 37 °C and 5% CO_2. Hereafter, cells were washed with PBS and then treated for 48 hours with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium on a shaker (200 rpm, Thermo Scientific™ CO_2 resistant orbital shaker) in an incubator at 37 °C and 5% CO_2. After treatment, cells were incubated for 24 hours with cholesterol-deprived micelles on the apical side and serum-free medium containing 1% human plasma on the basolateral side, again on a shaker (Thermo Scientific™ CO_2 resistant orbital shaker) set at 200 rpm in an incubator. Apical and basolateral supernatants were collected and centrifuged for 5 minutes at 13000 rpm (16060 x g) at 4 °C. Cells were washed with PBS and then incubated for 30 minutes with 150 μl NP40 lysis buffer. Cell lysates were then transferred into centrifuge tubes and centrifuged at 13000 rpm (16060 x g) and 4 °C for 20 minutes. Supernatants were transferred into new centrifuge tubes. A small aliquot from each sample was used for liquid scintillation counting on a Tri-carb® 2910TR liquid scintillation counter. Results were normalized to 1 ml volume and expressed as dpm/ml, before being normalized for interexperimental variance.

3.2.16 Resazurin conversion assay and subsequent crystal violet staining with Caco-2 cells

To assess the viability of differentiated Caco-2 cells after compound treatment, the resazurin conversion assay and a subsequent crystal violet staining was carried out. During cell death, adherent cells detach from the culture plates. This feature can be used for the indirect measurement of viability with the help of the crystal violet dye. This dye binds to DNA and proteins and can be eluted from the cells for quantification *via* absorbance. Dead cells that lost their adherence are washed away, thereby reducing the amount of crystal violet stain in the cell population under investigation [505].

Cells were cultivated and fully differentiated on transparent filter inserts as described in the corresponding section. Before starting experiments, TEER was measured to assure the integrity of the cell monolayer. Cells were washed once with PBS and then incubated with serum-free medium on the apical side and medium containing 0.5% BSA on the basolateral side for 24 hours. Cells were again washed with PBS and then treated for 48 hours with the different compounds or with the solvent control (DMSO 0.1%) in serum-free medium. Hereafter, cells were incubated with a dilution of resazurin in serum-free medium (10 μg/ml) for 2 hours in the filter insert (0.5 ml). Inserts were transferred into a new 12-well companion plate after the 2 hour-incubation, then 400 μl from the apical solution were removed and pipetted into a 48-well plate. Fluorescence (excitation 535 nm, emission 590 nm) was then measured on a Tecan Spark™ plate reader.

Remaining resazurin dilution in the filter inserts was carefully removed using a pipet tip. Then 500 μl crystal violet solution were added and the filter inserts were incubated for 10 minutes at room temperature. Hereafter, inserts were washed extensively with deionized water, remaining water was removed and inserts were dried overnight at room temperature. The next day, 500 μl sodium citrate:ethanol were added per insert and the plate with the inserts was shaken smoothly for 10 seconds. Another 500 μl of sodium citrate:ethanol were added per insert and the plate was again smoothly shaken. An aliquot of 100 μl was taken out of each insert and pipetted into the wells of a 96-well plate. OD was then measured at 595 nm on a Tecan Sunrise™ plate reader.

3.2.17 Data analysis and statistics

Data analysis was carried out using Microsoft Excel 2016. Analyzed data were then transferred to GraphPad Prism™ 6.01 for statistical analysis and graphics. Statistical tests used were two-tailed paired t-test (differences between two groups), one-way ANOVA with Dunnett post-test (multiple comparisons) or two-way ANOVA with Bonferroni post-test (analysis of the effect of two independent variables). A difference was considered as statistically significant when $P < 0.05$.

4 Results and Discussion

4.1 Caco-2 cells for measuring intestinal cholesterol transport

In this section, the establishment of the Caco-2 cell line in our working group is described. It is shown how conditions and assays were modified and optimized, and where we experienced limitations of this cell model. The final methodology can be found in the section "Materials and Methods".

The results described in this section, as well as the corresponding methods used, were submitted for publication as a manuscript entitled "Caco-2 cells for measuring intestinal cholesterol transport – possibilities and limitations" to the Journal *Biological Procedures Online*, and is currently in revision.

4.1.1 Maintenance of Caco-2 cells and cultivation on filter inserts with spontaneous differentiation

Since the Caco-2 cell line was a new cell line in our working group, we had to establish protocols for maintenance and subculturing of the cells, as well as for cultivating them on filter inserts. For the cultivation on filter inserts, several parameters like seeding density, membrane materials and differentiation period have to be considered in order to obtain a tight monolayer of cells over time. Several different protocols are already available in literature [457, 458, 460, 488, 489, 506–510], however, they differ quite a lot in regard to the above mentioned parameters. We therefore used the existing protocols as a starting basis and evaluated these parameters, leading to optimized protocols described in the methods section.

To identify the best conditions for the cultivation of Caco-2 cells on filter inserts, several quality control steps were necessary. A quality control that is routinely carried out during the cultivation and before the start of experiments is transepithelial electrical resistance measurement (TEER). TEER was measured at least once per week to observe the formation of a monolayer, and right before the start of an experiment, to guarantee the integrity

of the monolayer. In Figure 4.1A, a typical increase in TEER over the differentiation period is shown. We used inserts only with a TEER value of $\geq 200\ \Omega\ cm^2$ at 37 ℃ for experiments.

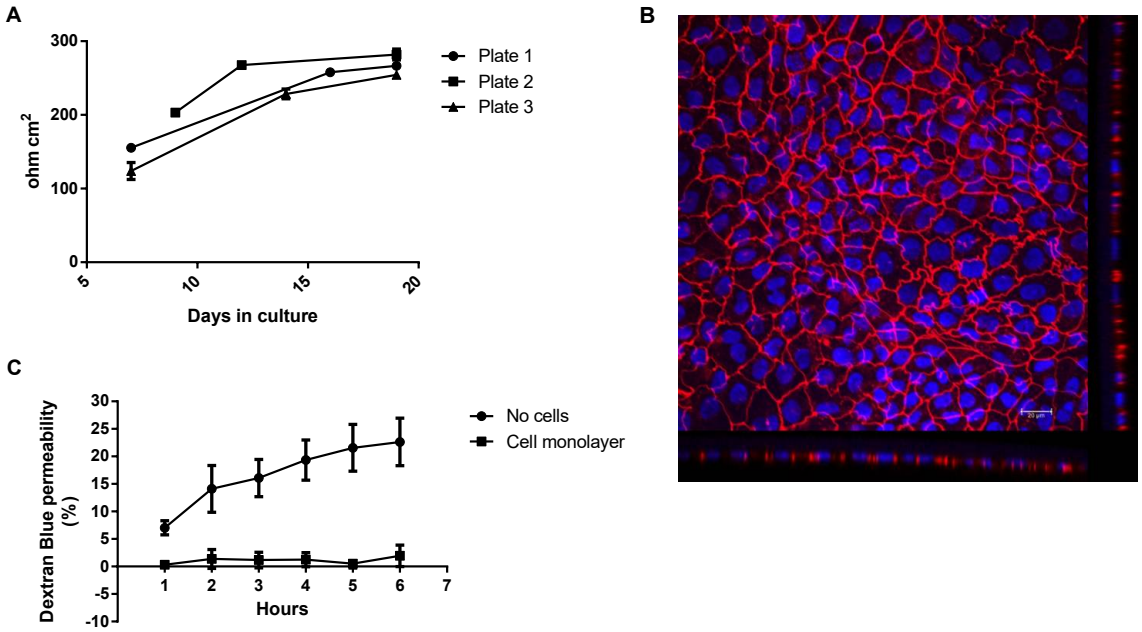

Figure 4.1: Quality control steps for the cultivation of Caco-2 cells on filter inserts. Increase in TEER over the cultivation period **(A)**. Caco-2 cells were seeded onto filter inserts and TEER was measured during the cultivation period. The data points represent mean ± SD of three inserts from the respective plate. Representative image of a Caco-2 cell monolayer grown on a filter insert for 21 days **(B)**. Depicted is a maximum projection of the top view and the optical cross sections obtained by CLSM. Cell nuclei are shown in blue (DAPI) and the tight junction protein ZO-1 in red (Alexa Fluor 594 conjugated anti ZO-1 antibody). Dextran Blue permeability of a filter insert without cells and a filter insert with a fully differentiated cell monolayer **(C)**. Filter inserts were incubated with Dextran Blue over a period of 6 hours and sampling every hour. The data points represent mean ± SD of three to four independent experiments. Experiments for Figures B and C were performed by Daniel Schachner.

When we set up protocols for the cultivation of Caco-2 cells, confocal laser scanning microscopy was performed at the end of the differentiation period. In this way, we were able to evaluate certain conditions in regard to monolayer and tight junction formation, and to exclude that conditions lead to the formation of multilayers instead of the desired monolayer. We stained the cell nuclei with DAPI and the tight junction protein ZO-1 with a fluorescently labelled antibody, as detailed in the methods section. In Figure 4.1B, a representative picture of a Caco-2 cell monolayer grown on a transparent filter insert is shown.

Another method that we applied to observe the integrity of the formed monolayer is the investigation of the paracellular permeability of the markers Dextran Blue and [^{14}C]-mannitol. In regard to Dextran Blue, the paracellular permeability, i.e. the movement of this marker from the apical to the basolateral compartment, was investigated every hour for a total period of 6 hours. Results of the Dextran Blue permeability can be found in Figure 4.1C. The paracellular permeability of [^{14}C]-mannitol was observed over the course of 2 hours. In our laboratory, typical apparent permeability values for [^{14}C]-mannitol are $1.30 \pm 0.77 \times 10^{-6}$ cm s^{-1}.

4.1.2 Transfection of Caco-2 cells and luciferase reporter gene assay

Major regulators of cholesterol homeostasis are nuclear receptors [266, 323] and determining the activation of a particular nuclear receptor is of high interest in atherosclerosis research. A very prominent assay to determine nuclear receptor activation is the luciferase reporter gene assay. This assay is usually carried out in HEK293 cells [494], as they are easy to handle and can be easily transfected. However, cellular characteristics, like the expression of coregulators that are important for nuclear receptor activation, differ between cell lines, which could lead to a different assay outcome in different cell lines [272]. Therefore, we decided to establish a protocol for the transfection of Caco-2 cells and a subsequent luciferase reporter gene assay in this cell line. Several transfection reagents were tested, i.e. Fugene® HD, the calcium phosphate precipitation method [494, 511], and linear polyethylenimine (LPEI), but only Lipofectamine™ LTX led to an applicable transient transfection rate with the Caco-2 cells we used. Cells were co-transfected with either a human full-length LXRα or LXRβ expression plasmid, the ABCA1_Luc firefly luciferase reporter plasmid and an EGFP expression plasmid, as internal control. For the mammalian one-hybrid assay, cells were co-transfected with an expression plasmid coding for a chimeric protein consisting of the yeast Gal4-DBD and the human LXRα/β-LBD, the UAS_Luc firefly luciferase reporter plasmid and an EGFP expression plasmid, as internal control. Cells were then treated with increasing concentrations of the LXR agonist GW3965 (0.1 – 10 μM), and the results are shown in Figure 4.2. A concentration-dependent increase in luciferase activity was observed for all cases, except for LXRβ, where a plateau was already reached from 1 μM GW3965 upwards. These results indicate that Caco-2 cells can be sufficiently transfected and are a suitable cell line for a subsequent luciferase reporter gene assay.

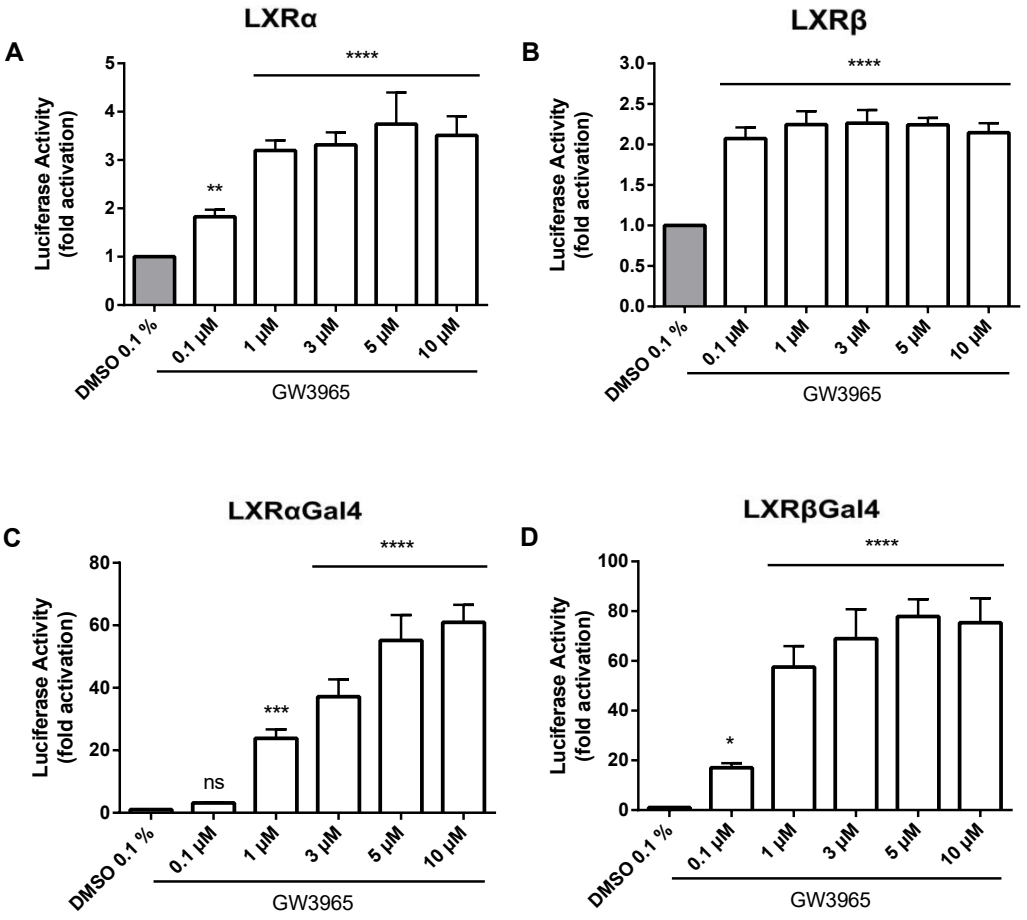

Figure 4.2: Luciferase reporter gene assays with Caco-2 cells. Caco-2 cells were transiently co-transfected with a human full-length LXRα **(A)** or LXRβ **(B)** expression plasmid, the firefly luciferase reporter plasmid ABCA1_Luc and an EGFP expression plasmid, in a ratio of 1:3:1. In the case of the mammalian one-hybrid assays, cells were transiently transfected with LXRαGal4 **(C)** or LXRβGal4 **(D)**, the reporter plasmid UAS_Luc, and the EGFP expression plasmid, in a ratio of 5:3:1. Cells were then treated with GW3965 (LXR agonist, 0.1 – 10 μM) or the vehicle control (DMSO 0.1%). The luminescence was normalized to the fluorescence, and set in relation to the solvent vehicle. Bar graphs represent mean ± SD of three independent experiments each performed in quadruplicate. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test).

4.1.3 Establishing functional assays with Caco-2 cells

Since the intestine is a very important interface in cholesterol uptake and excretion, we set up two functional assays for investigating the effect of a certain test compound or drug on intestinal cholesterol uptake and efflux. Several groups have already carried out uptake and efflux assays with Caco-2 cells, but unfortunately, the available protocols again differ

regarding several assay conditions, like incubation with/without micelles, composition of the micelles, incubation time, incubation with/without acceptors and differentiation of Caco-2 cells on plates with/without inserts beforehand [512–516]. Therefore, we also optimized these protocols in our laboratory.

4.1.3.1 Cholesterol uptake assay

Caco-2 cells were grown and fully differentiated on filter inserts for the cholesterol uptake assay. They were treated for 48 hours with the LXR agonist GW3965 (5 and 10 μM) or the RXR agonist bexarotene (1 μM). Cells were then incubated for 2 hours with fresh treatment and micelles mimicking postprandial conditions [517, 518] on the apical side, while serum-free medium containing 1% human plasma was added to the basolateral compartment. After this incubation period, cells were lysed and the uptake of radioactively labelled cholesterol was measured by liquid scintillation counting. The LXR agonist GW3965 significantly reduced cholesterol uptake at both concentrations applied (Figure 4.3), which is in accordance with already published data [301]. In contrast, bexarotene did not reduce cholesterol uptake in Caco-2 cells.

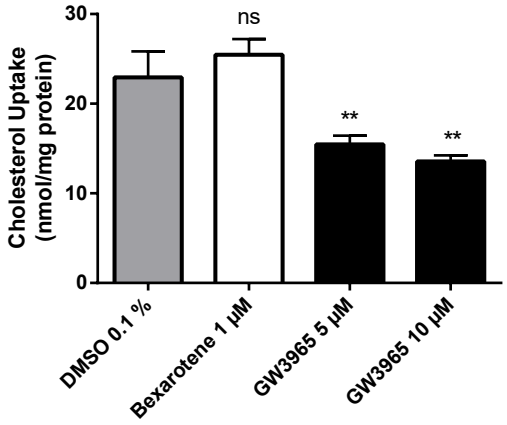

Figure 4.3: Cholesterol uptake assay with Caco-2 cells. Caco-2 cells cultivated on filter inserts were treated for 48 h with bexarotene (RXR agonist, 1 μM), GW3965 (LXR agonist, 5 and 10 μM) or the vehicle control (DMSO 0.1%). Cholesterol uptake was then determined by liquid scintillation counting. Protein concentration was measured and the results expressed as nmol cholesterol/mg protein before being normalized for interexperimental variance. Bar graphs represent mean ± SD of three independent experiments. ns not significant, $^{**}P < 0.01$ versus solvent vehicle (determined by paired t-test or one-way ANOVA with Dunnett post-test).

4.1.3.2 Cholesterol efflux assay

Cells were seeded and fully differentiated on filter inserts, as for the cholesterol uptake assay. Caco-2 cells were then loaded with cholesterol *via* incubation with micelles containing radioactively labelled cholesterol for 24 hours. Hereafter, cells were treated with different LXR agonists (GW3965 and T0901317, each at both 5 and 10 μM) for 48 hours. They were then incubated with cholesterol-deprived micelles on the apical, and serum-free medium containing 1% human plasma on the basolateral side for 24 hours. Effluxed cholesterol in the apical and basolateral supernatant was then measured by means of liquid scintillation counting. Figure 4.4 shows that both GW3965 and T0901317 led to a dose-dependent increase in cholesterol efflux, to both the apical and the basolateral compartment. This is in line with currently available literature that suggests that LXR agonists increase cholesterol efflux [300, 490].

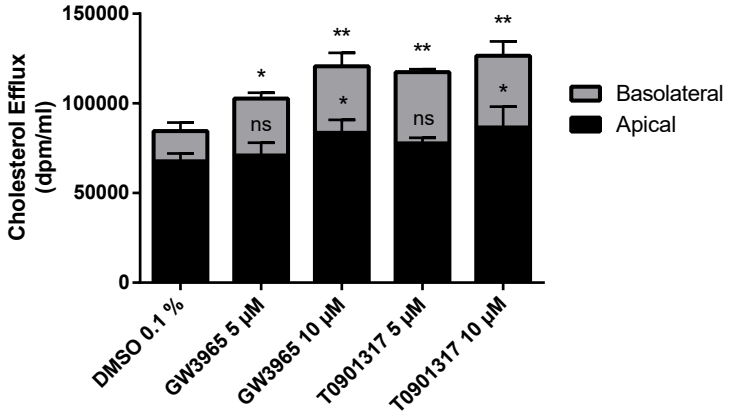

Figure 4.4: Cholesterol efflux assay with Caco-2 cells. Caco-2 cells cultivated on filter inserts were loaded with cholesterol and then treated for 48 h with GW3965 (LXR agonist, 5 and 10 μM), T0901317 (LXR agonist, 5 and 10 μM) or the vehicle control (DMSO 0.1%). Finally, cells were incubated with cholesterol-free micelles on the apical side, while being incubated with 1% human plasma on the basolateral side. Effluxed (apical and basolateral supernatant) cholesterol was then measured by liquid scintillation counting. The results in dpm were normalized to 1 ml volume, before being normalized for interexperimental variance. Bar graphs represent mean ± SD of three to four independent experiments. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test). Experiments for this Figure were performed by Daniel Schachner.

4.1.4 Detection of protein and mRNA levels of the cholesterol transporters ABCA1, ABCG5, NPC1L1 and ABCB1

Several transporters are responsible for the uptake and efflux of cholesterol in intestinal enterocytes. The most important ones already known are ABCA1, the heterodimer ABCG5/G8, NPC1L1 and ABCB1 (also known as P-glycoprotein) [48, 277]. Due to their role in cholesterol homeostasis, the effect of a compound on the protein and/or mRNA expression of these transporters is of high interest. The above mentioned cholesterol transporters are either up- or downregulated, either directly or indirectly, by the activation of nuclear receptors [277, 519].

4.1.4.1 Detection of protein levels of ABCA1, ABCG5, NPC1L1 and ABCB1

Caco-2 cells were differentiated on filter inserts and then treated with either GW3965 or T0901317 (LXR agonists, each at 10 μM, upregulate ABCA1 and ABCG5/G8, downregulate NPC1L1 [277]), bexarotene (RXR agonist, 5 μM, co-regulating LXR:RXR and CAR:RXR heterodimers [416]) or the solvent vehicle (DMSO 0.1%). Bexarotene was expected to upregulate ABCA1 and ABCG5/G8, as well as to downregulate NPC1L1 *via* the co-regulation of LXR:RXR heterodimers. As ABCB1 expression is regulated by CAR:RXR heterodimers [519], amongst others, we expected an upregulation of this transporter with bexarotene treatment.

We performed a treatment time course (from 4 h to 72 h) and a subsequent Western blot analysis, where an induction of ABCA1 of about 4-fold and 5-fold was detected when cells were treated for 48 h with GW3965 and T0901317, respectively (Figure 4.5).

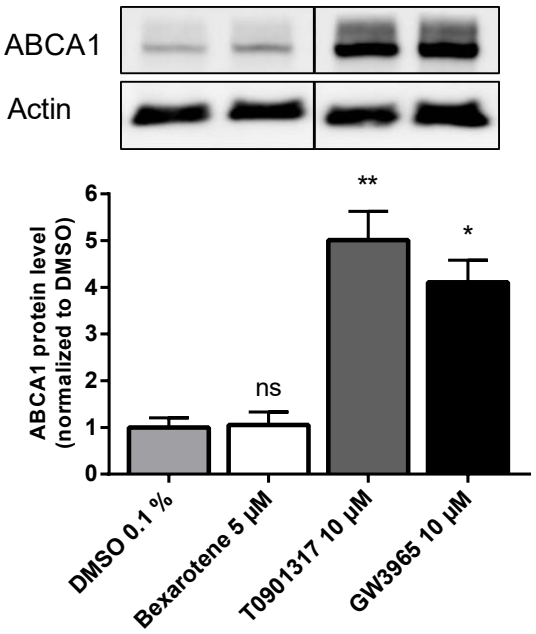

Figure 4.5: Western blot analysis of ABCA1 protein levels. Caco-2 cells cultivated on filter inserts were treated for 48 h with bexarotene (RXR agonist, 5 μM), T0901317 (LXR agonist, 10 μM), GW3965 (LXR agonist, 10 μM) or the vehicle control (DMSO 0.1%). Protein expression was then determined by Western blot analysis. Results were normalized for interexperimental variance and then set in relation to the vehicle control. Bar graphs represent mean ± SD of three independent experiments. A representative blot is shown - the bands stem from the same membrane and the vertical line indicates excised irrelevant bands. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$ versus solvent vehicle (determined by paired t-test).

However, no induction of this bona fide LXR target gene was observed by treatment with bexarotene, which is in contrast to findings in other cell types, like glial cells, where bexarotene significantly induced ABCA1 protein expression [520]. Western blot results regarding the protein expression of ABCG5, ABCG8, NPC1L1 and ABCB1 were inconsistent throughout all the conditions tested (data not shown, detailed discussion in the discussion chapter). For this reason, we then performed protein detection of these transporters with the help of CLSM. By CLSM, we were able to detect an upregulation of ABCG5 and ABCB1, as well as a downregulation of NPC1L1 when cells were treated with the respective positive controls for 48 h, as shown in Figure 4.6. At least for the Caco-2 strain we used and the conditions we tested in our laboratory, we conclude that Western blot analysis seems less suitable for detecting protein expression of these cholesterol transporters, whereas CLSM seems to be a suitable alternative approach.

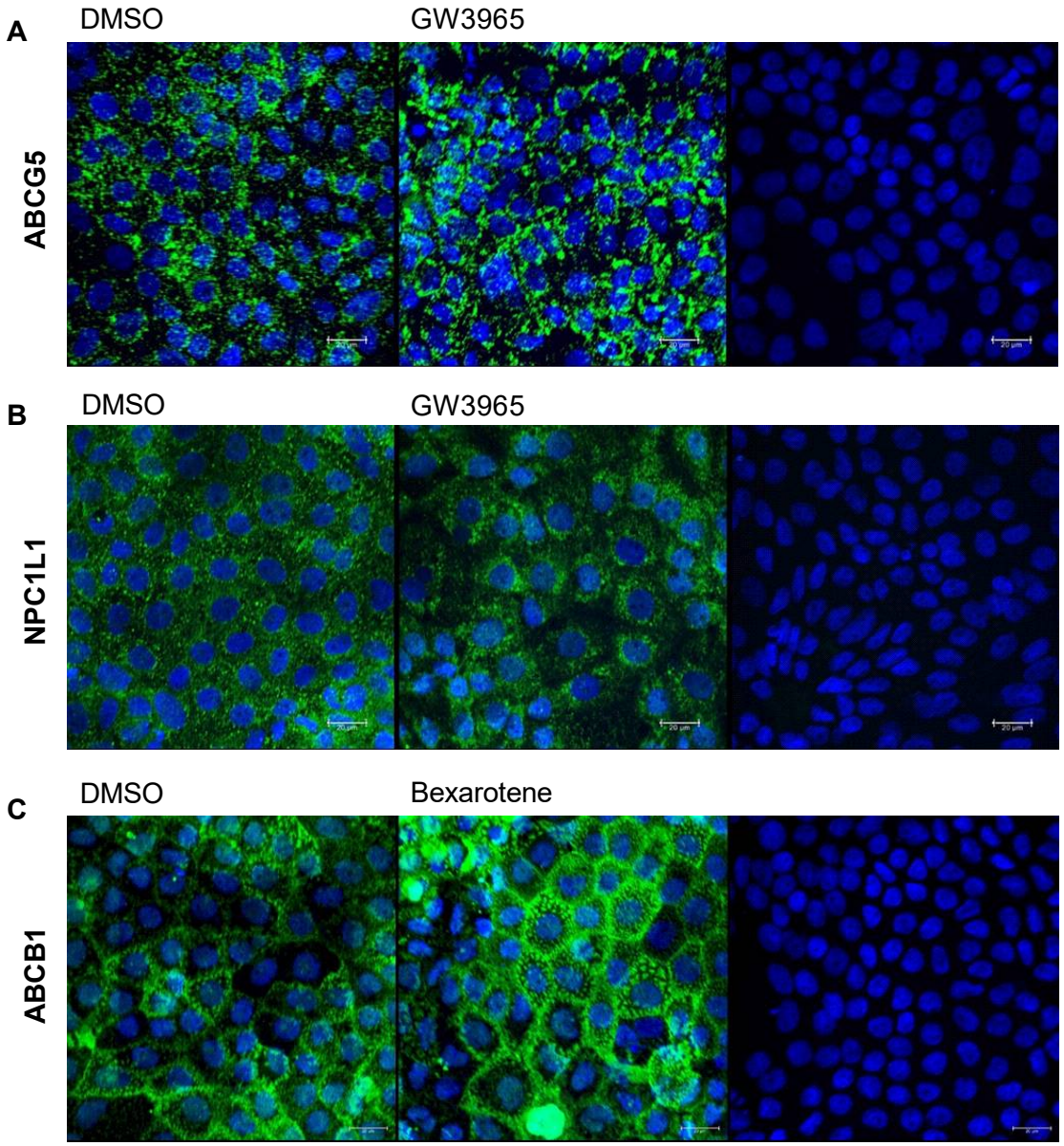

Figure 4.6: Confocal laser scanning microscopy for the detection of ABCG5, NPC1L1 and ABCB1 protein levels. Cell nuclei are shown in blue (DAPI) and ABCG5, NPC1L1 or ABCB1 in green (fluorescently labelled antibody). Representative images of ABCG5 **(A)**, treated with the vehicle control (DMSO 0.1%, left image) or GW3965 (LXR agonist, 10 μM, middle image). Representative images of NPC1L1 **(B)**, treated with the vehicle control (DMSO 0.1%, left image) or GW3965 (LXR agonist, 10 μM, middle image). Representative images of ABCB1 **(C)**, treated with the vehicle control (DMSO 0.1%, left image) or bexarotene (RXR agonist, 5 μM, middle image). The image on the right **(A - C)** is the respective control only stained with DAPI and the secondary antibody, showing no unspecific binding of the secondary antibody. Shown are maximum projections of the top views. Experiments for this Figure were performed by Daniel Schachner.

4.1.4.2 Detection of mRNA levels of ABCA1, ABCG5, NPC1L1 and ABCB1

Caco-2 cells were cultivated and fully differentiated on filter inserts, before being treated with either GW3965 (LXR agonist, 10 μM), bexarotene (RXR agonist, 5 μM) or the solvent vehicle (DMSO 0.1%). Treatment was carried out for different time intervals, i.e. 2 h, 4 h, 6 h, 8 h, 16 h, 24 h, and 30 h, with the purpose of detecting the time point of maximal up- or downregulation. Cells were finally subjected to RNA extraction and RT-qPCR in order to evaluate mRNA expression.

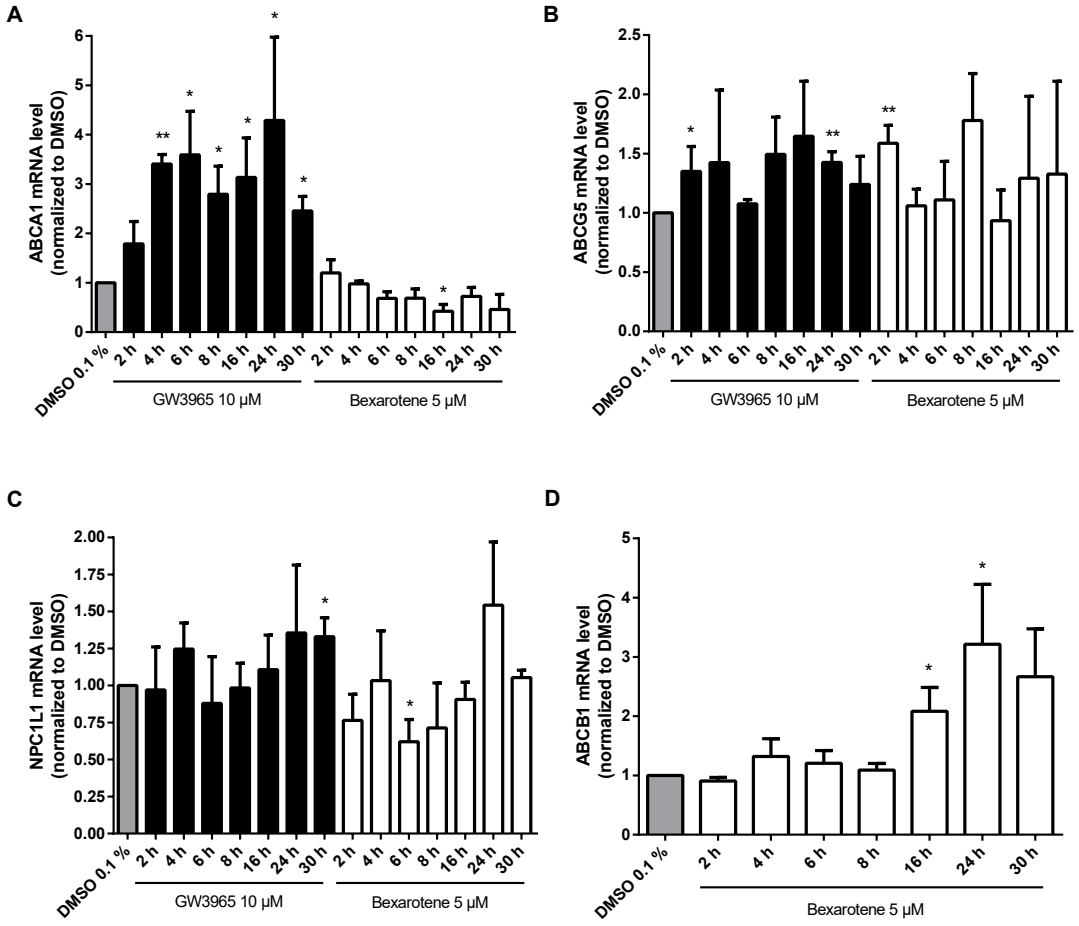

Figure 4.7: RT-qPCR with Caco-2 cells for ABCA1, ABCG5, NPC1L1 and ABCB1. Caco-2 cells cultivated on filter inserts were treated for the indicated time points with bexarotene (RXR agonist, 5 μM), GW3965 (LXR agonist, 10 μM) or the vehicle control (DMSO 0.1%). Quantification of mRNA expression was carried out by RT-qPCR. Results were expressed as the ratio of expression of the respective gene to that of GAPDH and were then normalized to the vehicle control of the respective time point (vehicle control not indicated for each time point). Bar graphs represent mean ± SD of three to four independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ versus solvent vehicle (determined by paired t-test).

ABCA1 mRNA expression was significantly increased by treatment with GW3965 already after 4 h, and a significant upregulation was maintained till the final treatment period of 30 h (Figure 4.7A). Interestingly, bexarotene did not elevate ABCA1 mRNA levels at any of the time points investigated. In contrast, there was a trend towards a decrease of ABCA1 expression, that reached significance after 16 h treatment. This observation is in contrast to findings in other cell types, like e.g. brain-like endothelial cells [521].

A significant induction of ABCG5 mRNA expression was observed by treatment with GW3965 for 2 h and for 24 h, as well as by bexarotene treatment for 2 h (Figure 4.7B). The other treatment periods showed either a decline in ABCG5 mRNA level or a high variation in mRNA expression, therefore not reaching statistical significance. The induction of ABCG5 was in general low, with about 1.4-fold after treatment with GW3965 for 24 h compared to the vehicle control.

NPC1L1 mRNA was not downregulated by the LXR agonist GW3965 at any of the treatment periods investigated (2 h – 30 h, Figure 4.7C). This is in contrast to already published work [154], where a significant downregulation of NPC1L1 was shown in Caco-2/TC7 cells after 24 h treatment with GW3965. Interestingly, NPC1L1 mRNA expression was decreased by treatment with bexarotene. Treatment for 2 h, 8 h and 16 h showed a tendency towards downregulation, and 6 h treatment with bexarotene significantly reduced NPC1L1 mRNA levels (Figure 4.7C).

After 16 h and 24 h treatment, bexarotene significantly upregulated ABCB1 mRNA expression (Figure 4.7D). Maximal induction of ABCB1 was observed after 24 h of treatment, with about 3-fold compared to the DMSO control.

4.1.5 Viability assays with Caco-2 cells cultivated on filter inserts – resazurin conversion assay and subsequent crystal violet staining

In order to assure that a certain compound does not affect the viability of the cells, viability assays are a standard procedure conducted. To the best of our knowledge, no protocols are currently available for viability assays with Caco-2 cells differentiated on filter inserts. Since most of the assays we performed with Caco-2 cells were, however, carried out with cells on filter inserts, we set up a protocol for a resazurin conversion assay and a subsequent crystal violet staining with Caco-2 cells cultivated on filter inserts.

Caco-2 cells were fully differentiated on transparent filter inserts and were then either left untreated in serum-free DMEM or were treated for 48 hours with the highest concentrations of the compounds used in previous experiments, i.e. GW3965 (10 μM), T0901317 (10 μM),

bexarotene (5 μM), or DMSO 0.1%. As a positive control, the natural cytotoxic product digitonin was applied at 50 μg/ml. After treatment, cells were washed once with PBS, followed by incubation with a resazurin dilution (10 μg/ml in serum-free DMEM) for 2 hours. Fluorescence (excitation 535 nm, emission 590 nm) was then measured in aliquots of the apical medium with the help of a plate reader. Digitonin significantly reduced fluorescence in the apical medium, indicative for reduced cell viability, compared to untreated cells (Figure 4.8A). All other compounds investigated did not affect cell viability. After measurement of fluorescence, inserts were incubated for 10 minutes with a crystal violet solution. Hereafter, cells were extensively, but carefully, washed with deionized water and the inserts were then dried overnight. Crystal violet was then solubilized with a sodium citrate:ethanol solution and absorbance was measured at 595 nm. OD was significantly reduced by digitonin, whereas the other compounds had no significant effect (Figure 4.8B). These results suggest that none of the compounds is cytotoxic in the applied concentrations.

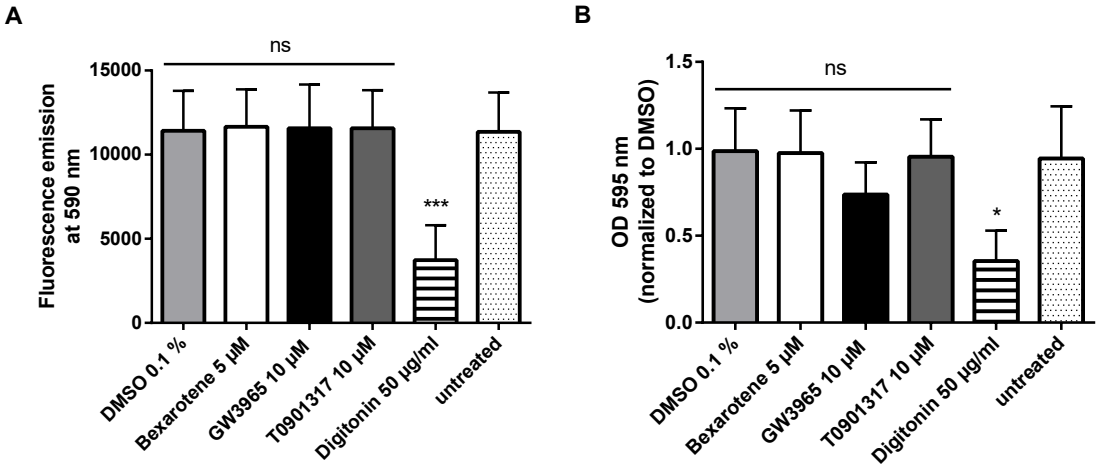

Figure 4.8: Resazurin conversion assay and subsequent crystal violet staining with Caco-2 cells grown on filter inserts. Caco-2 cells were differentiated on filter inserts and were then either left untreated (negative control) or were treated for 48 h with GW3965 (LXR agonist, 10 μM), T0901317 (LXR agonist, 10 μM), bexarotene (RXR agonist, 5 μM), digitonin (positive control, 50 μg/ml) or the vehicle control (DMSO 0.1%). Cells were then incubated with a dilution of resazurin. Hereafter, fluorescence emission was measured in the apical medium **(A)**. The inserts were then incubated with crystal violet solution. After washing steps and drying overnight, crystal violet was solubilized and the OD was measured at 595 nm and set in relation to the vehicle control **(B)**. Bar graphs represent mean ± SD of three independent experiments. ns not significant, $^{*}P < 0.05$, $^{***}P < 0.001$ versus untreated (determined by paired t-test). Experiments for this Figure were performed by Daniel Schachner.

4.1.6 Discussion

The Caco-2 cell line is a useful *in vitro* model of the intestinal epithelial barrier, suitable for studying cholesterol homeostasis and transport. Caco-2 cells differentiate spontaneously when kept in post-confluent cultures [457–459]. Cultivation of Caco-2 cells on permeable filter inserts is known to lead to an improved functional and morphological differentiation [458], which emphasizes the use of filter inserts for cultivation of these cells. Moreover, the use of filter inserts gives access to both the apical and the basolateral side of the cells, enabling transport experiments and further the use of different media for these two sides, closer resembling the physiological situation. Cells seeded and differentiated on filter inserts build a dense and intact monolayer of polarized cells [458]. Several quality control steps are, however, required to assure the integrity of the formed monolayer and to avoid the formation of multilayers. CLSM is a suitable approach for checking the presence of a monolayer and can further be used to monitor tight junction formation. To evaluate the quality of the formed monolayer, TEER measurement should routinely be carried out. Moreover, measurement of the paracellular permeability for compounds like Dextran Blue and [^{14}C]-mannitol are standard techniques to assess the quality of the monolayer. Nonetheless, it has to be mentioned that the Caco-2 cell line is heterogeneous, meaning that TEER and apparent permeability values vary highly with the clone or strain used and with the passage number of the cells [506, 522–525]. We only used monolayers on filter inserts with a TEER value of $\geq 200\ \Omega\ \text{cm}^2$ at 37 °C for experiments. In the permeability assay with Dextran Blue, we used 2.5% human plasma as acceptor in the basolateral compartment. Human plasma was also used as acceptor on the basolateral side in both the cholesterol uptake and the cholesterol efflux assay, although at a lower concentration of 1%. Publications using up to 2.5% human plasma as acceptor exist [526], which is why we decided to test this concentration, and to thereby exclude that human plasma has an influence on monolayer integrity.

A luciferase reporter gene assay was established, in order to determine nuclear receptor activation also in this cell line. Since transfection is required for this assay, several transfection reagents were tested, namely FuGENE® HD, the calcium phosphate precipitation method [494, 511], LPEI and Lipofectamine™ LTX. Only Lipofectamine™ LTX led to an acceptable transfection efficiency in our hands, as judged by the green fluorescence provided by the internal control EGFP. We evaluated the transactivation of the nuclear receptors LXRα and LXRβ in our assay conditions by using the synthetic LXR agonist GW3965. We transfected expression plasmids for the LXRs only, although LXRs function as heterodimers with RXRs [323]. Endogenous RXR protein expression was confirmed by Western blot analysis (data not shown). Additionally, results from experiments shown

in Figure 4.2 prove that LXR overexpression is sufficient to achieve activation by LXR agonists.

Two functional assays, namely cholesterol uptake and cholesterol efflux assay, were established and show the suitability of this cell line for investigating cholesterol transport. In both assays, LXR agonists were shown to be appropriate positive controls, decreasing cholesterol uptake and increasing cholesterol efflux in this intestinal cell line.

The expression of ABCA1 protein in Caco-2 cells was confirmed by Western blot analysis and by the use of relevant control compounds. However, treatment time course experiments regarding other transporter proteins were not able to produce convincing and consistent Western blot results, regardless of the lysis buffer used (NP40 versus RIPA). In most of the experiments, blots showed blurred and/or multiple bands, hampering semi-quantitative analysis. A possible explanation for this could be the fact that the transporters we wanted to detect are all glycoproteins, which exist in different glycosylated and non-glycosylated isoforms during the process of maturation [120, 527–529].

In regard to ABCG5/G8, LXR response elements that regulate gene expression were found in two evolutionary conserved regions distal of the intergenic promoter [127]. It was reported that a different transcription factor, LRH-1, binds to the intergenic promoter [129]. We, therefore, treated Caco-2 cells with an LRH-1 agonist (DLPC) [530], which, however, did not lead to a consistent increase in ABCG5 protein expression either. The application of antibodies targeting both partners in this heterodimer, i.e. ABCG5 and ABCG8, also did not improve the results. As ABCG5/G8 are glycosylated proteins, sugar residues were cleaved by treating the samples with the endoglycosidase PNGase F, but this also did not overcome the obstacles. Studies addressing ABCG5/G8 protein expression stem mainly from *in vivo* experiments in mice. Only a few studies were found using Caco-2 cells, but most of these lack suitable positive controls, i.e. LXR agonists. To the best of our knowledge, we found only two studies that included positive controls [513, 531], but unfortunately did not mention where the Caco-2 cells were obtained from. Moreover, these studies did not differentiate Caco-2 cells on filter inserts, which could account for a different outcome and makes it difficult to directly compare their results to ours.

In intestinal cells, activation of PPARβ/δ leads to a downregulation of NPC1L1 [151, 152]. We, therefore, treated cells with the synthetic PPARβ/δ agonist GW0742 and determined NPC1L1 protein levels by Western blot analysis. Unfortunately, the obtained data were still not convincing. Like for ABCG5/G8, studies showing Western blot analysis of NPC1L1 originate mainly from experiments in mice and the few studies performed with Caco-2 cells did in most cases not include appropriate positive controls. Concerning NPC1L1, two studies were found that included positive controls [531, 532], but again the origin of the Caco-2 cells was not given and cells were not cultivated on filter inserts.

In regard to ABCB1, valproic acid was applied as an alternative positive control, as valproic acid was reported to increase ABCB1 expression *via* CAR [187]. Valproic acid, however, did not yield consistent and convincing results either.

These results made us switch to CLSM, as shown in Figure 4.6. With CLSM, detection of all isoforms at once is possible without separation *via* gel electrophoresis, saving the trouble of cleaving sugar residues by an enzymatic reaction. Moreover, specific software tools allow a semi-quantitative analysis of the detected fluorescence.

For RNA extraction, we first used a commercially available kit (peqGOLD total RNA kit), which, however, only yielded RNA with poor quality, which is probably due to the material of the filter inserts interfering with this extraction method. For that reason, we switched to RNA extraction with the TRIzol reagent. With this reagent, RNA extraction and detection of mRNA levels of ABCA1 and ABCB1 can easily be done and shows consistent results (Figures 4.7A and D). Identifying the best time point for detecting ABCG5 and NPC1L1 up- and downregulation, respectively, is more challenging. The induction of ABCG5 provided by treatment with GW3965 was generally low. A general trend towards upregulation was visible, but most of the time points did not reach statistical significance. Similar to Western blot analyses, the few studies addressing ABCG5/G8 mRNA expression in Caco-2 cells do not include positive controls in most cases. One study that included positive controls [513], did not state the source of the Caco-2 cells and cultivated cells without filter inserts. Importantly, intestinal cells, including Caco-2 cells, were reported to express several important clock genes [533–537], and are therefore subjected to a circadian rhythm. In the liver, a circadian regulation of ABCG5/G8 expression has already been shown [538], which will likely also be the case for AGCG5/G8 expression in the intestine. This circadian regulation could explain the high variability in ABCG5/G8 mRNA expression that we detected.

In contrast to already published work [154], treatment with the LXR agonist GW3965 did not lead to a downregulation of NPC1L1 mRNA expression in our hands at any of the time points investigated (Figure 4.7C). One possible reason for the discrepancy between our and the published study is the usage of different Caco-2 clones/strains. As already mentioned, the expression of coactivators and corepressors differs between different cell types, which can have an influence on the regulation of gene expression provided by nuclear receptors. We thus cannot exclude that the expression of coregulatory proteins in the Caco-2 strain we used is different from the Caco-2/TC7 clone used by Duval et al. [154]. Nevertheless, treatment with bexarotene for 6 h significantly decreased NPC1L1 mRNA expression. The exact mechanisms of how NPC1L1 expression is regulated by nuclear receptors, including LXR, are still not clear. Not many studies exist measuring NPC1L1 mRNA levels in Caco-2 cells, as most studies were performed in mice. Again, most of

the studies that were carried out in Caco-2 cells lack appropriate positive controls. From the five studies we found that included positive controls [154, 531, 532, 539, 540], only the study by Duval et al. [154] cultivated and differentiated Caco-2 cells on filter inserts. Notably, NPC1L1 expression is also subjected to a circadian regulation [541, 542], which could be the reason for the high variability that we observed.

Despite the obstacles we experienced, it still seems more appropriate to differentiate Caco-2 cells on filter inserts also for protein and mRNA expression analyses of cholesterol transporters, since it closer resembles the physiological situation and the conditions of the two functional assays.

Interestingly, cholesterol uptake was not decreased by bexarotene treatment, showing that bexarotene is not suitable as positive control in this assay. Moreover, neither ABCA1 protein nor ABCA1 mRNA levels were increased by bexarotene. ABCA1 mRNA expression was even decreased. This is in contrast with findings in other cell types, such as macrophages, brain-like endothelial cells and glial cells [436, 520, 521]. It was reported, however, that NPC1L1 and ABCA1 mRNA expression were reduced in the jejunum and duodenum of bexarotene-treated mice [436], which supports our findings and underscores the plausibility that bexarotene acts in a tissue- and gene-specific manner.

Finally, we also established viability assays with Caco-2 cells differentiated on filter inserts. To the best of our knowledge, no protocol is currently available for a resazurin conversion assay and a subsequent crystal violet staining with Caco-2 cells cultivated on filter inserts. We show here that both resazurin conversion assay and crystal violet staining are feasible with Caco-2 cells on filter inserts. However, care has to be taken not to detach still viable and adherent cells during the washing steps after crystal violet staining. Since most of the assays are carried out with Caco-2 cells cultivated on filter inserts, it seems more appropriate to also conduct the viability assays with cells on inserts.

4.2 Soraphen A enhances macrophage cholesterol efflux *via* indirect LXR activation and ABCA1 upregulation

In this section, the effect of the polyketide soraphen A on cholesterol homeostasis in macrophages is described. Further, the underlying mechanisms are investigated and explained by the results obtained.

An exhausting amount of data in regard to soraphen A was provided by Dr. Dongdong Wang, who performed parts of his PhD work and post-doctoral work in our group. Many of the results described here rely on the work he performed and are linked to the results he produced. Therefore, his results will not only be mentioned but also shown in some cases, which is then indicated in the corresponding figure legend.

Most of the work and results described in this section are part of a manuscript entitled "Soraphen A enhances macrophage cholesterol efflux *via* indirect LXR activation and ABCA1 upregulation", which is in preparation for publication in the Journal *Biochemical Pharmacology*.

4.2.1 Soraphen A reduces total neutral lipid content in THP-1-derived macrophage foam cells

Soraphen A is a known ACC inhibitor [470], thereby affecting fatty acid metabolism [471, 473]. Lipid metabolism plays an important role in macrophages, since a dysbalance can facilitate the formation of foam cells. Therefore, we decided to investigate the effect of the natural compound soraphen A on total neutral lipid content in macrophage foam cells *via* Oil Red O staining. As shown in Figure 4.9, soraphen A (0.1 μM) significantly reduced total neutral lipids in macrophage foam cells. Another ACC inhibitor, ND-630 (0.5 μM), which was used as control, also significantly reduced total neutral lipids.

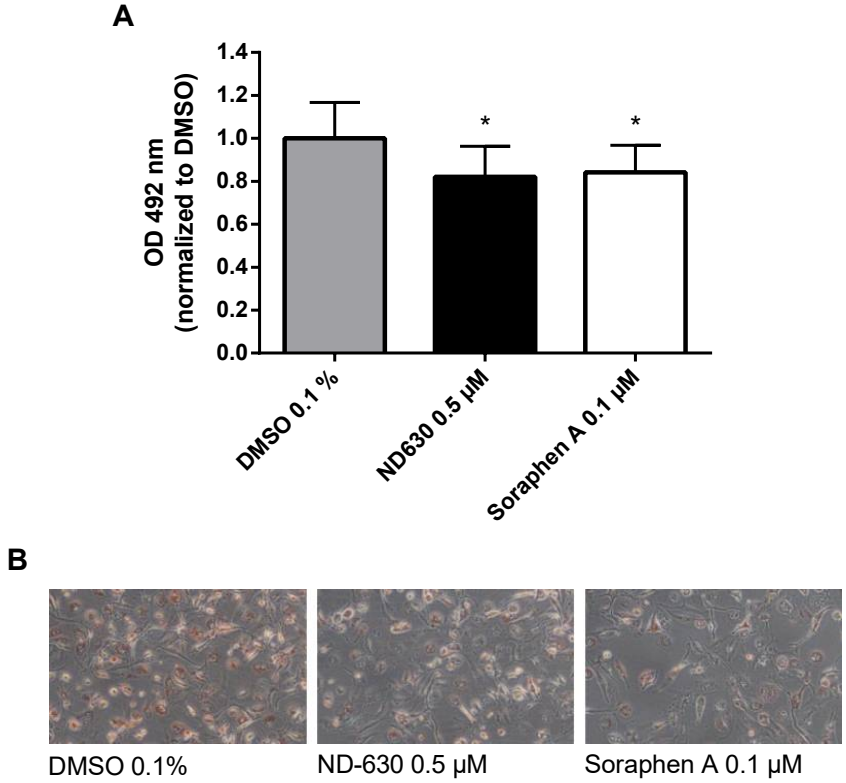

Figure 4.9: Soraphen A reduces total neutral lipid content in THP-1-derived macrophage foam cells. THP-1 cells were differentiated for 72 h with 200 nmol/L PMA and then loaded with cholesterol for 24 h. Cells were treated with soraphen A (0.1 μM), ND-630 (ACC inhibitor, 0.5 μM) or the solvent vehicle (0.1 % DMSO) for another 24 h. Cells were then fixed and stained. Oil Red O was eluted and OD was measured at 492 nm and set in relation to the vehicle control **(A)**. The bar graphs represent mean ± SD of three to four independent experiments each performed in duplicate. $^*P < 0.05$ versus solvent vehicle (determined by paired t-test). Representative images are shown in **(B)**.

4.2.2 Soraphen A increases cholesterol efflux from macrophage foam cells mainly *via* ABCA1

Work performed by Dr. Dongdong Wang demonstrated that cholesterol efflux from macrophages was increased upon treatment with soraphen A, both when mediated by the addition of 1% human plasma as well as by the addition of 10 μg/ml apoA1. It was further shown that among the cholesterol transporters relevant in cholesterol efflux from macrophages [543], only the protein levels of ABCA1 were increased, whereas ABCG1 and SR-B1 protein levels were not influenced by soraphen A treatment in macrophage foam cells. It was therefore tempting to speculate that the increase in cholesterol efflux provided by soraphen A is dependent on the ABCA1 transporter, since the expression of the other cholesterol transporters was not affected by soraphen A. To clarify if the increase in

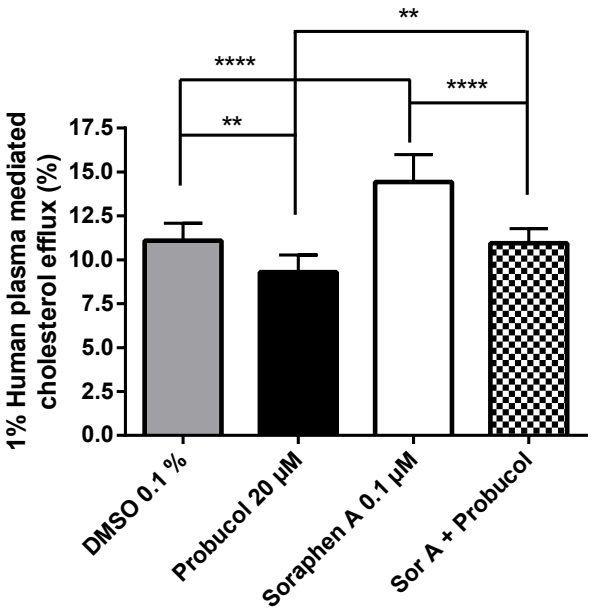

Figure 4.10: Soraphen A increases cholesterol efflux from macrophage foam cells mainly *via* ABCA1. THP-1 cells were differentiated for 72 h with 200 nmol/L PMA and then loaded with cholesterol and radioactive cholesterol tracer ([^{3}H]-cholesterol) for 24 h. Cells were treated with soraphen A (0.1 μM), probucol (ABCA1 inhibitor, 20 μM), soraphen A and probucol (0.1 μM and 20 μM, respectively) or the solvent vehicle (0.1 % DMSO) for another 24 h and cholesterol efflux was determined. The bar graphs represent mean ± SD of five independent experiments. $^{**}P < 0.01$, $^{****}P < 0.0001$ (determined by two-way ANOVA with Bonferroni post-test).

cholesterol efflux is indeed mediated *via* ABCA1, soraphen A was co-applied with probucol, which inhibits ABCA1 membrane localization and ABCA1-mediated transport [497], and cholesterol efflux was again measured. Co-treatment of macrophages with soraphen A (0.1 μM) and probucol (20 μM) decreased cholesterol efflux to the basal, i.e. DMSO level (Figure 4.10). These results indicate that the increase in cholesterol efflux provided by soraphen A is mainly mediated *via* ABCA1.

4.2.3 Soraphen A increases ABCA1 expression by regulation of nuclear receptor activity

Results from experiments by Dr. Dongdong Wang indicated that in addition to ABCA1 protein levels also mRNA levels of this transporter were increased by soraphen A treatment. Moreover, it was shown that ABCA1 protein stability is enhanced upon treatment with soraphen A, whereas there was no influence of soraphen A on ABCA1 mRNA stability

(data not shown). These data suggest a transcriptional as well as a post-translational regulation of ABCA1 expression by soraphen A.

ABCA1 gene expression is increased by the activation of the nuclear receptors LXR, RXR and PPARγ [4]. To support our hypothesis that nuclear receptors are involved in the effect of soraphen A, luciferase reporter gene assays in HEK293 cells were performed by Dr. Dongdong Wang. He found that both LXRα and LXRβ are activated in a concentration-dependent manner upon soraphen A treatment (Figure 4.11). RXRα activity was not significantly influenced by soraphen A, whereas PPARγ activity was decreased (data not shown).

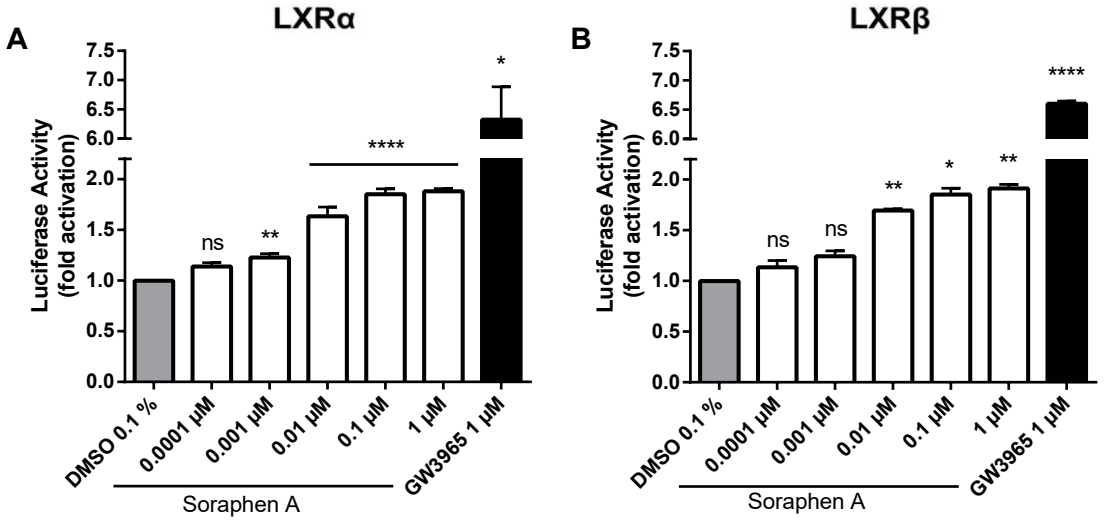

Figure 4.11: Soraphen A increases ABCA1 expression by regulation of nuclear receptor activity. Soraphen A increases activities of both LXRα **(A)** and LXRβ **(B)**. HEK293 cells were transiently co-transfected with a plasmid encoding full-length human LXRα or LXRβ, a reporter plasmid containing ABCA1_Luc, and an EGFP expression plasmid as internal control. Cells were treated with different concentrations of soraphen A (0.0001 - 1 μM), the positive control (GW3965, 1 μM) or the solvent vehicle (0.1% DMSO) for 18 h. The luminescence was normalized to the fluorescence, and the results expressed as fold induction compared to the solvent vehicle. Bar graphs represent mean ± SD of three independent experiments each performed in quadruplicate. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test or paired t-test). Figure kindly provided by Dr. Dongdong Wang.

To examine whether the effects on nuclear receptors occur through their ligand binding domain, luciferase reporter gene assays using the Gal4-UAS system were performed in HEK293 cells. LXRβGal4 activity was increased by soraphen A in a concentration-dependent manner (Figure 4.12B). Moreover, the activities of RXRαGal4 and PPARγGal4 were concentration-dependently decreased by treatment with soraphen A (Figure 4.13A and

C). In regard to activation of LXRαGal4 and RXRβGal4, results are not consistent among the different concentrations applied. Only one concentration of soraphen A (0.0001 μM) significantly increased LXRαGal4 activity (Figure 4.12A), and RXRβGal4 activity was only decreased significantly by one concentration (0.1 μM, Figure 4.13B). Despite being significant, the effects at these concentrations are very minor, indicating overall no relevant effect on LXRαGal4 and RXRβGal4.

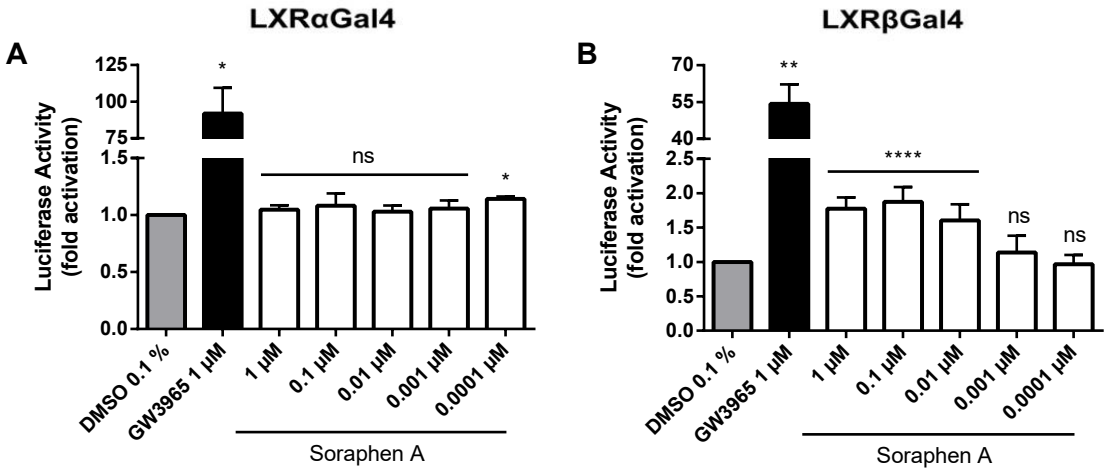

Figure 4.12: Soraphen A concentration-dependently increases LXRβGal4 activity (B), but not LXRαGal4 activity (A). HEK293 cells were transiently co-transfected with an expression plasmid encoding LXRα/βGal4, the reporter plasmid UAS_Luc, and an EGFP expression plasmid, which served as an internal control. Cells were treated with different concentrations of soraphen A (0.0001 - 1 μM), GW3965 (LXR agonist, 1 μM), or the solvent vehicle (0.1% DMSO). The luminescence was normalized to the fluorescence, and the results are expressed as fold induction compared to the solvent vehicle. Bar graphs represent mean ± SD of three independent experiments each performed in quadruplicate. ns not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test or paired t-test).

To investigate whether it is soraphen A itself that binds to the LXRβ-LBD, Dr. Dongdong Wang performed a TR-FRET LXRβ Coactivator Assay. Soraphen A did not induce coactivator recruitment in this assay, making it rather unlikely that soraphen A itself binds to the LXRβ-LBD (Figure 4.14). In contrast, the synthetic LXR agonist T0901317 led to a concentration-dependent increase in the recruitment of the coactivator, indicating a binding to the LBD of LXRβ.

Taken together, these results suggest that the soraphen A-increased activities of LXRα and LXRβ are mediated indirectly, since soraphen A itself seems not to bind to the LXRβ-LBD. Nonetheless, results of the LXRβGal4 luciferase assay indicate that the activation of LXRβ occurs through its ligand binding domain.

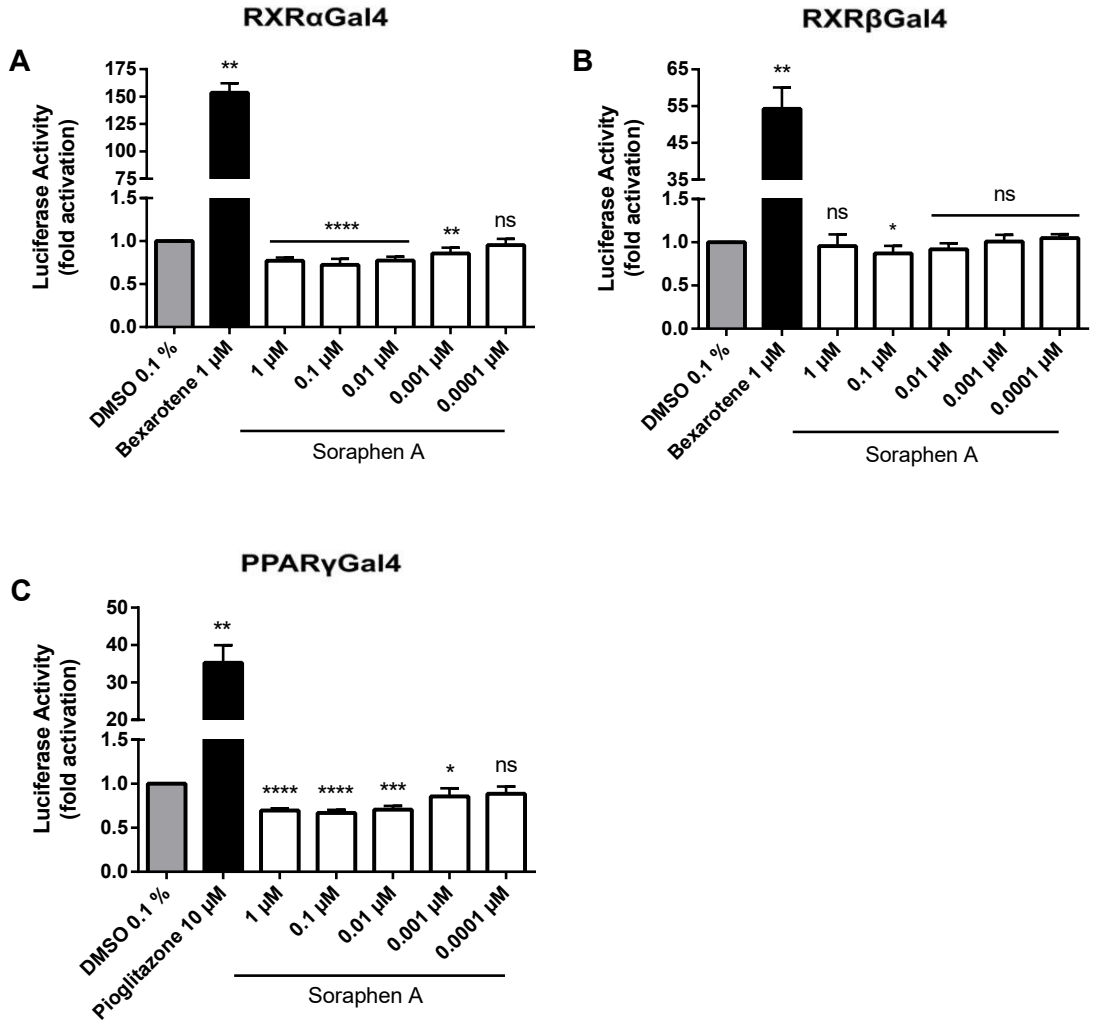

Figure 4.13: Soraphen A decreases RXRαGal4 (A) and PPARγGal4 (C) activities in a concentration-dependent manner, whereas it decreases RXRβGal4 (B) activity only at one concentration tested. HEK293 cells were transiently co-transfected with an expression plasmid encoding RXRα/βGal4, or PPARγGal4, the reporter plasmid UAS_Luc, and an EGFP expression plasmid, which served as an internal control. Cells were treated with different concentrations of soraphen A (0.0001 - 1 μM), bexarotene (RXR agonist, 1 μM), pioglitazone (PPARγ agonist, 10 μM), or the solvent vehicle (0.1% DMSO). The luminescence was normalized to the fluorescence, and the results are expressed as fold induction compared to the solvent vehicle. Bar graphs represent mean ± SD of three independent experiments each performed in quadruplicate. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test or paired t-test).

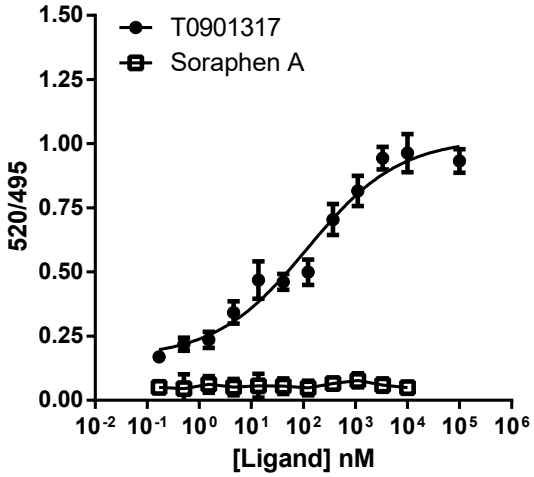

Figure 4.14: Soraphen A shows no binding activity at the LXRβ-LBD in a TR-FRET LXRβ Coactivator Assay. The TR-FRET Coactivator Assay was performed according to the manufacturer's instructions. T0901317 (0.0001 - 100 μM) was used as positive control. Soraphen A was tested in this assay at concentrations ranging from 0.0001 - 10 μM. The data points represent mean ± SD of three independent experiments. Figure kindly provided by Dr. Dongdong Wang.

4.2.4 Soraphen A-mediated activation of LXRα and LXRβ occurs indirectly by an increase in free cholesterol levels possibly *via* inhibition of fatty acid synthesis

4.2.4.1 Soraphen A increases the ratio of free to total cholesterol in THP-1-derived macrophage foam cells

Aiming to conciliate the weak binding of soraphen A to LXR-LBDs in the cell-based assays with the deficient coactivator recruitment in the TR-FRET assay, we hypothesized that soraphen A treatment leads to an increase in cellular cholesterol metabolites that act as endogenous low affinity LXR ligands. Soraphen A inhibits ACCs, leading to decreased levels of malonyl-CoA, and thereby inhibits *de novo* lipogenesis and increases fatty acid β-oxidation [470, 473]. This could lead to a reduced level of free fatty acids, which could in turn affect the esterification of cholesterol and thereby levels of free cholesterol in the cell. An increased level of free cholesterol could not only lead to the increased cholesterol efflux we observed, but could also account for the production of cholesterol metabolites mediating the activation of LXRα and LXRβ provided by soraphen A treatment. To test our hypothesis that soraphen A increases free cholesterol levels in macrophages, we measured both free and total cholesterol levels in macrophages that were treated for 24 h with soraphen A by means of the Amplex™ Red Cholesterol Assay Kit. As shown in

Figure 4.15, soraphen A indeed significantly increased the ratio of free to total cholesterol at a concentration of 0.1 μM.

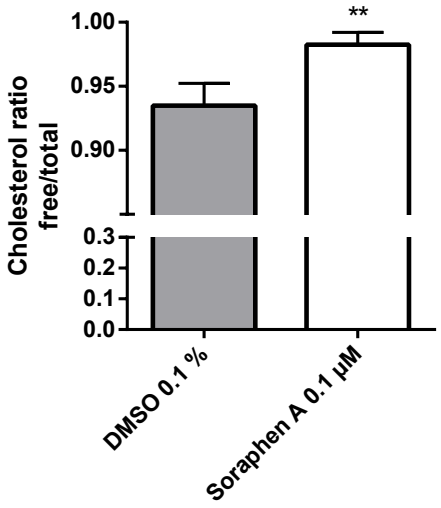

Figure 4.15: Soraphen A increases the ratio of free to total cholesterol in THP-1-derived macrophage foam cells. THP-1 cells were differentiated and loaded with cholesterol as described in Figure 4.9. Cells were treated with soraphen A (0.1 μM) or the solvent vehicle (0.1 % DMSO) for another 24 h. Free and total cholesterol levels were then determined with the Amplex™ Red Cholesterol Assay Kit according to the manufacturer's instructions. The bar graphs represent mean ± SD of four independent experiments. $^{**}P < 0.01$ versus solvent vehicle (determined by paired t-test). Figure obtained with technical assistance of Daniel Schachner.

4.2.4.2 The soraphen A-mediated activation of LXRα and LXRβ is significantly reduced upon the addition of different fatty acids in HEK293 cells

It was reported that soraphen A inhibits the formation of several different fatty acids, among them arachidonic acid (AA) and oleic acid (OA) [473]. We next evaluated whether the exogenous addition of fatty acids can overcome the effects of soraphen A on LXR activation. We, therefore, repeated the luciferase reporter gene assays on LXRα and LXRβ, co-applying soraphen A (0.1 μM) and either AA, eicosapentaenoic acid (EPA), or OA, each at 50 μM. Regarding LXRα, AA and EPA significantly reduced the activation provided by soraphen A (Figure 4.16A). However, this was not true for OA. In regard to LXRβ, again AA and EPA abolished the soraphen A-induced activation (Figure 4.16B). Also OA moderately, but significantly, decreased LXRβ activity induced by soraphen A treatment.

These results suggest that soraphen A causes an activation of LXRα and LXRβ by depleting cells from fatty acids with a subsequent rise in free cholesterol and potential ligands for LXR.

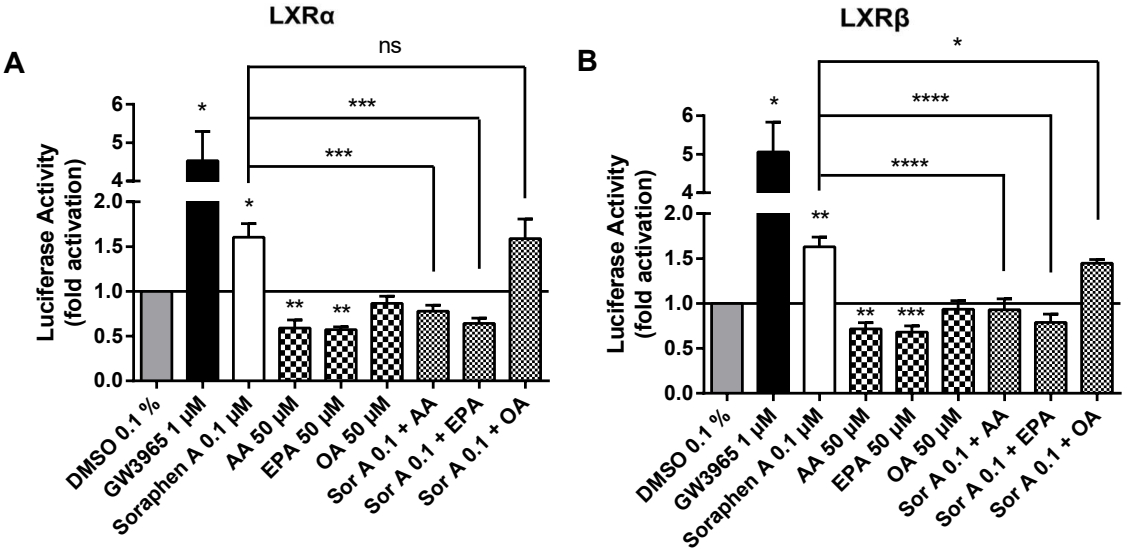

Figure 4.16: The soraphen A-mediated activation of LXRα and LXRβ is significantly reduced upon the addition of different fatty acids. HEK293 cells were transiently co-transfected with a plasmid encoding full-length human LXRα/β, a reporter plasmid containing ABCA1_Luc and an EGFP expression plasmid as internal control. Cells were then treated with soraphen A (0.1 μM), arachidonic acid (AA), eicosapentaenoic acid (EPA), or oleic acid (OA) (each at 50 μM), soraphen A (0.1 μM) and arachidonic acid/eicosapentaenoic acid/oleic acid (each at 50 μM), GW3965 (positive control, 1 μM), or the solvent vehicle (0.1% DMSO) for 18 h. The luminescence was normalized to the fluorescence, and the results are expressed as fold induction compared to the solvent vehicle. Bar graphs represent mean ± SD of three independent experiments each performed in quadruplicate. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus solvent vehicle or soraphen A (determined by two-way ANOVA with Bonferroni post-test or paired t-test).

4.2.5 Discussion

In this study, we demonstrated that soraphen A reduces total neutral lipid accumulation in macrophages and increases cholesterol efflux *via* the ABCA1 transporter. We could show that the increase in ABCA1 expression is mediated at both the transcriptional and post-translational level, since both the protein stability was enhanced in the presence of soraphen A and ABCA1 mRNA levels were increased. It was found in a previous study that fatty acids released from lipoprotein hydrolysis can decrease the mRNA levels of ABCA1 and ABCG1 and concomitantly the efflux of cholesterol [544]. Moreover, it was

reported that high expression levels of ABCA1 are related to reduced fatty acid synthase and ACC1 mRNA expression [545]. These data support our finding that ACC inhibition and subsequent inhibition of fatty acid synthesis by soraphen A leads to increased levels of ABCA1 and enhanced cholesterol efflux.

We found that soraphen A moderatley increases both LXRα and LXRβ transcriptional activity, which we concluded to be an indirect effect since soraphen A did not recruit coactivators in a TR-FRET LXRβ coactivator recruitment assay, indicating that it does not bind to the LXRβ-LBD in a cell-free setting. ACC inhibition can lead to reduced levels of fatty acids, due to inhibition of fatty acid synthesis and increased fatty acid oxidation, and to higher levels of free cholesterol [546]. The latter is due to increased usage of acetyl-CoA for *de novo* cholesterol synthesis or due to less esterification with fatty acids. Enhanced free cholesterol levels could give rise to potential LXR ligands, like oxysterols [292]. Indeed, soraphen A treatment increased free cholesterol levels and the addition of different exogenous fatty acids reversed the soraphen A-mediated activation of both LXRα and LXRβ, further corroborating that alterations in free cholesterol and available fatty acids are the cause of LXR activation by soraphen A.

In mammalian one-hybrid assays, we detected an increase in LXRβGal4 activity, but only a very mild activation of LXRαGal4. It was shown in a previous study that the interaction between the corepressor NCoR and LXRβ is much weaker compared to the interaction with LXRα [547], which could explain the different outcome on LXRαGal4 and LXRβGal4. Moreover, some endogenous ligands have already been identified that differentially regulate the two LXR isotypes, for instance 5,6-24*(S)*,25-diepoxycholesterol [548]. However, to elucidate if and how soraphen A treatment changes the cholesterol metabolite profile, lipidomics analysis would be necessary. A reduction in the conversion of acetyl-CoA to malonyl-CoA could also offer more substrate for histone and non-histone acetylation [549], which could also account for increased LXR activation. Further studies are needed to resolve this issue.

LXR activation by e.g. the synthetic agonist GW3965 was shown to induce hepatic lipogenesis, leading to liver steatosis [550–552]. Since soraphen A does not only increase the transcriptional activity of LXRs, but further inhibits fatty acid synthesis, this could help to avoid the development of hepatic steatosis. Indeed, it has been shown that ACC inhibition decreases liver steatosis in both mice and humans [553]. These data indicate that inhibition of ACC and subsequent inhibition of fatty acid synthesis might be a safe approach for enhancing macrophage cholesterol efflux.

Besides the activation of LXRs, we found that soraphen A decreases the activity of PPARγ, RXRα/βGal4 and PPARγGal4. This is in line with the results of a study reporting decreased PPARγ mRNA levels in 3T3-L1 cells upon soraphen A treatment

[474]. As fatty acids are ligands of both PPARγ and RXRs [272], the decreased activity of these nuclear receptors could be explained by reduced levels of endogenous fatty acids upon soraphen A treatment.

In our study, AA and EPA completely reversed the effects of soraphen A on LXR activation, while OA only showed moderate effects, and two reasons could account for this difference. First, AA and EPA are weak LXR antagonists [277] and this could additionally contribute to their effect on LXR activity. Second, soraphen A inhibits the synthesis of very long chain PUFAs, like AA and EPA, derived from linoleate (C18:2, cf. OA C18:1) [473]. It is within the bounds of possibility that soraphen A also inhibits formation of AA and EPA from OA, which could explain the greater potential of AA and EPA to overcome the LXR activation provided by soraphen A.

Several *in vivo* studies have indicated the improvement of risk factors for metabolic diseases by ACC inhibition. Loss of weight, tissue fat reduction, increased insulin sensitivity and improvement of dyslipidemia were reported in ACC2-knockout mice as well as in animals receiving ACC inhibitors [554]. In mice on a high-fat diet, soraphen A treatment reduced plasma total cholesterol and improved insulin sensitivity without changing hepatic LXRα mRNA expression [555]. A different ACC inhibitor, ND-630 (GS-0976), decreased hepatic *de novo* lipogenesis and liver steatosis in patients suffering from nonalcoholic fatty liver disease [556, 557].

In conclusion, we provide here a link of ACC inhibition by soraphen A with increased macrophage cholesterol efflux *via* a previously unknown implication of the LXR-ABCA1 pathway. Whether soraphen A can increase ABCA1 protein expression and reverse cholesterol transport *in vivo* remains to be elucidated.

4.3 The piperine derivative LAU398 exerts beneficial effects on cholesterol homeostasis in macrophages and intestinal cells

A series of 31 piperine derivatives was synthesized by the Vienna University of Technology (group of Univ.-Prof. Dr. Marko D. Mihovilovic). These piperine derivatives were screened on ABCA1 protein expression in THP-1-derived macrophages by Dr. Limei Wang, a former PhD student of our group, who identified the derivative LAU398 as the most potent one. Work described in this section shows the effects of the piperine derivative LAU398 on cholesterol homeostasis in macrophages and intestinal cells. The molecular mechanisms are investigated and the potential implications of TRPVs in the modulation of cholesterol homeostasis provided by LAU398 are discussed.

A manuscript including most of the results described in this section is currently in preparation, with the preliminary title "The piperine derivative LAU398 exerts beneficial effects on macrophage cholesterol homeostasis – implications of TRPV1".

4.3.1 LAU398 increases ABCA1 protein expression in a concentration-dependent manner

Since work performed by Dr. Limei Wang demonstrated that ABCA1 protein expression was increased by the piperine derivative LAU398, it was tested in regard to a concentration-dependent effect on ABCA1 protein expression and compared to the parent compound, piperine.

As shown in Figure 4.17, LAU398 increased ABCA1 protein levels in THP-1-derived macrophage foam cells in a concentration-dependent manner, with a significant effect at 15 μM. At this concentration, the increase in ABCA1 protein expression was more pronounced than with 1 μM of the LXR agonist and positive control GW3965. The parent compound piperine (50 μM) showed a tendency of increasing ABCA1 protein levels, which, however, did not reach statistical significance.

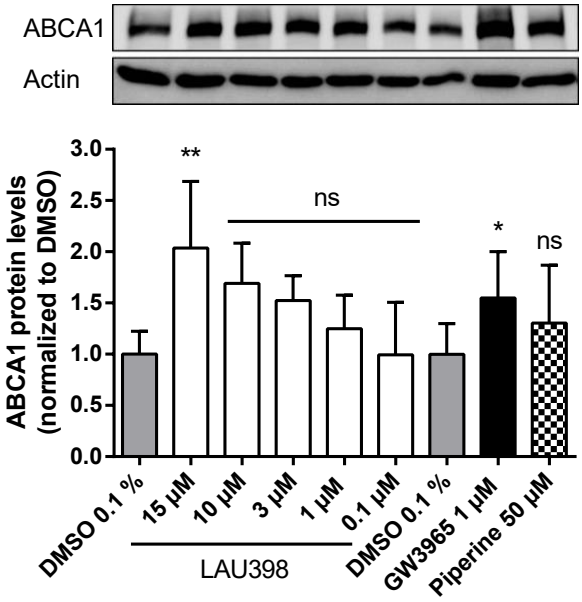

Figure 4.17: The piperine derivative LAU398 increases ABCA1 protein expression. THP-1 cells were differentiated for 72 h with 200 nmol/L PMA and then loaded with cholesterol for 24 h. Cells were treated with increasing concentrations of the piperine derivative LAU398 (0.1 – 15 μM), with GW3965 (LXR agonist and positive control, 1 μM), piperine (50 μM), or the solvent vehicle (0.1 % DMSO) for another 24 h. ABCA1 protein levels were detected by Western blot analysis. Bar graphs represent mean ± SD of four to five independent experiments. A representative blot is shown. Results were normalized for interexperimental variance and then set in relation to the respective DMSO control from the corresponding plate. ns not significant, $^*P < 0.05$, $^{**}P < 0.01$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test or paired t-test). Figure obtained with technical assistance of Scarlet Hummelbrunner.

4.3.2 Cholesterol efflux from THP-1-derived macrophage foam cells is increased by LAU398

Since the ABCA1 protein is one of the major cholesterol efflux transporters in macrophages [543], we tested whether LAU398 can increase cholesterol efflux concomitantly with the increase in ABCA1 protein expression. As shown in Figure 4.18A, LAU398 increases human plasma-mediated cholesterol efflux in a concentration-dependent manner. However, it seems that a plateau is reached from a concentration of 5 μM upwards, as no further increase in cholesterol efflux was observed at higher concentrations. Importantly, LAU398 does not affect cell viability at the concentrations applied in this assay, as shown by resazurin conversion (Figure 4.18B). Only concentrations of 20 μM or higher show a cytotoxic effect to THP-1-derived macrophage foam cells.

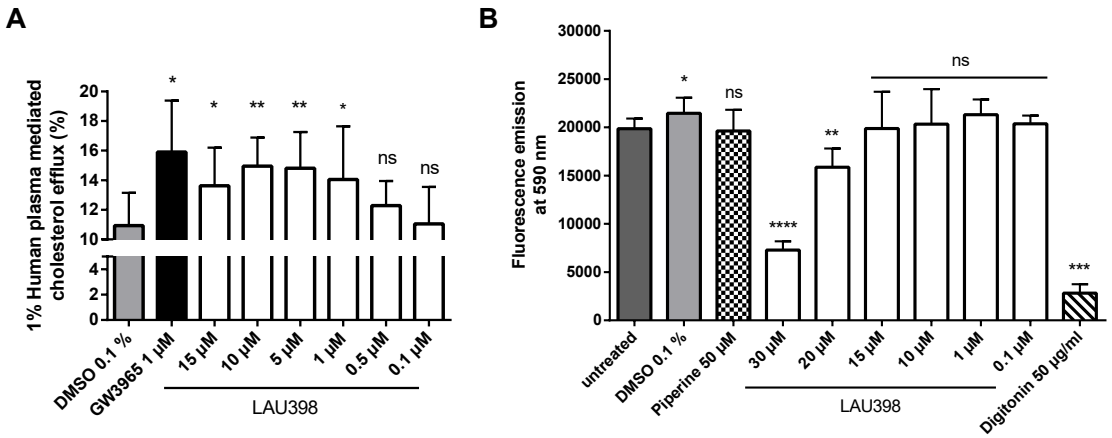

Figure 4.18: Cholesterol efflux from THP-1-derived macrophage foam cells is increased by LAU398 (A), without affecting cell viability (B). **(A)** THP-1 cells were differentiated as described in Figure 4.17. They were then loaded with cholesterol and radioactive cholesterol tracer ([^{3}H]-cholesterol) for 24 h. Cells were treated with the piperine derivative LAU398 (0.1 – 15 μM), GW3965 (LXR agonist, 1 μM), or the solvent vehicle (0.1 % DMSO) for another 24 h and cholesterol efflux was determined. **(B)** LAU398 does not affect cell viability, as determined by a resazurin conversion assay. THP-1 cells were differentiated and loaded as described in Figure 4.17. Cells were either left untreated (negative control) or treated with increasing concentrations of LAU398 (0.1 – 30 μM), piperine (50 μM) or the vehicle control (DMSO 0.1%) for 24 h. Treatment with the positive control digitonin (50 μg/ml) was carried out for 4 h. Cells were then incubated with a dilution of resazurin in PBS (10 μg/ml) for 4 h and fluorescence emission was measured at 590 nm. The bar graphs represent mean ± SD of three independent experiments. ns not significant, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus solvent vehicle (A) or versus untreated (B) (determined by one-way ANOVA with Dunnett post-test or paired t-test). Figure B obtained with technical assistance of Scarlet Hummelbrunner.

4.3.3 LAU398-increased cholesterol efflux from macrophage foam cells is mainly mediated *via* ABCA1

To investigate whether the increase in cholesterol efflux provided by LAU398 is dependent on the increase in ABCA1 protein expression, the cholesterol efflux assay was repeated and the piperine derivative was co-applied with probucol, which inhibits ABCA1-mediated transport [497]. Both concentrations of LAU398 (10 and 3 μM) significantly increased human plasma-mediated cholesterol efflux (Figure 4.19), as already shown in previous experiments. Co-application with probucol (20 μM) reversed the effects of LAU398 on cholesterol efflux, indicating that the LAU398-induced cholesterol efflux is mainly mediated *via* ABCA1.

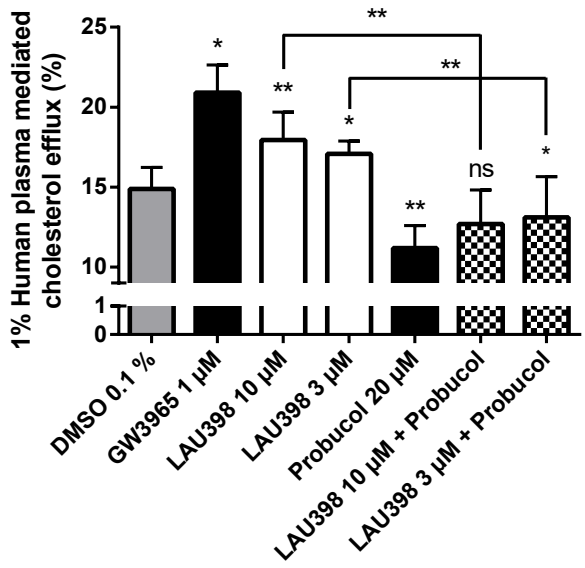

Figure 4.19: LAU398-increased cholesterol efflux from macrophage foam cells is mainly mediated *via* ABCA1. THP-1 cells were differentiated and loaded as described in Figure 4.18A. Cells were treated with the piperine derivative LAU398 (10 and 3 μM), GW3965 (LXR agonist, 1 μM), probucol (ABCA1 inhibitor, 20 μM), LAU398 and probucol (10 or 3 μM and 20 μM, respectively) or the solvent vehicle (0.1 % DMSO) for another 24 h and cholesterol efflux was determined. The bar graphs represent mean ± SD of three independent experiments. ns not significant, $^*P < 0.05$, $^{**}P < 0.01$ (determined by two-way ANOVA with Bonferroni post-test or paired t-test). Figure obtained with technical assistance of Daniel Schachner.

4.3.4 LAU398 does not activate nuclear receptors relevant in lipid homeostasis

Several nuclear receptors regulate the expression of cholesterol transporters in both macrophages and intestinal cells [265, 266, 270]. To test the possibility that LAU398 activates nuclear receptors, luciferase reporter gene assays were performed in HEK293 cells. As shown in Table 4.1, none of the tested nuclear receptors was activated by 10 μM of the piperine derivative LAU398.

Table 4.1: LAU398 does not activate nuclear receptors relevant in lipid homeostasis in HEK293 cells.

Nuclear receptor	LAU398 at 10 μM (fold activation, mean ± SD)	number of experiments
RXRα	1.04 ± 0.95	2
RXRαGal4	1.00 ± 0.36	2
RXRβGal4	0.98 ± 0.28	2
LXRα	0.78 ± 0.02	2
LXRβ	1.12 ± 0.17	2
PPARβ/δ	0.65 ± 0.04	2
PPARγ	1.42 ± 0.18	3
FXRGal4	1.06 ± 0.07	2

It was shown that LAU398 enhances capsaicin-induced currents through TRPV1 channels in *Xenopus laevis* oocytes [487]. We cannot exclude that TRPV1 is implicated in the regulation of cholesterol homeostasis provided by LAU398. Since HEK293 cells do not express TRPV1 channels [558, 559], we repeated the luciferase reporter gene assays regarding RXRα, LXRα and LXRβ activity in Caco-2 cells, which were shown to express TRPV1 channels [560, 561]. Also in Caco-2 cells, we could not detect nuclear receptor activation (Table 4.2).

Table 4.2: LAU398 does not activate RXRα and LXRs in Caco-2 cells.

Nuclear receptor	LAU398 at 10 μM (fold activation, mean ± SD)	number of experiments
RXRα	0.91 ± 0.02	2
LXRα	1.11 ± 0.01	2
LXRβ	1.27 ± 0.01	2
LXRαGal4	1.00 ± 0.06	2
LXRβGal4	0.85 ± 0.03	2

4.3.5 LXRα protein expression is increased in macrophage foam cells upon treatment with LAU398

Since not only the activation of a nuclear receptor is relevant, but also its expression levels, we analyzed LXRα protein expression in THP-1-derived macrophage foam cells upon treatment with LAU398. The positive control GW3965 (LXR agonist, 1 μM) induced a massive increase in LXRα protein expression (Figure 4.20). Treatment with the piperine derivative (0.1 – 15 μM) led to a concentration-dependent increase in LXRα protein levels, with a maximum induction of about 2.5-fold at 10 μM compared to the vehicle control. Likewise, piperine increased LXRα protein expression (about 2.1-fold compared to DMSO), although at a higher concentration (50 μM).

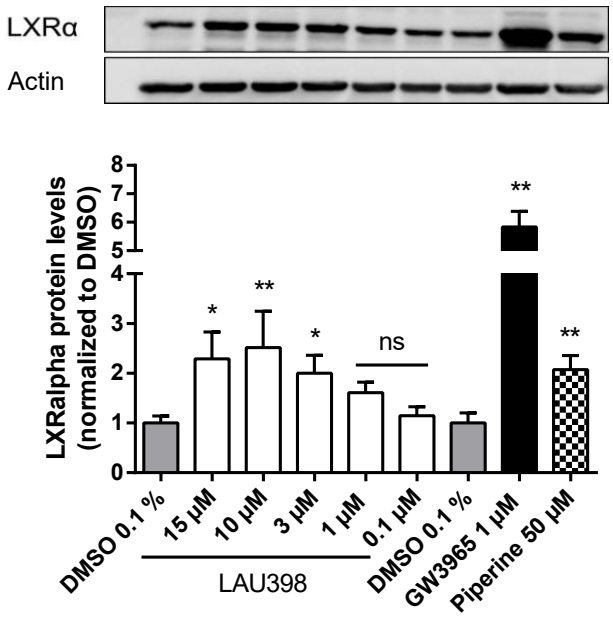

Figure 4.20: LXRα protein expression is increased in macrophage foam cells upon treatment with LAU398. THP-1 cells were differentiated and loaded with cholesterol as described in Figure 4.17. Cells were treated with increasing concentrations of the piperine derivative LAU398 (0.1 – 15 μM), with GW3965 (LXR agonist and positive control, 1 μM), piperine (50 μM), or the solvent vehicle (0.1 % DMSO) for another 24 h. LXRα protein levels were detected by Western blot analysis. Bar graphs represent mean ± SD of three independent experiments. A representative blot is shown. Results were normalized for interexperimental variance and then set in relation to the respective DMSO control from the corresponding plate. ns not significant, $^*P < 0.05$, $^{**}P < 0.01$ versus solvent vehicle (determined by one-way ANOVA with Dunnett post-test or paired t-test). Experiments for this Figure were performed by Scarlet Hummelbrunner.

4.3.6 Cholesterol uptake in Caco-2 cells is decreased by LAU398

Due to the promising effects of the piperine derivative on cholesterol homeostasis in macrophages, we next investigated whether it can have an influence on intestinal cholesterol homeostasis as well. The intestine is a very important interface in cholesterol turnover, and reducing intestinal cholesterol uptake could help to lower plasma cholesterol levels and could be a strategy to prevent atherosclerosis. We, therefore, tested the piperine derivative LAU398 in the cholesterol uptake assay in Caco-2 cells. We treated cells with concentrations ranging from 0.5 to 10 μM and found a concentration-dependent decrease in cholesterol uptake (Figure 4.21A). At 10 μM, LAU398 was as potent as the positive control GW3965 in reducing cholesterol uptake in Caco-2 cells. At this concentration, cell viability was not affected as shown by both a resazurin conversion assay and a subsequent crystal violet staining with Caco-2 cells cultivated on filter inserts (Figure 4.21B and C).

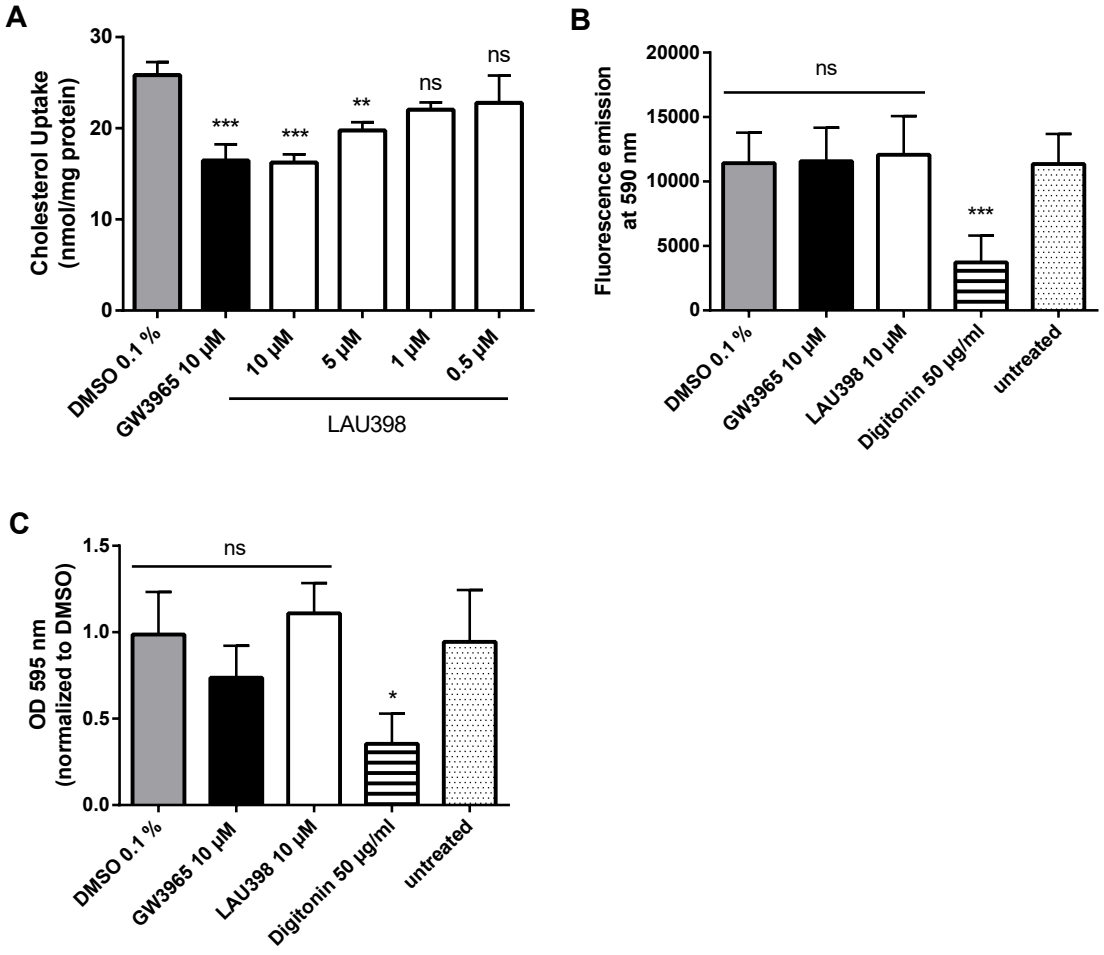

Figure 4.21: Cholesterol uptake in Caco-2 cells is decreased by LAU398 (A), without affecting cell viability (B and C). (A) Caco-2 cells cultivated on filter inserts were treated for 48 h with GW3965 (positive control, 10 μM), increasing concentrations of LAU398 (0.5 – 10 μM) or the vehicle control (DMSO 0.1%). Cholesterol uptake was then determined by liquid scintillation counting. Protein concentration was measured and the results expressed as nmol cholesterol/mg protein before being normalized for interexperimental variance. **(B)** Caco-2 cells cultivated on filter inserts were either left untreated (negative control) or were treated for 48 h with GW3965 (LXR agonist, 10 μM), LAU398 (10 μM), digitonin (positive control, 50 μg/ml) or the vehicle control (DMSO 0.1%). Fluorescence emission after resazurin incubation was measured in the apical medium. **(C)** The inserts were then incubated with crystal violet solution. Crystal violet was then solubilized and the OD was measured at 595 nm and set in relation to the vehicle control. The bar graphs represent mean ± SD of three independent experiments. ns not significant, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus solvent vehicle (A) or versus untreated (B and C) (determined by paired t-test or one-way ANOVA with Dunnett post-test). Experiments for Figures B and C were performed by Daniel Schachner.

4.3.7 A TRPV1 antagonist and an LXR antagonist cannot reverse cholesterol uptake-reduction mediated by LAU398

As treatment with LAU398 resulted in an increased expression of LXRα and further enhanced capsaicin-induced currents through TRPV1 channels in *Xenopus laevis* oocytes [487], we wanted to test whether antagonists for these two receptors can reverse the effect of LAU398 on cholesterol uptake in Caco-2 cells. For this purpose, we co-applied LAU398 (10 μM) with either the TRPV1 antagonist capsazepine (10 μM) or with the LXR antagonist GSK2033 (10 μM), and assessed cholesterol uptake in differentiated Caco-2 cells (Figure 4.22). Capsazepine did not have an influence on the reduction in cholesterol uptake mediated by LAU398, but surprisingly, we observed a significant reduction in cholesterol uptake by single treatment with capsazepine. Co-treatment with GSK2033 slightly reduced the effects of LAU398 on cholesterol uptake, however, it did not reach statistical significance.

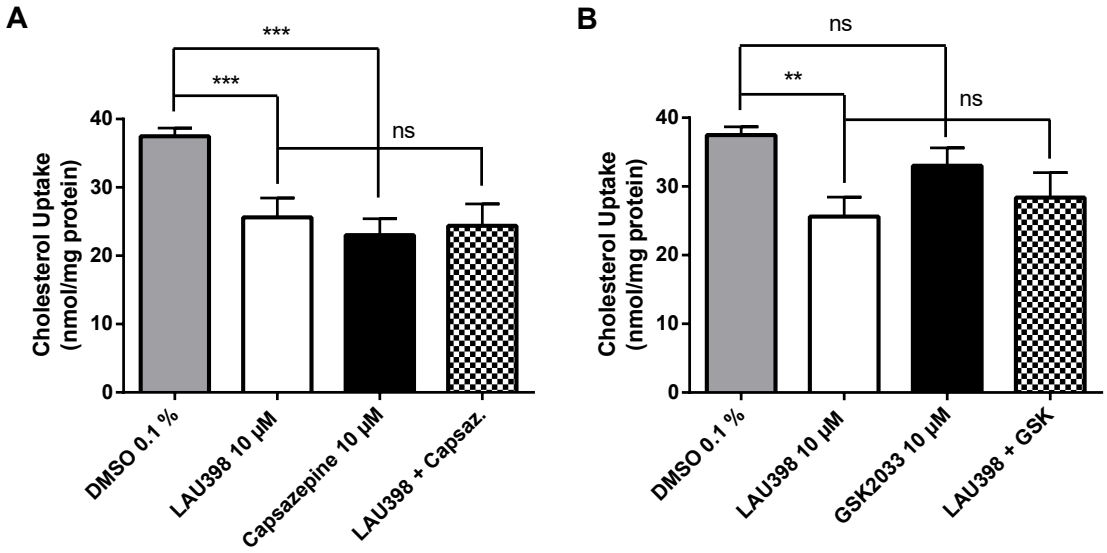

Figure 4.22: LAU398-decreased cholesterol uptake in Caco-2 cells is not reversed by a TRPV1 antagonist (A), or an LXR antagonist (B). Caco-2 cells cultivated on filter inserts were treated for 48 h **(A)** with LAU398 (10 μM), capsazepine (TRPV1 antagonist, 10 μM), co-treated with LAU398 and capsazepine, or with the vehicle control (DMSO 0.1%), or **(B)** with LAU398 (10 μM), GSK2033 (LXR antagonist, 10 μM), co-treated with LAU398 and GSK2033, or with the vehicle control (DMSO 0.1%). Cholesterol uptake was then determined by liquid scintillation counting. Protein concentration was measured and the results expressed as nmol cholesterol/mg protein before being normalized for interexperimental variance. Bar graphs represent mean ± SD of three independent experiments. ns not significant, $^{**}P < 0.01$, $^{***}P < 0.001$ (determined by two-way ANOVA with Bonferroni post-test).

4.3.8 Discussion

In this study, we describe the piperine derivative LAU398 as a modulator of macrophage and intestinal cholesterol homeostasis. We found that LAU398 increases cholesterol efflux from THP-1-derived macrophage foam cells, while at the same time reducing cholesterol uptake in the intestinal Caco-2 cell line. The increase in cholesterol efflux from macrophage foam cells mediated by LAU398 is accompanied by a significant upregulation of ABCA1 protein expression, and we could show that the increased cholesterol efflux is dependent on the ABCA1 transporter. The parent compound piperine slightly upregulated ABCA1 protein levels, without reaching statistical significance, indicating that the piperine derivative LAU398 is more potent than its lead compound. In an already published study using THP-1 macrophages, piperine at 50 μM achieved approximately a 1.5-fold induction of ABCA1, which is in line with our results [479].

ABCA1 expression can be regulated at the transcriptional, post-transcriptional and post-translational level. In regard to transcriptional control, activation of nuclear receptors, including LXR, PPARγ and RXR, was shown to cause an upregulation of ABCA1 [4]. We, therefore, tested a possible activation of nuclear receptors by LAU398 in HEK293 cells, however, none of the nuclear receptors investigated were activated. In a previous study, LAU398 was found to increase capsaicin-induced currents through TRPV1 channels [487], similar to its parent compound piperine. TRPV1 is a cation channel, that is mainly permeable for calcium, but also for sodium. Activation of TRPV1 elicits calcium influx that can lead to membrane depolarization or activate downstream cellular signaling cascades [562, 563]. There are publications implicating the importance of TRPV1 in cholesterol homeostasis *via* affecting nuclear receptors [564]. However, in this study LAU398 could not activate any of the tested nuclear receptors in Caco-2 cells, known to express TRPV1 [560, 561]. Luciferase reporter gene assays in Caco-2 cells for PPARγ are currently in progress. Moreover, PPARα is an important metabolic nuclear receptor that we did not yet examine in regard to activation by LAU398. When we performed luciferase reporter gene assays with PPARα, we realized that the expression plasmid for this nuclear receptor is not functional. Assays will be repeated with a new, functional plasmid to clarify a possible involvement of PPARα in the effects mediated by LAU398.

Nuclear receptor activation regulates the expression of target genes. Nevertheless, target gene expression cannot only be modulated by the activity, but also by changing the expression level of a certain nuclear receptor [565, 566]. In our study, we found that LAU398 significantly increases LXRα protein expression in macrophage foam cells from 3 μM upwards, with a maximum induction of 2.5-fold at 10 μM. The parent compound piperine likewise significantly increased the protein expression of LXRα, achieving 2.1-fold

induction at a higher concentration of 50 μM, indicating greater potency of its derivative LAU398 (Figure 4.20). The finding that piperine increases LXRα protein expression is in contrast to a study in murine hepatocytes, that reported reduced activity and protein expression of LXRα upon treatment with piperine [483]. However, since coregulator expression is different between different cell types, this could account for the different outcome [272]. In a study using bone marrow derived macrophages, it was shown that treatment with two other TRPV1 agonists, capsaicin and evodiamine, raises nuclear levels of LXRα protein, supporting the results of our study. The same study could, however, also show increased LXRα activity upon capsaicin or evodiamine treatment [564], which we could not detect for our piperine derivative in intestinal cells expressing TRPV1. This could either be a different mode of action exerted by LAU398 in comparison to other TRPV1 agonists, or it could be a tissue-specific effect, that is absent in intestinal cells, but present in macrophages. Therefore, luciferase reporter gene assays will also be repeated in THP-1 macrophages, to clarify this issue.

Several other experiments are currently in progress, including the measurement of both LXRα and ABCA1 mRNA levels, as well as the determination of LXRα and ABCA1 protein stability in macrophage foam cells. These experiments can help to elucidate whether the effects of LAU398 on LXRα and ABCA1 expression are occurring at the transcriptional or the post-translational level. Interestingly, it has been shown that increased levels of intracellular calcium can modulate the activity of protein kinases, like protein kinase A, and protein phosphatases, like calcineurin, which are important regulators of post-translational modifications [567–569] and therefore also protein stability. Depending on the outcomes of the above mentioned experiments, the activity of these kinases and phosphatases will also be investigated.

We demonstrated in our study that cholesterol uptake is reduced in LAU398-treated intestinal cells, and that the piperine derivative LAU398 was equally potent as the positive control GW3965 (Figure 4.21). In a previously published study, it was found that also the lead compound piperine reduces cholesterol uptake in Caco-2 cells [570], which would suggest a similar mechanism of action and could stand for an implication of TRPV1 in this effect. Therefore, we investigated whether a TRPV1 antagonist, capsazepine, can overcome the effects on cholesterol uptake provided by LAU398. Capsazepine did not reverse the effects of LAU398 on cholesterol uptake, in contrast, capsazepine itself significantly decreased cholesterol uptake. Since capsazepine is not specific for TRPV1 channels, but also blocks other calcium channels [571], we concluded that this might be an off-target effect. Co-application of the piperine derivative with an LXR antagonist led to a slight, but not significant, reversal of cholesterol uptake inhibition, indicating a partial involvement of LXR in the activities of LAU398.

In conclusion, several further experiments are required to elucidate the exact implications of TRPV1 and LXRα in the effects mediated by the piperine derivative LAU398.

5 Summary and Conclusion

Stroke and myocardial infarction are leading causes of death in industrialized countries. The underlying cause of these complications is atherosclerosis, representing a chronic disease marked by the development of lipid-rich plaques in the arteries [1, 2]. Various treatment strategies have been developed, targeting elevated plasma cholesterol levels that constitute one of the major risk factors for this disease [9–11]. Targeting the rate-limiting enzyme in cholesterol biosynthesis, HMG-CoA reductase, by statins has proven effective in lowering of lipids, however, a residual risk of cardiovascular events remains even in patients under statin treatment [14–16]. Therefore, the quest for new lipid-lowering strategies is still ongoing.

The intestine is an important interface in cholesterol turnover, as highlighted by its role in cholesterol uptake and TICE, making it an attractive target tissue for therapeutic interventions. Furthermore, influencing macrophage cholesterol homeostasis represents an interesting treatment strategy, since the generation of foam cells drives atherosclerotic lesion formation [2].

The nuclear receptors LXR, FXR, PPAR and their heterodimeric partner RXR are crucial regulators of lipid homeostasis. Their target genes include major cholesterol transporters in the intestine and in macrophages, e.g. ABCA1, ABCG1, ABCG5/G8 and NPC1L1, highlighting their great potential as drug targets [277].

The initial work of this study describes the establishment of the human Caco-2 cell line for the use as a model of intestinal cholesterol transport in our laboratory. It shows the suitability of this cell line in studying both intestinal cholesterol uptake and efflux. Except for ABCA1, Western blot analysis appeared largely unsuitable for the detection of protein levels of cholesterol transporters. Nonetheless, we show that CLSM is an appropriate alternative with respect to ABCG5, ABCB1 and NPC1L1. Measuring mRNA expression of cholesterol transporters can also pose some challenges when cells are differentiated on filter inserts. For NPC1L1 and ABCG5, both time point and positive control have to be carefully chosen. In summary, functional assays for measuring intestinal cholesterol transport seem to be more meaningful than the measurement of mRNA and protein levels of cholesterol transporters, since they give an overall impression of how cholesterol handling in intestinal cells is influenced by compound treatment.

The second part of this work describes the effects of the polyketide natural product soraphen A on macrophage cholesterol homeostasis. It was revealed that soraphen A increases cholesterol efflux from macrophage foam cells, which was accompanied by a rise in mRNA and protein levels of ABCA1. Further study demonstrated the dependency of the soraphen A-increased cholesterol efflux on the ABCA1 transporter. Examination of the mechanisms of ABCA1 upregulation revealed an increased transcriptional activity of both LXRα and LXRβ, but also indicated an increased protein stability of ABCA1. Soraphen A is a known ACC inhibitor and thereby inhibits *de novo* fatty acid synthesis and increases fatty acid β-oxidation [470, 473]. Further investigations in our study showed that soraphen A increases levels of free cholesterol in macrophages, which is likely due to the inhibition of fatty acid synthesis. A cell-free assay indicated that soraphen A does not bind to the LXRβ-LBD, suggesting that the activation of LXRα and LXRβ were the result of increased free cholesterol levels and, presumably, subsequently increased levels of cholesterol metabolites acting as low affinity ligands. However, the exact changes in the cholesterol metabolite profile have not been investigated by lipidomics analysis. In summary, we show a previously unknown mechanism for the regulation of macrophage cholesterol efflux by inhibition of ACC with soraphen A, which is depicted in Figure 5.1.

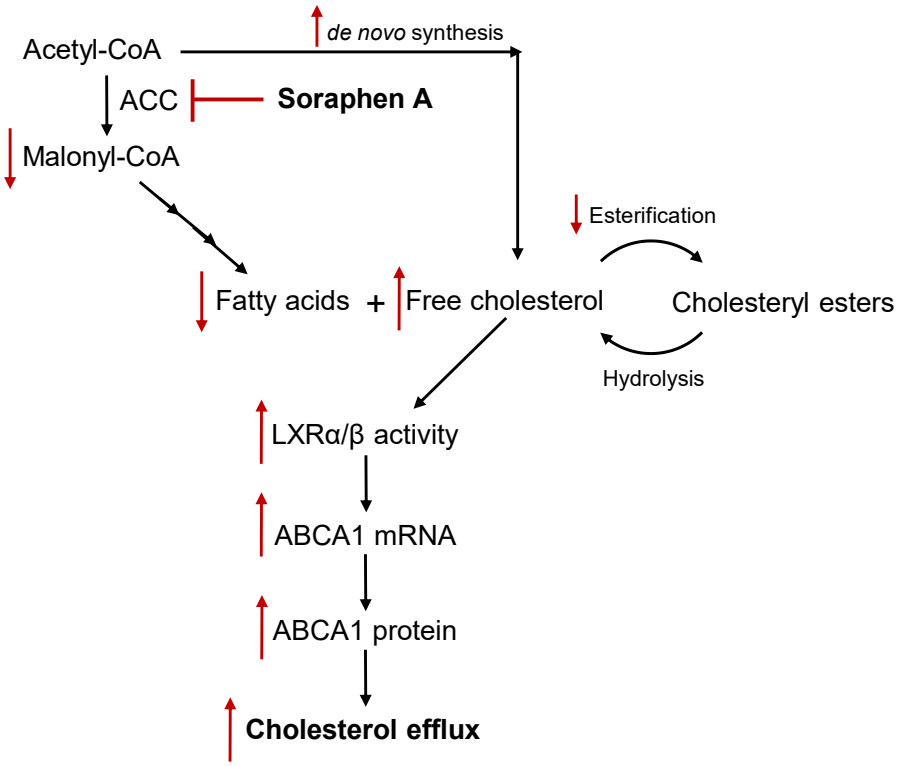

Figure 5.1: ACC inhibition by soraphen A and potential subsequent molecular mechanisms leading to increased cholesterol efflux. For further details, see text.

The final part of this work dealt with the characterization of the piperine derivative LAU398 in regard to cholesterol homeostasis in intestinal cells and macrophages. LAU398 reduced cholesterol uptake in intestinal cells, but increased cholesterol efflux from macrophages, suggesting a favorable effect of this compound on cholesterol homeostasis. Similar effects were previously shown for the lead compound piperine [479, 570]. The enhanced macrophage cholesterol efflux was dependent on an increase in ABCA1 protein expression. LAU398 did not activate nuclear receptors relevant in lipid homeostasis, but significantly increased LXRα protein expression. Notably, this study demonstrated that LAU398 was more potent than the lead compound piperine in regard to ABCA1 and LXRα protein expression. It was shown in a previous study that LAU398 enhances capsaicin-induced currents through TRPV1 channels [487], however, neither co-administration with a TRPV1, nor with an LXR antagonist could reverse the effects of LAU398 on cholesterol uptake. To explore the particular involvement of LXRα and TRPV1, further experiments will be performed, including the measurement of LXRα and ABCA1 protein stability and mRNA levels under application of TRPV1 antagonists. In summary, the piperine derivative LAU398 has a beneficial effect on cholesterol homeostasis in macrophages and intestinal cells, however, the exact implications of LXRα and TRPV1 remain to be clarified.

6 Abbreviations

12/15-LO	12/15-lipoxygenase
3'-UTR	3'-untranslated region
AA	arachidonic acid
ABC	ATP-binding cassette transporter
ACAT	acyl-CoA-cholesterol acyltransferase
ACC	acetyl-CoA carboxylase
ACTH	adrenocorticotropin
AMPK	AMP-activated protein kinase
AP-1	activator protein-1
apo	apolipoprotein
BSA	bovine serum albumin
C/EBPs	CCAAT/enhancer-binding proteins
CA	cholic acid
CAR	constitutive androstane receptor
CD36	scavenger receptor class B type 3
CDCA	chenodeoxycholic acid
CDX2	*caudal*-related Homeobox 2
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CLSM	confocal laser scanning microscopy
CYP7A1	cholesterol 7α-hydroxylase
CYP8B1	cytochrome P450 8B1
DAPI	4',6-diamidine-2'-phenylindole dihydrochloride

DBD	DNA-binding domain
DCA	deoxycholic acid
DLPC	dilauroyl phosphatidylcholine
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethylsulfoxide
DRIP205	vitamin D receptor interacting protein 205
DTT	dithiothreitol
ECD	extracellular domain
ECL	enhanced chemoluminescence
EGFP	enhanced green fluorescent protein
EMEM	Eagle's Minimum Essential Medium
EPA	eicosapentaenoic acid
ER	endoplasmic reticulum
ERK	extracellular signal-regulated protein kinase
FAS	fatty acid synthase
FBS	fetal bovine serum
FC	free cholesterol
FGF19	fibroblast growth factor 19
FGFR4	fibroblast growth factor receptor 4
FoxO1	Forkhead box protein O1
FXR	farnesoid X receptor
HBS	HEPES-buffered saline
HDL	high density lipoprotein
HMG-CoA reductase	3-hydroxy-3-methyl-glutaryl-coenzyme A reductase
HNF4α	hepatocyte nuclear factor 4α
HRP	horseradish peroxidase
HuR	human antigen R
IDOL	inducible degrader of the LDLR

IL-1β	interleukin-1β
LBD	ligand-binding domain
LDL	low density lipoprotein
LDLR	LDL receptor
lncRNA	long noncoding RNA
LPL	lipoprotein lipase
LRH-1	liver receptor homolog-1
LXR	liver X receptor
MDR	multidrug resistance
MED1	mediator complex subunit 1
miRNA	microRNA
MTP	microsomal transfer protein
NBD	nucleotide binding domain
NCoA2	nuclear receptor coactivator 2
NCoR	nuclear receptor corepressor
NEAA	non-essential amino acids
NFκB	nuclear factor kappa B
NPC	Niemann-Pick C1 protein
NPC1L1	Niemann-Pick C1-like protein 1
NR	nuclear receptor
OA	oleic acid
PBP	PPAR-binding protein
PBS	phosphate buffered saline
PDGF	platelet derived growth factor
PDZK1	PDZ-containing kidney protein 1
PET	polyethylene terephthalate
PGC-1α	peroxisome proliferator activated receptor γ coactivator-1α

P-gp	P-glycoprotein
PKC	protein kinase C
PLTP	phospholipid transfer protein
PMA	phorbol-12-myristate-13-acetate
PPAR	peroxisome proliferator activated receptor
PUFA	polyunsaturated fatty acid
PVDF	polyvinylidene difluoride
PXR	pregnane X receptor
RAR	retinoic acid receptor
RBP	RNA-binding protein
RCT	reverse cholesterol transport
RE	response element
RPMI-1640	Roswell Park Memorial Institute
RXR	retinoid X receptor
SCD-1	stearoyl-CoA desaturase-1
SF-1	steroidogenic factor-1
SHP	small heterodimer partner
SIRT1	sirtuin 1
SMC	smooth muscle cell
SR	scavenger receptor
SRC-1	steroid receptor coactivator-1
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
SSD	sterol sensing domain
SUMO	small ubiquitin-like modifier protein
TBST	TRIS-buffered saline with Tween-20
TCF/LEF	T-cell factor/lymphoid enhancer factor
TEER	transepithelial electrical resistance

TICE	transintestinal cholesterol efflux
TMD	transmembrane domain
TNF	tumor necrosis factor
TRAP220	thyroid hormone receptor-associated protein 220
TRPV1	transient receptor potential cation channel subfamily V member 1
UAS	upstream activating sequences
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoprotein
YB-1	Y-box binding protein-1
ZO-1	zonula occludens-1

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8 Appendix

8.1 Conference contributions

Hiebl, V., Wimmer, L., Latkolik, S.L., Ladurner, A., Mihovilovic, M.D., and Dirsch, V.M. The piperine derivative LAU398 reduces cholesterol uptake in Caco-2 cells. *PhytoValley - First Austrian Summit on Natural Products*, Seefeld, Austria, 14 – 16 January, 2018. **Poster presentation**.

Hiebl, V., Wimmer, L., Wang, L., Latkolik, S.L., Ladurner, A., Mihovilovic, M.D., and Dirsch, V.M. The piperine derivative LAU398 reduces cholesterol uptake in a human intestinal cell line. *66th Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA)*, Shanghai, China, 26 - 29 September, 2018. **Oral presentation**.

Hiebl, V., Wang, D., Heiss, E.H., Mueller, R., Atanasov, A.G., and Dirsch, V.M. The polyketide soraphen A exerts beneficial effects on cholesterol homeostasis in macrophages. *67th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research (GA)*, Innsbruck, Austria, 1 - 5 September, 2019. **Poster presentation**.

8.2 Publications

Hiebl, V.*, Ladurner, A.*, Latkolik, S., and Dirsch, V.M. (2018). Natural products as modulators of the nuclear receptors and metabolic sensors LXR, FXR and RXR. *Biotechnol Adv 36*, 1657-1698.

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Hiebl, V., Schachner, D., Hummelbrunner, S., Iorio, M.T., Wang, L., Blazevic, T., Heiss, E.H., Mihovilovic, M.D., and Dirsch, V.M. The piperine derivative LAU398 exerts beneficial effects on macrophage cholesterol homeostasis – implications of TRPV1. Manuscript in preparation, preliminary title, 2020.

*These authors contributed equally.