Regulation of low-density lipoprotein receptor expression by AKT signaling

Katrine Bjune

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the
Unit for Cardiac and Cardiovascular Genetics
Department of Medical Genetics
Oslo University Hospital
“Du har ikke dårlig tid, bare veldig kort tid.”

Soheil Naderi
Abstract

Institute of Clinical Medicine
Faculty of Medicine

Doctor of Philosophy

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by Katrine Bjune

The presence of high blood cholesterol levels, hypercholesterolemia, is a significant contributing factor to the development of coronary heart disease, a condition that is the leading cause of death for both women and men in industrialized countries. Low-density lipoprotein receptor (LDLR) plays a crucial role in the uptake of cholesterol from the blood, and up-regulation of LDLR is therefore a useful strategy for the treatment of hypercholesterolemia. We have investigated the AKT signal pathway, and how inhibition of AKT increases LDLR protein levels and LDL cholesterol uptake by two different mechanisms, by both increasing transcription of the LDLR gene and decreasing the LDLR mRNA decay rate.
Acknowledgements

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Oslo, September 2018

Katrine Bjune
List of Publications


• Bjune, K., Wierød, L., and Naderi, S. "Inhibitors of AKT kinase increase LDL receptor mRNA expression by two different mechanisms". Submitted (2018)
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<tbody>
<tr>
<td>3’UTR</td>
<td>3’untranslated region</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter A1</td>
</tr>
<tr>
<td>ADH</td>
<td>Autosomal dominant hypercholesterolemia</td>
</tr>
<tr>
<td>Apo</td>
<td>Apolipoprotein</td>
</tr>
<tr>
<td>ARE</td>
<td>Adenylate-uridydate rich elements</td>
</tr>
<tr>
<td>ARH</td>
<td>Autosomal recessive hypercholesterolemia</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosin-5’-triphosphate</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>COP</td>
<td>Coat protein</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FP1</td>
<td>Footprint 1</td>
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<tr>
<td>HDL</td>
<td>High-density lipoprotein</td>
</tr>
<tr>
<td>HepG2</td>
<td>Hepatoma cell line G2</td>
</tr>
<tr>
<td>HMG-CoA</td>
<td>3-hydroxy-3-methyl-glutaryl-coenzyme A</td>
</tr>
<tr>
<td>HMGCR</td>
<td>3-hydroxy-3-methyl-glutaryl-coenzyme A reductase</td>
</tr>
<tr>
<td>hnRNP</td>
<td>Heterogeneous nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>IDL</td>
<td>Intermediate-density lipoprotein</td>
</tr>
<tr>
<td>IDOL</td>
<td>Inducible degrader of the LDL receptor</td>
</tr>
<tr>
<td>INSIG</td>
<td>Insulin-induced gene</td>
</tr>
<tr>
<td>KHSRP</td>
<td>KH-type splicing regulatory protein</td>
</tr>
<tr>
<td>LCAT</td>
<td>Lecithin-cholesterol acetyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>LDLR</td>
<td>Low-density lipoprotein receptor</td>
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<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LRP</td>
<td>Low-density lipoprotein receptor-related protein</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>miRNA</td>
<td>Micro RNA</td>
</tr>
<tr>
<td>MTP</td>
<td>Microsomal triglyceride transfer protein</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>---------------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>mTORC</td>
<td>Mechanistic target of rapamycin complex</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>PCSK9</td>
<td>Proprotein convertase subtilisin/kexin type 9</td>
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<td>PDK1</td>
<td>Phosphoinositide-dependent kinase 1</td>
</tr>
<tr>
<td>PH</td>
<td>Pleckstrin homology 1</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>PIP2</td>
<td>PI 4,5 bisphosphate</td>
</tr>
<tr>
<td>PIP3</td>
<td>PI 3,4,5 triphosphate</td>
</tr>
<tr>
<td>PRAS40</td>
<td>Proline-rich AKT substrate of 40 kDa</td>
</tr>
<tr>
<td>RHEB</td>
<td>Ras homolog enriched in brain</td>
</tr>
<tr>
<td>RTK</td>
<td>Receptor tyrosine kinases</td>
</tr>
<tr>
<td>S1P</td>
<td>Site-1 protease</td>
</tr>
<tr>
<td>S2P</td>
<td>Site-2 protease</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage-activating protein</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SIRE</td>
<td>Sterol-independent regulatory element</td>
</tr>
<tr>
<td>SM</td>
<td>Squalene monoxygenase</td>
</tr>
<tr>
<td>SP1</td>
<td>Specificity factor 1</td>
</tr>
<tr>
<td>SR-B1</td>
<td>Scavenger receptor class B type 1</td>
</tr>
<tr>
<td>SRE</td>
<td>Sterol regulatory element</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>TSC</td>
<td>Tuberous sclerosis complex</td>
</tr>
<tr>
<td>TFRC</td>
<td>Transferrin receptor</td>
</tr>
<tr>
<td>Ub</td>
<td>Ubiquitine</td>
</tr>
<tr>
<td>UPR</td>
<td>Unfolded protein response</td>
</tr>
<tr>
<td>VLDL</td>
<td>Very-low density lipoprotein</td>
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</table>
Dedicated to my loving grandfather and father - Thank you for giving me the gift of optimism and positivity …
1 Introduction

1.1 Cholesterol

Cholesterol is a vital sterol synthesized by all animal cells, and it plays a key role in maintaining normal cell structure and function. It is a crucial component of the cell membrane by modulating membrane fluidity, and is an important component of membrane-based signaling systems. In addition to its importance at the cellular level, cholesterol also serves as a precursor for other essential molecules such as vitamin D, bile acids and steroid hormones [1].

1.2 Cholesterol transport

The organism’s need for cholesterol is provided either by diet or by de novo cholesterol synthesis. Humans obtain approximately 30 % of the cholesterol through diet, while the remaining 70 % is synthesized mostly by organs such as the liver, intestine, and skin [2]. Due to its hydrophobic nature, cholesterol has minimal solubility in water and blood. Therefore, cholesterol is transported as part of water-soluble lipoprotein particles. Lipoproteins are made up of an outer layer of polar phospholipids, cholesterol and apolipoproteins and have a hydrophobic core of cholesteryl esters and triglycerides (Figure 1) [3]. Based on increasing density and decreasing size, lipoproteins can be categorized into five major classes. These are: chylomicrons, very-low density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL).

The different lipoproteins represent dynamic structures that are continuously being synthesized, modified and degraded. After synthesis,
FIGURE 1: Lipoprotein particle - The surface of the lipoprotein particle is composed of hydrophilic phospholipids, unesterified cholesterol and lipoprotein-specific apolipoproteins, while the core contains hydrophobic triglycerides and esterified cholesterol. The figure is adapted from Servier Medical Art

The lipoprotein particles are found in plasma, where they constantly exchange lipids and apolipoproteins with cells and other lipoprotein classes. This process trims the different lipoproteins into new classes, ultimately allowing them to undergo cellular uptake and degradation [4, 5]. The lipoprotein metabolism can be divided into four pathways (Figure 2):

- **The exogenous pathway** refers to intestinal absorption of dietary fat and cholesterol and their transport in chylomicrons to the liver.
- **The endogenous pathway** by which the liver produces and secretes triglyceride-rich VLDL to provide peripheral cells with lipids.
• **The reverse cholesterol transport pathway** refers to the process by which excess cholesterol is transported in HDL particles from peripheral cells back to the liver.

• **The enterohepatic circulation** by which cholesterol and bile acids that have been secreted from the liver to the intestine, are partly being re-absorbed in the terminal part of the small intestine and transported back to the liver.

### 1.2.1 The exogenous pathway

This pathway involves the absorption and transport of dietary fat to the liver and starts in the intestine. Here, dietary triglycerides are hydrolyzed into monoglycerides and free fatty acids, while cholesteryl esters are hydrolyzed into free cholesterol and free fatty acids by intestinal lipases and cholesteryl ester hydrolase, respectively [6]. Free cholesterol and other lipids, including monoglycerides and free fatty acids, are then incorporated into micelles which are translocated into intestinal enterocytes by Niemann-Pick C1-like 1 protein and fatty acid binding proteins.

Once inside the enterocytes and localized in the endoplasmic reticulum (ER), monoglycerides and free fatty acids are converted into triglycerides while some of the free cholesterol is re-esterified by acyl-CoA cholesterol acetyltransferase. The biogenesis of chylomicrons takes place in the ER lumen, where nascent Apolipoprotein B48 (APOB48) is enriched with triglycerides by the action of microsomal triglyceride transfer protein (MTP) and added cholesteryl esters, free cholesterol, phospholipids and vitamins A and E [7].

It is the availability of lipids that dictates the chylomicron production. When lipids are in short supply, the newly synthesized APOB48 is rapidly degraded, whereas an adequate pool of lipids allows APOB48 to proceed with formation of chylomicrons. Chylomicrons are then exported out of enterocytes in secretory vesicles for release into lymphatic vessels for further transport to the bloodstream. Once inside the bloodstream, chylomicron particles interact with HDL resulting in the transfer of Apolipoprotein C-II (APOC-II) and Apolipoprotein E (APOE) from HDL to the chylomicrons [8]. APOC-II functions as a cofactor for lipoprotein
lipase (LPL), an enzyme that is located at the surface of the endothelial cells of the blood vessel. LPL hydrolyzes the triglycerides in chylomicrons into glycerol and fatty acids which are then taken up by peripheral tissues, such as skeletal muscle and adipose tissue. The resulting smaller particles are referred to as chylomicron remnants which are rapidly recognized by hepatic APOE-specific receptors and taken up by the liver [8].

1.2.2 The endogenous pathway

The liver plays a pivotal role in the body’s regulation of lipid metabolism. Therefore, it is not surprising that the liver, in addition to its ability to extract lipids from chylomicron remnants, can also produce lipids via de novo synthesis [6]. Regardless of their origin, the liver utilizes triglycerides and cholesterol to synthesize VLDL particles that are released into the bloodstream to provide peripheral cells with lipids. This constitutes the endogenous lipoprotein pathway.

In contrast to the chylomicrons, which contain APOB48, VLDL particles contain APOB100 as their main structural protein. APOB100 and APOB48 are encoded by a single mRNA that is transcribed from the APOB gene [9]. In hepatocytes, this transcript is translated into APOB100, whereas in enterocytes, RNA editing introduces a premature stop codon in the APOB transcript, resulting in a protein that contains the N-terminal 48% of APOB100, hence the name APOB48 [10].

VLDL particles are produced when newly synthesized APOB100 is translocated to the ER and combined with triglycerides and cholesteryl esters by the action of MTP. These lipidated particles are then exported out of the cell into the bloodstream in the same manner as described for chylomicrons. As with the chylomicrons, VLDL also encounters HDL which transfers APOC-II and APOE to VLDL. This allows VLDL to be hydrolyzed by LPL expressed by endothelial cells, a process that converts VLDL into IDL. IDL can either be cleared from plasma through APOE-binding receptors, or it can be further hydrolyzed by hepatic lipase to release glycerol and fatty acids, thus leaving an LDL particle that contains a relatively high amount of cholesterol. Approximately 70% of plasma cholesterol is carried in LDL [3–5]. LDL circulates in the
bloodstream until it is bound and internalized by the LDL receptor (LDLR) either in the liver or in peripheral cells.

1.2.3 The reverse cholesterol transport pathway

Non-hepatic cells cannot dispose of cholesterol. Because the liver is the only organ that can remove cholesterol from the body, a multistep process, referred to as the reverse cholesterol transport, mediates the transfer of surplus cholesterol from peripheral tissues to the liver. A central component of this pathway is HDL that is synthesized mainly by the liver and intestine, and is secreted as a poorly lipidated particle with a high protein content. This native HDL particle consists of Apolipoprotein AI (APOAI), as well as other proteins such as lecithin-cholesterol acyltransferase (LCAT), APOC-II and APOE [8].

Once in the bloodstream, the poorly lipidated HDL exchanges APOC-II and APOE for triglycerides with chylomicrons and VLDL. APOAI on HDL enables the binding of HDL to the ATP-binding cassette transporter A1 (ABCA1) on the cell membrane of peripheral cells. ABCA1 then transports lipids such as phospholipids and unesterified cholesterol out of the cell and onto the HDL particle [11]. The free cholesterol on the HDL particle surface is then converted to cholesteryl ester by LCAT and stored in the HDL core. This process not only enriches the HDL particle with cholesterol but also induces it to adopt a spherical shape. This maturation process is associated with inactivation of LCAT and release of the HDL particles from ABCA1 and the cell membrane [12].

HDL disposes of the cholesteryl esters from peripheral cells by different means. Cholesteryl esters can be either returned to the liver by the action of hepatic scavenger receptor class B type 1 (SR-B1), be exchanged for triglycerides from other lipoproteins such as VLDL, IDL or LDL by a process mediated by cholesteryl ester transfer protein, or be cleared by the kidney after being trimmed by hepatic lipase.
FIGURE 2: Pathways of lipid metabolism - Dietary fat and cholesterol are incorporated into chylomicrons in the exogenous pathway (blue arrows). The chylomicrons are hydrolyzed by LPL on endothelial cells in the blood vessels to form chylomicron remnants, which are rapidly cleared by the liver. In the endogenous pathway (black arrows) the liver provides peripheral cells with lipids by incorporating triglycerides and cholesteryl esters into VLDL. VLDL receives apolipoproteins and additional cholesteryl esters after encountering HDL in the bloodstream. This allows triglycerides in VLDL to be hydrolyzed by LPL. Hydrolysis and removal of triglycerides turn VLDL into IDL. IDL may be further hydrolyzed by hepatic lipase to form LDL, which is internalized by the liver or peripheral cells by LDLR. HDL is a protein-rich lipoprotein that is produced by the liver or intestine and transports excess cholesterol from peripheral cells back to the liver in the reverse cholesterol transport pathway (red arrows). HDL also functions by maturing other lipoproteins by transferring apolipoproteins and exchanging cholesteryl esters for triglycerides in the bloodstream. HDL is ultimately removed from the bloodstream by SR-B1 mediated liver uptake or clearance by the kidneys. The last pathway is the enterohepatic circulation (gray arrow) where bile acids are secreted from the liver and reabsorbed from the small intestine for transport back to the liver. The figure is modified from Servier Medical Art.
1.2.4 The enterohepatic circulation

Cholesterol transported back to the liver from peripheral tissues is either retained for internal use, for instance by incorporation into VLDL, or excreted from the body via bile, either directly or following conversion into bile acids[13]. Bile is produced in the liver and is stored in the gallbladder before it is discharged into the small intestine. Bile consists of approximately 0.7 % bile acids, 0.2 % bilirubin and 0.5 % fat. The bile functions not only in the regulation of cholesterol homeostasis but also has other important physiological functions such as promoting digestion and absorption of dietary fats, especially cholesterol and fat-soluble vitamins, as well as exerting an antimicrobial effect in the gut. However, a large proportion of the bile acids secreted into the intestine are re-absorbed from the terminal part of the small intestine for transport back to the liver. Thus, the enterohepatic circulation is a cyclic process that involves biliary excretion followed by their intestinal reabsorption and delivery back to the liver.

1.3 Atherosclerosis

The transport of cholesterol in the body is a finely tuned process that serves important biological functions. However, cholesterol transported in the blood may also form the basis for a pathological process called atherosclerosis. An early step in this process is the entry of blood cholesterol into the arterial wall, an event that leads to accumulation of cholesterol-loaded macrophages, referred to as foam cells, that are the basis for an atherosclerotic plaque. The ensuing narrowing of the arterial lumen may subsequently lead to occlusion of the arterial lumen by a thrombus, which then will precipitate a myocardial infarction (Figure 3). LDL is the main source of cholesterol deposited in these plaques. Therefore, high levels of LDL cholesterol in the blood constitute the major risk factor for coronary heart disease. Other contributing risk factors for developing atherosclerosis are hypertension, smoking, inflammation and high blood glucose levels [4].

The initiating step in atherogenesis is believed to involve damage to arterial endothelium triggered by one or more of the above-mentioned risk factors. Because damaged endothelium can no longer function
FIGURE 3: The evolution of atherosclerosis - LDL particles entering into the intima of the arterial wall induce an inflammatory process and production of adhesion proteins that attract inflammatory leukocytes such as monocytes. Following recruitment to the artery wall, monocytes differentiate into macrophages that take up modified lipoproteins to become lipid-laden foam cells. This lesion, referred to as a fatty streak, is also characterized by secretion of inflammatory cytokines and growth factors by leukocytes and vascular wall cells that attract more leukocytes and cause migration and proliferation of smooth muscle cells. The intermediate lesion is characterized by the presence of foam cells as well as small and scattered extracellular lipid pools. An increase in the size and number of these lipid pools leads to formation of a lipid core. At this stage, the lesion is referred to as an atheroma. The lipid core is covered by fibrous connective tissue that forms a fibrous cap between the lipid core and the bloodstream. Complicated lesions arise when rupture of the fibrous cap leads to hematoma and/or thrombus formation.

The figure is modified from Servier Medical Art.

optimally, plasma components, including LDL particles, move more readily across the endothelial surface to the subendothelial space (intima). Once in the intima, LDL can become oxidized by reactive oxygen species secreted by damaged cells in the artery wall or macrophages [14]. Due to its cytotoxic effect, oxidized LDL triggers the activation of several repair processes that induce the surface expression of a number of adhesion proteins on the endothelial cells. This in turn promotes the adhesion of leukocytes such as monocytes and T cells, facilitating their movement into the intima. Here, monocytes differentiate into macrophages that remove
oxidized LDL through phagocytosis by the action of scavenger receptors [15]. Although this process serves a beneficial role to remove oxidized LDL, it results in a build-up of foam cells in the intima. Foam cell formation is characteristic of an early stage of atherosclerosis, the so-called fatty streak [4]. The more LDL particles that enter the intima, the more foam cells are produced, and the more severe is the atherosclerotic lesion.

Foam cells have various functions in the atherosclerosis process, including sending out signals in the form of chemokines and growth factors. These not only recruit more leukocytes to the fatty streak lesion but also induce the aberrant proliferation and phenotypic transformation of vascular smooth muscle cells [16]. These vascular smooth muscle cells together with the migrated leukocytes promote further buildup of material in the artery wall. Excess accumulation of cholesterol in macrophages induces death and release of their cellular content, an event that recruits even more leukocytes and further amplifies the inflammatory milieu. This vicious circle promotes plaque formation and further narrowing of the blood vessel.

The necrotic core of the plaque is protected from rupture by a proteoglycan-collagen fibrous cap [17]. Over time, foamy macrophages and other inflammatory mediators degrade the collagen and the extracellular matrix, leading to thinning of the protective cap. This stage of the atherosclerotic process is called fibroatheroma. If the cap ruptures, the necrotic core will be exposed to the bloodstream which initiates coagulation and thrombus formation. The thrombus can clog the artery and prevent blood cells from passing through, leading to a myocardial infarction and potentially death.
1.4 The LDLR

As described above, maintaining plasma LDL cholesterol homeostasis is crucial to prevent the onset of atherosclerosis. This is achieved primarily through the action of the LDLR, a single-pass transplasma membrane protein that interacts with the APOB100 component of LDL, and promotes LDL particle uptake by endocytosis [18]. Approximately 60-70% of all LDLRs in the body are located in the liver, which demonstrates the key role of the liver in lipid metabolism. Individuals who fail to express functional LDLR have a 5-6 fold increase in plasma LDL concentration and typically contract myocardial infarction in their teens or early twenties [19]. Nevertheless, in addition to LDLR, other factors also influence the level of plasma LDL, such as the rate of VLDL synthesis and the activity of lipoprotein lipase.

The LDLR is a highly conserved membrane glycoprotein consisting of 839 amino acids arranged into five domains (Figure 4) [20]. In the N-to C-terminal direction, these domains are:

- The ligand-binding domain
- The epidermal growth factor precursor (EGFP) homology domain
- The O-linked sugar domain
- The transmembrane domain
- The cytoplasmic domain

The ligand-binding domain contains seven repeats of approximately 40 amino acid residues. Each repeat contains six disulfide-bound cysteine residues that enable it to assume a compact conformation, and also has clusters of negatively charged residues that are thought to mediate ligand binding [21]. The seven repeats are separated from each other by a short stretch of linker peptide, a feature that offers conformational flexibility for the ligand binding domain. Adjacent to the ligand-binding domain is the EGFP homology domain. This domain consists of approximately 400 amino acids and exhibits a 35% homology to the EGFP. The EGFP homology domain can be further divided into three growth factor-like repeats and a 280 amino acid long tandem repeat that has a conformation of a six-bladed β-propeller [11]. The O-linked sugar domain is located
immediately outside the plasma membrane and consists of a stretch of 58 amino acids which is rich in serines and threonines that are glycosylated [22]. O-linked sugars are added onto the LDLR during synthesis and maturation in ER and Golgi apparatus, and these are thought to be required for stable expression of LDLR at the cell surface [22, 23]. At the C-terminal end of the O-linked sugar domain is the 22 amino acid long hydrophobic transmembrane domain that anchors the LDLR into the plasma membrane. The cytoplasmic domain of 50 residues contains the NPxY internalization sequence required for the LDLR to concentrate in clathrin-coated pits [22].

There are approximately 15,000 LDLRs exposed on the surface of hepatocytes [18]. The majority of these LDLRs are localized in specialized regions of the cell membrane called clathrin-coated pits which comprise approximately 2% of the plasma membrane. Within the clathrin-coated pit, the NPxY motif on the cytoplasmic tail of LDLR interacts with the autosomal recessive hypercholesterolemia (ARH), a modular adapter protein that connects the LDLR to clathrin [24]. The clathrin-coated pits invaginate and pinch off to form LDLR-containing clathrin-coated vesicles that are released into the cell interior. Within seconds after being formed, these vesicles shed their clathrin coat and merge with other endosomes in a process that is accompanied by acidification of the lumen. The acidic pH of these early endosomes induces a conformational change in the LDLR making it fall back on itself to adopt a closed conformation [25]. This event leads to dissociation of the LDL particle from the receptor, allowing it to be transported down the endosomal track to the lysosomes for degradation, while the receptor is recycled back to the cell surface. The recycling of LDLR from endocytosis back to the cell surface takes between 10 to 15 minutes. Given the observation that LDLR has a lifespan of about 20 hours, it can be estimated that LDLR can be reused nearly 100 times before it is degraded [26].
FIGURE 4: Domain organization of the LDLR - The five domains of LDLR from the N-terminus to the C-terminus are: The ligand-binding domain, characterized by seven cysteine-rich repeats consisting of clusters of negatively charged amino acids that act cooperatively in binding APOB100. The EGFP homology domain consisting of about 400 amino acids has three repeats (A, B and C) and a β-propeller. The O-linked sugar domain consisting of 58 amino acids rich in serine and threonine residues containing O-linked carbohydrates. The transmembrane domain consisting of hydrophobic residues spanning the membrane, and The cytoplasmic domain with its 50 amino acids contains an internalization sequence.
1.4.1 Regulation of LDLR levels and cholesterol homeostasis

Because of its ability to internalize cholesterol into the cells, LDLR plays a vital role in regulation of both the extracellular and intracellular cholesterol pools. Cholesterol taken up by the hepatocytes mediates a complex series of feedback control mechanisms that protect the cell from over-accumulation of cholesterol. These feedback loops regulate cholesterol levels in the cell not only by modulating the synthesis and degradation of the LDLR, but also through control of de novo cholesterol synthesis.

1.4.2 Transcriptional regulation of LDLR and the genes involved in cholesterol synthesis

The LDLR gene and many of the genes that encode enzymes involved in cholesterol biosynthesis are transcriptionally regulated by sterol-regulatory element binding proteins (SREBPs) [27]. This family of transcription factors consists of three isoforms, SREBP-1a, -1c and -2, which are initially translated as inactive precursor proteins anchored in ER membrane [27]. All SREBPs are organized into three well-defined domains oriented in a hairpin formation with the N- and C-termini extending into the cytoplasm [27]. The N-terminal domain contains a helix-loop-helix leucine zipper motif and is the functionally active portion of the SREBP precursor protein. This part is followed by a membrane attachment domain, containing two hydrophobic membrane-spanning segments interrupted by a short hydrophilic loop that extends into the lumen of ER, while the C-terminal domain exerts a regulatory function. Immediately after synthesis, SREBP forms a heterodimeric complex via its C-terminal domain with SREBP cleavage-activating protein (SCAP) in the ER membrane. SCAP is a sterol-sensing escort protein that promotes the activation of SREBPs when ER cholesterol levels are low [28].

When ER cholesterol levels drop to below 5% of total ER lipids, SCAP adopts a conformation that exposes a coat protein complex II (COPII) recognition site known as the MELADL motif, allowing the SREBP-SCAP complex to be incorporated in a COPII vesicle for transport to the Golgi apparatus. In the Golgi apparatus, sequential proteolytic cleavage of SREBP by site-1 protease (S1P) and site-2 protease (S2P), releases its active
FIGURE 5: The SREBP pathway - In the presence of cholesterol and oxycholesterols the SREBP-SCAP complex is retained in the ER together with INSIG. In the absence of sterols, SCAP changes conformation which disrupts the binding to INSIG, that becomes ubiquitylated (Ub) by the E3 ligase, GP78, and is then rapidly degraded. After transport to the Golgi apparatus, SREBP is cleaved at two sites by S1P and S2P, releasing the N-terminal transcription factor for translocation to the nucleus.
N-terminal transcription factor domain into the cytosol which is then transported to the nucleus with the aid of importin β. Once inside the nucleus, the N-terminal domain of SREBP binds to the sterol regulatory element (SRE) within the promoter of target genes such as the LDLR gene and genes involved in cholesterol biosynthesis, and activates gene transcription. In contrast, when the ER cholesterol level exceeds 5% of the level of ER membrane lipids, cholesterol binds to SCAP and causes a conformational change that on one hand conceals the MELADL motif and prevents COPII from associating with SCAP and on the other hand allows SCAP to interact with insulin-induced genes (INSIGs) [29, 30]. INSIG serves to retain the SCAP-SREBP complex in the ER membrane in order to prevent further transcriptional activation of cholesterol-accumulating genes (Figure 5) [31, 32].

Despite the high degree of sequence similarity in the N-terminal transactivation domains of the SREBP isoforms, they exhibit differences in their target gene specificity [28]. SREBP-2 preferentially activates transcription of the LDLR gene and other genes that are involved in cholesterol synthesis and uptake, whereas SREBP-1c activates transcription of genes involved in fatty acid and triglyceride synthesis. SREBP-1a, on the other hand, appears to be a potent activator of all SREBP-responsive genes [28].

**LDLR promoter**

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**Figure 6:** A schematic illustration of the LDLR gene promoter - Regulation of LDLR transcription requires the concerted action of SP1 and SREBP-2. Footprint 1 confers maximal inducibility on the LDLR promoter while SIRE mediates the oncostatin M-induced transcription of LDLR gene.
1.4.3 **LDLR gene promoter**

Promoter mapping studies have identified a stretch of 177 nucleotides from -142 to +35, relative to the LDLR transcription start site, as the region that controls both the basal and the sterol-regulated transcription of the LDLR gene. Contained within this region are three 16 bp imperfect direct repeats that are designated as repeats 1, 2 and 3 [33]. Two of these, repeats 1 and 3, harbor the binding sites for SP1 transcription factor and support the basal expression of the LDLR gene. Embedded within repeat 2 is the 10 bp SRE (5′-ATCACCCAC-3′). The SRE serves as the binding site for SREBP-2 that in cooperation with Sp1 bound to the neighboring sites confers sterol-responsiveness and high level of LDLR gene expression [34].

In addition to repeats 1-3, the LDLR promoter also contains two other cis-acting elements referred to as footprint 1 and sterol-independent regulatory element (SIRE) [35]. Footprint 1 appears to be required for maximal induction of the LDLR gene, whereas SIRE mediates transcription of the LDLR gene in response to oncostatin M in a sterol-independent manner (Figure 6).

1.4.4 **Post-transcriptional regulation of LDLR**

Modulation of LDLR gene expression is a major mechanism for regulating the LDLR levels in a sterol-responsive fashion. However, LDLR is also subject to regulation at multiple post-transcriptional levels that allow fine-tuning of LDLR levels. An overview of these regulatory networks is provided below.

![LDLR 3'UTR](Image)

**FIGURE 7:** The LDLR 3’UTR - Schematic representation of the 3’UTR of the LDLR mRNA, which contains four AU-rich elements ARE1-4, two miR148 and one miR27a binding site. These elements regulate the LDLR mRNA’s half-life.
1.4.5 Regulation of LDLR mRNA stability

The human LDLR gene consists of 18 exons which are transcribed into a 5.3 kb mRNA strand [36, 37]. Approximately half of this sequence encodes the actual protein, while the remaining consists of non-translated sequences including the 2.5 kb 3’untranslated region (3’UTR) (Figure 7). Generally, the length of the 3’UTR is inversely correlated with mRNA stability and gene expression [38]. mRNAs with shorter 3’UTR are more stable because they escape mechanisms that degrade mRNA. Hence the long 3’UTR in LDLR mRNA confers a relatively high turnover rate ($t_{1/2} = 2$ hours) of the LDLR transcript. The instability of LDLR mRNA allows modulation of its turnover rate, allowing the cells to regulate their production of LDLR protein in an efficient and rapid manner. Although the underlying mechanism responsible for regulation of LDLR mRNA turnover have not been mapped out in detail, results form a number of studies paint an emerging picture of a complex regulatory network consisting of several mRNA-interacting proteins and their targets.

The short half-life of LDLR mRNA is mainly due to the presence of four adenylate-uridylate-rich elements (AU-rich elements or AREs), referred to as ARE1-4, in its 3’UTR [39, 40]. AREs are typically found in 3’UTR of short-lived mRNAs and are present in 5 to 10 % of all mRNAs, making them the most common regulatory motifs for mRNA stability in mammalian cells. A hallmark of AREs is the presence of the pentameric motif “AUUUA” which occurs with variable number of repeats in AU-rich regions. AREs serve as the docking sites for cytoplasmic ARE-binding proteins. Once tethered to 3’UTR, ARE-binding proteins recruit deadenylases to target mRNA to promote rapid deadenylation and decapping, resulting in subsequent degradation of the mRNA strand [41].

Several ARE-binding proteins have been identified to interact with and regulate the stability of LDLR mRNA [42]. For instance, heterogeneous nuclear ribonucleoprotein (hnRNP) D, hnRNP1 or KH-type splicing regulatory protein (KHSRP) have been shown to increase LDLR mRNA turnover rate, while association of human antigen R (HuR) with LDLR 3’UTR exerts a stabilizing effect on LDLR mRNA. However, ARE-directed mRNA turnover is not the sole mechanism governing LDLR mRNA stability. LDLR mRNA has also been shown to be targeted by the miRNA-mediated degradation pathway. LDLR 3’UTR contains at least
three miRNA seeds, two of which are targeted by miR148 while the third one is recognized by miR27a [43, 44].

1.4.6 Post-translational regulation of LDLR

Regulation of LDLR expression at the post-transcriptional level is mediated mainly by the inducible degrader of low-density lipoprotein receptor (IDOL) and proprotein convertase subtilisin/kexin type 9 (PCSK9).

IDOL is a RING domain E3 ubiquitin ligase that interacts with the cytoplasmic domain of LDLR and triggers lysine 63-specific ubiquitination [45, 46]. Ubiquitinated LDLR is then internalized by clathrin-independent but epsin-mediated endocytosis that directs the LDLR for degradation in lysosomes [47]. IDOL is a target gene for liver X receptor (LXR) that induces its transcription in response to increasing intracellular sterol levels [48, 49]. In other words, sterol-dependent activation of LXR works on the LDLR antagonistic to SREBP-2 by inducing the expression of IDOL and reducing LDLR levels.

PCSK9 is synthesized as a 692 amino acid pro-protein, referred to as pro-PCSK9, with a molecular weight of approximately 72 kDa that consists of five segments: a signal peptide, a prodomain, a catalytic domain, a hinge region and a cysteine- and histidine-rich C-terminal domain [50]. Following its synthesis in the ER, PCSK9 undergoes an autocatalytic cleavage, transforming it to a 62 kDa protein that remains non-covalently bound to its cleaved prodomain. PCSK9 is predominantly expressed in the liver and to a lesser extent in the intestine and kidney [51, 52]. As a target gene for SREBP-2, PCSK9 is co-regulated with other SREBP-2-regulated genes, such as the LDLR and HMGCR, in response to cellular cholesterol status. Consequently, a decrease in cellular cholesterol levels induces the expression of the PCSK9 gene resulting in increased PCSK9 synthesis. Following its secretion, PCSK9 binds to the EGFP homology domain of the LDLR at the cell surface. The LDLR-PCSK9 complex is then taken up into endosomes whose acidic milieu increases the binding affinity of PCSK9 to LDLR, an event that disrupts the normal recycling of the LDLR [53]. As a consequence of disrupted recycling, LDLR undergoes intracellular degradation, resulting in a decrease of
LDLR levels at the cell surface, and subsequently a reduction in cellular uptake of cholesterol. The relationship between LDLR and PCSK9 is a dynamic one that provide the cell with yet another layer of regulation to finely modulate the cellular cholesterol content. While, the newly synthesized LDLR takes up cholesterol into the cells, secretion of the newly synthesized PCSK9 acts to counteract this process.

1.5 Cholesterol synthesis

Cholesterol is vital for normal cell function. All animal cells are therefore capable of synthesizing cholesterol by a complex 37-step process [2]. This intricate process is extremely energy-expensive and requires 18 acetyl-coenzyme A (acetyl-CoA) molecules, 36 ATPs and 16 NADPHs to make a single cholesterol molecule. For this reason synthesis of cholesterol is primarily aimed to supplement the exogenous supply, and cells have therefore evolved elaborate feedback mechanisms to tightly control their cholesterol synthesis.

The cholesterol biosynthetic pathway begins with two molecules of acetyl-CoA and involves a large number of intermediates and a few side branches (Figure 8) [2]. Following two condensation reactions, acetyl-CoA is converted to 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA), an intermediate that upon reduction by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) is converted into mevalonic acid. HMGCR is the rate-limiting enzyme in the early steps of the cholesterol synthesis pathway, and its activity is tightly regulated at the transcriptional and post-transcriptional levels in a cellular cholesterol level-dependent manner [54, 55]. Following the processing of mevalonic acid by an eight-step enzymatic reaction, the substrate encounters the second rate-controlling enzyme in the cholesterol synthesis pathway, squalene monoxygenase (SM). The relative activity of SM determines whether its substrate continues along the cholesterol synthesis pathway or is diverted into a shunt pathway to be converted into 24,25-epoxycholesterol. Interestingly, 24,25-epoxycholesterol itself contributes to fine-tuning of cholesterol synthesis through its effect on various cellular mechanisms. For instance, 24,25-epoxycholesterol is a
FIGURE 8: A simplified presentation of the cholesterol synthesis pathway - This overview shows key intermediates, enzymes, side branches and products in the cholesterol synthesis pathway. Cholesterol synthesis starts with acetyl-CoA which through many conversions becomes lanosterol. Lanosterol can in turn go through either the Bloch or Kandutch-Russell pathway to end up as cholesterol.
potent LXR agonist, a transcription factor that regulates the expression of
genes whose products are involved in cholesterol transport.

The production of lanosterol marks the end of the single pathway route in
cholesterol synthesis. After lanosterol, the pathway diverges into either
the Bloch or Kandutsch-Russell pathway. These two routes are not
exclusive, and the enzymes acting in each branch of the pathway are
identical, although the order of the enzyme reactions is different. A cell’s
preference for the two pathways depends on different physiological
settings, although the mechanism for this is not well understood.

1.6  Autosomal dominant hypercholesterolemia

Due to its ability to remove a major portion of circulating LDL
(approximately 75 %) [4], LDLR plays a key role in regulation of plasma
LDL-cholesterol levels. Thus, factors that affect the function or the amount
of LDLRs cause hypercholesterolemia. These factors include mutations in
genes encoding LDLR, APOB or PCSK9 which cause autosomal dominant
hypercholesterolemia (ADH), a condition that is characterized by high
blood LDL-cholesterol levels and premature coronary heart disease [56].

Heterozygous ADH is the most common form of the disease, with affected
individuals exhibiting cholesterol levels in the range of 7-15 mmol/L. The
prevalence of heterozygous ADH in the general population has long been
believed to be 1:500 [57, 58]. However, results from more recent studies
suggest the carrier frequency for the ADH-causing mutations may be as
high as 1:250 [59–61]. Homozygous ADH occurs with a frequency of
approximately 1:1,000,000 in most populations and is characterized by
severely elevated blood cholesterol levels of 20-25 mmol/L [19] and
coronary heart disease that may present during childhood or adolescence.

1.6.1  Treatment of hypercholesterolemia

If left untreated, heterozygous ADH often leads to development of
coronary heart disease in approximately 50 % of males and 30 % of
females before the age of 60. As the atherosclerotic process depends on
the LDL-cholesterol levels and exposure time, early detection and early
start of treatment are important in reducing the risk of coronary heart
disease. Management of hypercholesterolemia consists of a drug-based intervention along with lifestyle changes. Currently, statins are the drug of choice for treatment of hypercholesterolemia. Statins are competitive inhibitors of HMGCR, which as previously mentioned, is the rate-limiting enzyme for de novo cholesterol synthesis. Inhibition of HMGCR blocks the hepatic synthesis of cholesterol, an event that consequently stimulates the proteolytic activation of SREBP-2 and results in upregulation of LDLR levels. Increased expression of LDLR promotes hepatic uptake of plasma LDL and thus a reduction in plasma LDL-cholesterol concentration. Unfortunately, the LDLR-inducing potency of statins is mitigated by the very factor that enables them to increase the expression of LDLR, namely SREBP-2. This is because PCSK9, similar to the LDLR, is an SREBP-2 target gene and is, therefore, co-induced with LDLR by statins. Despite the induction of PCSK9 by statins, a net increase in LDLR levels and LDL uptake is the outcome of statin treatment. Typically, statins lower the blood cholesterol levels by an average of 1.8 mmol/L which may result in a 60% reduction in the risk of coronary heart disease [62].

Different strategies are used to lower plasma LDL-cholesterol levels. In addition to statins, drugs like ezetimibe and resins that inhibit absorption in the small intestine of cholesterol and bile acid, respectively, are commonly used. The latter two drugs are often used in combination with statins to achieve a desirable reduction in cholesterol levels.

PCSK9 inhibitors are a new class of injectable drugs that have been shown to lower the LDL-cholesterol levels by up to 60%. These inhibitors are monoclonal antibodies that bind to and prevent PCSK9 from binding to the LDLR. Neutralization of PCSK9 then blocks the PCSK9-mediated degradation of LDLR, leading to an increase in cell-surface LDLR levels and thus reduction of plasma LDL-cholesterol levels. These PCSK9 inhibitors are generally well-tolerated and have few side effects. However, they are expensive with an annual cost of approximately USD 14,000, which severely limits their use. [63]
1.7 The AKT signaling pathway

AKT (also known as protein kinase B or PKB) is a serine/threonine protein kinase, which in mammalian cells is expressed as one of three highly homologous and structurally similar isoforms, AKT1, AKT2 and AKT3 [64]. AKT1 exhibits a ubiquitous pattern of expression, while the expression of AKT2 is predominant in insulin-responsive tissues and that of AKT3 is mostly restricted to brain and testes [65]. Each AKT isoform consists of three functional domains: an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal hydrophobic domain [66]. AKT functions as a major signaling hub that integrates and relays a multitude of input signals to downstream effectors that are involved in various cellular processes including metabolism, growth, proliferation, survival, transcription and protein synthesis [65]. AKT can be activated by a wide range of membrane receptor tyrosine kinases (RTKs) such as epidermal growth factor receptor or insulin receptor, but also other receptors such as G protein-coupled receptors have been shown to activate AKT. The initial step in activation of AKT involves the ligand-induced dimerization and autophosphorylation of the RTK, an event that promotes activation of phosphoinositide 3-kinase (PI3K). Activated PI3K in turn phosphorylates the membrane-bound PI 4,5 bisphosphate (PIP2) to generate PI 3,4,5 triphosphate (PIP3) [67]. PIP3 functions as a docking site for proteins that harbor a pleckstrin-homology (PH) domain, such as AKT and phosphoinositide-dependent kinase 1 (PDK1) [68]. Interaction of AKT and PDK1 with PIP3 brings the two proteins into close proximity, allowing PDK1 to phosphorylate AKT at threonine 308 leading to partial activation of AKT [69]. AKT may then be fully activated by phosphorylation at Serine 473 by the mechanistic target of rapamycin complex 2 (mTORC2) (Figure 9) [70, 71].

Active AKT promotes phosphorylation of a large number of substrates, leading to either activation or inhibition mainly depending on the substrate (Figure 10). Among these substrates are glycogen synthase kinase-3β (GSK-3β), proline-rich AKT substrate of 40 kDa (PRAS40) and tuberous sclerosis complex proteins 1 and 2 (TSC1/2) [72–75]. The three latter proteins play an important role in activation of mechanistic target of rapamycin complex 1 (mTORC1) [76]. PRAS40 is an mTORC1-interacting protein that inhibits mTORC1 signaling. Upon phosphorylation by AKT,
FIGURE 9: A schematic drawing of AKT activation - Stimulation of RTK by ligands such as growth factors or insulin activates PI3K leading to PIP3 production at the plasma membrane. Cytosolic inactive AKT is recruited to the membrane and engages PIP3 through PH-domain binding. This binding leads to phosphorylation of AKT by PDK1 and mTORC2 at threonine 308 and Serine 473, respectively, resulting in full activation of AKT.
PRAS40 dissociates from mTORC1 thus relieving its inhibitory effect on mTORC1. In addition to phosphorylation of PRAS40, AKT also phosphorylates and inhibits TSC1/2. Inactive TSC1/2 can no longer inhibit Ras homolog enriched in brain (RHEB) which can then proceed to activate mTORC1 through phosphorylation. Active mTORC1 has many substrates of its own; one of them is LIPIN-1 [77].

1.7.1 AKT signaling regulates SREBP

SREBPs were originally assumed to be regulated only by cholesterol- or oxysterols-mediated negative feedback control. However, a number of studies during the last decade have reported changes in SREBP activity following treatment of cells with modulators of the AKT signaling pathway.

For instance, insulin receptor-mediated activation of AKT has been found to influence the proteolytic processing of SREBP-1c in at least two different ways. The first involves AKT-mediated phosphorylation and degradation of INSIG, allowing SCAP-SREBP-1 to move from ER to Golgi apparatus where it is proteolytically activated [78]. In addition to inhibition of INSIG, insulin receptor-activated AKT phosphorylates CREB-regulated transcription coactivator 2 (CRTC2) at an inhibitory site, leading to alleviation of the inhibitory effect of CRTC2 on SEC31A, a component of COPII vesicles [79]. This in turn allows assembly of a functional COPII complex which facilitates translocation of SREBP-1c from ER to the Golgi apparatus. Thus, AKT induces the proteolytic activation of SREBP-1 by not only untethering it from ER but also by facilitating its COPII-mediated transport to the Golgi apparatus.

Modulation of SREBP proteolytic cleavage is not the sole mechanism by which AKT regulates the activity of SREBP. This is exemplified by LIPIN-1, a phosphatidic phosphatase, which is primarily known for its ability to convert phosphatidic acid into diacylglycerol, a precursor for the synthesis of phospholipids and triglycerides. In addition, LIPIN-1 has a non-enzymatic role allowing it to enter the nucleus and bind mature nuclear SREBP, resulting in SREBP to be sequestered to the nuclear lamina, thus preventing the binding of SREBP-1c to SRE of its target genes [80, 81]. Phosphorylation of LIPIN-1 by mTORC1 prevents its nuclear
FIGURE 10: A schematic drawing over substrates downstream of AKT - Phosphorylated and active AKT has many downstream substrates that are involved in regulation of diverse cellular functions. AKT phosphorylates and inhibits GSK-3β, PRAS40, TSC1 and TSC2, of which the three latter proteins work by activating mTORC1. mTORC1 again has many substrates; one of them being LIPIN-1.
translocation and thereby restores the SREBP-1c activity [80]. Another example is provided by the effect of GSK-3β, a downstream target of AKT, on nuclear SREBP-1c stability. GSK-3β is a serine/threonine protein kinase that is involved in a wide variety of cellular processes including glycogen metabolism [82, 83]. Upon binding to DNA, SREBP-1c adopts an altered conformation that allow it to be phosphorylation by GSK-3β at three different sites [84, 85]. In this phosphorylated form, SREBP-1c recruits the SCFFbw7 ubiquitin ligase, which results in ubiquitination and the subsequent proteasomal degradation of SREBP-1c [85]. Because GSK-3β activity is inhibited by AKT-mediated phosphorylation, activation of AKT prevents SCFFbw7-mediated degradation of nSREBP-1c, resulting in elevation of nuclear SREBP-1 levels and an increase in the expression of SREBP-1 target genes [82, 86].

As a result of the presence of SRE in their promoters, SREBP genes are responsive to transactivation by SREBPs. Therefore, regardless of the underlying mechanism involved, a stimulation of SREBP activity would be expected to further increase SREBP levels and subsequent activation, thus perpetuating an endless feedback loop. To prevent such a harmful event, cells have superimposed a negative feedback loop on SREBP activity. Similar to SREBP, the expression of the ER-tethering protein, INSIG-1, is responsive to SREBP activity and expression of INSIG-1 anchors SREBPs to the ER and thereby blocks its transcriptional activity.

The majority of studies aimed at elucidating the relationship between AKT signaling and SREBP activity have provided convincing evidence that AKT signaling stimulates SREBP-1a and SREBP-1c activities. This, together with the structural similarities between the SREBP isoforms have prompted researchers to presume that AKT may also stimulate the activation of SREBP-2. However, convincing evidence supporting this notion is lacking and the few studies that examined the effect of AKT on SREBP-2 activity have yielded conflicting results [81, 87–92].
2 Summary of results

2.1 Publication I: MK-2206, an allosteric inhibitor of AKT, stimulates LDLR expression and LDL uptake: a potential hypocholesterolemic agent

During the course of our research, which aimed to delineate the molecular events that exert a regulatory effect on LDLR expression, we noted that MK-2206, a highly selective allosteric inhibitor of AKT, exhibits an inducing effect on LDLR levels. Therefore, we initiated this study to investigate how MK-2206 and AKT regulate the expression of the LDLR. Using the hepatoma cell line HepG2, we found that MK-2206 potently increases LDLR mRNA levels, an event that resulted in upregulation of the cell-surface LDLR levels and increased cellular uptake of LDL. MK-2206-mediated induction of LDLR was independent of cell type and HMGCR activity, in contrast to the cholesterol-lowering drugs statins. Moreover, MK-2206 was found to potentiate the LDLR-inducing effect of statins. Mechanistically, we found that MK-2206 enhances the proteolytic cleavage of nascent SREBP-2, resulting in accumulation of its transactivation-competent N-terminal fragment that induces the expression of genes, such as LDLR and PCSK9, whose promoters harbor its cognate binding site.
2.2 Publication II: Triciribine increases LDLR expression and LDL uptake through stabilization of LDLR mRNA

The results obtained with MK-2206 implied that inhibition of AKT kinase activity induces the LDLR gene expression. To examine the validity of this inference, we decided to examine the effect of triciribine on LDLR expression. Triciribine is a highly AKT-selective small molecule that inhibits AKT activity by blocking its recruitment to the plasma membrane. Similar to MK-2206, triciribine induced LDLR mRNA and LDLR protein expression and increased the cellular LDL uptake. Triciribine was also found to potently enhance the ability of a statin to induce LDLR expression and increase the cellular uptake of LDL. Further experiments showed that triciribine, despite its above-mentioned similarity to MK-2206, affects the expression of LDLR in a different manner. First, we observed that, in contrast to MK-2206, triciribine induced LDLR expression in a hepatic lineage-specific manner. Furthermore, we found that triciribine, while reducing the SREBP-2-mediated gene expression, stabilizes LDLR mRNA, thus leading to its accumulation and subsequent translation into high levels of LDLR protein. There was no single element within the 3’UTR that mediated the stabilization effect of triciribine, but it was partially dependent on the integrity of a distal portion of LDLR mRNA 3’UTR that we have named IVS (intervening sequence between ARE3 and ARE4). Furthermore, our results showed the absolute requirement of ERK activity for the stabilizing effect of triciribine on LDLR mRNA. Importantly, a two-week treatment of mice with triciribine was found to lead to upregulation of hepatic LDLR protein levels, suggesting the potential of triciribine as a plasma cholesterol-lowering agent.
2.3 Publication III: Inhibitors of AKT kinase increase LDL receptor mRNA expression by two different mechanisms

Results from publications I and II suggested that while cells respond to inhibition of AKT by upregulating the expression of LDLR, they do so by two distinct mechanisms. This finding led us to hypothesize two possibilities: (1) induction of LDLR expression by MK-2206 or triciribine could be the result of an off-target effect of at least one of the inhibitors, or (2) AKT affects the expression of LDLR at one of two regulatory levels, i.e., gene expression or mRNA stability, in a kinase activity-independent but conformation-dependent manner. This notion was based on modes by which MK-2206 and triciribine bind to AKT. MK-2206 binds to and stabilizes the closed (PH-in) conformation of AKT, whereas triciribine interacts with AKT when it is in an open (PH-out) state. Assuming that it is unlikely that different AKT inhibitors can produce the same off-target artifact, i.e., LDLR induction, we examined two allosteric inhibitors of AKT, ARQ-092 and AKT inhibitor VIII, one AKT PH-interacting inhibitor, perifosine, and two ATP-competitive AKT inhibitors, AT7876 and CCT128930, for their ability to increase LDLR expression. We found that all the five AKT inhibitors induced LDLR protein expression. This indicated that AKT is indeed a kinase whose inhibition elicits an LDLR-inducing response. Interestingly, while all the five inhibitors increased the transcriptional activity of the LDLR promoter, only CCT128930 had an additional stabilizing effect on LDLR mRNA. Given the reported in vitro specificity of CCT128930 for the AKT2 isoform, we considered the possibility that AKT affect distinct LDLR-regulating mechanisms in an isoform-specific manner. To gain an indication of the validity of this notion, we examined the effect siRNA-mediated ablation of AKT1 or AKT2, the major AKT isoform expressed in hepatic cells, on LDLR promoter activity and LDLR mRNA stability. Interestingly, whereas either AKT1 or AKT2 knockdown induced LDLR promoter activity, only AKT2 knockdown increased the stability of LDLR mRNA additionally. In sum, these results corroborate our prediction that AKT inhibition exerts an inducing effect on LDLR levels and that the different AKT isoforms may play distinctive roles in this process.
3 Discussion

3.1 The effect of MK-2206 on LDLR expression

Members of the SREBP transcription factor family are master regulators of lipid homeostasis, with SREBP-1 activating genes that participate in fatty acid synthesis and SREBP-2 driving the transcription of genes that are involved in cholesterol metabolism [27]. In contrast to SREBP-1 whose sterol-dependent and sterol-independent regulation of activity is fairly well characterized, the literature on sterol-independent modulation of SREBP-2 activity is scarce, forcing investigators to rely on the homology between SREBP-1 and SREBP-2 and assume that SREBP-2 is subject to the same regulatory mechanisms as SREBP-1. Therefore, given our interest in mapping the novel regulatory mechanisms that govern LDLR expression, we directed our attention to investigating whether AKT activity, which is reported to induce SREBP-1 activation [93], affects the activity of SREBP-2.

To study the effect of AKT on SREBP-2, we exposed HepG2 cells to MK-2206, an allosteric and highly specific AKT inhibitor, and analyzed them for LDLR protein levels as an indicator of SREBP-2 activity. Interestingly, we observed that MK-2206 potently induced the activity of LDLR promoter, an event that led to upregulation of LDLR mRNA and LDLR protein, and ultimately an increase in cellular uptake of LDL (see Figure 1 and 2A-C, publication I). The induction of LDLR promoter activity required an intact SRE sequence in the LDLR promoter and was dependent on expression of SREBP-2, indicating that MK-2206 utilizes the activity of SREBP-2 to induce the expression LDLR. This implication was indeed validated by our result showing that MK-2206 induces the proteolytic cleavage of SREBP-2, an event that is a prerequisite for activation of SREBP-2.
3.1.1 The LDLR-inducing effect of MK-2206 is independent of cellular cholesterol level

The current model for the control of SREBP-2 activation, derived from studies aimed at understanding the sterol-regulated proteolytic cleavage of SREBPs portrays SCAP as a central player in cholesterol-induced cleavage of SREBP-2. Under cholesterol-rich conditions, direct binding of cholesterol to SCAP promotes its interaction with the ER membrane-resident INSIG protein, an event that sequesters the SCAP-accompanying protein, SREBP-2, away from the Golgi-localized SREBP-2-cleaving enzymes, S1P and S2P [31, 32]. Upon cholesterol depletion, SCAP is liberated from INSIG, allowing it to escort SREBP-2 to the Golgi apparatus for processing and activation. A basic prediction of this model is that MK-2206 induces SREBP-2 activation by decreasing intracellular cholesterol levels. In the following paragraphs, we propose that MK-2206-mediated activation of SREBP-2 occurs independent of cholesterol levels.

Although *de novo* cholesterol synthesis was found to be decreased moderately in MK-2206-treated cells, the resulting reduction of intracellular cholesterol levels cannot provide a satisfactory explanation for the SREBP-2-activating effect of MK-2206. This conclusion is based on comparison of the effects of MK-2206 on endogenous cholesterol synthesis and LDLR levels with those of the HMGCR-inhibiting agent, mevastatin. Whereas exposure of sterol-starved cells to 5 μM MK-2206 led to reduction of endogenous cholesterol synthesis by approximately 40 %, treatment of cells with 10 μM mevastatin inhibited *de novo* cholesterol biosynthesis by roughly 95 % (Figure 3A in publication I). This observation indicates that MK-2206-treated cells contain higher levels of cholesterol, and thus lower levels of SREBP-2 activity in comparison with mevastatin-treated cells. Consequently, cells that are treated with MK-2206 are expected to express less LDLR than mevastatin-exposed cells. However, we have observed that MK-2206-treated cells increase their expression of LDLR by approximately six-fold (Figure 1A in publication I), whereas treatment of cells with mevastatin induces LDLR levels by about two and a half-fold.
If MK-2206 were dependent on inhibition of cholesterol biogenesis and thus reduction of intracellular cholesterol levels to induce the expression of LDLR, then absence of cholesterol biosynthesis would be expected to compromise the LDLR-inducing ability of MK-2206. However, we have observed that MK-2206, similar to its effect on wild-type cells, potently induces the expression of LDLR in UT-2 cells, an HMGCR-deficient cell line unable to synthesize cholesterol (Figure 3B in publication I).

The above-mentioned observations together with the rapid kinetics of LDLR induction by MK-2206 (within 2 hours) and its independence of protein synthesis (publication I, Figures 3C and 3E) raise the possibility that MK-2206 bypasses the requirement for low cholesterol levels by engaging a signal transduction cascade that directly influences the SREBP-2 processing machinery. This suggestion implies that MK-2206 can induce activation of SREBP-2 and consequently increase LDLR levels under conditions in which SREBP-2 processing is usually suppressed by the sterol-activated negative feedback mechanism. The observation that MK-2206 promotes the proteolytic processing of SREBP-2 and induces LDLR expression in sterol-fed cells lends credence to this notion.

### 3.1.2 Possible mechanisms underlying the effect of MK-2206 on SREBP-2

Apart from its activation in a sterol-dependent manner, SREBP-2 has been shown to be proteolytically activated under ER stress or apoptotic conditions independently of intracellular cholesterol content, and several different components have been shown to increase LDLR levels by promoting ER-stress or apoptosis [94–98]. Proteins with a prominent role during ER-stress utilize the same proteases (SP1 and SP2) as SREBP-2 for proteolytic activation [28, 99, 100], and induce degradation of INSIG-1 resulting in activation of SREBP-2 [95, 101]. Moreover, studies have shown that SREBP-2 is positively regulated by the apoptosis protein caspase -7 and that activated caspases-1 and -3 cleave SREBP-2 during apoptosis [94, 102, 103]. Since 15 μM of MK-2206 was found to induce apoptosis in HepG2 cells [104], which is a concentration only three-fold higher than the concentration used in our studies, experiments were performed to determine if the effect of MK-2206-induced processing of SREBP-2 and the subsequent upregulation of LDLR occurs as a result of...
ER stress or apoptosis. To this end, CHO and HepG2 cells were treated with various concentrations of MK-2206 and then analyzed for the splicing of XBP1 mRNA and PARP cleavage as indicators of ER stress and apoptosis, respectively. However, no cleavage of PARP and XBP1 were observed after treatment with MK-2206 indicating that the observed increase SREBP-2 is not secondary to ER-stress or apoptosis.

As mentioned in section 1.4.2, cholesterol, upon accumulation in cells, binds to SCAP, altering its conformation and thereby allowing its interaction with INSIG, an event that ensures inhibition of SCAP-SREBP-2 translocation to the Golgi apparatus. Thus, SCAP conformation dictates the activation status of SREBP-2, a barrier that MK-2206 needs to overcome in order to induce activation of SREBP-2. One plausible mechanism by which MK-2206 could alter the INSIG-interacting conformation of SCAP is through post-translational modification of SCAP. Modulation of SREBP-2 processing through post-translational modification of SCAP is not without precedence. Luteinizing hormone has recently been reported to promote SREBP-2 activation in murine cells, at least in part, by inducing protein kinase A-mediated phosphorylation of SCAP at serine 821 (equivalent to human serine 822) [105]. This, together with identification of several serine residues in SCAP by phosphoproteomic analysis of human cancer cell lines [106] argue in favor of assessing whether MK-2206 affects the post-translational modification status of SCAP and if so whether it influences the interaction of SCAP with INSIG and/or the COPII machinery. It is equally plausible that MK-2206 induces a conformational change in INSIG that reduces its affinity for SCAP, thereby allowing SCAP to escort SREBP-2 to the Golgi apparatus. Integrity of the conserved DRSR tetrapeptide in the fourth extramembrane loop of INSIG, particularly that of the aspartic acid, is proposed to play a key role in maintaining the protein in an SCAP-interacting conformation [107]. Interestingly, we have noted that computational analysis of the amino acid sequence in this loop (DRSRSG) using the Group-based Prediction System 3.0 predicts the serine in DRSR as a potential target for a number of AGC kinases. This observation raises the possibility that MK-2206 might affect the conformation of INSIG and thus its association with SCAP by altering the phosphorylation status of the serine residue in the DRSR tetrapeptide. Regardless of whether MK-2206 alters the conformation of SCAP, INSIG or increases SREBP-2
activity by other mechanisms, the subsequent dissociation of INSIG from SCAP is expected to lead to reduction of INSIG protein levels because SCAP, when in complex with INSIG, acts as an INSIG-stabilizing factor [108]. Indeed, we will prioritize testing whether MK-2206 degrade INSIG in our future experiments.

3.2 A reflection on the mechanisms by which triciribine induces LDLR expression

To confirm the target specificity of MK-2206 with regard to its effect on LDLR levels, we exposed cells to another highly specific AKT inhibitor, triciribine, and then examined them for LDLR expression. Similar to MK-2206, triciribine was found to induce the accumulation of LDLR mRNA and LDLR protein and increase the cellular LDL uptake (see Figure 1, 2A and 2B, publication II). Surprisingly, unlike MK-2206, the triciribine-mediated induction of LDLR mRNA levels was associated with an increase in LDLR mRNA half-life and not LDLR promoter activity. To obtain a mechanistic explanation for why triciribine decreases the rate of LDLR mRNA turnover, we directed our attention to the 3’UTR of LDLR mRNA because, as mentioned in the introduction, this region contains four cis-acting regulatory AREs, and a cluster of UCAU repeats that influence LDLR mRNA decay rate. To interrogate the role of these ARE motifs in triciribine-mediated stabilization of LDLR mRNA, we constructed a plasmid where the LDLR 3’UTR was inserted downstream of the ZsGreen1 gene. The cis-acting elements in the LDLR 3’UTR act as binding sites for proteins and miRNAs that could regulate the LDLR mRNA half-life. We deleted and mutated multiple sites known to affect the mRNA stability both alone and in combination, the triciribine effect, however, was never completely lost except when almost the entire 3’UTR was deleted. This is perhaps not a surprising result as it is indeed becoming increasingly clear that various aspects of post-transcriptional mRNA metabolism are controlled by cis-regulatory elements that act in a combinatorial and redundant manner and by the dynamic array of trans-acting factors [109–111]. For instance, destabilization of IL-17 mRNA by tristetapoline has been shown to be dependent on a cluster of several ARE motifs within the IL-17 3’UTR [112]. Similarly, it has been
reported that p38α targets three distinct AREs in *IL-6* mRNA [113]. For instance, full-scale, stress-induced stabilization of the cyclin-dependent kinase inhibitor 1 (p21CIP1) mRNA, has been shown to depend on the mutual cooperation between RNPC1 and HuR, two trans-acting factors that bind to distinct AREs in *p21CIP1* mRNA [114]. Therefore, a conclusive determination of whether the AREs or the cluster of UCAU repeats are involved in *LDLR* mRNA-stabilizing effect of triciribine requires examination of the effect of triciribine on transcripts which harbor combinatorial deletions of these *cis*-regulatory elements.

### 3.2.1 Do miRNAs play a role in triciribine mediated stabilization?

Underscoring the complexity of post-transcriptional regulation of *LDLR* mRNA, a number of recent studies have reported a role for microRNAs (miRNAs) in control of *LDLR* mRNA stability. For instance, miR-27a and miR-148 have been shown to promote *LDLR* mRNA degradation by targeting its 3’UTR [115, 116]. Therefore, we wished to assess whether miR-27a or miR-148 contributed to triciribine-mediated stabilization of *LDLR* mRNA. To this end, we examined the effect of triciribine on the expression of ZsGreen1 fluorescence from pmR-ZsGreen1-LDLR-3’UTR constructs in which the seed sequences of miR-27a or miR-148 in *LDLR* 3’UTR were mutationally inactivated. Similar to the results obtained with ARE or UCAU deletions, we found that inhibition of the binding of miR-27a or miR-148 to *LDLR* 3’UTR did not diminish the effect of triciribine on *LDLR* mRNA stability (unpublished results). Considering the results obtained from both computational and cell-based analyses concluding that mRNA fates are controlled by substantial collaboration between RNA-binding proteins and miRNAs [117], we are inclined not to draw a definite conclusion from the above experiment. Determination of the potential involvement of miRNAs in *LDLR* mRNA-stabilizing effect of triciribine should await the results of experiments in which the role of miRNAs is examined in the context of other regulatory factors that drive *LDLR* mRNA stability.
3.2.2 Could N6-methyladenosine play a role in triciribine mediated mRNA stability?

The effect of triciribine on LDLR mRNA stabilization may possibly involve post-transcriptional modification of the 3’UTR. Reverse and dynamic methylation of mRNA can in many cases add another layer of sophisticated regulation of mRNA stability [118–120]. N6-methyladenosine (m6A) is the most prevalent internal (non-cap) modification present in mRNA of all higher eukaryotes [121]. m6A is an internal modification in mRNA generated post-transcriptionally by m6A methyltransferase on the consensus sequence G (m6A) C or A (m6A) C [122]. m6A methylation is essential for cell viability and development [123–125], but the exact role remains to be determined. LDLR mRNA has many m6A sequences in its 3’UTR, and the LDLR 3’UTR has been shown to be subject to m6A modification, which facilitates the binding of the YTH-domain family member 2, thus resulting in LDLR mRNA decay [126]. The region between ARE3 and ARE4, in the triciribine paper referred to as the intervening sequence (IVS), plays a partial role in both basal LDLR mRNA turnover, as well as the mRNA stabilization effect of triciribine. This region contains several sites that closely match the consensus signal for the m6A methylation [127], raising the possibility that triciribine may inhibit methylation of LDLR mRNA in the IVS region to protect it from binding to the mRNA-destabilizing m6A readers. It would therefore be interesting to investigate whether triciribine inhibits m6A writers or stimulates m6A erasers to abrogate methylation of LDLR mRNA.

3.2.3 The role of ARE-binding proteins in the effect of triciribine on LDLR mRNA stability

As mentioned above, cis-regulatory elements of RNA function as landing pads for trans-acting regulatory RNA-binding proteins that serve as nexus between diverse incoming signals and RNA fate, thereby ensuring a rapid and finely tuned regulation of local gene expression in response to various stimuli [128, 129]. The majority of RNA-binding proteins involved in regulation of mRNA stability fall within the group of ARE-binding proteins, that is made up of approximately 20 members [130]. Of these, only a handful has been shown to interact with and
regulate the stability of \( LDLR \) mRNA [39]. For instance, HuR has been shown to bind \( LDLR \) 3’UTR and reduce \( LDLR \) mRNA turnover [39, 131] while interaction of KHSRP, ZFP36L1 or ZFP36L2 with \( LDLR \) 3’UTR was found to promote \( LDLR \) mRNA degradation [39, 132]. Consequently, triciribine may affect the stability of \( LDLR \) mRNA by modulating the activity of mRNA decay-regulating proteins, such as ARE-binding proteins, that interact with \( LDLR \) 3’UTR. Triciribine could increase \( LDLR \) mRNA stability either by increasing the activity of mRNA-stabilizing trans-factors, such as HuR, by protecting it from decay-promoting trans-factors such as KHSRP or by a combination of the two events. Given the dependence of triciribine on ERK activity to stabilize \( LDLR \) mRNA, it is reasonable to assume that triciribine may target those \( LDLR \) 3’UTR-binding trans-factors whose activity is subject to regulation by ERK. One such factor is HuR which is reported to mediate the stabilizing effect of 5-Aminoimidazole-4-carboxamide ribonucleoside on \( LDLR \) mRNA in an ERK activity-dependent manner [131]. Another example is provided by ZFP36L1 and ZFP36L2 proteins that bind to \( LDLR \) 3’UTR and destabilize \( LDLR \) mRNA in an ERK-inhibitable fashion [132]. Hence, a plausible possibility is that triciribine, through the post-translational modification process, promotes or inhibits the interaction of HuR or ZFP36L1 and KHSRP, respectively, with \( LDLR \) 3’UTR.

### 3.2.4 Suppression of SREBP-2 transcription by triciribine

The observation that triciribine alleviated the SREBP-2-dependent transcription (Figures 2b and 3a, publication II) was unanticipated and prompted us to examine whether triciribine exerts an inhibitory effect on the activation process of SREBP-2. No change was observed in transcription of the SREBP-2 gene. However, triciribine was found to inhibit the expression of the Golgi-located C-terminal fragment of SREBP-2 (Supplementary Figure S3, publication II), indicating that it indeed suppresses the proteolytic activation of SREBP-2. The decrease in SREBP-2 activity was independent of whether the cells were sterol-feed or sterol-starved, making reduction in intracellular cholesterol content an unsatisfactory explanation of our findings. Given our current incomplete understanding of the signaling events that emanate from triciribine, we feel that an attempt to thoroughly discuss the possible mechanism(s) that
could underlie the inhibitory effect of triciribine on SREBP-2 activation would amount to mere speculation. However, with the little knowledge at hand, we would like to propose the possibility that triciribine might inhibit cleavage of SREBP-2 by blocking its translocation from ER to the Golgi apparatus. This suggestion is based on research indicating that insulin-induced transcription of INSIG-1 requires ERK activation [133]. This, together with our finding that triciribine induces ERK activity, raise the possibility that triciribine upregulates INSIG-1, an event that translates into detainment of SREBP-2 in ER.

3.3 Specificity and mechanism of AKT-mediated regulation of LDLR

Relying on LDLR protein expression as the readout parameter, MK-2206 and triciribine exhibit perfect phenotypic congruence, indicating that inhibition of AKT kinase activity induces LDLR protein levels. However, as demonstrated in publications I and II, this similarity does not extend to the regulatory mechanism by which these two inhibitors promote LDLR protein expression. This lack of alignment could be attributed to an off-target effect produced by non-specific interaction of either MK-2206 or triciribine with kinase(s) other than AKT. However, since early on in our research, we have viewed this possibility as unlikely for the following reasons: Despite the lack of a comprehensive analysis of MK-2206 selectivity, we believe that MK-2206 is a highly potent and selective inhibitor of AKT. This assumption is based on the binding mode of MK-2206 to AKT. MK-2206 is a non-ATP competitive kinase inhibitor that does not compete with cellular ATP for the ATP-binding pocket of AKT [134–136]. This allows MK-2206 to be used at concentrations that are closer to its biochemical Ki, thus making it unlikely that it would bind to off-target kinases. In addition, MK-2206 binds to a less evolutionarily conserved allosteric site on AKT [137], a feature that contributes to its improved selectivity.
Similar to MK-2206, there exists no selectivity profile for triciribine. However, several lines of evidence suggest that triciribine exerts a high degree of selectivity for inhibition of AKT activity. Triciribine phosphate, the biologically active metabolite of triciribine, has been shown to interact with the PH-domain of AKT in the vicinity of PIP3 binding pocket with a relatively high potency [138]. Because only a minor portion of PH-containing proteins (approximately 40), of which there are over 500 in the human proteome [139], exhibit high affinity for PI [140], the specificity of triciribine for a PI-binding PH-domain drastically reduces the number of PH-domain containing off-target kinases to roughly 40. Importantly, triciribine has been found to be selective for AKT over PDK1, another PH-domain containing protein that, similar to AKT, binds PIP3 [141]. Moreover, triciribine has been shown to be ineffective in inhibiting the activity of several members of the AGC family of kinases to which AKT belongs [141]. Finally, in line with its selectivity for AKT, triciribine inhibits cell growth and promotes apoptosis much more potently in cells that express aberrantly activated AKT [141].

3.3.1 LDLR-regulatory divergence of AKT isoform

What would then be a plausible mechanism explaining the divergent effects of MK-2206 and triciribine on the LDLR expression machinery? To answer this question, we mulled over the possibility that AKT might regulate LDLR expression in a kinase activity-independent but conformation-dependent manner and hypothesized that different conformations regulate different aspects of the LDLR expression machinery. For this notion to be potentially valid, at least two conditions need to be fulfilled: AKT adopts different conformations upon its binding to MK-2206 or triciribine, and AKT, in addition to its role as a kinase, performs non-catalytic functions.

Under basal conditions, AKT is in complex with PDK1 in a compact, inactive conformer (PH-in) [142, 143]. Upon stimulation, AKT is recruited to the plasma membrane through interaction of its PH-domain with PIP3, an event that induces AKT to assume an open PH-out conformation [142, 143]. Given the close similarity between the binding of PIP3 and triciribine to AKT PH-domain [138, 144, 145], we believe it is reasonable to infer that association of AKT PH domain with triciribine induces AKT to
Figure 11: A schematic representation of how AKT is inhibited by MK-2206 and triciribine – MK-2206 is known to specifically target the PH-in conformation of AKT through binding to the PH domain-induced cavity in the kinase domain, while triciribine is believed to bind AKT in a PH-out conformation.

With regard to a kinase-independent function of AKT, there is ample evidence for the fact that the function of kinases is not limited to phosphorylation but extends to various non-catalytic, structure-dependent roles, such as allosteric regulation of other enzymes or scaffolding of protein complexes [146]. For instance, ERK2 kinase has been shown to displace retinoblastoma protein from lamin A in a kinase activity-independent manner or allosterically activate the dual-specificity phosphatase MKP3 [147, 148]. More specifically, it was found that the
ability of AKT PH domain to bind PIP3 is controlled by the conformation but not the activity of its kinase domain [149]. Recently, Vivanco et al. observed that inhibition of AKT activity by MK-2206 was significantly more potent in blocking AKT-mediated survival than the ATP-competitive AKT inhibitor GSK690693 and provide evidence suggesting that AKT conformation, but not kinase activity, serves as a platform for a kinase-independent survival signal [150].

On the basis of the above arguments, we concluded that the aforementioned conditions were fulfilled and therefore set out to experimentally determine the validity of our hypothesis. To this end, we argued that if the mode of regulation of LDLR expression by AKT is primarily determined by its conformation, then inhibitors that induce AKT to adopt the PH-in conformation should behave as MK-2206 and increase the expression of LDLR by inducing the activity of LDLR promoter. Similarly, inhibitors that lock AKT in the PH-out conformation would be expected to induce the expression of LDLR by a mechanism similar to that employed by triciribine. Accordingly, as reported in paper III, we examined the effect of the following AKT inhibitors on LDLR expression:

- AKT inhibitor VIII and ARQ-092, two highly selective allosteric inhibitors of AKT with a mode of inhibition similar to that of MK-2206 [136, 151–153].
- Perifosine, an alkylphospholipid resembling naturally occurring phospholipids, that is assumed to inhibit the activation of AKT by targeting its PH-domain, thereby resembling triciribine in promoting the accumulation of AKT PH-out conformer in the cytoplasm [154, 155].
- The ATP-competitive AKT inhibitors, CCT128930 and AT7867, that inhibit the catalytic activity of AKT PH-out conformer at the plasma membrane.

Comparison of the effect of these inhibitors on LDLR promoter activity in terms of inhibitor-induced AKT conformation showed that inactive AKT, both in PH-in and membrane-bound PH-out conformations, promoted LDLR promoter activity, whereas the cytoplasmic PH-out conformer of AKT had either an inhibitory or stimulating effect on LDLR promoter.
(Figure 2B, paper III). Similarly, we did not observe an association between the inhibitor-induced AKT conformation and LDLR mRNA stability (Figure 3, paper III). These results falsified our hypothesis, which predicts that the conformation of inactive AKT dictates the mode of LDLR induction. However, the serendipitous use of CCT128930, reported to exhibit specificity towards AKT2 [156], encouraged us to consider the possibility that different AKT isoforms regulate different aspects of LDLR expression machinery. Interestingly, use of an siRNA-based approach to eliminate each AKT isoform in isolation revealed that while knockdown of AKT1 stimulated LDLR promoter activity, loss of AKT2 increased both LDLR promoter activity and LDLR mRNA stability. Assuming that these effects are caused by loss of AKT enzymatic activity and not its structural function, the results obtained with AKT siRNAs need to be confirmed by overexpression of different AKT1 or AKT2 mutants. For instance, increase in LDLR promoter activity alone or in combination with induction of LDLR mRNA stability by overexpression of dominant-negative AKT1 or AKT2 mutants, respectively, provide strong support for the results arrived at with the isoform-specific AKT siRNAs.

It should be noted that AKT isoforms, despite their high homology, have been found to regulate distinct cellular processes. The clearest example is provided by the phenotypes of AKT-specific knockout mice. Whereas AKT1 knockout mice exhibit growth retardation and increased apoptosis [157, 158], AKT2 knockout mice exhibit a type 2 diabetes-like phenotype and only a mild growth deficiency [159, 160]. Another example is provided by the observations that specific AKT isoforms are hyper-activated in certain tumors. For instance, aberrated expression of AKT1 has been found in 47 % and about 50 % of prostate carcinomas [161]. Similarly, 25 % of primary breast carcinomas have been shown to express elevated levels of AKT2 kinase activity [162]. The mechanisms proposed to contribute to AKT isoform-specific signaling include distinct tissue distribution and substrate specificity of AKT isoforms and isoform-specific subcellular compartmentalization [163].

Finally, it is important to highlight that the results obtained through use of AKT isoform-specific siRNAs have interesting implications for the isoform specificity of the AKT inhibitors that we have used in our studies. SiRNA-mediated loss of AKT1 solely induces LDLR promoter activity
alluding to AKT1 as the major intracellular target of MK-2206, ARQ-092, perifosine and AT7867, inhibitors that increase the expression of LDLR through induction of LDLR promoter activity. Following the same logic, because siRNA-mediated knockdown of AKT2 leads to stabilization of LDLR mRNA, triciribine and CCT128930, AKT inhibitors that reduce turnover rate of LDLR mRNA would be expected to primarily target AKT2 in the cell, with the caveat that triciribine, in addition to inhibition of AKT2, influences a cellular process that exerts an adverse effect on AKT2 inhibition-mediated induction of LDLR promoter activity. We intend to assess the validity of this conjecture by examining the effect of these AKT inhibitors on the kinase activity of AKT isoforms.

3.3.2 In vivo effect of MK-2206 and triciribine

Encouraged by the potent effect of MK-2206 and triciribine on LDLR levels and cellular LDL uptake, we felt it was important to assess their therapeutic potentials. To this end, we conducted an exploratory evaluation of the effectiveness of MK-2206 and triciribine in induction of the hepatic expression of LDLR and reduction of plasma cholesterol levels. We found that a two-week treatment of six C57BL/6J mice with MK-2206 led to reduction of plasma LDL-cholesterol levels by 26% at a significance level of 0.05. This was associated with an increase in hepatic LDLR levels with a $p$ value of 0.067, which, based on our choice of a < 5% significance level, was not statistically significant and was therefore not reported. Because LDLR is the major determinant of plasma LDL cholesterol levels, we chose not to include the result on the effect of MK-2206 on mouse LDL cholesterol levels in the absence of a statistically significant increase in hepatic LDLR expression. Interestingly, exposure of four C57BL/6J mice to triciribine for two weeks led to a significant upregulation of hepatic LDLR levels (Figure 7, publication II), an event that was associated with a statistically insignificant ($p = 0.078$) downregulation of plasma LDL-cholesterol levels. These results provide a heartening indication, but not an unequivocal proof, that MK-2206 and triciribine exert an LDLR-inducing and LDL-cholesterol-lowering effect in vivo. At this point, it is important to emphasize that these in vivo studies were conducted in an exploratory mode, the results of which warrant examination of the in vivo effects of MK-2206 and triciribine in
confirmatory studies that not only employ sufficiently large sample sizes to minimize the effect of random variations, but also use the appropriate mouse model to study cholesterol metabolism. Murine cholesterol metabolism is fundamentally different from human cholesterol metabolism. For instance, mice carry a large fraction of their plasma cholesterol in HDL whereas in humans most of plasma cholesterol is associated with LDL particles [164, 165]. For this reason, mice is not a perfect model to study the human cholesterol metabolism. This species-specific difference is alleviated by introduction of human APOE*3-Leiden and CETP gene constructs into C57BL/6J mice [166, 167]. As the result, the APOE*3-Leiden.CETP mice exhibit a humanized lipid metabolism with reduced clearance of triglyceride-rich lipoprotein and a cholesterol profile that is more similar to that of humans [167]. Importantly, APOE*3-Leiden.CETP mice respond well to lipid-lowering strategies and develop diet-induced atherosclerotic plaques that show characteristics similar to those of human lesions. Therefore, it will be interesting to examine the effect of MK-2206 and triciribine on diet-induced elevation of plasma LDL-cholesterol levels and formation and progression of atherosclerotic lesions in APOE*3-Leiden.CETP mice.

3.3.3 Clinical relevance of AKT inhibitors as cholesterol-lowering agents

Given the robust inducing effect of MK-2206 and triciribine on LDLR expression and cellular LDL uptake, we feel warranted in suggesting that MK-2206 and triciribine deserve careful evaluation as hypocholesterolemic drugs. Perhaps, similar to PCSK9 antibodies, MK-2206 or triciribine could be used to treat FH patients that exhibit sub-optimal response to statin-therapy. Alternatively, MK-2206 or triciribine could be used in combinatorial therapeutic strategies to either maximize the effect of or reduce the dose of cholesterol-lowering drugs such as statins. However, with respect to our speculation on the possible clinical relevance of MK-2206 or triciribine, there is one note of caution that should be mentioned. AKT displays multi-faceted functions in cell physiology by regulating a wide range of cellular processes such as proliferation, apoptosis and insulin signaling. Therefore, inhibition or even reduction of AKT kinase activity might be of particular concern as it may
lead to adverse effects such as hyperinsulinaemia and hyperglycaemia. Nonetheless, given the hypocholesterolemic potential of MK-2206 and triciribine, we consider it worthwhile to proceed to assess the therapeutic as well as possible adverse effects of these AKT inhibitors in an *in vivo* setting.
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MK-2206, an allosteric inhibitor of AKT, stimulates LDLR expression and LDL uptake: A potential hypocholesterolemic agent

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ABSTRACT

Background and aims: Induction of low-density lipoprotein receptor (LDLR) plays a significant role in reduction of plasma LDL-cholesterol (LDL-C) levels. Therefore, strategies that enhance the protein level of LDLR provide an attractive therapeutic target for the treatment of hypercholesterolemia. With this aim in mind, we concentrated our effort on studying the role of AKT kinase in regulation of LDLR levels and proceeded to examine the effect of MK-2206, an allosteric and highly selective AKT inhibitor, on LDLR expression.

Methods: Cultured human hepatoma cells were used to examine the effect of MK-2206 on the proteolytic processing of sterol regulatory element-binding protein-2 (SREBP-2), the expression of LDLR and cellular internalization of LDL. We also examined the effect of MK-2206 on LDLR levels in primary human hepatocytes.

Results: MK-2206 induced the proteolytic processing of SREBP-2, upregulated LDLR expression and stimulated LDL uptake. In contrast to statins, induction of LDLR levels by MK-2206 did not rely on 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) inhibition. As a result, cotreatment of cells with MK-2206 and mevastatin potentiated the impact of mevastatin on LDLR. Importantly, MK-2206 stimulated the expression of LDLR by primary human hepatocytes.

Conclusions: MK-2206 is a novel LDLR-inducing agent that, either alone or in combination with statins, exerts a stimulating effect on cellular LDL uptake.

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insulin-like growth factor-1 or cholesterol-depletion has been reported to depend on AKT activity [12,13], another study has failed to find a link between AKT activation and SREBP-2 processing [14]. The AKT kinase family comprises three related and differentially expressed isoforms, AKT1, AKT2 and AKT3, that act as molecular hubs to link both extracellular and intracellular stimuli to various cellular processes such as cell proliferation, apoptosis and metabolism [15]. It is therefore not surprising that aberrant AKT activity underlies the pathophysiological properties of a variety of human diseases [15]. Accordingly, multiple AKT inhibitors, including MK-2206, are currently being explored in clinical trials [16]. MK-2206 is an orally active and highly selective allosteric inhibitor of all AKT isoforms that binds in a cavity formed at the interface of the catalytically active kinase domain and the regulatory pleckstrin homology domain, locking the kinase in a closed, inactive conformation [17–19]. MK-2206 has been examined as mono- or combination-therapy in a number of clinical trials [20–23]. The results of these studies show that MK-2206, although modest in its ability to achieve the desired clinical antitumor activity, is well tolerated.

In the course of our research on LDLR regulatory mechanisms, we found that MK-2206 affects the expression of LDLR, prompting us to focus our attention on the relationship between MK-2206 and LDLR. Here, we show that MK-2206 activates SREBP-2 and exerts an LDLR-inducing and LDL C-lowering effect in a manner that is independent of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR) inhibition, endoplasmic reticulum (ER) stress or apoptosis. Furthermore, we show that MK-2206 augments the effect of mevastatin on LDLR levels. Based on these results, we believe that further research is warranted to examine the feasibility and potential of using MK-2206 as a hypocholesterolemic drug.

2. Materials and methods

2.1. Reagents and antibodies

MK-2206 2HC and mevastatin were from Selleckchem (Houston, Texas). Actinomycin D (Act D), cycloheximide (CHX), 25-hydroxycholesterol (25-HC) and (−)-mevalonolactone (which turns to mevalonate in water) were obtained from Sigma-Aldrich (St. Louis, MO). LDLR antibodies (3839; for Western blot analysis) were purchased from R and D Systems (Minneapolis, MN). Antibodies directed against the C-terminus of SREBP-2 (557037) were obtained from BD Biosciences (San Jose, CA). Anti-GAPDH (Glyceraldehyde 3-phosphate dehydrogenase; C9295) was purchased from Sigma-Aldrich. Antibodies against pAKT (S473; AF887), AKT (MAB2055), pPRAS40 (T246; MAB6890) and PRAS40 (MAB6408) were obtained from R&D Systems (Minneapolis, MN). Anti-β-tubulin (T9154-05G) was purchased from USBiological (Swampscott, MA). Anti-pAKT1 (S473; 9018) and anti-HA (ab18181) were obtained from Cell Signaling (Danvers, MA) and Abcam (Cambridge, UK), respectively.

2.2. Cell culture and treatment

HepG2 and HeLa cells (European Collection of Cell Cultures, Salisbury, UK), human telomerase reverse transcriptase (hTERT)-immortalized human hepatocytes (IH;H; provided by Dr. Philippe Collas, University of Oslo, Oslo, Norway), SV40 large T antigen-immortalized mouse hepatocytes (IMH; obtained from Dr. Angela M. Valleve [Instituto de Investigaciones Biomédicas Alberto Sols, Madrid, Spain] were cultured on collagen-coated culture vessel (BD Biosciences, San Jose, CA) in HyClone Minimum Essential Medium (GE Healthcare Life Sciences, Pittsburg, PA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich) and non-essential amino acids (BioWest, Nuaillé, France). Chinese hamster ovary (CHO) cells (American Type Culture Collection, Manassas, VA) were maintained in Ham’s F-12 medium (BioWest) supplemented with 10% FBS. UT-2 cells (a gift from Dr. Joseph L. Goldstein, University of Texas Southwestern Medical Center, Dallas, TX) were cultured in the same media as CHO cells and supplemented with 0.2 mM mevalonate. Hepac1c7 cells (provided by Dr. Jørn Andreas Holme, Norwegian Institute of Public Health, Oslo, Norway) were cultured in MEM Alpha medium without Nucleosides (Thermo Fisher Scientific, Waltham, MA) containing 10% FBS. For culture of cells in the absence of exogenous lipoproteins, cells were first grown for 24 h in complete medium containing 10% FBS and then washed twice with phosphate-buffered saline (PBS) before being provided with complete medium supplemented with 5% lipoprotein-deficient serum (LPDS). All media were supplemented with 2 mL insulin-like growth factor-1 (Sigma-Aldrich), 50 U/ml penicillin and 50 µg/ml streptomycin (GE Healthcare Life Sciences). Freshly plated human hepatocytes were obtained from QPS Hepatic Biosciences (Research Triangle Park, NC). Upon arrival, Storage Medium was replaced with Fresh Maintenance Medium supplemented with 0.1% dexamethasone (QPS Hepatic Biosciences). After a 24 h acclimatization in a cell culture incubator, cells were exposed to reagents for 14 h before harvesting for Western blot analysis. To inhibit HMGCR activity without affecting prenylation of proteins, cells were incubated with a mixture of mevastatin and 20 µmol/L mevalonate. All cells were grown in monolayer cultures in 5% CO2 at 37 °C. All drugs were added in dimethyl sulfoxide (DMSO) with a constant DMSO concentration of 0.1% (v/v). To control for possible DMSO effects, control samples were treated with DMSO alone at final concentrations of 0.1%.

2.3. Cell fractionation

HepG2 cells were harvested by trypsinization and pellets were washed in PBS before incubation in hypotonic buffer (10 mM Tris [pH 7.6], 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) for 15 min on ice. Cells were then lysed by 25 passages through a 25-gauge needle and centrifuged at 1000 × g for 10 min at 4 °C to collect the nuclei. The nuclei pellet was resuspended in 100 µl RIPA buffer (for SREBP-2 detection; 50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na3VO4 and Complete Protease Inhibitor Cocktail [Sigma-Aldrich]) and the suspension was incubated on ice for 40 min with intermittent vortexing at 2000 rpm and then centrifuged at 20,000 × g for 30 min at 4 °C. The recovered supernatant was designated as nuclear extract. The supernatant recovered from the 1000 × g centrifugation was spun at 20,000 × g for 30 min at 4 °C to pellet membranes. The membrane pellet was then resuspended in 100 µl RIPA and incubated on ice for 40 min with intermittent vortexing at 2000 rpm to extract membrane proteins and then clarified by centrifugation at 20,000 × g for 30 min at 4 °C. The supernatant was designated as the membrane fraction.

2.4. Western blot analysis

For detection of SREBP-2, 25 µg of nuclei extracts or membrane fractions were separated on a 10% SDS-PAGE. For detection of other proteins, cells were lysed in Triton X-100 lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton X-100, 10 mM EDTA and Complete Protease Inhibitor Cocktail) and separated on a 4–20% SDS-PAGE. After transfer to PVDF membrane (Bio-Rad, Hercules, California), proteins were detected by use of standard immunoblotting.
procedures. For plotting of the results, the immunoblots were scanned and the intensity of the target protein was normalized to that of the internal loading control (GAPDH, β-actin or β-tubulin). The obtained values were then plotted relative to vehicle-treated values or the values obtained from cells at time 0, which were set at 1.

2.5. Quantitative real-time PCR

Total RNA was purified using the QiAamp RNA Isolation Kit (Qiagen, Hilden, Germany). cDNA was synthesized with the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, CA). Quantitative real-time PCR (qPCR) was performed using Brilliant III Ultra-Fast qPCR Master Mix on Mx3005P qPCR system (Agilent technologies). The assay id of the PrimeTime Predesigned qPCR Assays (Integrated DNA Technologies, Coralville, Iowa) are: GAPDH (Hs.PT.39a.22214836); HMCRG (Hs.PT.58.41105492); LDLR (Hs.PT.58.14599757), proprotein convertase subtilisin/kexin type 9 (PCSK9; Hs. PT.58.20317419) and transferrin receptor (TFRC; Hs. PT.39a.22214826). The experiments were carried out in duplicate for each data point. The housekeeping gene GAPDH was used for normalizing target mRNA expression. Relative mRNA expression was calculated using the 2-ΔΔCt method.

2.6. Analysis of cell-surface LDLR and LDL internalization by flow cytometry

For detection of the amount of cell-surface LDLR expression, HepG2 cells were harvested using Non-enzymatic Cell Dissociation Solution (Sigma-Aldrich), washed twice with Staining Buffer (PBS + 1% BSA) and incubated with anti-LDLR (1:20 dilution in Staining Buffer; Progen) at room temperature for 40 min. Cells were then washed three times and incubated with Alexa Fluor 647-conjugated anti-mouse (1:60 dilution in Staining Buffer; Abcam) at room temperature for 30 min in the dark. After antibody incubation, cells were washed twice with Staining Buffer, resuspended in PBS and analyzed on a FACS Canto flow cytometer (BD Biosciences) for quantification of Alexa Fluor 647 fluorescence. To measure LDLR internalization activity, human LDL was isolated and labelled with 1,1-diiodoacetacetyl-3,3',3'-tetramethyldiodocarcocyanine perchlorate (DiD; Invitrogen, Carlsbad, CA) as previously described [24]. Cells were incubated with DiD-LDL (10 μg/ml) at 37 °C for 2 h. At the end of the incubation period, cells were harvested and washed three times with PBS containing 0.5% BSA before analysis by flow cytometry.

2.7. Plasmid constructs, transfection and reporter assays

pcDNA3.1-HA-V5/His plasmid was constructed by inserting a synthetic oligonucleotides duplex encoding an HA tag between the KpnI and BamHI sites of pcDNA3.1-V5/His(A) (Thermo Fisher Scientific). pcDNA3.1-HA-AKT1 WT-V5/His was constructed by subcloning the BamHI-EcoRI fragment from pcDNA3-T7-AKT1 (a gift from Dr. William Sellers; Addgene plasmid #9003) into the corresponding sites of pcDNA3.1-HA-V5/His. The pcDNA3.1-HA-AKT1 DD-V5/His mutant was generated using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technologies) to mutate the ACC and TCC codons encoding T308 and S473, respectively, in pcDNA3.1-HA-AKT1 WT-V5/His to create Asp codons. Both pcDNA3.1-HA-AKT1 WT-V5/His and pcDNA3.1- HA-AKT1 DD-V5/His encode HA-tagged proteins without V5/His tag at their C-termini due to presence of a stop codon located at the end of the cell-coding sequences. plLR1563-luc was a gift from Dr. Youngmi Kim Pak (Asian Institute for Life Sciences, University of Ulsan College of Medicine, Seoul, Korea) [25]. To construct plLR1563/mutSRE-1-luc, CC (underlined) in the LDLR promoter SRE-1 motif (TACCCCCAC) in plLR1563-luc was replaced with GG using QuickChange II XL mutagenesis kit (Agilent Technologies). pcDNA3.1-2xFLAG-SREBP-2 (Addgene plasmid #26807) was a gift from Dr. Timothy Osborne (Molecular Biology and Biochemistry, University of California, Irvine, CA) [26]. For transfection with expression vectors, cells were transfected with 312 ng DNA/cm2 of culture vessel growth surface area at a 4.5:1 (for HepG2) or 3:0.1 (for HeLa) FuGENE HD transfection reagent:DNA ratio following the manufacturer’s instructions (Promega, Madison, WI). To monitor the effect of transfection process, control cells were transfected with empty vector. For gene knockdown studies, HepG2 cells were reverse transfected during plating with 24 nmol/L gene-specific or non-targeting (NT) AllStars negative siRNA (Qiagen) siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific) with cells at 70% confluence. Drug treatments were performed at 24 h post-transfection. For measurement of LDLR promoter activity, HepG2 cells were transfected with pRL-SV40 vector (Renilla luciferase as internal control; Promega) and plLR1563-luc or plLR1563/mutSRE-1-luc at a 1:10 ratio. Cells were subjected to drug treatment at 24 h post-transfection, and then harvested for analysis of reporter gene activities by Dual-Luciferase Reporter Assay (Promega) following the manufacturer’s instructions.

2.8. Measurement of cellular cholesterol synthesis

HepG2 cells were first cultured in complete growth medium containing 5% LPDS for 24 h. The medium was then replaced with fresh medium supplemented with 0.2 μCi/ml [3H]acetate (PerkinElmer, Waltham, MA). After 1 h, cells were treated with reagents and incubated for further 24 h. Cells were then washed twice with PBS and harvested by trypsinization. The cell pellet was mixed in 0.5 ml methanol and 1 ml hexane and incubated for 1 h with agitation at room temperature. The cellular lipid extract in the upper phase was evaporated to dryness, resuspended in 30 μl chloroform and then resolved by thin-layer chromatography (TLC) on silica gel 60-coated glass plates (2292974, Sigma-Aldrich) using a 1:1 (v/v) mixture of diethyl-ether and hexane as the mobile phase. A solution of 10 μg/ml cholesterol (Sigma-Aldrich) was used as standard. After staining the TLC plate with iodide vapor, the migrated spots were scraped off and then analyzed for the amount of radioactivity by liquid scintillation.

2.9. Statistical analysis

An unpaired, two-tailed Student’s t-test was used to determine the significance of differences between the means of two independent groups.

3. Results

3.1. MK-2206 increases the expression of LDLR

The role of AKT in activation of SREBP-2 and thus the expression of its target gene, LDLR, remains controversial, as there is conflicting evidence suggesting that AKT activity either has no effect on or is required for activation of SREBP-2[12-14]. We therefore considered it important to investigate the relationship between AKT and the SREBP-2/LDLR axis. As a first step towards this aim, we decided to focus on the effect of AKT inhibition on LDLR expression. For this purpose, we utilized MK-2206, an allosteric and highly selective pan-AKT inhibitor [17], and for a cellular model system for expression of LDLR, we used the human liver-derived cell line HepG2. We first cultured HepG2 cells in the presence of either PBS or LPDS for 24 h (hereafter referred to as sterol-fed and sterol-
sterol-starved, respectively). Sterol-starved cells have been shown to contain lower cholesterol levels relative to sterol-fed cells [27,28]. Cells were then exposed to various concentrations of MK-2206, harvested after 14 h and examined for the expression of LDLR protein. Fig. 1A shows that MK-2206 induced LDLR expression in a dose-responsive manner with 5 and 10 μmol/L of MK-2206 exhibiting a maximal effect on LDLR protein levels in sterol-starved and sterol-fed cells, respectively. Above 10 μmol/L, the LDLR-inducing

Fig. 1. MK-2206 induces the expression of LDLR regardless of intracellular cholesterol levels. (A) HepG2 cells were treated with the indicated doses of MK-2206 for 14 h and then examined by Western blotting. Upper panel: representative blot from seven experiments. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated, sterol-fed cells. (B) HepG2 cells were cultured as in A, treated with 5 μmol/L MK-2206 and then harvested at the indicated time points for immunoblot analysis. Upper panel: representative experiment of seven. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated, sterol-fed cells that were harvested at 0 h. (C) Sterol-fed HepG2 cells were treated with the indicated concentrations of MK-2206 and harvested at the indicated times for immunoblot analysis. One representative blot is shown (n = 3). pAKT, phosphorylated AKT; pPRAS40, phosphorylated PRAS40. (D) Sterol-fed HeLa cells were transfected with either empty vector, pcDNA3.1-HA-AKT1- WT-V5/His or pcDNA3.1-HA-AKT1-DD-V5/His. At 24 h after transfection, cells were exposed to MK-2206 for 14 h before harvesting for Western blot analysis. pAKT, phosphorylated AKT. One representative blot is shown (n = 3).
effect of MK-2206 was mitigated presumably as a result of the cytostatic effect of such high concentrations of MK-2206. Examination of the kinetics of the MK-2206-mediated induction of LDLR showed that 5 μmol/L MK-2206 significantly induced LDLR levels within 4 h (Fig. 1B). LDLR reached its maximum levels in sterol-starved and sterol-fed cells at 14 and 18 h post-treatment, respectively, followed by a slight decline by 24 h. In the remainder of the experiments of this study, cells were incubated with 5 μmol/L MK-2206 for 14 h unless otherwise noted. Importantly, MK-2206 upregulated LDLR levels at the cell surface and induced DiD-LDL uptake in both sterol-fed and sterol-starved cells (Fig. 2A–B). Finally, examination of the effect of MK-2206 on LDLR protein showed that there was a correlation between both the magnitude of LDLR inhibition of AKT, we observed that MK-2206 increases LDLR protein levels and activity under both normal and sterol-deficient conditions.

To validate that induction of LDLR by MK-2206 is mediated by inhibition of AKT, we first examined the effect of MK-2206 on phosphorylation of AKT at S473 and its downstream target protein, PRAS40, as indicators of AKT kinase activity [15]. As shown in Fig. 1C, a concentration as low as 2.5 μmol/L of MK-2206 inhibited phosphorylation of AKT and PRAS40 within 2 h, showing that MK-2206 potently inhibits AKT activity in HepG2 cells. Next, we assessed the effect of MK-2206 on LDLR levels in HeLa cells that overexpressed HA-tagged AKT1-T308D/S473D (AKT1-DD) mutant. Substitution of T308 and S473 with aspartic acid generates a phosphomimetic AKT1 that is locked into a constitutively active state, thus rendering the kinase refractory to the action of MK-2206 [15]. As expected, cells expressing HA-tagged AKT1-DD displayed decreased phosphorylation of the transgene concomitant with elevated levels of LDLR (Fig. 1D). In contrast, MK-2206 failed to induce the expression of LDLR in cells that expressed the constitutively active mutant of AKT1. This result indicates that MK-2206 increases LDLR levels through inhibition of AKT kinase.

To determine whether the inducing effect of MK-2206 is restricted to LDLR or extends to other genes, we examined the effect of MK-2206 on the expression of two sets of genes: 1) PCSK9 and a number of cholesterogenic genes that are co-regulated with LDLR, and 2) SREBP-1c, IDOL, ACACA, FASN and SCD1 whose patterns of expression differ from that of LDLR. As shown in Fig. 4A and S3, MK-2206 induced the expression PCSK9, HMGCR, SREBP-2 and HMGCS1 mRNAs, indicating that it affects the expression of genes that are controlled by a regulatory mechanism similar to that of LDLR. In contrast, whereas MK-2206 had no significant stabilizing effect on LDLR mRNA (Fig. 2D), suggested that MK-2206-mediated induction of LDLR protein was the result of enhanced LDLR gene transcription.

The rapid induction of LDLR mRNA within 2 h after MK-2206 treatment suggested that it might occur independently of de novo protein synthesis. To examine this idea, sterol-fed HepG2 cells were pretreated with the protein synthesis inhibitor cycloheximide (CHX) for 1 h before addition of MK-2206 and then harvested after 5 h for examination of LDLR protein and LDLR mRNA levels. Inhibition of protein synthesis, as judged by the inability of MK-2206 to induce LDLR protein levels in the presence of CHX, had no effect on MK-2206-mediated induction of LDLR mRNA (Fig. 2E). This result indicates that MK-2206-induced LDLR transcription does not require new protein synthesis.

Finally, examination of the effect of MK-2206 on LDLR in a panel of both hepatic and non-hepatic cell lines from different species showed that MK-2206 elevates its LDLR-inducing effect in a non-cell type-specific manner (Figs. 2F–3B).

3.2. MK-2206 stimulates the expression of LDLR through a mechanism distinct from that of statins

Statins induce the expression of LDLR as a result of their ability to block the endogenous cholesterol production. To examine whether MK-2206 utilizes a mechanism similar to that of statins to upregulate LDLR levels, we first examined the effect of MK-2206 on endogenous cholesterol synthesis. Fig. 3A shows that MK-2206 inhibited cholesterol biosynthesis, albeit with significantly less efficiency when compared to mevastatin or 25-hydroxycholesterol (25-HC). Given the potent LDLR-inducing effect of MK-2206, this observation implied the possibility that MK-2206 might regulate the expression of LDLR in a manner that is independent of the HMGCR activity and cholesterol biosynthesis. To assess the validity of this suggestion, we examined the effect of MK-2206 on the expression of LDLR protein in UT-2 cells, a mutant clone of CHO cells that is deficient in HMGCR and thus, in contrast to CHO cells, should be resistant to the LDLR-inducing effect of HMGCR inhibition [29]. As shown in Fig. 3B, MK-2206 induced the expression of LDLR in both CHO and UT-2 cells. These results show that MK-2206 affects the expression of LDLR independent of HMGCR activity.

The distinction between the mechanisms by which MK-2206 and statins increase the expression of LDLR suggested that MK-2206 might exert a complementary effect on statin-induced upregulation of LDLR. To test this hypothesis, sterol-starved HepG2 cells were exposed to the individual and combination of MK-2206 and mevastatin and examined for the expression of LDLR by immunoblotting. The results in Fig. 3C clearly showed that combination of MK-2206 and mevastatin markedly increased LDLR levels as compared with treatment with either agent alone.

3.3. MK-2206 stimulates the cleavage of SREBP-2 and increases the LDLR promoter activity

To determine whether the inducing effect of MK-2206 is restricted to LDLR or extends to other genes, we examined the effect of MK-2206 on the expression of two sets of genes: 1) PCSK9 and a number of cholesterogenic genes that are co-regulated with LDLR, and 2) SREBP-1c, IDOL, ACACA, FASN and SCD1 whose patterns of expression differ from that of LDLR. As shown in Fig. 4A and S3, MK-2206 induced the expression PCSK9, HMGCR, SREBP-2 and HMGCS1 mRNAs, indicating that it affects the expression of genes that are controlled by a regulatory mechanism similar to that of LDLR. In contrast, whereas MK-2206 had no significant stabilizing effect on LDLR mRNA (Fig. 2D), suggested that MK-2206-mediated induction of LDLR protein was the result of enhanced LDLR gene transcription.

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Fig. 2. Induction of LDLR activity by MK-2206. (A) HepG2 cells were exposed to MK-2206 and then harvested after 14 h for flow cytometric analysis of mean fluorescence intensity as an indicator of cell-surface LDLR expression. Results were then plotted relative to vehicle-treated, sterol-fed cells (n = 6). (B) HepG2 cells were cultured and treated as in A. Cells were then exposed to DiD-LDL (10 μg/ml) for the last 2 h of treatment before harvesting for flow cytometric analysis of the mean fluorescent intensity of internalized DiD-LDL. Results were then plotted relative to vehicle-treated, sterol-fed controls (n = 5). (C) Sterol-fed HepG2 cells were treated with or without 5 μmol/L MK-2206 before harvesting at the indicated times for determination of LDLR mRNA levels by qPCR. LDLR mRNA levels were then plotted relative to the value obtained at time 0 (n = 5). (D) Sterol-fed HepG2 cells were treated with vehicle or 5 μmol/L MK-2206 for 12 h before treatment with actinomycin D (Act D; 5 μg/ml). Cells were harvested at the indicated time points after Act D treatment for determination of LDLR mRNA levels by qPCR. LDLR mRNA levels were plotted relative to respective vehicle-treated controls (n = 4). (E) Sterol-fed HepG2 cells were preincubated with 10 μg/ml cycloheximide (CHX) for 1 h before treatment with vehicle or MK-2206 for 5 h. After harvesting the cells, each sample was split in two. Lysates prepared from one half of each sample were analyzed by immunoblotting. The immunoblots data were plotted relative to matched vehicle-treated controls (n = 3). The other halves of samples were processed for determination of LDLR mRNA levels by qPCR. LDLR mRNA levels were plotted relative to those obtained from matched vehicle-treated cells. ns, not significant. (F) Upper panel: sterol-fed immortalized human hepatocytes (IHII), HeLa, immortalized mouse hepatocytes (IMH) and Hepac1c7 cells were treated with vehicle or the indicated concentrations of MK-2206 for 14 h before analysis by immunoblotting. One representative experiment of three is shown. Lower panel: Western blot data were quantified and plotted relative to the value from vehicle-treated cells. mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. Two and three asterisks indicate p < 0.01 and p < 0.001, respectively, compared with matched vehicle-treated cells (A, B and E) or matched cells harvested at 0 h (C).
the LDLR promoter activity (Fig. 4B). Importantly, mutational inactivation of the sterol regulatory element-1 (SRE-1) [30] in LDLR promoter led to not only a reduction in basal luciferase expression but also loss of MK-2206-induced transactivation activity (Fig. 4B). Thus, MK-2206 stimulates LDLR promoter activity in a manner that is dependent on functional SRE-1. This conclusion is further supported by the observation that MK-2206 induces the expression of other SRE-regulated genes (Fig. 4A and S3). Because SRE-1 is the binding site for SREBP-2, this result implied that MK-2206 is dependent on functional SRE-1. This conclusion is further supported by the observation that MK-2206-induced transactivation activity (Fig. 4B).

Promoter led to not only a reduction in basal luciferase expression but also loss of MK-2206-induced transactivation activity (Fig. 4B). To examine this notion, we silenced SREBP-2 expression in HepG2 cells with SREBP-2-specific siRNAs and examined them for the expression of LDLR mRNA in response to MK-2206 treatment. Knockdown of SREBP-2 expression (Supplementary Fig. 6) significantly diminished the LDLR-inducing effect of MK-2206 (Fig. 4C). This result suggests that SREBP-2 mediates the effect of MK-2206 on LDLR gene expression.

Proteolytic cleavage of SREBP-2 in the Golgi apparatus produces two products: the transactivation-competent N-terminal fragment (hereafter “NTF-SREBP-2”) that upon entering the nucleus activates the transcription of its target genes, and the C-terminal fragment (hereafter “CTF-SREBP-2”) that remains associated with the Golgi [31]. The observation that MK-2206 requires the activity of SREBP-2 to induce the expression of LDLR predicted the possibility that MK-2206 may stimulate the proteolytic cleavage of SREBP-2 and thereby increase the level of NTF-SREBP-2. We evaluated this prediction by immunoblotting the membrane fractions and nuclear extracts from sterol-fed HepG2 cells that were treated with MK-2206 with anti-SREBP-2 antibodies. Western blot analysis of the membrane fractions with an antibody directed against the C-terminal region of SREBP-2 and the nuclear extracts with an antibody directed against the N-terminal SREBP-2 antibody revealed that MK-2206 increases the amounts of both CTF-SREBP-2 and NTF-SREBP-2 with a concomitant reduction in full-length (FL)-SREBP-2 levels (Fig. 4D). This result showed that MK-2206 stimulates the proteolytic cleavage of FL-SREBP-2. Once in the nucleus, NTF-SREBP-2s are rapidly degraded by the ubiquitin-proteasome system [32]. Therefore, MK-2206 could be assumed to induce the amount of NTF-SREBP-2 in the nucleus by not only enhancing FL-SREBP-2 processing but also through increasing its stability. To address this possibility, HepG2 cells transiently transfected with a FLAG-tagged NTF-SREBP-2 (2xFLAG-SREBP-2) were treated with MK-2206 and then examined by immunoblotting with anti-FLAG antibodies. As shown in Fig. 4E, MK-2206 did not affect the amount of exogenously expressed NTF-SREBP-2, suggesting that MK-2206 does not alter the stability of NTF-SREBP-2. Taken together, these results show that MK-2206 capacitates the cells for increased expression of LDLR by enhancing the processing of nascent SREBP-2 and thereby increasing the amount of NTF-SREBP-2 in the nucleus.

Apart from its activation in a sterol-dependent manner, SREBP-2 has been shown to be proteolytically activated under ER stress or apoptotic conditions independently of intracellular cholesterol content [33–36]. Therefore, we felt it important to examine whether MK-2206-induced processing of SREBP-2 and the subsequent upregulation of LDLR occurs as a result of induction of ER stress or apoptosis. To this end, CHO and HepG2 cells were treated with various concentrations of MK-2206 and then analyzed for the splicing of XBP1 mRNA and PARP cleavage as indicators of ER stress and apoptosis, respectively [37–39]. As shown in Supplementary Fig. 7 and 8, MK-2206 did not induce ER stress or apoptosis. Thus, neither ER stress nor apoptosis accounts for the MK-2206-mediated activation of SREBP-2 and induction of LDLR. Furthermore, the data in Supplementary Fig. 8 and 9 show that MK-2205 does not appreciably affect the viability of HepG2 cells under the experimental condition used.

3.4. MK-2206 increases LDLR expression in primary hepatocytes

To examine whether MK-2206 also induced the expression of LDLR in primary cells, we treated primary adult human hepatocytes with various concentrations of MK-2206 or 10 μmol/L mevastatin and then examined them for the expression of LDLR protein by Western blotting. As expected, mevastatin induced the expression of LDLR (Fig. 5). Importantly, similar to its effect on the cell lines used in this study, MK-2206 exerted a stimulating effect on the expression of LDLR by primary human hepatocytes.

4. Discussion

This study was initiated to investigate the potential role of AKT in regulation of LDLR expression. Here, we show that MK-2206, an allosteric inhibitor of AKT, enhances the cellular uptake of LDL-C through induction of LDLR mRNA and LDLR protein. Furthermore, our results show that although MK-2206-mediated induction of LDLR requires the activity of SREBP-2, it occurs independently of intracellular cholesterol status, a property that distinguishes MK-2206 from statins. Based on the rapid kinetics of MK-2206-induced induction of LDLR and the observation that it occurs in a protein synthesis-independent manner, we propose that MK-2206 circumvents the dependency on low intracellular cholesterol levels to induce the expression of LDLR by triggering a signaling cascade that promotes the activation of SREBP-2. Thus, this property of MK-2206 enables it to upregulate LDLR even when the expression of LDLR is subject to sterol negative feedback regulation.

The observation that MK-2206 activates SREBP-2 and induces the expression of LDLR was an unexpected finding that is in disagreement with the notion that AKT activity is required for activation of SREBP-2 and induction of LDLR levels [12,13,40]. By contrast, our finding is in line with the result of Portsmann et al. showing a lack of relationship between activation of AKT and SREBP-2 processing [14]. This, together with the high selectivity of MK-2206 for AKT [17] and the abrogation of the LDLR-inducing effect of MK-2206 by AKT1-DD expression, prompts us to speculate that allosteric inhibition rather than stimulation of AKT promotes the activation of SREBP-2.

Our results demonstrate that MK-2206 increases the amount of nuclear SREBP-2 by enhancing the proteolytic processing of nascent SREBP-2, in a manner that is independent of HMGCR inhibition, ER stress or apoptosis. A plausible mechanism for this effect of MK-2206 would be that MK-2206 facilitates incorporation of SREBP-2-SCAP into COPII-coated vesicles, thus enhancing the export of nascent SREBP-2 from ER to the Golgi for proteolytic cleavage. MK-2206-induced degradation of INSIG proteins could account for increased loading of SREBP-2-SCAP into COPII-coated vesicles. However, because induction of LDLR mRNA within 2 h after MK-2206 treatment indicates a rapid processing and activation of nascent SREBP-2, we favor the possibility that MK-2206 might influence the conformation of SCAP or INSIGs through a post-translational modification mechanism causing SREBP-2-SCAP to dissociate from INSIGs. We would like to emphasize that elucidation of MK-2206-triggered molecular events that culminate in activation of SREBP-2 is of paramount importance as a detailed understanding of this signaling cascade may allow its modulation for the purpose of induction of LDLR.

AKT activity is crucial for normal functioning of several cellular processes, including, but not limited to, insulin signaling [15]. Therefore, it can be assumed that prolonged exposure to MK-2206 might produce serious adverse effects, such as hyperglycemia. This assumption is justified by the results of clinical trials with MK-2206 in cancer patients, showing incidence of hyperglycemia with a rate of approximately 9–30% [21,22,41,42]. Interestingly, this MK-2206-
Fig. 3. MK-2206 increases the expression of LDLR independent of the cholesterol biosynthesis pathway and improves the LDLR-inducing effect of mevastatin. (A) Sterol-starved HepG2 cells were treated with vehicle, 5 μmol/L MK-2206, 10 μmol/L mevastatin or 10 μmol/L 25-hydroxycholesterol (25-HC) for 14 h before processing for determination of cholesterol biosynthesis as described in materials and methods. The obtained values were then plotted relative to the value for vehicle-treated cells (n = 4). (B) CHO and UT-2 were treated with MK-2206 for 14 h before harvesting and analysis by immunoblotting. Left panel shows one representative blot (n = 3). Right panel: the immunoblots data were plotted relative to matched vehicle-treated controls. (C) Sterol-starved HepG2 cells were treated with vehicle or mevastatin for 24 h before exposure to the indicated concentrations of MK-2206 for a further 14 h. Cells were then harvested and subjected to Western blot analysis. Upper panel shows one representative blot from six experiments. Lower panel: the immunoblots data were plotted relative to vehicle-treated cells. Error bars represent SD. One, two and three asterisks indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with matched vehicle-treated cells (A and B).
Fig. 4. MK-2206-mediated induction of LDLR requires SREBP-2.
(A) Sterol-fed HepG2 cells were treated with 5 μmol/L MK-2206 before harvesting at the indicated times for determination of PCSK9, HMGCR and TFRC mRNA levels by qPCR. The obtained values were then plotted relative to the values of respective controls harvested at time 0 (n = 3). (B) Sterol-fed HepG2 cells were transfected with either wt (pLR1563-luc) or mutant (pLR1563/mut-SRE-1-luc) LDLR promoter and Renilla luciferase reporter. At 24 h after transfection, cells were treated with the indicated concentrations of MK-2206 for 14 h and then harvested for analysis of LDLR promoter activity. The plot shows the average of data pooled from four independent experiments relative to the value obtained from vehicle-treated cells that were transfected with pLR1563-luc. (C) Sterol-fed HepG2 cells were transfected with a non-targeting siRNA (NT siRNA) or an SREBP-2-specific siRNA. Cells were treated with MK-2206 at 24 h post-transfection and then harvested after 14 h for determination of LDLR mRNA levels by qPCR. LDLR mRNA levels were plotted relative to those obtained from vehicle-treated, NT siRNA-transfected cells (n = 4). (D) Left panel: sterol-fed HepG2 cells were treated with 5 μmol/L MK-2206 for the indicated time points, harvested and subjected to subcellular fractionation before being analyzed by Western blotting. Membrane fractions were blotted with an antibody raised against the C-terminus of SREBP-2 and anti-calreticulin. Nuclear extracts were blotted with an antibody directed against the N-terminus of SREBP-2 and anti-lamin B1. FL-SREBP-2, full-length SREBP-2; CTF-SREBP-2, C-terminal fragment of SREBP-2; NTF-SREBP-2, N-terminal fragment of SREBP-2. One representative blot of three is shown. Right panel: the western blots were analyzed by densitometry to obtain FL-SREBP-2/calreticulin, CTF-SREBP-2/calreticulin and NTF-lamin B1 ratios. The obtained values were then plotted relative to the values of respective controls harvested at time 0. mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. One, two and three asterisks indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with matched vehicle-treated cells (B and C) or matched cells harvested at 0 h (A and D). (E) Sterol-fed HepG2 cells were transfected with vector encoding the 2xFLAG-tagged N-terminal domain of SREBP-2 (pcDNA3.1-2xFLAG-SREBP-2). At 24 h post-transfection, cells were treated with MK-2206 for 14 h and then harvested for analysis by Western blotting with anti-FLAG and anti-β-actin antibodies. One representative blot is shown (n = 3).
MK-2206 induces the expression of LDLR in primary human hepatocytes. Primary human hepatocytes were treated with vehicle, MK-2206 or 10 μmol/L mevatatin for 14h before analysis by Western blotting. After quantification by densitometry, the LDLR/GAPDH ratio was calculated to obtain the LDLR band relative intensity. One representative experiment of two is shown.

related hyperglycemia was mainly mild and transient. Nonetheless, while appreciating the concern over the suitability of MK-2206 as a potential therapeutic option for hypercholesterolemia, we believe that the results from clinical studies supporting the favorable safety profile of MK-2206 [21,23,43], together with the results presented in this study, merit in vivo studies to examine whether concentrations of MK-2206 that exert a cholesterol-lowering effect, both singularly and in combination with statins, elicit any side effects, and if so, whether they are of such severity that overwhelm the therapeutic effect of MK-2206.

Conflicts of interest
SN is listed as inventor on a patent application relating to MK-2206. The other authors do not have anything to disclose.

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Author contributions
KB designed the research, performed experiments, analyzed data, and contributed to the writing of the manuscript. HS performed experiments. TPL helped with data review and writing of the manuscript. SN provided the concept, designed the research, performed experiments, analyzed data, and wrote the first draft and the final version of the manuscript.

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Appendix A. Supplementary data
Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosis.2018.07.009.

References


MK-2206, an allosteric inhibitor of AKT, stimulates LDLR expression and LDL uptake: a potential hypocholesterolemic agent

Supplementary materials

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**Acronyms**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
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<tbody>
<tr>
<td>25-HC</td>
<td>25-hydroxycholesterol</td>
</tr>
<tr>
<td>ACACA</td>
<td>Acetyl-CoA carboxylase alpha</td>
</tr>
<tr>
<td>Act D</td>
<td>Actinomycin D</td>
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<tr>
<td>CHO</td>
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<td>CHX</td>
<td>Cycloheximide</td>
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<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
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<td>DiD-LDL</td>
<td>1,1’-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FASN</td>
<td>Fatty acid synthase</td>
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<td>FL</td>
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<td>High-density lipoprotein cholesterol</td>
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<tr>
<td>HMGCR</td>
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<tr>
<td>HMGCS1</td>
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<td>hTERT</td>
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<td>IDOL</td>
<td>Inducible degrader of the LDL receptor</td>
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<td>PARP</td>
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**SREBP-1c** Sterol regulatory element binding protein-1c

**SREBP-2** Sterol regulatory element binding protein-2

**TC** Total cholesterol

**TFRC** Transferrin receptor

**TLC** Thin-layer chromatography

**XBP1** X-Box Binding Protein 1

**XBP1s** Spliced XBP1

**XBP1u** Unspliced XBP1
I. Supplementary materials and methods

Analysis of mRNA expression by real-time PCR

MK-2206 2HCl, triciribine, AT7867, ARQ-092 and CCT1298930 were obtained from Selleckchem (Houston, TX). AKT inhibitor VIII and perifosine were from AdooQ Bioscience (Irvine, CA). The kinase inhibitors were dissolved in dimethyl sulfoxide (DMSO; Sigma Aldrich, St. Louis, MO), except from perifosine which was dissolved in ethanol. Actinomycin D and dithiothreitol (DTT) were from Sigma-Aldrich. Antibodies against LDLR (3839-100) and β-tubulin (T9154-05G) were purchased from BioVision (Milpitas, CA) and Nordic BioSite AB (Täby, Sweden), respectively. Antibodies against AKT1 (2938) and AKT2 (2964) were obtained from Cell Signaling (Danvers, MA). siRNAs against AKT1 (Hs_AKT1_7 FlexiTube siRNA) and AKT2 (Hs_AKT2_5 FlexiTube siRNA) were obtained from Qiagen (Hilden, Germany).

Determination of XBP1 splicing

HepG2 cells (European Collection of Cell Cultures, Salisbury, UK), were cultured on collagen-coated culture vessels (BD Biosciences, San Jose, CA) in HyClone Minimum Essential Medium (GE Healthcare Life Sciences, Pittsburg, PA) containing 10% fetal bovine serum (Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U/ml penicillin (GE Healthcare Life Sciences), 50 μg/ml streptomycin (GE Healthcare Life Sciences) and non-essential amino acids (Biowest, Nuaillé, France). The cells were grown in monolayer in an atmosphere of 5% CO2 at 37°C. CHO T-REx cells (Invitrogen, Carlsbad, CA) were cultured as previously described [?]. All drugs, except perifosine, were added in DMSO with a constant DMSO concentration of 0.1% (v/v). To control for possible DMSO effects, control samples were treated with DMSO alone at final concentrations of 0.1%.

CCK-8 viability assay

Cells were lysed in Triton X-100 lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton X-100, 10 mM EDTA and Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland). Equal amounts of proteins were separated by 4-20% sodium dodecyl sulfate polyacrylamide gel electrophoresis. After transfer to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA), proteins were detected by use of standard immunoblotting procedures. The band intensities were quantified by the use of Chemidoc Touch Imaging System (Bio-Rad).
II. Supplementary figures and figure legends

**Figure 1:** Effect of LDLR knockdown on MK-2206-induced DiD-LDL uptake – Sterol-fed HepG2 transfected with non-targeting (NT) siRNA or an LDLR-specific siRNA (5’-AAGGACAAUUCUGACGAGGAA-3’ [sense strand]) as described in Materials and Methods in the main text. At 24 h post-transfection, MK-2206 was added to the media and cells were incubated for 8 h before addition of DiD-LDL (10 μg/ml). After 2 h of incubation with DiD-LDL, cells were harvested and each sample was split in two. One half of samples were analyzed by flow cytometry for DiD-LDL internalization (see Materials and Methods in the main text). Results were then plotted relative to vehicle-treated, NT siRNA-transfected cells (n = 4). The other halves of samples were processed for determination of LDLR mRNA levels by qPCR. LDLR mRNA levels were plotted relative to those obtained from vehicle-treated, NT siRNA-transfected cells (n = 4). mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. One, two and three asterisks indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with matched vehicle-treated, NT siRNA-transfected cells.
**Figure 2:** Induction of LDLR mRNA in sterol-starved cells by MK-2206 – Sterol-starved HepG2 cells were treated with or without 5 μmol/L MK-2206 before harvesting at the indicated times. Cells were then processed for isolation of mRNA and determination of LDLR and GAPDH mRNA levels by qPCR assay. LDLR mRNA levels were then plotted relative to the value obtained at time 0 (n = 4). Error bars represent the 95% confidence interval. Three asterisks indicate p < 0.001 compared with cells harvested at 0 h.
Figure 3: Regulation of SREBP-2 and HMGCS1 mRNA levels by MK-2206 – Sterol-fed HepG2 cells were treated with or without 5 μmol/L MK-2206 before harvesting at the indicated times. Cells were then processed for isolation of mRNA and determination of SREBP-2, HMGCS1 and GAPDH mRNA levels by qPCR assay. SREBP-2 and HMGCS1 mRNA levels were then plotted relative to the value obtained at time 0 (n = 4). Error bars represent the 95% confidence interval. One, two and three asterisks indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with cells harvested at 0 h.
**Figure 4:** Effect of MK-2206 on the expression of key genes of the lipogenic pathway – Sterol-fed HepG2 cells were treated with or without 5 μmol/L MK-2206 before harvesting at the indicated times. Cells were then processed for isolation of mRNA and determination of SREBP-1c, IDOL, ACACA, FASN, SCD1 and GAPDH mRNA levels by qPCR assay. SREBP-1c, IDOL, ACACA, FASN and SCD1 mRNA levels were then plotted relative to the value obtained at time 0 (n = 4). Error bars represent the 95% confidence interval. One, two and three asterisks indicate p < 0.05, p < 0.01 and p < 0.001, respectively, compared with matched cells harvested at 0 h.
Figure 5: MK-2206 reduces HMGCR protein levels in an MG-132-sensitive manner – Sterol-fed HepG2 cells were treated with vehicle, the indicated concentrations of MK-2206 or 25-hydroxycholesterol (25-HC) for 14 h in the presence or absence of MG-132 for the last 8 h of treatment. After harvesting and fractionation of the cells, the membrane fractions were subjected to Western blot analysis with the indicated antibodies. The blot shows one of four independent experiments. Anti-HMGCR (ABS229) and anti-calreticulin (12238) were obtained from Merck Millipore (Burlington, MA) and Cell Signaling (Danvers, MA), respectively.
Figure 6: Knockdown efficiency of siRNA targeting SREBP-2 in HepG2 cells – mRNA preparations in Fig. 4C were used for determination of SREBP-2 and GAPDH mRNA levels by qPCR assay. SREBP-2 mRNA levels were then plotted relative to the value obtained at time 0 (n = 4). Error bars represent the 95% confidence interval. Three asterisks indicate $p < 0.001$ compared with vehicle-treated cells.
Figure 7: Evaluation of ER stress in response to MK-2206 – Sterol-fed CHO cells were treated with vehicle or the indicated concentrations of MK-2206 for 14 h. As a positive control for ER stress, cells were treated with 5 mM DTT for 14 h. Total RNA was isolated, subjected to RT-PCR with XBP1-specific primers and the PCR products were then resolved on 2% agarose gel for separation of unspliced XBP1 (XBP1u) and spliced XBP1 (XBP1s) mRNAs. One representative experiment out of three is shown. DTT (43815) was obtained from Sigma-Aldrich (St. Louis, MO).

Figure 8: Evaluation of apoptosis in response to MK-2206 – (Sterol-fed HepG2 cells were treated with vehicle or the indicated concentrations of MK-2206 or okadaic acid (OA). Cells treated with OA served as positive control for apoptosis-associated PARP cleavage. At 14 h post-treatment, cells were harvested and subjected to anti-PARP Western blot analysis to detect full-length (116 kDa) and caspase-cleaved (89 kDa) PARP. The blot shows one of three independent experiments. Okadaic acid (OA) and anti-PARP (9542) were from Santa Cruz Biotechnology (Dallas, TX) and Cell Signaling (Danvers, MA), respectively.
**Figure 9:** Examination of the effect of MK-2206 on cell viability – Sterol-fed HepG2 cells were treated with vehicle or the indicated concentrations of MK-2206 for 72 h before analysis of cell viability using CCK-8 assay as described in materials and methods. Results were then plotted relative to vehicle-treated control (n = 4). Two asterisks indicate p < 0.01 compared with vehicle-treated cells.
Triciribine increases LDLR expression and LDL uptake through stabilization of LDLR mRNA

Katrine Bjune, Lene Wierød & Soheil Naderi

Low-density lipoprotein receptor (LDLR) is a key regulator of the metabolism of plasma low-density lipoprotein cholesterol (LDL-C), the elevated levels of which are associated with an increased risk of cardiovascular disease. Therefore, enhancing LDLR expression represents a potent treatment strategy for hypercholesterolemia. Here, we report that in cultured human hepatoma cells, triciribine, a highly selective AKT inhibitor, increases the stability of LDLR mRNA, an event that translates into upregulation of cell-surface LDLR levels and induction of cellular LDL uptake. This effect of triciribine requires ERK activity and is partially dependent on the intervening sequence between the AU-rich elements ARE3 and ARE4 in LDLR 3′UTR. We also show that triciribine downregulates the expression of PCSK9 mRNA and blunts the secretion of its protein. Notably, triciribine was found to potentiate the effect of mevastatin on LDLR protein levels and activity. We also show that primary human hepatocytes respond to triciribine by increasing the expression of LDLR. Furthermore, a pilot experiment with mice revealed that a two-week treatment with triciribine significantly induced the hepatic expression of LDLR protein. These results identify triciribine as a novel LDLR-elevating agent and warrant further examination of its potential as a hypocholesterolemic drug either as monotherapy or in combination with statins.

Elevated plasma low-density lipoprotein cholesterol (LDL-C) is associated with the risk of developing atherosclerotic cardiovascular disease (ASCVD), a condition that accounts for a large proportion of deaths worldwide. Therefore, lowering of plasma LDL-C levels is the most effective method to prevent development of ASCVD. Low-density lipoprotein receptor (LDLR) is an integral membrane protein that is most abundantly expressed in the liver, where it binds to and removes LDL-C from the circulation by endocytosis. Therefore, the amount of LDLR expressed in hepatocytes inversely correlates with the level of plasma LDL-C.

LDLR levels are controlled by a multi-layered regulatory mechanism: At the transcriptional level, LDLR gene expression is controlled by a cholesterol-responsive negative feedback mechanism through sterol regulatory element-binding protein-2 (SREBP-2). Post-translational regulation of LDLR is primarily governed by PCSK9 that upon binding to cell-surface LDLR mediates its degradation. The post-transcriptional regulation of LDLR is mainly achieved through modulation of its mRNA stability. LDLR mRNA is a labile transcript that contains a 2.5 kb-long stretch of 3′ untranslated region (3′UTR). Embedded within the LDLR 3′UTR are several cis-regulating elements that modulate the rate of LDLR mRNA degradation through their interaction with trans-regulatory RNA binding proteins. Certain trans-regulating factors act as mRNA stabilizers, while others promote mRNA decay. Both types of factors have been reported to interact with LDLR mRNA. For instance, LDLR 3′UTR has been shown to associate with not only the mRNA-stabilizing factor, human antigen R (HuR), but also with decay-promoting factors, such as ZFP36 ring finger protein-like 1 and –like 2 proteins (ZFP36L1 and ZFP36L2), KH-type splicing regulatory protein (KSRP) as well as the D and I members of the heterogeneous nuclear ribonucleoprotein family (hnRNP D and hnRNP I). These and similar observations raise the interesting possibility that the stability of LDLR mRNA is determined by the sum of the positive and negative effects these trans-regulating factors exert on its turnover rate.

The AKT family of phospholipid-binding serine/threonine protein kinases consists of three highly homologous members, AKT1, AKT2 and AKT3, which exhibit a differential tissue-specific expression. The AKT isoforms share a common structure that consists of an N-terminal pleckstrin homology (PH) domain, a central kinase domain and a C-terminal hydrophobic motif. The first prerequisite for activation of AKT is interaction...
of its PH domain with phosphatidylinositol (3,4,5)-trisphosphate (PIP3), an event that recruits AKT to the mem-
brane where it is activated through phosphorylation. AKT proteins regulate a wide range of cellular functions,
such as cell cycle progression, apoptosis and intermediary metabolism24. Consequently, abnormal AKT kinase
activity is implicated in the etiology of a number of diseases, such as cancer, making it an attractive target for
kinase inhibitor-based drug development25. One such inhibitor is triciribine, a tricyclic analogue of purine which
was initially identified as an inhibitor of DNA synthesis26. In its capacity as an inhibitor of AKT, the phosphoryl-
ated form of triciribine, triciribine phosphate, is currently in clinical testing as an anti-neoplastic agent27,28. Once
inside the cell, triciribine is converted to triciribine phosphate by adenosine kinase29. Triciribine phosphate exerts
it kinase inhibitory effect by binding to the PH domain of AKT, thereby preventing its recruitment to the plasma
membrane24.

Research in our lab centers on the mechanisms that modulate the expression of LDLR. Because the regulation
of LDLR by sterols is relatively well-characterized, we recently decided to focus our efforts on the investigation of
other, less-characterized LDLR regulatory pathways. To this end, we chose to study the interaction between AKT
and LDLR expression, because although AKT signaling has been reported to impinge on the LDLR expression
machinery20,24,25, the mechanism by which it does so remains to be elucidated. During the course of our investiga-
tion into the effect of AKT inhibition on LDLR expression, we discovered that triciribine exerts a potent inducing
effect on LDLR levels and decided to explore in more detail this property of triciribine. Here, we report that
triciribine, while mitigating SREBP-2 transactivation activity, increases the stability of LDLR mRNA. The net
result of these effects is a significant induction of LDLR protein levels that is accompanied by reduction in PCSK9
expression. We also show that triciribine enhances the effects of mevastatin on LDLR levels. Importantly, we found
that triciribine increases the expression of LDLR in both primary human hepatocytes and mice liver.

Results

Stimulation of LDLR expression and function by triciribine. To examine the effect of AKT inhibition
on LDLR expression, we chose triciribine, a tricyclic nucleoside that inhibits all AKT isoforms with a high degree
of selectivity26. Using HepG2 cells, a human hepatoma cell line, we selected whether triciribine influences LDLR
protein levels and if the effect is influenced by the presence or absence of sterols in the culture medium. To this
end, HepG2 cells were first cultured in medium containing fetal bovine serum (FBS) or lipoprotein-deficient
serum (LPDS; henceforth, sterol-fed and sterol-starved, respectively). Sterol-starved cells have lower cholesterol
content than sterol-fed cells27,28. Cells were then treated with different concentrations of triciribine for 14 hours
and examined for LDLR expression by Western blotting. Figure 1a shows that triciribine elevated the levels of
LDLR in both sterol-fed and sterol-starved cells in a dose-responsive manner up to a concentration of 5 μM.
The sub-maximal concentration of 1 μM was used for the remainder of this study unless otherwise indicated. To
examine the induction kinetics of LDLR in response to triciribine, sterol-fed and sterol-starved HepG2 cells were
treated with 1 μM triciribine and harvested at different times up to 24 hours for analysis of LDLR levels by immu-
noblotting. LDLR was significantly induced within 4 hours after triciribine treatment and continued to increase
until 14 hours after which triciribine exhibited a slight loss of its LDLR-inducing effect (Fig. 1b). Importantly,
short time after exposure to triciribine, triciribine exhibited a slight loss of its LDLR-inducing effect (Fig. 1b).
Importantly, examination of the phosphorylation status of AKT on S473, which serves as an indicator of its kinase
activity, showed that the onset of LDLR induction by triciribine was preceded by a robust inhibition of AKT activity
(Supplementary Fig. S1). The immunoblotting results for LDLR were further confirmed by the results obtained
with fluorescence microscopy showing that cells exposed to triciribine expressed higher amount of LDLR pro-
tein (Fig. 1c). Thus, triciribine increases the expression of LDLR under both basal and sterol-deficient culture
conditions. To assess the impact of triciribine on the function of LDLR, we analyzed the effect of triciribine on
cell-surface LDLR expression. Fig. 1d shows that treatment with triciribine potently increased cell-surface LDLR
levels. Importantly, the induction of LDLR surface expression by triciribine correlated with enhanced internaliza-
tion of DiD-LDL (Fig. 1e). These results show that the induction of LDLR by triciribine translates to an increase
in LDLR function.

Finally, to ascertain that the observed effect is not specific to HepG2 cells, we examined the effect of triciribine
on LDLR expression in hTERT-immortalized human hepatocytes hepatocytes (IHH) and the non-hepatic cell
line, HeLa. Similar to HepG2, IHH cells responded to triciribine treatment with an increased expression of LDLR
(Fig. 1f). In contrast, triciribine failed to increase LDLR levels in HeLa cells. Taken together, these results suggest
that the LDLR-inducing effect of triciribine may be restricted to cells of hepatic origin.

Triciribine represses the turnover of LDLR mRNA. As a first step towards understanding the mech-
anism that underlies the triciribine-mediated induction of LDLR, we examined the effect of triciribine on the
expression of LDLR mRNA. As shown in Fig. 2a, LDLR mRNA was rapidly induced by triciribine in sterol-fed
HepG2 cells, and reached a maximum level at about 6 hours after which it gradually declined to approximately
half its peak level by the end of the experiment. Because LDLR expression is mainly regulated at the level of tran-
scription, we wished to assess whether triciribine induces LDLR gene transcription. To this end, we first trans-
fected sterol-fed HepG2 cells with pLR1563-luc vector, a construct that encodes the firefly luciferase gene under
the control of the human LDLR promoter region +58 to −156325, and examined them for luciferase activity
after exposure to triciribine. Surprisingly, treatment of cells with triciribine led to reduction, rather than induc-
tion, of luciferase activity (Fig. 2b), suggesting that activation of LDLR gene expression does not account for
triciribine-mediated induction of LDLR mRNA levels. To determine whether this suggestion holds true in the
endogenous cellular setting, we examined the effect of triciribine on LDLR mRNA levels in cells whose SREBP-2
was knocked down by siRNA. If induction of LDLR gene is responsible for triciribine-mediated upregulation
of LDLR mRNA, then inhibition of SREBP-2, which confers regulability on LDLR gene expression24, should
abrogate the inducing effect of triciribine on LDLR mRNA levels. As shown in Fig. 2c, triciribine retained its
Figure 1. Induction of LDLR expression and activity by triciribine. (a) Sterol-fed or sterol-starved HepG2 cells were treated with vehicle or the indicated doses of triciribine for 14 hours and then harvested for examination by Western blotting. Left panel: One representative blot is shown (n = 5). Unprocessed blots are shown in Supplementary Fig. S7. The graph in the right panel shows quantification of the Western blots, relative to similarly cultured, vehicle-treated cells. (b) HepG2 cells were cultured as in a and then treated with or without 1 μM triciribine, harvested at the indicated time points and then analyzed by immunoblotting. Left panel: One representative blot is shown (n = 5). Unprocessed blots are shown in Supplementary Fig. S8. The graph in the right panel shows quantification of the Western blots, relative to similarly cultured cells harvested at 0 hour. (c) Sterol-fed HepG2 cells were treated with 1 μM for 14 hours and then examined by fluorescence microscopy as described in Materials and Methods. Green: LDLR, Alexa fluor 488, blue: nuclei, DAPI. One representative experiment is shown (n = 3). (d) Sterol-fed HepG2 cells were exposed to vehicle or 1 μM triciribine for 14 hours before analysis for mean fluorescence intensity (as a measure of cell-surface LDLR levels) by flow cytometry. The graph shows the results plotted relative to vehicle-treated control (n = 4). (e) Sterol-fed HepG2 cells
were cultured and treated as described in d. After exposure of cells to DiD-LDL (10 μg/ml) during the last 2 hours of treatment, cells were harvested and analyzed by flow cytometry to determine the mean fluorescent intensity of internalized DiD-LDL. The graph shows the results plotted relative to vehicle-treated control (n = 4). (f) Sterol-fed immortalized human hepatocytes (IHH) and HepA cells were treated with vehicle or the indicated concentrations of triciribine for 14 hours and then harvested and analyzed by Western blotting. One representative blot is shown (n = 3). Unprocessed blots are shown in Supplementary Fig. S9. Error bars represent SD. *p < 0.05. **p < 0.01 and ***p < 0.001 compared with matched vehicle-treated cells (a, d and e) or matched cells harvested at 0 hour (b).

ability to induce LDLR mRNA levels in the absence of SREBP-2. These results show that triciribine utilizes a post-transcriptional mechanism to increase LDLR mRNA levels.

Stabilization of mRNA provides a plausible explanation for the triciribine-mediated accumulation of LDLR mRNA in the absence of LDLR gene induction. To examine the validity of this notion, we assessed the effect of triciribine on LDLR mRNA stability using actinomycin D (Act D). As shown in Fig. 2d, triciribine significantly increased the stability of LDLR mRNA. Estimation of LDLR mRNA half-life showed that triciribine increased the half-life of LDLR mRNA from about 1.9 hours to approximately 4.8 hours. Thus, triciribine induces the accumulation of LDLR mRNA through induction of its stability.

Mammalian cells utilize a number of different mRNA decay pathways to regulate the stability of mRNA39. One such mechanism relies on the cis-regulatory elements that reside in the 3′ UTR of mRNA. The LDLR 2.3 kb long 3′ UTR harbors four AU-rich elements (AREs) and a region encompassing four clustered UCUA repeats that have been shown to impact the turnover rate of LDLR mRNA41,31,33. In addition, close inspection of LDLR 3′ UTR sequence showed the region between ARE3 and ARE4, referred to herein as intervening sequence (IVS), to be disproportionately AU-rich. To determine whether these sequences in the 3′ UTR of LDLR mRNA play a role in the stabilizing effect of triciribine on LDLR mRNA, we cloned wt as well as different deletion mutants of LDLR 3′ UTR into the pmR-ZsGreen1 reporter plasmid. These constructs express the fluorescent ZsGreen1 protein from a ZsGreen1-LDLR-3′ UTR chimeric mRNA. Translation of these transcripts produces ZsGreen1 proteins whose fluorescence serves as an indicator for the stability of the chimeric transcript. As expected, inclusion of wt LDLR 3′ UTR in pmR-ZsGreen1 plasmid potently reduced the amount of ZsGreen1 fluorescence, confirming the destabilizing effect of LDLR 3′ UTR in this experimental system (Fig. 2e). Importantly, cells that were transfected with pmR-ZsGreen1-LDLR-3′ UTR-wt exhibited a one-fold increase in fluorescence when treated with triciribine. However, neither ARE1, ARE2, ARE3, ARE4 nor UCUA deletions had an appreciable impact on the detected fluorescence signal in triciribine-treated cells. In contrast, deletion of IVS in LDLR 3′ UTR not only augmented ZsGreen1 levels in untreated cells, but also partially alleviated (30%) the effect of triciribine on the expression of ZsGreen1 fluorescence. To exclude the possibility that the effects observed with pmR-ZsGreen1-LDLR-3′ UTR-Del-IVS result from shortening of LDLR 3′ UTR, we replaced the IVS region (1202 bases) in pmR-ZsGreen1-LDLR-3′ UTR-wt with a 1155 bases long portion of the transferrin receptor (TFRC) coding region to generate pmR-ZsGreen1-LDLR-3′ UTR-IVS-TFRC plasmid. This vector expresses a ZsGreen1-LDLR-3′ UTR chimeric mRNA that is of similar size to ZsGreen1-LDLR-3′ UTR-wt but lacks the IVS region. As shown in Supplementary Fig. S2, the level of fluorescence expression generated by pmR-ZsGreen1-LDLR-3′ UTR-IVS-TFRC was similar to that of pmR-ZsGreen1-LDLR-3′ UTR-Del-IVS. Furthermore, replacement of IVS with the TFRC coding sequence had an inhibitory effect (25%) on triciribine-mediated induction of fluorescence expression that was similar to that of IVS deletion. Taken together, these results suggest that triciribine exerts its LDLR mRNA-stabilizing effect in part through one or several destabilizing regulatory elements that are located between ARE3 and ARE4.

Triciribine inhibits PCSK9 expression. PCSK9 plays an important role in cholesterol homeostasis as a result of its ability to reduce cell-surface LDLR levels7. The promoter of PCSK9 gene contains a serum response element (SRE) that makes its transcription subject to regulation by SREBP-217. Given the above observation that triciribine inhibited the SREBP-2-dependent transcription, we set out to investigate the effect of triciribine on PCSK9 mRNA levels. Fig. 3a shows that triciribine reduced the levels of PCSK9 mRNA. Similarly, expression of HMGCR mRNA whose gene is also subject to regulation by SREBP-232,33 was inhibited by triciribine (Fig. 3a). These results suggest that inhibition of SREBP-2-regulated transcription is a general feature of triciribine. PCSK9 exerts its inhibitory effect on LDLR following its secretion into the extracellular milieu. Therefore, we wished to examine whether the triciribine-mediated inhibition of PCSK9 expression is associated with a decrease in the level of secreted PCSK9. To this end, we carried out ELISA measurements of PCSK9 secreted into the media of HepG2 cells that were cultured in the absence or presence of triciribine and found that PCSK9 secretion from cells that were exposed to triciribine was significantly attenuated (Fig. 3b).

Triciribine augments the mevastatin-mediated upregulation of LDLR levels and LDL uptake. The ability of triciribine to increase the LDLR mRNA pool size through mRNA stabilization suggested that in a combinatorial treatment, it would potentiate the LDLR-inducing effect of statins. To examine this assumption, we treated sterol-starved HepG2 cells with triciribine and mevastatin, either alone or in combination, and analyzed them for LDLR levels and LDL uptake. As shown in Fig. 4a, treatment of cells with both triciribine and mevastatin enhanced the mevastatin-mediated induction of LDLR protein. Similarly, examination of cellular uptake of DiD-LDL showed that triciribine significantly enhanced the effect of mevastatin on LDL internalization.
Figure 2. Triciribine induces LDLR mRNA levels in the absence of SREBP-2 activity. (a) Sterol-fed HepG2 cells were treated with or without 1 μM triciribine before harvesting at the indicated times. Cells were then processed for isolation of mRNA and determination of LDLR, TFRC and GAPDH mRNA levels by qPCR assay. The plot shows LDLR and TFRC mRNA levels plotted relative to cells harvested at time 0 (n = 4). (b) Sterol-fed HepG2 cells were transfected with firefly luciferase reporter constructs containing either wt (pLR1563-luc) or mutant (pLR1563/mutSRE-1-luc) LDLR promoter and Renilla luciferase reporter. At 16 hours after transfection, cells were exposed to the indicated concentrations of triciribine for 14 hours and then harvested for determination of LDLR promoter activity. The graph shows the results plotted relative to vehicle-treated cells that were transfected with pLR1563-luc (n = 4). (c) Sterol-fed HepG2 cells were transfected with a non-targeting siRNA (NT siRNA) or an SREBP-2-specific siRNA. Twenty four hours after transfection, vehicle or triciribine was added to the cultures and cells were incubated for another 14 hours before harvesting for qPCR analysis of LDLR, SREBP-2 and GAPDH mRNA levels. The levels of LDLR and SREBP-2 mRNAs were then plotted relative...
to vehicle-treated, NT siRNA-transfected cells (n = 4). (d) At 14 hours after treatment of sterol-fed HepG2 cells with vehicle or 1 μM triciribine, actinomycin D (Act D; 5 μg/ml) was added to the cultures and then harvested at the indicated time points for qPCR analysis of LDLR and GAPDH mRNA levels. The plot shows LDLR mRNA levels relative to GAPDH mRNA levels relative to respective vehicle-treated controls (n = 4). (e) Upper panel: schematic presentation of the chimeric ZsGreen1-LDLR-3′UTR constructs. Lower panel: at 24 hours after transfection of sterol-fed HepG2 cells with one of the constructs, cells were treated with 1 μM triciribine and then harvested for determination of mean ZsGreen1 fluorescence intensity (as an indicator of ZsGreen1 transcript levels) by flow cytometry. The graph shows the results plotted relative to vehicle-treated cells that were transfected with ZsGreen1-LDLR-3′UTR-wt construct (n = 4). AREs are represented with numerically labelled rectangles. Letter U refers to UCAU repeats. IVS, intervening sequence. mRNA data are displayed with error bars representing the 95% confidence interval. Otherwise, error bars represent SD. *p < 0.05, **p < 0.01 and ***p < 0.001 compared with matched cells harvested at time 0 (a and d), vehicle-treated cells that were transfected with pLR1563-luc (b), vehicle-treated cells that were transfected with NT siRNA (c) or matched vehicle-treated cells (e).

Stimulation of mevastatin-mediated induction of LDLR by triciribine suggests that triciribine ameliorates the mevastatin-induced activation of SREBP-2, an implication that contrasts with the result that triciribine exerts an inhibitory effect on SREBP-2 activity (Fig. 2b and Supplementary Fig. S3). To address this contradiction, we set out to examine the LDLR promoter activity and mRNA levels in cells that were co-treated with triciribine and mevastatin. To this end, we treated HepG2 cells that were transfected with pLR1563-luc vector with triciribine and mevastatin, either alone or in combination, and examined them for luciferase activity and LDLR mRNA levels. As expected, luciferase activity was partially inhibited in cells that were treated with triciribine, whereas it was induced in mevastatin-treated cells (Fig. 4b). Importantly, although cells that were co-treated with triciribine and mevastatin contained somewhat lower levels of luciferase activity than cells that were treated with mevastatin alone, they expressed higher levels of LDLR mRNA, apparently as a consequence of the LDLR mRNA-stabilizing effect of triciribine. This result indicates that the LDLR mRNA-stabilizing effect of triciribine not only compensates for its hampering effect on SREBP-2 activity, but also contributes to its stimulating effect on mevastatin-mediated induction of LDLR mRNA.

Induction of LDLR by triciribine requires ERK activity. The effects of triciribine on LDLR mRNA stability and PCSK9 levels were reminiscent of those elicited by berberine and 5-Azacytidine (5-AzaC)11,34,35. This similarity suggested that triciribine might use a mechanism analogous to that of berberine or 5-AzaC to increase the stability of LDLR mRNA. We therefore proceeded to examine whether ERK was involved in the stabilizing effect of triciribine on LDLR mRNA. We first assessed the effect of triciribine on phosphorylation of ERK as an indicator of its kinase activity. Treatment of cells with triciribine led to a rapid induction of ERK phosphorylation (within 1 hour) which reached its peak value at 3 hours after which it decreased to levels slightly above basal values (Fig. 5a). Of note is the observation that induction of LDLR mRNA is preceded by the kinetics of ERK phosphorylation (see Fig. 1b). To examine whether ERK plays a role in the inducing effect of triciribine on LDLR mRNA, we treated the cells with the MEK/ERK inhibitor, U0126, in the absence or presence of triciribine for 5 or 12 hours and then examined them for ERK phosphorylation and LDLR protein levels by Western blotting. U0126 was found to abrogate the phosphorylation of ERK both in the absence and presence of triciribine, an event that correlated with inhibition of triciribine-mediated induction of LDLR protein and LDLR mRNA (Fig. 5b,c). Finally, to ascertain the nature of this inhibitory effect of U0126, we examined the effect of ERK inhibition on the stabilizing effect of triciribine on LDLR mRNA. As shown in Fig. 5d, U0126 significantly mitigated the triciribine-induced stabilization of LDLR mRNA. Taken together, these results show that triciribine stabilizes LDLR mRNA in an ERK activity-dependent manner.

Triciribine phosphate mimics the effect of triciribine on LDLR. Once inside the cell, triciribine is converted to its active phosphorylated derivative, triciribine phosphate that is currently in phase II human clinical trials37,38,39. Consequently, we considered it important to examine whether the effect of triciribine phosphate on LDLR levels and LDL uptake were similar to those produced by triciribine. For this purpose, we treated HepG2 cells with different concentrations of triciribine phosphate and then examined them for the expression of LDLR protein. As shown in Fig. 6a, treatment of cells with triciribine phosphate strongly upregulated LDLR protein levels. Next, we extended our analysis to the effect of triciribine phosphate on LDLR mRNA stability and found that triciribine phosphate exerted a potent stabilizing effect on LDLR mRNA (Fig. 6b). Finally, to assess the effect of triciribine phosphate on LDLR functionality, we analyzed internalization of DiD-LDL following exposure of cells to triciribine phosphate. As shown in Fig. 6c, treatment of cells with triciribine phosphate led to a significant induction of cellular DiD-LDL uptake. In sum, these results demonstrate that triciribine phosphate affects LDLR expression and LDL uptake in a fashion similar to that of triciribine.

Triciribine stimulates LDLR expression in primary human hepatocytes and mouse liver. To extend our findings obtained in cell lines to primary cells, we exposed cultured primary adult human hepatocytes to different concentrations of triciribine and analyzed them for LDLR protein levels by immunoblotting. Triciribine induced LDLR levels in primary human hepatocytes, albeit at concentrations higher than those that were needed to exert a similar effect in cell lines (Fig. 7a). Next, we sought to obtain an indication of whether triciribine exerts an LDLR-inducing effect in an in vivo setting. To this end, we first treated C57BL/6j mice with two different daily doses of triciribine (1 mg/kg or 2 mg/kg) for two weeks and then analyzed them for hepatic LDLR levels. Fig. 7b shows that treatment of mice with either doses of triciribine for two weeks significantly increased
the levels of hepatic LDLR. Interestingly, triciribine, similar to its effect on ERK phosphorylation in HepG2 cells, induced the phosphorylation of hepatic ERK (Supplementary Fig. S4). Taken together, these results show that triciribine exerts an LDLR-inducing effect in both primary human hepatocytes and mouse liver.

Discussion
As a result of its ability to internalize cholesterol into the cells, LDLR plays a crucial role in modulation of circulating levels of cholesterol4,5. It is therefore unsurprising that drugs that increase LDLR levels by either inducing its expression or inhibiting its PCSK9-mediated degradation have proven to be the most effective treatments for hypercholesterolemia39–41. Here, we report identification of a new agent, triciribine, as an effective LDLR-inducing agent that not only induces the expression of LDLR but also reduces PCSK9 levels.

Using in vitro cultures of the human hepatoma cell line HepG2 as a model system to study modulation of LDLR expression, we found that triciribine, a highly specific inhibitor of AKT kinases, induces the expression of LDLR through augmentation of its mRNA stability. Examination of ZsGreen1 fluorescence in cells that expressed chimeric ZsGreen1 mRNAs containing either wt or deletion mutants of LDLR 3′ UTR revealed that none of the four LDLR mRNA AREs or the cluster of UCAU repeats alone is utilized by triciribine to exert its mRNA-stabilizing effect. Although this result suggests that these cis-regulatory elements are not targeted by triciribine in isolation, it does not preclude the possibility that these elements mediate the stabilizing effect of triciribine on LDLR mRNA in a combinatorial manner. Indeed, evidence suggests that cis-regulatory elements in 3′ UTR often act in a combinatorial and perhaps redundant manner to control mRNA stability42–44. For instance, destabilization of IL-17 mRNA by tristetrapolin has been shown to depend on a cluster of several ARE motifs within the IL-17 3′ UTR45. Similarly, it has been reported that p38α targets 3 distinct AREs in IL-6 3′ UTR to stabilize IL-6 mRNA46. Therefore, it is reasonable to suggest that triciribine might depend on the concomitant action of several cis-acting elements in LDLR 3′ UTR for its mRNA-stabilizing effect. Interestingly, examination of ZsGreen1 fluorescence in cells that expressed the chimeric ZsGreen1-LDLR 3′ UTR-Del-IVS or ZsGreen1-LDLR 3′ UTR-IVS>TFRC revealed that the intervening region between ARE3 and ARE4 plays an partial role in both basal LDLR mRNA turnover, as well as the mRNA-stabilizing effect of triciribine. LDLR 3′ UTR has been shown to be subject to m6A modification, an event that facilitates its binding to the YTH-domain family member 2 (YTHDF2), thus resulting in LDLR mRNA decay47. This report, combined with our observation that the IVS region contains several sites that closely match the consensus signal for m6A methylation48, raise the possibility that triciribine may inhibit methylation of LDLR mRNA in the IVS region to protect it from binding to the mRNA-destabilizing m6A readers. If so, it will be interesting to investigate whether triciribine inhibits m6A writers or stimulates m6A erasers to abrogate methylation of LDLR mRNA.
The finding that triciribine reduces LDLR promoter activity suggests that it exerts an inhibitory effect on SREBP-2 activity. This idea is corroborated by the inhibitory effect of triciribine on HMGCR and PCSK9 mRNA levels, whose genes are also regulated by SREBP-2. Because these results were obtained with cells that were cultured in the presence of FBS, it may be suggested that through induction of cellular uptake of cholesterol, triciribine increases intracellular cholesterol levels, an event that is expected to trigger the feedback inhibition of SREBP-2. However, triciribine was found to also decrease HMGCR and PCSK9 mRNA levels in cells that were cultured in the presence of LPDS indicating that inhibition of SREBP-2 activity occurs as a direct response to triciribine. This notion is substantiated by the finding that triciribine inhibits the expression of the C-terminal fragment of cleaved SREBP-2 (Supplementary Fig. S3).

Interestingly, triciribine shares a number of similar traits with berberine and 5-AzaC: 1) inhibition of DNA synthesis, 2) induction of ERK activity, 3) stabilization of LDLR mRNA and 4) inhibition of PCSK9.
These similarities raise the possibility that triciribine exerts the effects that are presented in this report in its capacity as an inhibitor of DNA synthesis. Although we cannot formally disprove this suggestion, we are inclined to believe that triciribine-mediated induction of LDLR expression occurs as a result of its ability to inhibit AKT activity. This is primarily because GS K690693, another selective AKT inhibitor, also increases the expression of LDLR (Supplementary Fig. S5). Furthermore, triciribine exhibits a number of functional dissimilarities with berberine and 5-AzaC. For instance, while berberine has no effect on proteolytic activation of SREBP-2\(^{11}\), triciribine appears to inhibit cleavage of SREBP -2 (Supplementary Fig. S3). Similarly, induction of LDLR\(^{18}\) mRNA by 5-AzaC has been shown to be associated with increase in IRE1\(^{34}\) levels, whereas triciribine inhibits the expression of IRE1\(^{34}\) (Supplementary Fig. S6).

Berberine is reported to exert its LDLR mRNA-stabilizing effect by modulating the activity of mRNA-destabilizing factors hnRNP I and KSRP\(^{18}\). Therefore, we believe that future experiments designed to identify trans-regulatory proteins that mediate the stabilizing effect of triciribine on LDLR mRNA should consider hnRNP I and KSRP as plausible targets of triciribine. Equally important is assessment of the possible role of ZFP36L1 and ZFP36L2 proteins in the effect of triciribine on LDLR mRNA stability. ZFP36L1 and ZFP36L2 destabilize LDLR mRNA in an ERK activity-sensitive manner\(^{17}\). Therefore, it can be envisioned that triciribine, through its ability to increase the activity of ERK, induces phosphorylation of ZFP36L1 and ZFP36L2, an event that is reported to result in increase in LDLR mRNA stability.

The involvement of ERK in regulation of LDLR mRNA stability and the dependence of triciribine on ERK activity to stabilize LDLR mRNA promise us to put forward a simple mechanistic explanation for the stabilizing effect of triciribine on LDLR mRNA. AKT inhibits ERK activation by phosphorylating inhibitory sites in Raf, a kinase that functions as an upstream activator of ERK\(^{25-34}\). Inhibition of AKT by triciribine is then expected to alleviate the inhibitory effect of AKT on Raf, thus allowing downstream activation of ERK and the subsequent stabilization of LDLR mRNA.

**Figure 5.** Stabilization of LDLR mRNA by triciribine is dependent on ERK activity. (a) Sterol-fed HepG2 cells were treated with vehicle or 1 μM triciribine and harvested at the time points indicated for Western blot analysis. One representative blot from four independent experiments is shown. Unprocessed blots are shown in Supplementary Fig. S11. (b) sterol-fed HepG2 cells were pretreated with U0126 for 1 hour before exposure to triciribine. Cells were harvested at the indicated time points after addition of triciribine and analyzed by Western blotting. One representative blot is shown (n = 4). Unprocessed blots are shown in Supplementary Fig. S12. (c) Sterol-fed HepG2 cells were treated as in b, harvested 14 hours after addition of triciribine and then processed for isolation of mRNA and determination of LDLR and GAPDH mRNA levels by qPCR assay. The plot shows LDLR mRNA levels relative to vehicle-treated cells (n = 4). (d) Sterol-fed HepG2 cells were treated as in b. At 14 hours after addition of triciribine, cells were treated with actinomycin D (Act D; 5μg/ml) and then harvested after 6 hours for qPCR analysis of LDLR and GAPDH mRNA levels. The plot shows LDLR mRNA levels relative to respective vehicle-treated controls that were harvested before addition of Act D (0 hour), the values of which was set at 1 (n = 4). Error bars represent the 95% confidence interval. ***p < 0.001.
Figure 6. Triciribine phosphate mimics the effect of triciribine on LDLR expression and function. (a) Sterol-fed HepG2 cells were treated with vehicle or the indicated doses of triciribine phosphate. At 14 hours after incubation, cells were harvested and subjected to Western blot analysis. The left panel shows one representative blot from four independent experiments. Unprocessed blots are shown in Supplementary Fig. S13. The graph in the right panel shows quantification of the Western blots, relative to vehicle-treated cells. (b) Sterol-fed HepG2 cells were treated with vehicle or 0.5 μM triciribine phosphate for 14 hours before addition of actinomycin D (Act D; 5 μg/ml). At the indicated time points after addition of Act D, cells were harvested and analyzed by qPCR for quantification of LDLR and GAPDH mRNA levels. The plot shows LDLR mRNA levels relative to cells at time 0 (n = 4). (c) Sterol-fed HepG2 cells were treated with vehicle or triciribine phosphate for 14 hours. After exposure of cells to DiD-LDL (10 μg/ml) during the last 2 hours of treatment, cells were harvested and analyzed by flow cytometry to determine the mean fluorescence intensity of internalized DiD-LDL. The graph shows the results plotted relative to vehicle-treated control (n = 4). Error bars represent SD (a and c) or the 95% confidence interval (b) ***p < 0.001 when compared with vehicle-treated cells (a, c) or matched, vehicle-treated cells harvested at 0 hour (b).
Of importance is our finding that triciribine increases LDLR levels in both primary human hepatocytes and mouse liver. Therefore, we would like to suggest that triciribine represents a novel class of LDLR-inducing drugs with a therapeutic potential. The ability of triciribine to increase LDLR levels in cells that were cultured in either lipoprotein-containing medium or lipoprotein-deficient medium that were supplemented with statins (Fig. 4), a treatment reported to decrease cellular free cholesterol content\(^5\), suggests that triciribine is able to induce LDLR expression irrespective of cellular cholesterol status, raising the possibility that it can upregulate LDLR levels and consequently enhance cellular uptake of circulating cholesterol even under conditions where LDLR is inhibited by feedback inhibition. Furthermore, the ability of triciribine to enhance the effect of mevastatin on LDLR expression and cellular uptake of LDL suggests that it may also prove to exert an effective hypocholesterolemic effect when used in combination with statins. In this capacity, triciribine can not only be used to maximize the effect of statins, but it can also serve a statin dose-sparing function. Finally, we would like to note that given the involvement of AKT in a multitude of cellular events such as proliferation, apoptosis and insulin signaling\(^2\), it is justified to assume that inhibition of AKT by triciribine could carry a potential for adverse side effects. Therefore, we believe that prospective in vivo experiments should be carried out to determine whether triciribine, either alone or in combination with statins, evokes side effects which can negatively affect its potential as a hypocholesterolemic agent.

**Materials and Methods**

**Reagents and antibodies.** Triciribin and mevastatin were purchased from Selleckchem (Houston, Texas). Triciribine phosphate, U0126, actinomycin D (Act D), (±)-mevalonolactone, paraformaldehyde and saponin were from Sigma-Aldrich (St. Louis, Missouri). Antibodies against LDLR (3839; for Western blot analysis and immunofluorescence microscopy) and β-actin (ab8227) were obtained from BioVision (Milpitas, California) and Abcam (Cambridge, UK), respectively. Anti-LDLR (61087; for detection of cell-surface LDLR by flow cytometry) was obtained from Progen (Heidelberg, Germany). Anti-GAPDH (G9295) was from Sigma-Aldrich. Anti-β-tubulin (T9154-05G) was purchased from USBiological (Swampscott, Massachusetts).

**Cell culture and treatments.** HepG2 cells (European Collection of Cell Cultures, Salisbury, UK), human telomerase reverse transcriptase (hTERT)-immortalized human hepatocytes (IIH; a gift from Dr. Philippe Collas, University of Oslo, Oslo, Norway) and HeLa cells (European Collection of Cell Cultures, Salisbury, UK)
were cultured on collagen-coated culture vessel (BD Biosciences, San Jose, California) in HyClone Minimum Essential Medium (GE Healthcare Life Sciences, Pittsburg, Pennsylvania) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine (Sigma-Aldrich), 50 U/mL penicillin (GE Healthcare Life Sciences), 50 μg/mL streptomycin (GE Healthcare Life Sciences) and non-essential amino acids (BioWest, Nuaille, France). To obtain sterol-starved cells, cells were first cultured for 24 hours in complete medium supplemented with 10% FBS, washed twice in PBS and then cultured in complete medium containing 5% lipoprotein-deficient serum (LPDS). Freshly plated hepatocytes were purchased from QPS Hepatic Biosciences (Research Triangle Park, North Carolina). Immediately on arrival, cells were provided with Maintenance Medium containing 0.1% dexamethasone (QPS Hepatic Biosciences) and incubated for 24 hours before treatment with triciribine.

All cells were cultured at 37°C in an atmosphere with 5% CO2 and 95% humidity. All drugs were dissolved in DMSO and added to cell cultures such that the final concentration of DMSO was kept at 0.1% (v/v). Control cultures were treated with DMSO alone at 0.1% (v/v).

Western blot analysis. Cells were harvested by trypsinization and lysed in Triton X-100 lysis buffer (20 mM Tris [pH 7.5], 100 mM NaCl, 1% Triton X-100, 10 mM EDTA and Complete Protease Inhibitor Cocktail). After protein concentration determination using Pierce BCA protein assay (Thermo Fischer Scientific), equal amounts of proteins were resolved on a 4–20% SDS-PAGE, transferred to PVDF membrane (Bio-Rad, Hercules, California) and subjected to immunoblotting. After densitometric scanning of the immunoblots, the band intensity of the protein of interest was normalized to that of GAPDH or β-tubulin and plotted relative to the control value (vehicle-treated cells or cells harvested at 0 hour).

Quantitative real-time PCR. Total RNA was isolated with the QIAamp RNA Isolation Kit (Qiagen, Hilden, Germany) and converted to cDNA using the AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies, Santa Clara, California). The cDNA was then used as the template for quantitative real-time polymerase chain reaction (qPCR) using Brilliant III Ultra-Fast qPCR Master Mix on MX3000P qPCR system (Agilent technologies) and the following PrimeTime Predesigned qPCR Assays (Integrated DNA Technologies, Coralville, Iowa): human LDLR (Hs.PT.58.14599757), human GAPDH (Hs.PT.39a.22214836), human HMGCR (Hs.PT.58.41105492), human PCSK9 (Hs.PT.58.203171419), SREBP-2 (Hs.PT.56a.2651954) and human TFR (Hs.PT.39a.22214826). qPCRs were run in duplicate and GAPDH was used as the normalizing gene. The 2ΔΔCt method was used to calculate relative mRNA levels.

Analysis of cell-surface LDLR and LDL internalization. The levels of LDLR on the cell surface and internalization of LDL were measured by flow cytometry as previously described.

Analysis of LDLR expression by confocal fluorescence microscopy. Samples were fixed in 4% paraformaldehyde followed by permeabilization using 0.1% saponin. Cells were then incubated with anti-LDLR (1:100 dilution; BioVision) overnight. Alexa Fluor 488-conjugated anti-rabbit (1:200 dilution; 715–546–150 Jackson ImmunoResearch, Pennsylvania) was used for visualization. DAPI (ThermoFischer Scientific) was used to stain DNA. All pictures were taken with a Zeiss LSM 700 confocal system.

Determination of PCSK9 in cell culture medium. The amount of PCSK9 secreted by cells into culture medium was measured using Circulex Human PCSK9 ELISA Kit (MBL International, Woburn, MA) according to the manufacturer's instructions.

DNA constructs, transfection and luciferase reporter assays. The amount of PCSK9 secreted by cells into culture medium was measured using Circulex Human MCSK9 ELISA Kit (MBL International, Woburn, MA) according to the manufacturer's instructions.

Triciribine in vivo activity. Six to eight week old male C57BL/6J mice (Janvier Labs, Le Genest-Saint-Ise, France) were housed in a controlled environment with a 12 hour light/dark cycle at 22°C. All mice were acclimated for 7 days before a 2-weeks treatment period and were fed a normal Chow diet (801700; SDS, Essex, UK) during both acclimatization and treatment periods. Mice were separated into three treatment groups each consisting of 4 mice. One group received i.p. triciribine at 1 mg/kg daily. A second group received daily i.p. injections...
of triciribine at 2 mg/kg. A third group was administered daily i.p. injections of vehicle (0.1% DMSO in PBS). Injection volumes were 100 µl per mouse. At the end of the treatment period, mice were sacrificed and livers were processed into a powder by grinding in liquid nitrogen, lysed in RIPA buffer and then analyzed by Western blotting. Experiments were performed in accordance with institutional guidelines and were approved by The Norwegian Animal Research Authority.

**Statistical analysis.** The difference between the means of the two independent groups was examined by unpaired, two-tailed Student’s t-test and a p value of <0.05 was considered statistically significant.

**References**


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Author Contributions
Katrine Bjune and Lene Wierød designed the research, performed experiments, analyzed data, and contributed to the writing of the manuscript. Soheil Naderi provided the concept, designed the research, performed experiments, analyzed data, and wrote the first draft and the final version of the manuscript.

Additional Information
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Competing Interests: Soheil Naderi is listed as an inventor on a patent application relating to triciribine. Katrine Bjune and Lene Wierød declare no potential conflict of interest.

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Triciribine increases LDLR expression and LDL uptake through stabilization of LDLR mRNA

Supplementary materials

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I. Supplementary materials and methods

Reagents and antibodies

DTT, 25-hydroxycholesterol (25-HC) and 5-Azacytidine (5-AzaC) were obtained from Sigma-Aldrich (St. Louis, Missouri). GSK690693 was obtained from selleckchem (Houston, Texas). Anti-phospho-AKT (pAKT, S473; AF887) and anti-AKT (MAB2055) antibodies were from R&D Systems (Minneapolis, MN). Antibodies raised against the C-terminus of SREBP-2 (557037) were purchased from BD Biosciences (San Jose, CA). Anti-calreticulin (12238), anti-IRE1α (3294) and anti-phospho-GSK-3β (pGSK-3β, S9) were from Cell Signaling (Danvers, MA).

Determination of XBP1 splicing

Total RNA (1 μg) isolated from CHO cells was reverse-transcribed with the Qiagen Onestep RT-PCR kit (Qiagen, Hilden, Germany) and the following X-Box Binding Protein 1 (XBP1)-specific primers [1]; forward primer: 5’-CCTTGTAGTTGAGAACCAGG-3’; reverse primer: 5’-GGGGCTTTGATATATGTGGG-3’. The reaction conditions consisted of a 15 minute polymerase activation step at 95°C followed by 40 tri-step cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. The final PCR products were then separated on a 2% agarose gel containing GelRed Nucleic Acid Gel Stain (Biotium, Fermont, CA) along with DNA markers.

Cell fractionation

HepG2 cells were harvested by trypsinization and pellets were washed in PBS before incubation in hypotonic buffer (10 mM Tris [pH 7.6], 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT) for 15 min on ice. Cells were then lysed by 25 passes through a 25-gauge needle and centrifuged at 1000xg for 10 min at 4°C to collect the nuclei. The nuclei pellet was resuspended in 100 μl RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5 mM EDTA, 10 mM NaF, 5 mM β-glycerophosphate, 0.1 mM Na3VO4 and Complete Protease Inhibitor Cocktail [Sigma-Aldrich]) and the suspension was incubated on ice for 40 min with intermittent vortexing at 2000 rpm and then centrifuged at 20,000xg for 30 min at 4°C. The recovered supernatant was designated as nuclear extract. The supernatant recovered from the 1000xg centrifugation was spun at 20,000xg for 30 min at 4°C to pellet membranes. The membrane pellet was then resuspended in 100 μl RIPA and incubated on ice for 40 min with intermittent vortexing at 2000 rpm to extract membrane proteins and then clarified by centrifugation at 20,000xg for 30 min at 4°C. The supernatant was designated as the membrane fraction.
Figure 1: Effect of triciribine on AKT phosphorylation – Sterol-fed HepG2 cells were treated with or without 1 μM triciribine for the indicated times before harvesting for analysis by immunoblotting with the antibodies against phospho-AKT (pAKT) and total AKT. One representative blot of three is shown.
Figure 2: Effect of IVS deletion on LDLR mRNA. — Upper panel: schematic presentation of the chimeric ZsGreen1-LDLR-3'UTR constructs. Lower panel: at 24 hours after transfection of sterol-fed HepG2 cells with one of the constructs, cells were treated with 1 μM triciribine and then harvested for flow cytometric analysis of mean ZsGreen1 fluorescence intensity as an indicator of ZsGreen1 transcript levels. Results were then plotted relative to vehicle-treated cells that were transfected with ZsGreen1-LDLR-3'UTR-wt construct (n = 4). Error bars represent SD. **p < 0.01 and ***p < 0.001 compared with matched vehicle-treated cells. AREs are represented with numerically labelled rectangles. Letter U refers to UCAU repeats. IVS, intervening sequence; TFRC, transferrin receptor.
Figure 3: Effect of triciribine on levels of the C-terminal fragment of SREBP-2. — Sterol-fed HepG2 cells were treated with 1 μM triciribine for the indicated times before harvesting. Sterol-starved cells that were treated with mevastatin or mevastatin + 25-hydroxycholesterol (25-HC) served as controls for induction and inhibition of SREBP-2 cleavage, respectively. Cells were then subjected to subcellular fractionation before Western blot analysis for expression of SREBP-2 and calreticulin. The blot shows one of three independent experiments. FL-SREBP-2, full-length SREBP-2; CTF-SREBP-2, C-terminal fragment of SREBP-2. Unprocessed blots are shown in Supplementary Fig. S17.
**Figure 4:** Effect of triciribine on ERK phosphorylation in mouse liver – Equal amount of mouse liver lysates (50 μg) for the experiment of Fig. 7b were run on duplicate gels and transferred to PVDF membrane. One membrane was immunoblotted with anti-phospho-ERK (pERK) and the duplicate membrane was immunoblotted with anti-total ERK.

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<thead>
<tr>
<th>Mouse No.</th>
<th>Vehicle</th>
<th>Triciribine (1 mg/kg)</th>
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**Figure 5:** Induction of LDLR levels by GSK690693 – Sterol-fed HepG2 cells were treated with the indicated concentrations of GSK690693 for 14 hours and then harvested for immunoblot analysis with antibodies against LDLR, phospho-GSK-3β (pGSK-3β) and GAPDH. One representative experiment of three is shown.
Figure 6: Effect of triciribine on expression of ER stress markers – (a) Sterol-fed HepG2 cells were treated with vehicle or triciribine for 14 h. As a positive control for ER stress, cells were treated with DTT for 14 hours. Total RNA was isolated, subjected to RT-PCR with XBP1-specific primers and the PCR products were then resolved on 2% agarose gel for separation of unspliced XBP1 (XBP1u) and spliced XBP1 (XBP1s) mRNAs. One representative experiment of two is shown. Unprocessed image is shown in Supplementary Fig. S20. (b) Sterol-fed HepG2 cells were treated with the indicated concentrations of triciribine or 5-AzaC (5-AzaCytidine). Cells treated with 5 mM DTT served as positive control for expression of IRE1α. At 16 hours post-treatment, cells were harvested and subjected to immunoblotting with the indicated antibodies. One representative blot of three independent experiments is shown.
Bibliography