Cholesterol: The Good, the Bad, and the Ugly – Therapeutic Targets for the Treatment of Dyslipidemia

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Abstract

Maintaining cholesterol and triglyceride (TG) levels within healthy limits is critical for decreasing the risk of heart disease. Dyslipidemia refers to the abnormal levels of lipids in the blood, including low high-density lipoprotein cholesterol (HDL-C), also known as good cholesterol, high low-density lipoprotein cholesterol (LDL-C), also known as bad cholesterol, and/or high TG levels that contribute to the development and progression of atherosclerosis. In this article we reviewed some of the current therapeutic targets for the treatment of dyslipidemia, with a primary focus on endothelial lipase and lecithin cholesterol acyl transferase for raising HDL-C, and the proprotein convertase subtilisin-like kexin type 9 (PCSK9), microsomal triglyceride transfer protein, and the messenger RNA of apolipoprotein B for lowering LDL-C. In addition, we reviewed the role of apolipoprotein AI (apoAI) in raising HDL-C, where we discuss three apoAI-based drugs under development. These are its mutated dimer (apoAI-Milano), a complex with phospholipids, and a mimetic peptide. Atherosclerosis, mainly because of dyslipidemia, is a leading cause of cardiovascular disease. Regarding the title of this article, the 'good' refers to HDL-C, the 'bad' refers to LDL-C, and the 'ugly' refers to atherosclerosis.

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Introduction

Of the estimated 57 million global deaths in 2008, 36 million (63%) were due to noncommunicable diseases (NCD) [1–3]. The largest proportion of NCD deaths is caused by cardiovascular disease (48%), followed by cancers (21%) and chronic respiratory diseases (12%). Diabetes is directly responsible for 3.5% of NCD deaths. Behavioral risk factors, including tobacco use, physical inactivity, an unhealthy diet, and harmful use of alcohol, are estimated to be responsible for about 80% of coronary heart disease (defined as myocardial infarction, coronary death, or coronary revascularization) and cerebrovascular disease cases [1, 4]. Although heart disease is more common among people aged 65 years or older, the number of sudden deaths from heart disease among people aged 15–34 years has increased [5]. The economic impact of cardiovascular diseases on the global health care system continues to grow as the population ages. For example, the total cost of heart disease in the USA alone in 2010 was estimated to be USD 444 billion, including health care expenditures and lost productivity from death and disability [6]. According to the American Heart Association [7], atherosclerosis is a leading cause of cardiovascular disease. A large number of histological studies have shown that atherosclerosis begins in youth, making primary prevention efforts necessary from childhood. By the

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time the heart problems are detected, the underlying cause is usually quite advanced, having progressed for decades. Therefore, primary prevention of atherosclerosis must begin in childhood or adolescence [8].

Atherosclerosis is an inflammatory condition resulting from multiple and cumulative risk factors, each of which contributes in varying ways to the development and severity of the condition. Known factors that contribute to the development of atherosclerosis include high low-density lipoprotein cholesterol (LDL-C), low high-density lipoprotein cholesterol (HDL-C), high triglycerides (TG), obesity, a poor diet, physical inactivity, hypertension, genetics, smoking, diabetes mellitus, and the environment [9, 10]. All of the major lipoprotein classes have an impact in some way on the inflammatory process that leads to the development of atherosclerosis; LDL are proinflammatory, whereas HDL are anti-inflammatory [9, 10].

Although it is not possible to control some of the factors that contribute to the development of atherosclerosis, such as genetics and the environment, other factors, such as physical inactivity, smoking, and diet, are controllable. For example, the lower occurrence of cancer and cardiovascular disease in the population around the Mediterranean basin has been linked to the dietary habits of the region [11]. Such a diet is rich in nuts, fruits, vegetables, legumes, whole-wheat bread, fish, and olive oil [11]. Components of the Mediterranean diet are an important source of antioxidant and anti-inflammatory molecules, among which omega-3 fatty acids, oleic acid, and phenolic compounds are prominent [11].

HDL: The Good

HDL as a Risk Factor for Heart Disease

HDL plays an important role in removing unesterified (free) cholesterol from peripheral cells and delivering it to the liver through the interaction of HDL with the hepatic HDL receptor. This process is known as reverse cholesterol transport (RCT) [10, 12]. This is in addition to its antiatherosclerotic, anti-inflammatory, and endothelial protective effects [1, 10]. Several studies have shown an inverse relationship between HDL blood levels and heart disease [13–15]. It is estimated that >40% of coronary events occur in individuals with HDL <40 mg/dl. These and several other epidemiological studies emphasize that the risk factor associated with low levels of HDL is totally independent of LDL-C, i.e. no matter how low the LDL level is, a decrease in the HDL level would increase the risk of coronary artery disease.

HDL Is a Primary Participant in RCT

The importance of RCT for the removal of cholesterol from peripheral tissue for excretion through the liver is well known [16–20]. A critical step in RCT, a multistep process, is maturation of the pre-β_1-HDL formed through the acquisition of free cholesterol (FC) and phospholipids (PL) by apolipoprotein AI (apoAI) into α-migrating HDL. The FC is converted into cholesteryl ester (CE) by lecithin cholesterol acyl transferase (LCAT) and migrates into the interior of the HDL particle, thus enabling the transfer of more FC to the cell surface. Plasma pre-β_1-HDL levels have been reported to be increased in patients with coronary artery disease and dyslipidemia. Elevation of the plasma pre-β_1-HDL level is associated with the atherosclerotic phase of coronary artery disease and may be useful for the identification of patients with unstable angina pectoris. High pre-β_1-HDL concentrations and low LCAT activities are strong positive risk markers for ischemic heart disease and are independent of HDL-C.

HDL Is Anti-Inflammatory

Atherosclerosis is currently thought to be triggered by initial inflammatory events [21]. A plethora of proinflammatory molecules (e.g. cytokines) in a variety of cell types assist in eventual plaque formation [21]. The formation of oxidized LDL (ox-LDL) amplifies the inflammatory response [22–26]. HDL are anti-inflammatory and inhibit inflammation, as demonstrated in a variety of in vitro and in vivo animal models and human studies [9, 27, 28], due to their protein constituents, including several paraoxonases [29], platelet-activating factor acetylhydrolase [30], LCAT [31], and glutathione peroxidase [32], as well as apoAI [33]. apoAI has been shown to extract the lipid hydroperoxides [13-hydroperoxyoctadecadienoic acid (HPODE) and 15-hydroperoxyeicosatetraenoic acid (HPETE)] called 'seeding molecules' from ox-LDL [34]. The anti-inflammatory role of HDL is further underscored by studies showing severe degradation of this property during oxidative stress and in the acute phase response to infections [35], in which HDL anti-inflammatory proteins get displaced by proinflammatory molecules such as serum amyloid A [36]. Enzyme systems such as paraoxonases and platelet-activating factor acetylhydrolase are also displaced from HDL [37, 38] and, in addition, HDL acquire the proinflammatory protein ceruloplasmin [39]. These changes rapidly shift the HDL profile from RCT supportive and anti-inflammatory to cholesterol accumulative and proinflammatory under such stressful stimuli [9, 40].

Pleiotropic Effects of HDL

HDL may exert several potentially important antiatherosclerotic, anti-inflammatory, antithrombotic, and endothelial protective effects [for a review, see 41]. In particular, the promotion of RCT has been proposed as an antiatherogenic effect of HDL that may promote regression of atherosclerotic lesions [16–20]. Moreover, endothelial dysfunction is thought to play a critical role in the development and progression of atherosclerosis. Several studies have suggested that HDL exerts direct endothelial protective effects, such as stimulation of endothelial production of the antiatherogenic molecule nitric oxide. These studies also suggest that the antioxidant effect of HDL prevents endothelial dysfunction and cell death induced by ox-LDL and tumor necrosis factor-alpha (TNF-α). HDL inhibits secretion of the potent vasoconstrictor endothelin, oxidation of LDL, adhesion of monocytes to endothelial cells, and thrombosis. Furthermore, it has been observed that HDL may stimulate endothelial repair processes involving mobilization and promotion of the endothelial repair capacity of endothelial progenitor cells. All of the above-mentioned studies have conclusively demonstrated that individuals with low levels of HDL-C have a much greater risk of coronary heart disease outcomes than those without them. In clinical trials involving lowering LDL-C, scientists have studied the effect of HDL-C on atherosclerosis and heart attack rates. They have found that even small increases in HDL-C could reduce the frequency of heart attacks. For each 1 mg/dl increase in HDL-C, there is a 2–4% reduction in the risk of coronary heart disease. Although there are no formal National Cholesterol Education Program (NCEP) target treatment levels of HDL-C, as indicated above, an HDL level <40 mg/dl is considered undesirable and measures should be taken to increase it.

Current Drugs for Raising HDL and Their Limitations

Much of the current therapeutic efforts, centered on reducing LDL-C levels through the administration of statins [42], do not eliminate the cardiovascular risk, necessitating other approaches to eliminate the residual risk [42], especially for diabetic patients. In addition, limited success has been achieved in providing good therapy for individuals with low HDL-C. Niacin (nicotinic acid) and fibric acid derivatives have been used to increase HDL-C. Niacin decreases LDL-C by about 10%, it increases HDL-C by about 20%, and it decreases TG by about 25%. However, major side effects of niacin therapy have been observed. These include flushing, pruritus, nausea, vomiting, gastrointestinal irritation, and rare hepatotoxicity

[43]. Fibrates such as gemfibrozil (Lopid) and fenofibrate (TriCor) that are PPARα agonists are known to increase HDL-C by 10–20% and decrease TG by 20–50%. Side effects of fibrate therapy are dyspepsia, myopathy, and gallstones [44].

One mechanism for increasing HDL is to inhibit the CE transfer protein (CETP), also called plasma lipid transfer protein. CETP, a protein made in the liver, facilitates the transport of CE and TG between the lipoproteins. CETP is also closely involved in the metabolism of cholesterol, β-lipoproteins, apolipoprotein B (apoB), and apolipoprotein E (apoE) in type 2 diabetes patients [45]. Partial inhibition of CETP is associated with an increase in HDL-C of up to 100% and might also decrease the level of LDL. Several recent HDL-raising strategies in clinical trials centered around the CETP inhibitors (torcetrapib and dalcetrapib) were stopped because of death in phase III or a lack of efficacy in reducing cardiovascular events [46, 47]. The only CETP inhibitor currently in a phase III clinical trial is anacetrapib.

Recently, the US National Institutes of Health (NIH) stopped the AIM-HIGH clinical trial 18 months earlier than planned. The trial revealed that adding high-dose, extended-release niacin to statin treatment in people with heart and vascular disease did not reduce the risk of cardiovascular events, including heart attacks and stroke [48]. The unfortunate failure of the AIM-HIGH clinical trial has caused significant confusion and apprehension in the field since the study showed that the levels of HDL were elevated in patients under treatment. This study contradicted the thousands of in vitro and in vivo studies in laboratory animals and in humans that strongly suggested a host of cardioprotective benefits for high HDL levels. The AIM-HIGH clinical trial was criticized for having a small cohort and inappropriate controls [49]. These data put together, however, suggest that raising HDL levels, although necessary, may not be sufficient for reducing the risk of cardiovascular events, and that higher HDL levels must positively correlate with enhancement of the many beneficial properties of HDL such as cholesterol efflux as a biomarker for HDL functionality.

Endothelial Lipase as a Therapeutic Target for Dyslipidemia

Endothelial lipase (EL) is a member of the TG lipase gene family [50]. It has both phospholipase and TG lipase activity, but it is more active as a phospholipase than as a TG lipase (phospholipase-to-TG lipase ratio: 1.6) [51]. The link between EL and HDL-C was established following mouse studies suggesting that changes in EL levels

influence HDL-C metabolism. EL cloned into an adeno-viral vector and transfected into COS cells demonstrated that in vitro HDL-C particles are the preferred source of EL substrate for all lipoprotein fractions [51]. Furthermore, a significant increase in plasma HDL-C in mice was observed when the EL gene was knocked out [52, 53]. Using genetic mouse models with altered levels of EL expression, Ishida et al. [53] reported a strong inverse correlation between HDL levels and EL expression. Furthermore, recent studies showed that targeted EL deletion increases HDL particles with anti-inflammatory properties both in vitro and in vivo [54–58]. This was further supported by the finding that inhibition of EL activity in mice using an EL antibody [59] resulted in a significant increase in HDL-C. Conversely, overexpression of EL in transgenic animals resulted in a significant decrease in HDL-C [52]. These data suggested that EL, at least in mice, plays an important role in HDL-C metabolism. Further genetic association studies in humans demonstrated inverse correlations between EL and HDL-C levels [60–62].

Structure of EL

Human EL is a protein of about 500 amino acids, with 5 potential N-glycosylation sites. The size of the expressed mature protein is 68 kDa. EL has 45, 40, and 27% amino acid sequence identity with lipoprotein lipase (LPL), hepatic lipase (HL), and pancreatic lipase (PL), respectively. The locations of the 10 cysteine residues, as well as the 19-amino acid lid region, are conserved. The catalytic pocket of EL has the same conserved catalytic triad found in other members of the lipase family. The GXSXG lipase motif surrounding the active site serine is conserved. In addition, there are two conserved potential lipid-binding domains (GLDPAGP and RSFGLSIGIQM), as well as a conserved heparin-binding region. Although the crystal structure of EL is not available, the high sequence identity of EL with other members of the LPL gene family, the conserved disulfide bonds, and other similarities all strongly suggest a similar three-dimensional fold. The only member of this family for which crystal structures are available is PL [63]. Therefore, it is possible to generate a homology model of EL based on the crystal structures of PL and related proteins. The validity of such a model can be easily tested by docking known inhibitors of EL [64].

Unlike LPL and HL, EL is synthesized by endothelial cells and functions at the site where it is synthesized. Furthermore, its tissue distribution is different from that of LPL and HL. As a lipase, EL has primarily phospholipase A1 activity, and it hydrolyzes effectively and specifically the HDL PL in vitro and ex vivo. Unlike LPL and HL, EL is located in the vascular endothelial cells and its expression is highly regulated by cytokines and physical forces, suggesting that it may play a role in the development of atherosclerosis [53–58, 65].

Inhibitors of Human EL

Recently, GlaxoSmithKline reported a series of EL irreversible inhibitors featuring a sulfonylfuran urea core identified in a high-throughput screening campaign [64]. A lead optimization effort was undertaken to improve the potency and selectivity, leading to inhibitors with improved LPL selectivity (up to 15-fold). O'Connell et al. [66] more recently reported the synthesis of alkyl, aryl, and acyl-substituted phenylboronic acids that inhibit EL, many with near equal potency against both EL and LPL, but several compounds exhibited moderate to good selectivity for EL (up to 42-fold). Clearly, the development of highly EL-selective inhibitors is desired. The discovery of such a molecule would provide a novel means by which to specifically raise HDL-C.

LCAT as a Therapeutic Target for Dyslipidemia

Overexpression of human LCAT in rabbits and co-overexpression with CETP in mice has shown protection against diet-induced atherogenesis [67, 68]. Conversely, several mutations in apoAI that impair its LCAT activation are strongly correlated with reduced HDL levels [69]. Physiologically, LCAT binds FC and phosphatidylcholine (PC), transferring the acyl chain at the sn-2 position of PC to FC, producing CE and lyso-PC. LCAT, an interfacial enzyme [70], functions on the surface of HDL particles. Binding to the HDL surface activates the enzyme, but optimal activity is reached only after association with a cofactor. The most potent activator of LCAT is apoAI, and the mechanism of activation is thought to be similar to that of PL by colipase which forms a 1:1 complex with PL [71]. A three-dimensional structure for LCAT is currently unavailable, although a computational model has been proposed [72]. The model suggests that LCAT has an architecture similar to that of the family of α/β hydrolases [73]. Consistent with this hypothesis, mutational experiments that changed the catalytic residues Ser181 [74], His377, and Asp345 [72] into Ala resulted in complete loss of, or severe reductions in, enzyme activity.

Studies with apoAI model peptides have shown that although several molecules bind strongly to HDL and activate LCAT, not all are capable of protection against atherosclerosis. Strong lipid binding is necessary, but not

sufficient, for protection [75]. It has also been shown recently that apoAI peptides with little or no effect on LCAT in vitro are nevertheless able to increase LCAT activity and HDL-C in apoE-null mice [76]. Furthermore, overexpression of LCAT, even when coexpressed with scavenger receptor class B type I and CETP, failed to increase RCT in macrophages although HDL-C was significantly enhanced [77]. Measurement of the arterial intima media thickness in patients with LCAT genetic deficiencies, as well as other parameters, compared to normal controls, have been reported to both show [78, 79] and not show [80] signs of atherosclerosis in the former. On the other hand, it has long been known that mutations in the LCAT gene in humans cause familial LCAT deficiency or fish eye disease [81, 82]. Several animal models also emphasize the need for a functional LCAT for protection against atherosclerosis. For example, apoE-null mice develop an early atherogenic phenotype, including lesions, concomitant with decreases in LCAT activity [30]; LCAT overexpression is also antiatherogenic in rabbits, in the presence of normal LDL receptors (LDLR) [67], and in mice with LDLR–/– [83]. Conversely, LCAT deficiency increases atherosclerosis in LDLR–/– and apoE–/– double knockout mice [66]. Recent studies in humans have demonstrated that carriers of LCAT gene mutations exhibit increased cardiac atherosclerosis [84, 85]. In addition, gene transfer using apoAI and LCAT induced cholesterol unloading in complex atherosclerotic lesions in a rabbit model [86]. Recently, small-molecule activators of LCAT have been reported, supporting the potential of this approach [87]. These data strongly suggest that activators of LCAT should be effective in raising HDL levels and treating dyslipidemia.

apoAI

Another potential therapy for raising HDL is increasing apoAI levels. Experimental data indicate that overexpression of apoAI or infusion apoAI in animal models increases HDL-C levels and decreases atherosclerosis. Several apoAI mimetic peptides are being developed; these include apoAI-Milano [88] (also known as ETC-216 or MDCO-216), CER-001 [89], and D-4F [90].

apoAI-Milano is a naturally occurring mutated variant of apoAI. It is a disulfide homodimer resulting from a cysteine for arginine substitution at position 173 on the surface of apoAI. The mutation alters the characteristics of the protein, resulting in apoAI-Milano being functionally more effective than normal apoAI. apoAI-Milano has been shown to significantly reduce cardiovascular disease, even though it causes a reduction in HDL levels and

an increase in TG levels [91]. Currently, apoAI-Milano in complex with palmitoyl-oleoyl-phosphatidylcholine (POPC), known as MDCO-216, is being developed by The Medicines Company [92].

CER-001 is a synthetic HDL comprised of recombinant human apoAI complexed with PL and designed to mimic the beneficial properties of natural, nascent pre-β HDL [93]. Cerenis Therapeutics reported the completion in May 2010 of a phase I clinical trial of CER-001 for acute coronary syndrome. This randomized, double-blind, placebo-controlled, crossover, single-rising-dose study of 32 healthy dyslipidemic volunteers showed that CER-001 is safe and well tolerated at dosages up to 45 mg/kg [89]. Cerenis Therapeutics also reported the initiation in March 2011 of a phase II clinical trial for CER-001. This double-blind, randomized, placebo-controlled, safety and efficacy study was designed to assess the ability of CER-001 to regress coronary atherosclerotic plaque as measured by intravascular ultrasound [89].

D-4F is an orally bioavailable 18-D-amino acid apoAI mimetic peptide [94]. Similar to apoAI, D-4F binds nonoxidized lipids but has a structure that enhances its ability to bind and sequester fatty acid hydroperoxides and proinflammatory oxidized PL [90, 95]. Oral administration of D-4F was shown to improve HDL anti-inflammatory properties in mice and monkeys and dramatically reduced lesions in mouse models of atherosclerosis despite no change in plasma levels of HDL cholesterol [90, 96].

Other Therapeutic Targets

PPAR agonists, such as the glitazones, are known to have modest HDL-boosting effects although their main action is in reducing insulin resistance. PPAR agonists have been postulated to increase the macrophage cholesterol efflux through increasing ABCA1 and ABCG1 expressions via the PPARγ/LXRα pathway [97].

LDL: The Bad

LDL as a Risk Factor for Heart Disease

LDL carries about 60–70% of serum cholesterol [98]. It transports cholesterol from the liver to the peripheral tissues. High levels of LDL-C are harmful because it can build up on the arterial walls to initiate the formation of atherosclerotic plaques. Unlike the other lipoproteins, each LDL particle contains mostly one apoB-100 which is responsible for the selective binding of LDL to the LDLR. The binding of LDL to its receptor in the liver is the major

mechanism used to remove LDL from circulation. Using this mechanism, about 70% of LDL are removed by the liver, releasing FC [99]. The increase in intracellular cholesterol can affect blood cholesterol levels by inhibiting de novo synthesis of cholesterol, decreasing the synthesis of the LDLR, and increasing the activity of an enzyme that facilitates cholesterol storage [98].

The term hypercholesterolemia usually refers to elevations in the serum LDL level. Primary hypercholesterolemia includes familial hypercholesterolemia (FH), familial ligand-defective apoB, and familial combined hyperlipoproteinemia. Primary hypercholesterolemia is a type IIa dominant disorder that involves mutations in the LDLR gene. Homozygotes usually have a more severe case than heterozygotes. Familial ligand-defective apoB is a type IIa disorder that results in a mutation in apoB-100 that disrupts the binding of LDL to the LDLR, thereby decreasing the metabolism of LDL. Both of these disorders decrease LDLR-mediated endocytosis in the liver and thus increase the serum LDL.

LDL Is Inflammatory

High levels of LDL, and to a lesser extent very-low-density lipoprotein, cause the accumulation of LDL-C in the arterial wall. After LDL accumulates, oxidation of LDL occurs [98]. The ox-LDL can cause extensive damage to the arterial wall, including provocation of an inflammation response, promotion of coagulation, an increase in the activity of some substances that cause vasoconstriction, and inhibition of some substances that cause vasodilation [98]. ox-LDL recruits monocytes which enter the arterial wall and are activated to become macrophages. The macrophages ingest the ox-LDL through the macrophage scavenger receptor to become foam cells or fatty streaks. The foam cells propagate further inflammatory responses, as well as more ox-LDL deposition. Further, microcalcification of the vascular smooth muscle cells takes place, which progresses toward the development of atherosclerosis. Fatty streaks, which are cholesterol-filled macrophages, are the first stage of atherosclerosis formation. Plaques (deposition of fatty substances, cholesterol, calcium, cell components, and others) will then form. Plaques will gradually increase inside the artery, narrowing the arterial walls and thus decreasing the blood flow and oxygen supply to tissues. Plaques are usually kept in check by fibrous caps, which protect and stabilize the lesion. If the plaques rupture, then thrombosis will occur and the damage will spread to other areas, leading to diseases such as ischemic heart disease, myocardial infarction, stroke, peripheral arterial disease, other cardiovascular diseases, and death. Therefore, it is crucial that dyslipidemia, primary or secondary, be treated.

Current Drugs for Lowering LDL and Their Limitations

The primary objective for treating high cholesterol is to lower LDL levels in the blood; the goal is for LDL cholesterol to be <100 mg/dl and for very-high-risk individuals <70 mg/dl [100]. The current drugs for lowering LDL-C, and their limitations, are listed in table 1. The first drugs of choice are the statins. Statins are inhibitors of HMG CoA reductase, a rate-limiting enzyme of the mevalonate pathway of cholesterol synthesis. Fibrates, bile acid-binding resins, and nicotinic acid are also used to lower blood cholesterol levels. Fibrates are PPARα agonists, while bile acid-binding resins bind to bile acids in the intestine and prevent their absorption. To compensate for the loss of bile acids, the liver increases the conversion of cholesterol to bile acids. The conversion of cholesterol to bile acids reduces the cholesterol in the body, and the levels of blood cholesterol drop. In addition, ezetimibe, a lipid-lowering compound that selectively inhibits the intestinal absorption of cholesterol and related phytosterols, although it decreases cholesterol levels, has not been shown to improve cardiovascular disease outcomes. However, it has been reported [106] that ezetimibe monotherapy may be associated with a greater reduction in remnant-like particle cholesterol levels in subjects with metabolic syndrome than in those without it.

There are numerous cholesterol drugs on the market, but they all have limitations as shown in table 1 due to possible adverse effects. Pearson et al. [107] studied 4,888 patients receiving lipid-lowering therapy. They found that only 38% of these patients were achieving the LDL-C goals set by the NCEP. Their findings indicate that more aggressive treatment of dyslipidemia is needed to attain the goals established by the NCEP guidelines. Such treatment may come from modulation of a pathway other than the mevalonate pathway. One such pathway is the proprotein convertase subtilisin-like kexin type 9 (PCSK9) pathway [108].

Structure of PCSK9

PCSK9 is a protease that belongs to the subtilisin family of kexin-like proconvertases [109]. It contains a signal sequence of 30 amino acids followed by a prodomain of 122 amino acids, a catalytic domain, and a 279-amino acid cysteine- and histidine-rich C-terminal region known as the V domain. Unlike other proprotein con-

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vertases, PCSK9 lacks a classical P domain that is required for folding and the regulation of protease activity [110].

The protein is synthesized as a 72-kDa precursor that undergoes zymogen processing between the prodomain and the catalytic domain [111, 112]. The prodomain (14 kDa) remains bound to the mature protein (63 kDa) as it traverses the secretory pathway. The site of intramolecular cleavage in PCSK9 (Val-Phe-Ala-Gln↓Ser-Ile-Pro) differs from most other proconvertases in that cleavage does not occur after a basic residue [112]. Obtaining a robust in vitro assay for PCSK9 activity has proven difficult and little is known about the requirements for catalytic activity. In contrast to other proprotein convertases, autocatalytic cleavage of PCSK9 does not require calcium [113]. The mature PCSK9 and the associated prodomain both undergo tyrosine sulfation in the late Golgi complex before secretion [113, 114]. Sulfation of tyrosine residues in other proteins enhances protein-protein interactions, but the role of this posttranslational modification in PCSK9 has not been defined [109]. Different groups have reported the high-resolution crystal structure of PCSK9 [109, 115]. The crystal structure reveals that PCSK9 has subtilisin-like pro- and catalytic domains, and a V domain with a novel fold. Although the full-length protein was crystallized, the crystal structure was found to be of the processed enzyme [109], with residues 152 and 153 separated by about 25 Å. The core of the prodomain of PCSK9 closely resembles the prodomain of subtilisin, with the C-terminal tetrapeptide of the prodomain (Val-Phe-Ala-Gln) bound in the active site, forming an antiparallel β sheet with a strand from the catalytic domain.

PCSK9 as a Therapeutic Target for Dyslipidemia

The establishment of a link between PCSK9 and cholesterol metabolism was rapidly followed by the discovery that selected mutations in the PCSK9 gene caused autosomal dominant hypercholesterolemia [111], suggesting that the mutations confer a gain of function [116] by increasing the normal activity of PCSK9. This was supported by an experiment in which wild-type and mutant PCSK9 (S127R and F216L) were expressed at high levels in the livers of mice; hepatic LDLR protein levels fell dramatically in mice receiving either the wild-type or the mutant PCSK9 [113, 117]. No associated reductions in LDLR mRNA levels were observed, indicating that overexpression of PCSK9, whether mutant or wild type, reduces LDLR through a posttranscriptional mechanism.

Table 1. Current drugs for lowering LDL-C and their limitations

Drug class	Drugs	Possible adverse effects	References
HMG CoA reductase inhibitors (statins)	Atorvastatin Fluvastatin Lovastatin Pitavastatin Pravastatin Rosuvastatin Simvastatin	Liver injury, memory loss, diabetes, muscle damage	100–105
Fibrates (PPARα agonists)	Clofibrate Fenofibrate Gemfibrazil	Dyspepsia, gallstones, muscle damage	100
Bile acid-binding resins	Cholestyramine Colestipol Colesevelam	Gastrointestinal distress, constipation, decreased absorption of other drugs	100
Niacin (nicotinic acid)	Nicotinic acid	Flushing, hyperglycemia, gout, upper gastrointestinal distress, hepatotoxicity	100

Given that gain-of-function mutations in PCSK9 cause hypercholesterolemia, it was reasonable to ask whether loss-of-function mutations would have the opposite effect and result in hypocholesterolemia. Three loss-of-function mutations in PCSK9 (i.e. Y142X, L253F, and C679X) have been identified in African-Americans [118]. These mutations reduce LDL-C levels by 28% and have been shown to decrease the frequency of coronary heart disease by 88%. Rashid et al. [119] studied the mechanism of loss-of-function mutations in mice where PCSK9 was inactivated. They reported that these knockout mice showed increased hepatic LDLR protein (but not mRNA), increased clearance of circulating lipoproteins, and reduced plasma cholesterol levels. Structure-function relationship analysis of the naturally occurring mutations in PCSK9 has also provided insight into the mechanism of action of PCSK9. Interestingly, mutations in PCSK9 that have been found to be associated with the greatest reductions in LDL-C plasma levels are those that prevent the secretion of mature PCSK9 by disrupting its synthesis (Y142X), autocatalytic processing (L253F), or folding (C679X) [120]. The Y142X mutation produces no detectable protein because it occurs early in the transcript and is predicted to initiate nonsense-mediated mRNA decay.

Mutations in the catalytic domain (L253F) interfere with the autocatalytic cleavage of the protein. In cells expressing PCSK9-253F, the amount of mature protein was reduced compared to that in cells expressing PCSK-WT, suggesting that the mutation inhibits autocatalytic cleavage. The L253F mutation is near the catalytic triad (PCSK9 is a serine protease); therefore, it might disrupt the active site [120]. Inasmuch as autocatalytic cleavage of PCSK9 is required for the export of the protein out of the ER, the L253F mutation delays the transport of PCSK9 from the ER to the cell surface. The nonsense mutation (C679X) in PCSK9, which truncates the protein by 14 amino acids, did not interfere with protein processing, but the mature protein accumulated in the cells and none was secreted, suggesting that the protein is cleaved normally but is misfolded and retained in the ER [114, 120].

The PCSK9 Mechanism

The LDLR is a multidomain protein that consists of a ligand-binding domain, an epidermal growth factor (EGF) precursor homology domain, an O-glycosylated domain, a membrane-spanning domain, and a cytoplasmic domain. Upon LDL binding to the LDLR, the receptor/ligand complex is endocytosed, the ligand is released into the acidic environment of the endosome, and the LDLR is recycled to the cell surface. The LDL then undergoes lysosomal degradation. The binding of PCSK9 to the LDLR on the cell surface causes the eventual degradation of the LDLR. Reduced LDLR levels result in decreased metabolism of LDL, which leads to hypercholesterolemia. Although the mechanism by which PCSK9 causes degradation of the LDLR has not been fully elucidated, it is clear that the protease activity of PCSK9 is not required for LDLR degradation [121, 122]. Li et al. [121] coexpressed the prodomain and the catalytic domain in *trans* and showed that the secreted PCSK9 was catalytically inactive yet functionally equivalent to the wild-type protein in lowering the cellular LDL uptake and LDLR levels. Similar studies were also reported by McNutt et al. [122]. Furthermore, Zhang et al. [123] mapped PCSK9 binding to the EGF-A repeat of the LDLR and showed that such binding decreases receptor recycling and increases its degradation. They also reported that binding to the EGF-A domain of the LDLR was calcium dependent and increased dramatically with a reduction in pH from 7 to 5.2. Kwon et al. [124] determined the crystal structure of PCSK9 in complex with LDLR-EGF-AB (EGF-A and EGF-B). The structure shows a well-defined EGF-A domain, but the EGF-B domain is disordered and absent from their electron density map. The EGF-A domain

binds to the PCSK9 catalytic domain at a site distant from the catalytic site and makes no contact with either the C-terminal domain or the prodomain [125].

Targeting PCSK9

Several strategies have been proposed for targeting PCSK9 [126, 127]. mRNA knockdown approaches include the use of antisense oligonucleotides or RNAi. Antisense oligonucleotides administered to mice reduced PCSK9 expression by >90% and lowered plasma cholesterol levels by 53% [128, 129]. A single intravenous injection of an RNAi delivered in lipidoid nanoparticles to cynomolgus monkeys reduced plasma PCSK9 levels by 70% and plasma LDL-C levels by 56% [130]. A second approach is to prevent the binding of PCSK9 to the LDLR on the cell surface with a small molecule, a peptide, or an antibody directed against PCSK9. Adding EGF-A fragments to cultured cells inhibits the ability of exogenously added PCSK9 to mediate LDLR degradation. A third approach is to develop small-molecule inhibitors of PCSK9 processing. Despite evidence that the catalytic activity of PCSK9 is not required for LDLR degradation [122], an intracellular inhibitor of PCSK9 catalytic activity should be effective since autocatalytic processing of PCSK9 is required for secretion of the protein from the ER. Following its synthesis, PCSK9 undergoes an autocatalytic cleavage reaction that clips off the prodomain, but the prodomain remains attached to the catalytic domain [109, 115]. The autocatalytic processing step is required for the secretion of PCSK9 [131], likely because the prodomain serves as a chaperone and facilitates folding. The continued attachment of the prodomain partially blocks the substrate-binding pocket of PCSK9 [109, 115]. McNutt et al. [132] demonstrated that antagonism of secreted PCSK9 increases LDLR expression in HepG2 cells. They showed that an FH-associated LDLR allele (H306Y) that results in a gain-of-function mutation is due to an increase in the affinity of PCSK9 to the LDLR, which would lead to enhanced LDLR destruction and decreased plasma LDL-C clearance. Furthermore, they were able to show elegantly that blocking the secreted PCSK9 with an LDLR (H306Y) subfragment resulted in an increase in the level of LDLR in cultured HepG2 cells. Therefore, PCSK9 acts as a secreted factor to cause LDLR degradation, and a small-molecule inhibitor that interferes with the autocatalytic process should decrease the amount of mature secreted PCSK9.

The above data strongly suggest that PCSK9 inhibitors should be effective lipid-lowering agents. PCSK9 as a therapeutic target appears to be well validated. This is

strongly supported by the low plasma LDL-C levels associated with loss-of-function mutations in the PCSK9 gene, which indicate that inhibition of autoprocessing and secretion of PCSK9 through small-molecule treatment should be an effective cholesterol-lowering strategy. In addition, no safety issues associated with inhibition of PCSK9 have been identified. Knockout mice lacking PCSK9 developed normally and had no gross neurological defects [120]. Humans heterozygous for loss-of-function mutations in PCSK9 seem to be healthy [119] and have a normal life span. In addition, human heterozygotes with two inactivating mutations in the PCSK9 gene (Y142X and ΔR97) and no circulating PCSK9 have very low levels of LDL-C (14–34 mg/dl) and normal hepatic and renal function [133].

The most promising approach thus far for targeting PCSK9 is the use of monoclonal antibodies (mAb) that interfere with the interaction of the PCSK9 catalytic domain with the LDLR on the cell surface [134]. Using nonhuman primates, intravenous infusion of 3 mg/kg of mAb1 against the catalytic domain of PCSK9 resulted in a significant reduction in plasma LDL-C of 80% on day 10 postinjection [135] and, similarly, injection of 3 mg/kg of J16 mAb in cynomologous monkeys resulted in a 64% reduction in LDL-C [136]. In addition, a further reduction in LDL-C was observed when these animals were treated with simvastatin (50 mg/day) and subsequently infused with the J16 antibody (3 mg/kg) [136]. Human clinical trials are underway and the results have been very promising. Pfizer-Rinat RN316 is currently in a phase II clinical trial. The mAb (AMG145) by Amgen was evaluated in a phase I ascending single-dose study which showed that LDL-C was dose-dependently decreased by up to 64% relative to the placebo when AMG145 was infused intravenously or subcutaneously in healthy subjects [137], with no adverse events. The effect of AMG145 administration will be evaluated in the LAPLACE-TIMI 57 (NCT01380730) clinical trials [138]. In phase I clinical trials, a single dose of Aventis/Regeneron SAR236553/ REGN727 antibody resulted in significant reduction of LDL-C (33–46%) from baseline when given subcutaneously [139]. SAR236553/REGN727 was also shown to be synergistic with statins [140]. In an 8-week phase II study of patients with LDL-C levels ≥100 mg/dl on a stable dose of atorvastatin (10 mg/day), SAR236553/REGN727 subcutaneously administered in combination with atorvastatin resulted in a 66% reduction in LDL-C [7]. In the same study, SAR236553/REGN727 as an add-on to atorvastatin (80 mg/day) resulted in a decrease of 73% in comparison with a reduction of 17% with atorvastatin (80 mg/day) alone. Furthermore, recent phase II clinical data revealed a drop of 40–60% in LDL-C after subcutaneous administration of the AMG145 PCSK9 mAb in statin-intolerant patients [141]. In view of the absence of overt toxicity associated with this treatment, a phase III clinical trial has been initiated with >20,000 individuals. Completion of this trial is planned for 2015. The anticipated success of the trial should support the concept that inhibition of circulating PCSK9 in combination with statins will result in sharply decreased plasma levels of LDL-C and will be well tolerated. Small-molecule inhibitors are also undergoing early preclinical testing [127].

Other Therapeutic Targets

Drugs targeting the microsomal triglyceride transfer protein (MTTP) and the messenger RNA for apoB have been approved in the USA. Lomitapide is an MTTP inhibitor that has been approved as an orphan drug for lowering LDL-C in patients with homozygous familial hypercholesterolemia. It is indicated as an adjunct therapy with other lipid-lowering treatments [142]. Mipomersen is an antisense therapeutic that targets the messenger RNA for apolipoprotein. It is a once-weekly subcutaneous injection. It is also a drug for the treatment of patients with homozygous familial hypercholesterolemia, and it is approved as an adjunct therapy with other lipid-lowering treatments [143].

Atherosclerosis: The Ugly

Endothelial dysfunction in atherosclerosis involves a series of early changes that precede lesion formation. The changes include greater permeability of lipoproteins, upregulation of leukocyte and endothelial adhesion molecules, and migration of leukocytes into the artery wall. Initiation of atherosclerosis involves the accumulation of LDL-C in the subendothelial extracellular space within the arterial wall. Vascular cells oxidize the accumulated LDL to a form that is able to stimulate the recruitment of monocytes that take up the ox-LDL to form macrophages. Further accumulation of the ox-LDL in the subendothelial extracellular space results in the formation of foam cells. Such cells form the earliest visible lesion of atherosclerosis: the fatty streaks. Fatty streak formation in atherosclerosis occurs early with infiltration by lipid-laden monocytes and macrophages along with T lymphocytes. Later lesions involve a complicated series of steps, including smooth muscle migration, T cell activation, foam cell formation, and platelet adherence and aggregation [9, 10].

Table 2. Novel dyslipidemia drugs

Drug class	Drug	Company	Status	Effect
apoAI variant	MDCO-216	The Medicines Company	Phase I/II	Raise HDL
apoAI	CER-001	Cerenis Therapeutics	Phase II	Raise HDL
MTTP inhibitor	Lomitapide	Aegerion Pharmaceuticals	Marketed	Lower LDL
apoB mRNA antisense	Mipomersen	Genzyme	Marketed	Lower LDL
PCSK9 antagonist (monoclonal antibody)	REGN727/SAR236553	Regeneron/Sanofi	Phase III	Lower LDL
PCSK9 antagonist (monoclonal antibody)	AMG-145	Amgen	Phase III	Lower LDL
PCSK9 antagonist (monoclonal antibody)	LGT209	Novartis	Phase II	Lower LDL
PCSK9 antagonist (monoclonal antibody)	RN316	Pfizer	Phase II	Lower LDL
PCSK9 antagonist (monoclonal antibody)	1D05	Merck	Development	Lower LDL

Conclusion

As stated by the American Heart Association: 'Keeping your cholesterol levels healthy is a great way to keep your heart healthy – and lower your chances of getting heart disease or having a stroke.' Today there are a number of effective drugs for lowering 'bad' cholesterol and decreasing the chance of developing 'ugly' atherosclerotic plaques. Unfortunately, these drugs are not suitable for everyone. Therefore, the development of novel drugs for lowering blood LDL levels is needed and, fortunately, is being pursued today by a number of companies.

Although there are considerable data to suggest that raising 'good' cholesterol levels is necessary to reduce the risk of cardiovascular events, it is now clear that higher HDL levels by themselves are not sufficient and must positively correlate with the enhancement of the many beneficial properties of HDL such as cholesterol efflux as a biomarker of HDL functionality. Therefore, the focus of new drugs aimed at raising HDL levels must now positively correlate the higher HDL levels with HDL functionality.

As indicated above, there are several novel drugs that have been recently marketed and that are being developed to raise HDL and lower LDL. These drugs are summarized in table 2. With these and other promising earlier-stage programs underway, the arsenal of therapeutic tools is expanding to combat and reduce the 'ugly' consequences of dyslipidemia and, ultimately, atherosclerosis.

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