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Mini review

PROPROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 9: A NEW TARGET MOLECULE FOR GENE THERAPY

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Abstract: Proprotein convertase subtilisin/kexin type 9 (PCSK9) has emerged as a novel target for controlling plasma levels of low-density lipoprotein cholesterol (LDL-C) and decreasing the risk of cardiovascular diseases. At present it is clear that the major classes of commonly prescribed lipid-lowering medications increase serum PCSK9 levels and fail to protect a significant percentage of patients from cardiovascular events. Therefore development of new LDL-C lowering medications that either do not increase circulating PCSK9 levels or work through inhibition of PCSK9 expression and protease activity is a highly desirable approach to overcome hypercholesterolemia. Since there are several agents which are being evaluated in human preclinical and clinical trials, this review summarizes current therapeutic strategies targeting PCSK9, including specific antibodies, antisense oligonucleotides, small interfering RNAs (siRNAs) and other small-molecule inhibitors.

Key words: PCSK9, LDL cholesterol, LDL receptor degradation, Statins, Fibrates, Ezetimibe, Ani-PCSK9 antibody, Antisense oligonucleotides, RNAi, Hypercholesterolemia

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Abbreviations used: ApoER2 – apolipoprotein E receptor 2; ASO – oligonucleotide inhibitors; CHD – coronary heart disease; dsRNA – double-stranded ribonucleic acid; EGF-A – epidermal growth factor-like repeat; GOF – gain of function; HDL-C – high-density lipoprotein cholesterol; HNF1 – hepatocyte nuclear factor 1; LDL-C – low-density lipoprotein cholesterol; LDLR – low-density lipoprotein receptor; LOF – loss of function; mVLDLR - mouse very low-density lipoprotein receptor, PCSK9 – proprotein convertase subtilisin kexin type 9; PPARα – peroxisome proliferator activated receptor alpha; RISC – RNA-induced silencing complex; RNAi – RNA interference; siRNA – small interfering RNA

INTRODUCTION

Proprotein convertase subtilisin kexin type 9 (PCSK9) is a circulating protein that accelerates degradation of hepatic low-density lipoprotein receptor (LDLR) and inhibits plasma LDL cholesterol (LDL-C) clearance. Genetic studies in humans have shown that some PCSK9 mutations are "gain-of-function" (GOF) mutations linked to hypercholesterolemia and are found in individuals with increased levels of low-density lipoprotein cholesterol (LDL-C) and with an increased risk of cardiovascular disease, while others are "loss-of-function" (LOF) mutations associated with hypocholesterolemia which manifested with significantly lower plasma LDL-C levels and reduced risk of coronary heart disease (CHD) [1, 2].

Since the LOF mutations in PCSK9 are not associated with any apparent side effects, inactivation of this protease has become an attractive strategy for lowering LDL-C levels and decreasing the risk of atherosclerotic cardiovascular diseases, either alone or in combination with lipid-lowering drugs. Rashid *et al*. [3] reported that knockout mice lacking PCSK9 not only exhibited hypersensitivity to statins, but also produced an exaggerated amount of low-density lipoprotein receptors (LDLR) in the liver, showed enhanced clearance of circulating lipoproteins from the bloodstream and had decreased plasma cholesterol levels. Notably, PCSK9 knockout mice exhibited no significant differences in terms of body and liver weights, hepatic cholesterol level, or triglyceride concentrations in comparison to control mice.

Moreover, studies in both heterozygous and homozygous *PCSK9* loss-of-function mutation carriers, who appear to be healthy, despite very low plasma LDL-C levels [4, 5], demonstrated that the inhibition of PCSK9 function could be therapeutically beneficial to effectively reduce the risk of cardiovascular diseases in patients, with no obvious side-effects. Cariou *et al*. [6] provided an additional argument for developing PCSK9 inhibitors by demonstrating that patients affected by PCSK9 LOF mutations exhibit accelerated LDL catabolic rates.

Therefore, it is a highly desirable approach to identify and develop efficient compounds modifying protein PCSK9 levels for the prevention, treatment or alleviation of PCSK9-mediated disorders with no serious adverse effects.

In fact, there are several agents being tested at an advanced stage. These are mainly specific antibodies directed against PCSK9 and PCSK9 silencing, namely antisense oligonucleotide inhibitors (ASO) and RNA interference (RNAi).

PCSK9 AND PHARMACOLOGY

The goal of atherosclerotic pharmacotherapy is to prevent or slow the development of atherosclerotic plaques, mainly by targeting LDL-C as a therapeutic endpoint. These days three commonly used classes of lipid-modulating drugs, namely statins, fibrates and ezetimibe, are used to treat lipid metabolism disorders. However, these agents additionally significantly modify plasma PCSK9 levels.

Statins

Statins are first line agents for cardiovascular prevention that have revolutionized the treatment of hyperlipidemia. However, as with most drugs, statin therapy is not suitable for all patients, because some of them do not respond to the treatment or require additional therapy, as statin treatment alone does not achieve sufficient LDL-C reduction [7].

Statins act through inhibiting HMG-CoA reductase, which plays a crucial role in cholesterol synthesis in the liver. Low cholesterol levels in the cells activate the transcription factor steroid response element binding protein 2 (SREBP2) and afterwards induce LDLR expression. SREBP2 also regulates the expression of many genes involved in cholesterol metabolism, among others PCSK9.

The research by Dubuc *et al*. [8] confirmed the statement that statin therapy upregulates PCSK9 expression, thereby disabling its basic function to reduce LDL-C level. In the research HepG2 cells were treated with 1 μmol/l of cerivastatin, atorvastatin, lovastatin, simvastatin, or pitavastatin. This dose of cerivastatin increased PCSK9 expression 3-fold, and other statins 1.5-fold. Additionally it has been shown that 10 μmol/l of atorvastatin increased *PCSK9* expression 7.5-fold, whereas under the same conditions LDLR expression increased only 2.5-fold in HepG2 cells.

Caresky *et al*. [9] demonstrated that atorvastatin treatment (10 mg/day) reduced LDL-C levels by 30%, with no significant effect on PCSK9 expression, whereas patients receiving 40 mg/day of atorvastatin displayed a total decrease of 42% in LDL-C level, while plasma PCSK9 concentration increased by 34%. However, Costet *et al*. [10] reported that the same dose of atorvastatin (10 mg/day) increased plasma PCSK9 by 24% by day 1, and by 14% after 6 weeks of administration in patients with type 2 diabetes mellitus. Later studies by Welder *et al*. [11] confirmed that high-dose atorvastatin treatment (80 mg/day) causes a 47% increase in serum PCSK9 after 4 weeks of administration, which was completely sustained at 8-week, 12-week, and 16-week time points. These results suggest the existence of a dose response effect for atorvastatin on circulating PCSK9 levels – the higher the doses of atorvastatin, the larger the percentage increases in plasma PCSK9 levels.

Fibrates

Besides statins, other commonly prescribed hypolipidemic agents are fibrates (peroxisome proliferator activated receptor alpha (PPARα) agonists). Used as an add-on to ongoing statin therapy, fibrates have been shown to enhance LDL-C lowering efficiency, compared with HMG-CoA reductase inhibitor (statin) treatment alone [12]. In clinical practice, fibrates are a class of drug that is used to treat primary hypercholesterolemia, mixed dyslipidemia and hypertriglyceridemia, since it improves lipid levels, in particular triglyceride and HDL-C levels. Moreover, fenofibrates are known for their pleiotropic effects, since they reduce fibrinogen, uric acid levels and C-reactive protein [13].

Fibrates have been shown to reduce PCSK9 mRNA levels in human hepatocytes [14] and in mice [15]. In humans Troutt *et al*. [16] reported that 12 weeks of fenofibrate monotherapy (200 mg/day) significantly increased plasma PCSK9 levels by 25% compared to baseline. Costet *et al*. [10] reported that three weeks of administration of fenofibrate (160 mg/day) in combination with atorvastatin (10 mg/day), apart from decreasing LDL cholesterol by 30% and triglyceride level by 31% and increasing HDL cholesterol by 13%, additionally increased PCSK9 concentration by 42%.

In contrast to these reports, Kourimate *et al*. [14] demonstrated that various fibrates (Wy14643, fenofibric acid – the active form of fenofibrate, clofibrate, gemfibrozil) repressed PCSK9 expression in human immortalized hepatocytes. This group also reported that each agonist (especially Wy14643 and gemfibrozil) dramatically reduces PCSK9 protein expression in hepatocytes. Furthermore, Lambert *et al*. [17] demonstrated that fenofibrate (200 mg/day for 6 weeks) slightly decreased plasma PCSK9 circulating levels by 8.5% in a cohort of 115 patients with type 2 diabetes from the FIELD (*Fenofibrate Intervention and Event Lowering in Diabetes*) study.

In another report, Mayne *et al*. [18] showed that plasma PCSK9 levels were either increased or decreased by fenofibrate and gemfibrozil in 19 individuals (overall significant increase of 17%). However, fenofibrate (up to 200 μM) did not significantly change the expression of PCSK9 or the LDLR in HepG2 cells. This heterogeneity in results may be due to the relatively small group of patients who were treated with gemfibrozil and fenofibrate.

Ezetimibe

Another lipid metabolism modifying agent is ezetimibe – a hypocholesterolemic drug reducing blood cholesterol by inhibiting intestinal uptake of dietary cholesterol [19]. Ezetimibe is commonly used as a monotherapy or as an add-on to statin therapy in patients who fail to maintain the proper LDL-C level.

Dubuc *et al*. [20] reported that patients on statin treatment alone had a 45% significantly higher plasma PCSK9 level than controls, whereas those treated with two cholesterol-lowering agents, statins and ezetimibe, exhibited 77% higher PCSK9 levels in comparison to control individuals. Similarly, Davignon *et al*. [21] found that patients treated with a statin-ezetimibe combination had even higher levels of plasma PCSK9 than patients treated with statin therapy alone. Going a step further, Ason *et al*. [22] analyzed the potential of PCSK9 knock down with ezetimibe, statin, and combination of statin with ezetimibe to lower the LDL-C level with the use of a mouse model with a human-like lipid profile. The research revealed that all three analyzed types of treatments lower serum cholesterol concentration, but induce the expression of PCSK9. Combined ezetimibe/rosuvastatin/*PCSK9* siRNA treatment additionally led to a significant reduction in serum non-HDL and APOB protein levels and serum triglycerides. Gouni-Berthold *et al*. [23] found that the combination of simvastatin with

ezetimibe increased PCSK9 gene expression in peripheral blood mononuclear cells, while ezetimibe treatment alone had no significant effect.

Berberine

Additional treatment to statin therapy may include the use of berberine – a plant alkaloid that upregulates LDLR coding gene expression while downregulating PCSK9 transcription. Berberine has been previously reported to reduce serum total cholesterol by 29%, triglycerides by 35%, and LDL-C by 25% in 32 hypercholesterolemic patients, and increased hepatic LDLR mRNA by 3.5-fold in hyperlipidemic hamsters [24]. Cameron *et al*. [25] reported that berberine reduced the amount of PCSK9 mRNA by 77% in HepG2 cells, indicating the possibility of reduction of secreted PCSK9.

Research carried out by Li *et al*. [26] described the mechanisms underlying the transcriptional suppression of PCSK9 by berberine in HepG2 cells. They reported that berberine suppresses PCSK9 transcription through decreasing the cellular abundance of the active form of SREBP2 and highly conserved hepatocyte nuclear factor 1 (HNF1α) protein, the newly identified key transactivator for PCSK9 gene expression. Since SREBP2 is crucial for LDLR transcription, it would be undesirable to use berberine in hyperlipidemic disorders. However, Kong *et al*. [24] reported that berberine upregulates LDLR expression regardless of SREBP2, hence elevating LDLR expression through a post-transcriptional mechanism that stabilizes the receptor mRNA.

Since PCSK9 diminishes the full potential effect of commonly used hypolipidemic therapy (statins, fibrates, ezetimibe), elimination or reduction of PCSK9 activity via monoclonal antibodies, antisense or RNAi may augment the hypolipidemic effects of these powerful drugs in patients who are unable to attain a balance of the lipid profile on pharmacological therapy alone. Moreover, it is hoped that new therapeutic agents inhibiting PCSK9 activity will work additively with widely used hypolipidemic drugs.

SUPPRESSION OF PCSK9

Other promising therapeutic strategies, such as monoclonal antibodies, antisense oligonucleotides (ASO), or small interfering RNA (RNAi), may represent the future of hypercholesterolemia treatment.

Anti-PCSK9 antibody

Recently, several groups have attempted to obtain neutralization of PCSK9 by using antibody or its antigen-binding fragment that specifically binds and inhibits human PCSK9. These high-affinity anti-PCSK9 antibodies are able to reduce serum total cholesterol and LDL-C levels, with little or no reduction in serum high-density lipoprotein cholesterol (HDL) in mice and non-human primates.

Shan *et al*. [27] reported for the first time that synthetic LDLR EGF-A peptide domain inhibits PCSK9-mediated degradation of LDL receptors in HepG2 cells. Furthermore, it has been shown that PCSK9 interacts with both EGF domain(s) of apolipoprotein E receptor 2 (ApoER2) and mouse very low-density

lipoprotein receptor (mVLDLR). Ni *et al*. [28] from Merck Research Laboratories also produced a neutralizing monoclonal PCSK9 antibody, 1D05-IgG2, suppressing the wild-type PCSK9 and two gain-of-function PCSK9 mutants, namely S127R and D374Y. The analysis revealed that 1D05-Fab, attached to an epitope on the PCSK9 catalytic site, covers the entire LDLR EGF-A binding site and directly inhibits PCSK9 from binding to the LDLR EGF-A domain. The antibody neutralized both wild-type PCSK9 and GOF mutants, and significantly decreased LDL-C and total cholesterol levels in mice and rhesus monkeys. After administration of the antibody to rhesus monkeys, LDL-C decreased by approximately 30-50% for 2 weeks, which was associated with more than 70% reduction in the level of plasma PCSK9.

Chan and Jackson from Amgen's Inc. [29] developed a neutralizing monoclonal antibody, mAb1, that binds to an epitope on PCSK9 adjacent to the region that is involved in the interaction with the LDLR and therefore disturbs the interaction of PCSK9 with LDLR. In wild-type mice, this antibody induced a 2.3-fold increase in hepatic LDLR protein levels and lowered total serum cholesterol by 36% after a single injection in comparison to control mice. When the antibody binding to PCSK9 was injected into cynomolgus monkeys, within 15 min the antibody had bound to more than 97% of circulating PCSK9 and significantly decreased serum LDL-C as early as 8 h after a single injection. The LDL-lowering effect persisted for about two weeks (~80% at day 10), even though the mAb1 had a relatively short half-life, 61 h. Incidentally, a minor decrease in HDL-C level was also observed (maximum: -18%). However, no effect on serum triglycerides was noted.

Recently, Amgen has started phase 2 of a clinical trial to evaluate the safety, tolerability and efficacy of multiple doses of their promising novel anti-PCSK9 antibody AMG 145 on LDL-C, when given as an add-on to stable statin therapy in individuals with familial hypercholesterolemia compared to placebo [30].

Another group from Pfizer-Rinat Inc. is evaluating a monoclonal antibody against the PCSK9 protein, RN316, as a treatment for cholesterol disorders. The antibody antagonizes the activity of extracellular PCSK9, neutralizing its interaction with the LDLR, and therefore can be used therapeutically to lower LDL cholesterol levels in blood, and to prevent and/or treat lipid metabolism disorders. The drug has been recently tested in phase 1 studies as a single agent tested alone and as a combination with statins in patients who do not respond well to statin therapy, as well as in those who do not tolerate statins at all. Pfizer-Rinat have reported that the tested agent significantly reduces LDL-C in hypercholesterolemia patients [31].

Regeneron Pharmaceuticals conducted an analysis of a dose-escalating, randomized, double-blind, placebo-controlled phase 1 trial in healthy volunteers with its fully human monoclonal antibody called REGN727, which is designed to bind to PCSK9 and to prevent LDLR degradation. During clinical development, REGN727 achieved a substantial, dose-dependent decrease of LDL-C. The highest tested dose significantly reduced LDL-C concentration by

> 60% for 1 month following a single dose [32]. In 2011 Regeneron Pharmaceuticals started phase 2 of their study in hyperlipidemic patients treated with REGN727 or placebo receiving stable doses of atorvastatin [33].

Antisense oligonucleotides

The term antisense oligonucleotides (ASO) refers to oligomers which comprise a contiguous nucleotide sequence of a total of 10-30 nucleotides, wherein the nucleotide sequence is at least 80% homologous to a coding sequence of the target gene. ASO reduce expression of the protein typically by inducing its mRNA degradation or suppression of the mRNA translation.

High effectiveness of antisense oligonucleotides directed towards *PCSK9* has been clearly demonstrated by Graham *et al*. [34] from Isis Pharmaceuticals Inc. They reported that administration of a second-generation antisense oligonucleotide complementary to the PCSK9 coding sequence to mice fed a high-fat diet for 6 weeks reduced total cholesterol and LDL-C levels by 53% and 38% respectively. Additionally, as a result of ASO treatment the level of *PCSK9* expression decreased by 92%, while the hepatic LDLR protein level increased 2-fold in comparison to control animals.

Similarly, Gupta *et al*. [35] from Santaris Pharma A/S reported a 60% reduction of the *PCSK9* mRNA level in human HepG2 and approximately 50% in HuH7 cells after a single treatment with ASO. They also observed that in mice sacrificed 24 h after injection, the hepatic PCSK9 mRNA content was found to be significantly reduced by the ASO in a dose-dependent manner. However, the almost 3-fold increased level of hepatic LDLR protein compared to the control mice was observed only in the high-dose groups.

In 2010, Santaris Pharma A/S announced that they were developing a cholesterol-lowering drug, SPC5001. The antisense oligonucleotide directed against PCSK9 seems to be an important new target for the treatment of high LDL-C levels. Preclinical data presented by Santaris Pharma A/S showed that SPC5001 provided potent, specific and long-lasting inhibition of PCSK9 and lowered LDL-C by 50% in non-human primates with a sustained reduction of 74% in the highest responder on average. Additionally, SPC5001 did not change HDL-C levels in the blood [36]. As of this year, Santaris Pharma A/S has been testing SPC5001 in phase 1 clinical trials in patients with familial hypercholesterolemia to assess drug safety, tolerability, pharmacokinetics and pharmacodynamics [37].

RNAi

The mechanism of RNA interference has become a solution in the therapy of many diseases based on the downregulation of gene expression using double-stranded ribonucleic acid (dsRNA) molecules. The construct has a homologous double-stranded region that is substantially complementary to at least a part of an mRNA-encoding target protein. The appearance of dsRNA in a cell activates the RNase III-like enzyme (Dicer), which generates the small (20-23 nucleotides long), double-stranded interfering RNA (siRNA), which allows activation of

a multi-protein complex called RNA-induced silencing complex (RISC). After incorporation into RISC, the siRNA unwinds, leaving the antisense strand to direct RISC to its target mRNA, for endonucleolytic degradation [38].

Frank-Kamenetsky *et al*. [39] from Alnylam Pharmaceuticals demonstrated that in transgenic mice expressing human *PCSK9*, application of two siRNAs (LNP-PCS-A2 and LNP-PCS-C2) resulted in a 70% reduction in *PCSK9* mRNA levels and a 500-fold decrease in the levels of circulating human PCSK9 protein, with an effect that lasts approximately 3 weeks after a single dose. This same group reported that siRNA-mediated reduction in *PCSK9* mRNA and protein was also possible in cynomolgus monkeys, by showing that a single injection of siRNA delivered in lipidoid nanoparticles successfully reduced plasma LDL-C levels by 50-60% within 48 h after administration. Again, a single siRNA injection produced long-lasting reductions in both serum PCSK9 and LDL-C levels, which remained significantly lower for 2 to 3 weeks.

Alnylam Pharmaceuticals is also developing ALN-PCS01, an RNAi therapeutic, to treat high levels of LDL-C in the blood. ALN-PCS01 works by silencing *PCSK9*, and it has the potential to treat hypercholesterolemia in a way that is unachievable by current therapies, such as statins. ALN-PCS01 is systemically delivered, and optimized siRNA is encapsulated in a cationic liposomal nanoparticle formulation. In 2007 Alnylam announced that they had advanced ALN-PCS01 in pre-clinical studies and had obtained very promising results, including a more than 50% reduction in levels of LDL-C in non-human primates, and that they intended to advance this program towards a phase 1 study in humans [40].

CONCLUSIONS

Taking these data together, pre-clinical and clinical studies show that the use of monoclonal antibodies, antisense oligonucleotides and short interfering RNA is effective in inhibiting the expression or activity of PCSK9 and reducing LDL-C levels. Therefore it is hoped that within the next few years useful and efficacious pharmacological compounds that can block the interaction between PCSK9 and LDLR will be available for patients with hypercholesterolemia. However, although the studies with active agents inhibiting PCSK9 have shown them to promote positive trends in the treatment of lipid metabolism disorders, further human clinical trials are required to completely understand the biology of PCSK9 and will be crucial to evaluate whether its inhibition may have any side effects in particular groups of patients.

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