

## Review

# Lowering serum lipids via PCSK9-targeting drugs: current advances and future perspectives

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### Abstract

Proprotein convertase subtilisin/kexin type 9 (PCSK9), also known as neural apoptosis regulated convertase (NARC1), is a key modulator of cholesterol metabolism. PCSK9 increases the serum concentration of low-density lipoprotein cholesterol by escorting low-density lipoprotein receptors (LDLRs) from the membrane of hepatic cells into lysosomes, where the LDLRs are degraded. Owing to the importance of PCSK9 in lipid metabolism, considerable effort has been made over the past decade in developing drugs targeting PCSK9 to lower serum lipid levels. Nevertheless, some problems and challenges remain. In this review we first describes the structure and function of PCSK9 and its gene polymorphisms. We then discuss the various designs of pharmacological targets of PCSK9, including those that block the binding of PCSK9 to hepatic LDLRs (mimetic peptides, adnectins, and monoclonal antibodies), inhibit PCSK9 expression (the clustered regularly interspaced short palindromic repeats/Cas9 platform, small molecules, antisense oligonucleotides, and small interfering RNAs), and interfere with PCSK9 secretion. Finally, this review highlights future challenges in this field, including safety concerns associated with PCSK9 monoclonal antibodies, the limited utility of PCSK9 inhibitors in the central nervous system, and the cost-effectiveness of PCSK9 inhibitors.

**Keywords:** PCSK9; LDL receptor; monoclonal antibodies; anti-sense oligonucleotide; mimetic peptides; adnectin; CRISPR/Cas9 platform; berberine; oleanolic acid; neuronal apoptosis; coronary artery disease; cholesterol metabolism

*Acta Pharmacologica Sinica* (2017) 38: 301–311; doi: 10.1038/aps.2016.134; published online 16 Jan 2017

### Introduction

Cardiovascular diseases that occur in patients who have high levels of low-density lipoprotein cholesterol (LDL-C) are a leading cause of death in developed countries<sup>[1, 2]</sup>. Increased levels of LDL-C are considered a major risk factor for coronary artery disease (CHD) and for the development of atherosclerotic plaques in arteries<sup>[3, 4]</sup>. Cardiovascular risk is decreased by 22% when LDL-C is reduced by 24 mg/dL or 38.5 mg/dL<sup>[5, 6]</sup>.

Loss-of-function mutations in the low-density lipoprotein receptor (LDLR) gene in patients with familial hypercholesterolemia (FH) are associated with high plasma LDL-C levels and early-onset CHD, which begins in childhood<sup>[7, 8]</sup>. The LDLR, which is localized to the cell membrane, degrades the plasma LDL-C concentration via the receptor-mediated uptake of

LDL-C into the cell. FH patients lacking LDLR mutations have apolipoprotein B (apoB) gene mutations<sup>[9–11]</sup>. Specific mutations in the apoB gene increase the LDL-C levels and induce early-onset CHD by blocking the interaction between LDLRs and apoB.

One of the greatest advances in the lipid-lowering field over the past decade was the development of a lipid-altering therapy targeting proprotein convertase subtilisin/kexin type 9 (PCSK9), which binds to LDLRs and targets them for lysosomal degradation<sup>[12, 13]</sup>. The administration of statins to reduce LDL-C has also effectively diminished the risk of cardiovascular disease over the past 20 years<sup>[14, 15]</sup>. However, some patients, especially those with FH, are unable to achieve normal LDL-C levels, even when they are treated with the maximum dose of statins<sup>[16]</sup>. This condition is due to the increase in the transcription of both PCSK9 and LDLR upon using statins, which leads to a decreased lipid restriction of statins<sup>[17–19]</sup>. Therefore, agents that target PCSK9 are promising therapeutic targets for the treatment of hypercholesterolemia and might improve the efficacy of statins in CHD.

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Received 2016-08-30 Accepted 2016-10-18

This paper summarizes current advancements in PCSK9 inhibitors, including blocking the combination of PCSK9 with hepatic LDLRs (mimetic peptides, adnectins, and monoclonal antibodies (mAbs)), inhibiting PCSK9 expression (the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 genome editing platform, small molecules, antisense oligonucleotides, and small interfering RNAs (siRNAs)), and interfering with PCSK9 secretion. This review also discusses the opportunities and challenges that are associated with the development of drugs that target PCSK9.

### Structure, function, and gene polymorphisms of PCSK9

PCSK9, also known as neural apoptosis-regulated convertase 1 (NARC-1), was first described in 2003<sup>[20]</sup>. The human PCSK9 gene is located on chromosome 1p32.3 and is 3617 bps in length. It contains 12 exons that encode 692 amino acids<sup>[21, 22]</sup>. The PCSK9 protein contains a signal peptide, a prodomain, a catalytic domain, and a C-terminal cysteine-histidine-rich domain that is composed of 3 modules (M1, M2, and M3)<sup>[23–25]</sup>. It has been shown that PCSK9 zymogen processing and secretion are inhibited by the absence of M1 and M3. By contrast, M2 is not required for PCSK9 maturation and release, but it is important for intracellular LDLR degradation. The molecular weight of the PCSK9 precursor protein is 75 kDa. After autocatalytic cleavage in the endoplasmic reticulum (ER), the prodomain is separated from the 62 kDa mature PCSK9 protein. The separated prodomain remains non-covalently bound to the mature PCSK9 protein, thus forming a prosegment-PCSK9 complex that forces the PCSK9 catalytic domain into an inactive conformation<sup>[26]</sup>. The cleaved complex is then transported from the ER to the Golgi apparatus and then released<sup>[27]</sup>. The secreted PCSK9 then interacts via its catalytic domain<sup>[28]</sup> and prodomain<sup>[29]</sup> with the epidermal growth factor-like repeat homology domain-A (EGF-A) and  $\beta$ -propeller domain, respectively, of the LDLR. The binding of PCSK9 to the LDLR is blocked by mutations in the EGF-A of the LDLR, which is a major binding site for PCSK9<sup>[12]</sup>. Recently, it has been reported that the interaction between the C-terminal domain (CTD) of PCSK9 and the ligand-binding domain (LBD) of the LDLR may be enhanced in an acidic milieu<sup>[30]</sup>. In addition, it has been shown that the PCSK9 CTD is necessary for the localization of PCSK9 to the trans-Golgi network (TGN) and that it increases the endocytosis of the PCSK9/LDLR complex<sup>[31]</sup>. To date, the mechanism by which the PCSK9/LDLR complex is targeted for degradation is unclear, although the available evidence suggests that at least two mechanisms are possible. First, the affinity between the PCSK9 catalytic domain and LDLR EGF-A is increased under acidic conditions; this could change the conformation of the LDLR, thereby promoting its degradation in lysosomes after formation of the PCSK9/LDLR complex<sup>[31]</sup>. Second, there is evidence to indicate that transmembrane protein X targets the PCSK9/LDLR complex to lysosomes for degradation by forming a bridge between the PCSK9 CTD and cytosolic adaptor proteins<sup>[32]</sup>.

Recently, new strategies for inhibiting PCSK9 for the treatment of hypercholesterolemia have been developed. Initially, mAbs that change the conformation of the catalytic domain of PCSK9 were discovered<sup>[32]</sup>. Other studies have shown that annexin A2<sup>[33]</sup> and a mAb<sup>[34]</sup> can inhibit PCSK9-induced LDLR degradation by binding specifically to the CTD of PCSK9.

The synthesis, secretion, and expression of PCSK9 occur primarily in the liver; PCSK9 is expressed at low levels in the gastrointestinal tract, kidneys, and central nervous system (CNS)<sup>[20, 21]</sup>. In hepatic cells, secreted PCSK9 can bind to LDLRs in the hepatocellular membrane through the EGF-A of the LDLR. The PCSK9/LDLR complex enters the endosomal system and is then degraded in the lysosomes before LDLR transport to the cell surface<sup>[35, 36]</sup>. Therefore, increased levels of PCSK9 can inhibit LDLR recycling to the cell surface<sup>[35–37]</sup>, which can in turn increase the LDL-C levels in the bloodstream. Recent studies have shown that PCSK9 might modulate LDLR transport by interacting with amyloid precursor-like protein 2, a protein that transports transmembrane proteins to the lysosomes<sup>[38–40]</sup> (Figure 1). In the small intestine, PCSK9 is closely associated with apoB, which is important in triglyceride and postprandial lipoprotein production<sup>[41]</sup>. In one study, the secretion of apoB48 and apoB100 was increased by 40% and 55%, respectively, in human enterocytes (Caco-2 cells) treated with human PCSK9<sup>[41]</sup>. Transintestinal cholesterol excretion is also modulated by PCSK9<sup>[42]</sup>. In addition to the LDLR, PCSK9 can mediate the degradation of other lipid receptors in the LDLR family, such as the very low-density lipoprotein receptor<sup>[43]</sup>, the apolipoprotein E receptor 2<sup>[43]</sup>, and LDL receptor-related protein 1. PCSK9 also appears to play an important role in the pathogenesis of diseases such as diabetes<sup>[44]</sup>, cancer<sup>[45]</sup>, and Alzheimer's disease<sup>[46, 47]</sup>.

A mutation in the PCSK9 gene was first detected in 2003 in French families<sup>[48]</sup> and PCSK9 was the third gene to be linked to autosomal dominant FH, after apoB and LDLR. In autosomal dominant FH, a gain-of-function mutation in the PCSK9 gene results in elevated levels of LDL-C<sup>[48–52]</sup>. In addition, overexpressing PCSK9 in the hepatic cells of mice elevated the levels of total plasma and non-high-density lipoprotein cholesterol<sup>[53]</sup> and decreased the levels of LDLR in hepatic cells<sup>[54]</sup>, which indicated that the increased risk of cardiovascular disease is due to PCSK9 gain-of-function mutations in humans. In 2005, loss-of-function mutations in PCSK9 were found in people who had lower LDL-C levels and a significantly reduced incidence of cardiovascular events. Two nonsense mutations (Y142X and C679X) of the coding region of PCSK9, which were associated with reduced LDL-C in 128 participants (50% African American), were reported in a study. The incidences of these two mutations were more common in African Americans compared with European Americans and were correlated with a 40% decrease in the plasma concentration of LDL cholesterol<sup>[55]</sup>. Moreover, PCSK9 loss-of-function patients were shown to have a heightened response to statin therapy. Thus, PCSK9 inhibitors are required to enhance the lowering action of LDL-C of statins<sup>[56]</sup>.

### PCSK9 as an emerging target for LDL-C-lowering therapy

Researchers have devoted much time and knowledge to examining PCSK9 as a significant therapeutic target. Such research primarily includes blocking the combination of PCSK9 and LDLR via mAbs, adnectins, and mimetic peptides, inhibiting PCSK9 expression with CRISPR/Cas9 genome-editing technology, antisense oligonucleotides (ASOs) and siRNA, and interfering with PCSK9 secretion from the ER (Figure 1, Table 1).

#### Blocking the PCSK9-LDLR combination

##### Mimetic peptides

Mimetic peptides that inhibit the interaction between PCSK9 and the LDLR include several short amino acid sequences that mimic the EGF-A<sup>[35, 57, 58]</sup>, catalytic domain, prodomain, or C-terminal domain of PCSK9<sup>[25, 59, 60]</sup>. These therapeutic

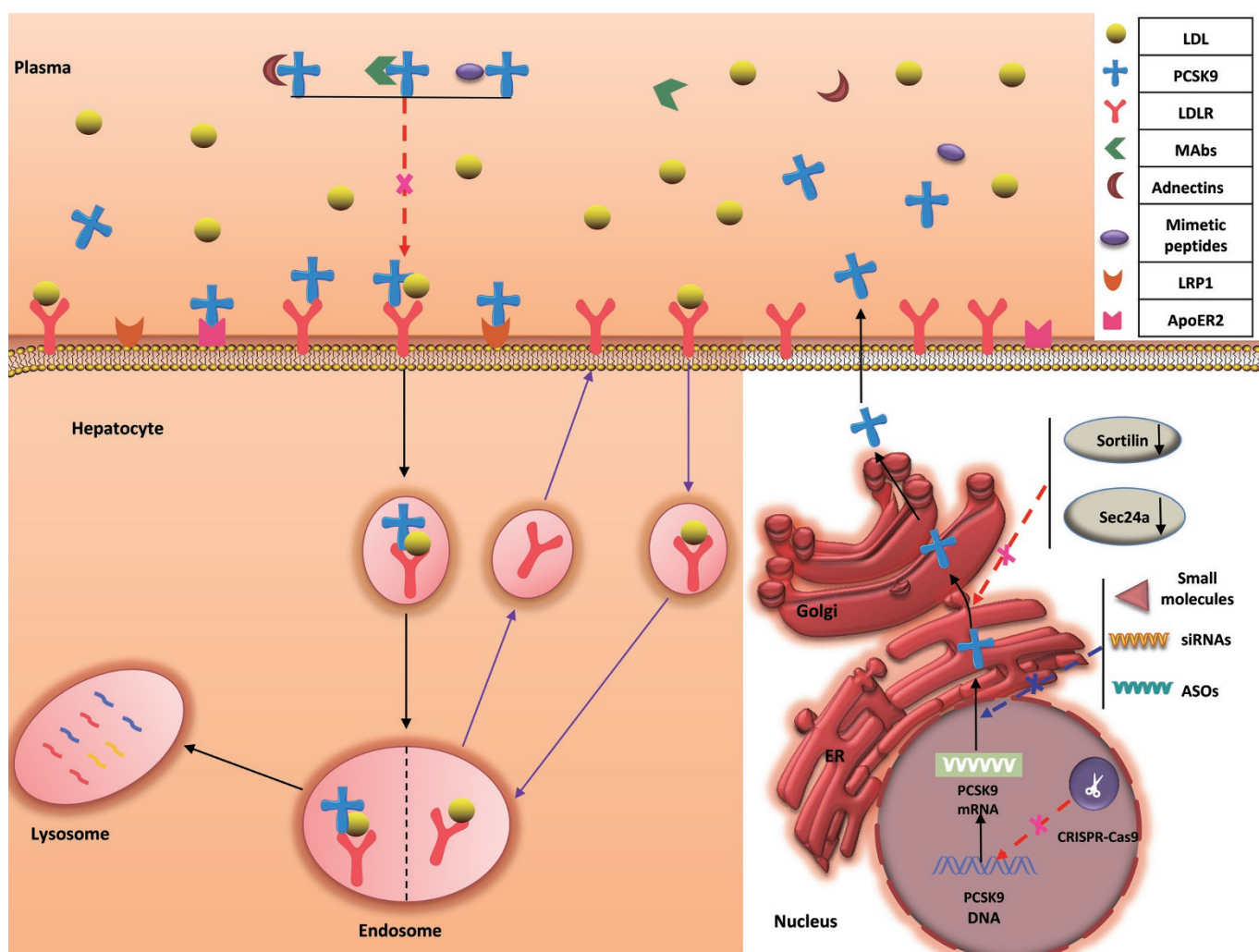
small peptides have high specificity, are easy to produce and modify, and are cheaper to produce than antibodies, but their routes of administration are limited.

##### Adnectins

Adnectins are a new type of therapeutic protein based on the 10th fibronectin type III domain. They bind to their target proteins with high specificity and affinity via transforming  $\beta$ -sheet loops, which provide structural stability. BMS-962476 (Bristol-Myers Squibb/Adnexus) is thought to prevent PCSK9-mediated LDLR degradation<sup>[61]</sup>.

##### Antibodies targeting PCSK9

Currently, mAb administration is an effective therapeutic way to suppress the interaction between PCSK9 and LDLR because of its high titer, high specificity, and long half-life. Primary



**Figure 1.** Steps in the LDLR metabolic pathway. After LDL binds to the LDLR at the cell membrane, the LDLR-LDL complex undergoes endocytosis. Within the endosome, LDL dissociates from the LDLR. The LDL then enters the lysosome where it is degraded, and the LDLR is recycled to the cell surface. The LDLR metabolic pathway under PCSK9 regulation. Secreted PCSK9 binds to the LDLR at the cell surface, and the PCSK9-LDLR complex is endocytosed, thereby promoting its lysosomal degradation. PCSK9-targeted therapies include mAbs, adnectins, and mimetic peptides (which block the interaction between PCSK9 and the LDLR), CRISPR/Cas9 genome editing technology, antisense oligonucleotides (ASOs), siRNAs and small molecules (which inhibit PCSK9 expression), and drugs that interfere with PCSK9 secretion from the ER.

**Table 1.** PCSK9-targeting mechanisms.

Mechanisms	Representative agents	References
Blocking the combination of PCSK9 and LDLR	Mimetic Peptides	[25, 35]; [57–60]
	Adnectins	[61]
	Monoclonal antibodies (alirocumab; evolocumab; bococizumab)	[62–75]
Inhibiting PCSK9 expression	CRISPR/Cas9 technology	[11]; [76–79]
	Small molecules (berberine; oleanoic acid)	[81–91]; [92–99]
	Antisense oligonucleotides	[100–102]
	Small interfering RNAs	[103, 104]; [58, 59]
Interfering PCSK9 secretion	Sortilin	[105, 106]
	Sec24a	

mAbs that have been developed against PCSK9 include alirocumab (REGN727/SAR236553), evolocumab (AMG 145), and bococizumab (RN316). Antibodies that target a single domain of PCSK9 are powerful inhibitors that mediate the degradation of the LDLR<sup>[62]</sup>.

#### Alirocumab (REGN727/SAR236553) developed by Sanofi/Regeneron

Alirocumab (REGN727/SAR236553), which is marketed by Sanofi/Regeneron under the brand name Praluent, is a human mAb that inhibits PCSK9<sup>[63]</sup>. Its weight is approximately 146 kDa. Within 4–8 h, alirocumab achieves maximum suppression and is dose-dependent. Within 3–7 d, the maximum serum concentration occurs via the subcutaneous injection of alirocumab, which is 75 mg or 150 mg every 2 weeks, and its bioavailability increases by approximately 85%. The half-life of alirocumab in patients who are intolerant to statins and have heterozygous FH (HeFH) is 17–20 d. In Phase II trials, LDL-C was reduced by 40%–70% through the combined treatment of alirocumab and statins<sup>[64–66]</sup>. The LDL-C levels were reduced to less than 100 mg/dL in all patients treated with SAR236553 compared with 52% of patients treated with 80 mg of atorvastatin plus placebo. Similarly, the LDL-C levels were reduced to less than 70 mg/dL in 90% of patients treated with SAR236553 compared with 17% of patients treated with 80 mg of atorvastatin plus placebo<sup>[65]</sup>. Three Phase III Odyssey FH studies found that LDL-C levels were reduced by an additional 49% in patients treated with alirocumab compared with untreated patients<sup>[67]</sup>. Across all trials, LDL-C levels of <70 mg/dL were achieved in the majority of patients treated with alirocumab<sup>[68]</sup>.

#### Evolocumab (AMG 145) developed by Amgen

Evolocumab (AMG 145), which is marketed by Amgen under the brand name Repatha, is a human monoclonal antibody that inhibits PCSK9<sup>[69]</sup>. Repatha has a molecular weight of 144 kDa. Evolocumab is administered by subcutaneous injection either biweekly or monthly, at a dose of 140 mg or 420 mg, respectively, and causes a dose-dependent reduction in

LDL-C. Evolocumab has a bioavailability of approximately 72%, and a half-life of 11–17 d. After 12 weeks of dosing, a steady state was reached. In Phase I and II trials, a subcutaneous injection of evolocumab reduced the LDL-C levels in HeFH and non-FH patients; the apoB and lipoprotein (a) levels were also reduced by 30%–59% and 18%–36%, respectively, in a dose-dependent manner<sup>[70]</sup>. In a meta-analysis of Phase II and III trials, evolocumab and alirocumab reduced LDL-C and lipoprotein (a) levels by 47% and 26%, respectively<sup>[71]</sup>. The Phase III Descartes study, which determined the efficacy and safety of evolocumab in hyperlipidemic patients, showed that evolocumab reduced LDL-C by 48.5%–61.6%<sup>[72]</sup>. Evolocumab reduced LDL-C by <70 mg/dL in 82% of subjects<sup>[73]</sup>. In Phase II and III trials, adverse reactions (including nasopharyngitis, headache, upper respiratory tract infection, diarrhea, rash, myalgia, back pain, and urticaria), were reported at similar levels among patients receiving evolocumab treatment or placebo. Currently, evolocumab is prescribed primarily to patients who have FH and are intolerant to statin therapy.

#### Bococizumab (RN316) developed by Pfizer

Bococizumab (RN316) is another humanized mAb that directly inhibits PCSK9. A Phase II, randomized, placebo-controlled study by Gumbiner *et al* determined the efficacy and safety of bococizumab in hypercholesterolemic patients receiving high-dose statin therapy<sup>[74]</sup>. After 12 weeks, bococizumab administration decreased LDL-C by 56%, compared with 4% in the placebo group. In several patients receiving bococizumab, LDL-C was reduced to levels below 25 mg/dL, leading to an interruption in treatment at week 4. Bococizumab is more potent than other LDL-C-lowering mAbs. In a randomized, placebo-controlled trial, 150 mg of bococizumab biweekly reduced the LDL-C levels by 53%<sup>[75]</sup>. Adverse events were reported at similar levels in patients receiving bococizumab or placebo. The SPIRE program is currently conducting five Phase III trials with bococizumab (SPIREHF, SPIRE-LDL, SPIRE-HR, SPIRE-1, and SPIRE-2).



## Inhibition of PCSK9 expression

### CRISPR/Cas9 platform

CRISPR-Cas9, a novel genome editing technology, is based on the CRISPR adaptive immune system of bacteria and comprises a guided RNA linked to an endonuclease (*ie*, Cas9). Recently, the CRISPR-Cas9 platform was found in a program to specifically target and cleave DNA using a single-stranded RNA molecule and has thus received considerable attention<sup>[11, 76]</sup>. Many bacteria use the CRISPR system to protect themselves against the invasion of foreign nucleic acids, including viruses and plasmids. Activation of the CRISPR-Cas9 mechanism induces double-stranded breaks in the DNA of host cells, followed by error-prone recombination and non-homologous end-joining. This process produces frameshift mutations and allele knockout following the infection of CRISPR-Cas9-containing host cells with viruses or plasmids<sup>[77]</sup>. The mutagenesis rate of PCSK9 in mouse liver was found to be >50% on days three through four, and off-target mutagenesis was minimized by treatment with adenovirus to express Cas9 and a CRISPR guide RNA targeting PCSK9. This approach reduced the PCSK9 levels, increased the hepatic LDL receptor levels, and reduced the plasma cholesterol levels by 35%–40%<sup>[77]</sup>. In a study using *Fah*<sup>-/-</sup>*Rag2*<sup>-/-</sup>*Il2rg*<sup>-/-</sup> (FRG KO) mice, which have chimeric, humanized livers, treatment with CRISPR-Cas9 targeting the human PCSK9 gene induced high levels of on-target mutagenesis of PCSK9, resulting in a 52% decrease in the level of human PCSK9 protein in the blood; off-target mutagenesis was not detected<sup>[78]</sup>. A compensatory mechanism within mouse hepatocytes, which increases the post-treatment blood levels of mouse PCSK9 protein more than twofold, has been found in transplanted FRG KO mice. Although the CRISPR-Cas9 platform that targets PCSK9, which can cause a permanent PCSK9 alteration, is a promising tool editing gene for use in humans, many questions remain unanswered. Considering the chimeric liver-humanized FRG KO mouse with default immunity and the loss of immune consequences of viral vectors targeting human PCSK9 in the mice liver, a future strategy may aim for the double humanization of mice-related FRG KO to both the liver and hematopoietic systems<sup>[79]</sup>.

### Small-molecule inhibitors

Small molecules have also received considerable attention. Unfortunately, efforts to develop small-molecule inhibitors of PCSK9, which are desirable because of their low cost, have been unsuccessful. Pep2-8, a peptide-based inhibitor of PCSK9 that mimics the secondary structural elements of the EGF-A domain of the LDLR, binds to PCSK9 and competitively inhibits the binding of PCSK9 to the LDLR. The major challenge associated with the development of PCSK9 small-molecule inhibitors is the lack of small-molecule-binding sites on PCSK9 due to the relatively flat surface of PCSK9; by contrast, antibody-binding sites are readily accessible. Regardless, other small molecules hold promise, such as berberine and oleanolic acid, which directly inhibit PCSK9 expression<sup>[80]</sup>.

### Berberine (BBR)

BBR is a type of protoberberine that is also known as an isoquinoline plant alkaloid. BBR elicits several pharmacological effects through diverse mechanisms, including antagonizing microbes and tumors, immunomodulation, and lowering both glucose and cholesterol<sup>[81, 82]</sup>. Accumulating evidence indicates that there is a close relationship between BBR and the PCSK9-LDLR pathway, an important mediatory of cholesterol depletion<sup>[83, 84]</sup>. In liver tissue, transcription factors, including members of the sterol regulatory element-binding protein (SREBP) family (which bind to the SRE motif in the proximal promoter) and hepatocyte nuclear factor 1 $\alpha$  (HNF1 $\alpha$ ; an essential trans-activator containing homeodomain)<sup>[85]</sup>, co-regulate the transcription of PCSK9<sup>[86]</sup>. The original study found that the SREBP pathway did not involve a reduction of the PCSK9 mRNA levels induced by BBR; specifically, the levels of SREBP-2 mRNA increased by 74%, and the question of whether BBR could decrease the activity of PCSK9 promoter was addressed<sup>[84]</sup>. In subsequent studies, BBR was found to simultaneously decrease the levels of HNF1 $\alpha$  (a transcription factor that specifically recognizes the site on the PCSK9 promoter between Sp1 and SRE) and SREBP2. Thus, the interaction of two transcription factor with their binding sequence of PCSK9 promoter is reduced, and PCSK9 transcription is repressed<sup>[87]</sup>. Recently, *in vivo* studies using hyperlipidemic mice suggested that the serum PCSK9 concentrations decreased by 50%, that the mRNA level of PCSK9 in all liver samples decreased by 46%, and that the LDLR protein levels increased by 67% in the BBR compared with the control, whereas the mRNA levels of LDLR and other SREBP2 target genes were unchanged<sup>[86]</sup>. BBR treatment reduced HNF1 $\alpha$  protein levels by 42% compared with control, whereas HNF1 $\alpha$  mRNA levels were unaffected by BBR treatment. These results were consistent with those obtained for a hyperlipidemic hamster. By blocking the ubiquitin proteasome system (UPS) and the autophagy-lysosomal pathway, BBR inhibited HNF1 $\alpha$ -mediated PCSK9 transcription via UPS to decrease the hepatic HNF1 $\alpha$  protein levels without affecting the mRNA levels<sup>[86]</sup>. Several studies have also reported that BBR has neuroprotective effects (such as suppressed neuronal apoptosis<sup>[88]</sup> and neuroinflammation<sup>[89]</sup>) and both anti-apoptotic and anti-inflammatory effects<sup>[90, 91]</sup>. Additional studies are needed to determine whether BBR exerts its effects through PCSK9.

### Oleanolic acid (OA)

OA is a pentacyclic triterpene that is widely distributed in plants and medicinal herbs as a free acid or as a saponin aglycone<sup>[92, 93]</sup>. OA has numerous beneficial properties, including anti-cancer, hepatoprotective, hypolipidemic<sup>[94, 95]</sup>, antioxidative, anti-inflammatory<sup>[96]</sup>, endothelial protective<sup>[97]</sup>, and anti-atherosclerotic effects.

In OA-treated *db/db* mice, serum triglycerides, total cholesterol (TC), LDL-cholesterol, free fatty acids, and the quantity of lipid droplets in hepatic cells were markedly reduced compared with untreated mice<sup>[98]</sup>. Furthermore, our previous

studies have shown that OA decreases the levels of PCSK9 protein and mRNA in HepG2 cells, in a time- and dose-dependent manner<sup>[99]</sup>. However, the underlying mechanism is unknown, and the OA efficiency is limited because of its low bioavailability and insolubility in water.

#### **Antisense oligonucleotides (ASOs)**

ASOs, which interfere with mRNA activation, consist of short, single-stranded nucleotide sequences. The successful delivery of ASOs to the hepatic nucleus has been reported<sup>[100]</sup>. By binding to their target mRNA, ASOs prevent protein translation and thereby reduce protein levels. In one study, the administration of an ASO (ISIS 394814) to hyperlipidemic mice for 6 weeks demonstrated that the levels of PCSK9 mRNA and LDL-C were reduced by 92% and 32%, respectively, that TC was reduced by 52%, and that the LDLR protein levels were increased twofold<sup>[101]</sup>. In addition, two locked antisense oligonucleotides (SPC5001 and SPC4061) targeting PCSK9 decreased the levels of plasma PCSK9 and LDL-C by 85% and 50%, respectively. A Phase I clinical trial on BMS-844421 was terminated because of safety concerns<sup>[67]</sup>. Both ends of ASO (SPC5001) DNA are locked with RNA nucleotides, which are composed of one monomer and are stable<sup>[102]</sup>. Even if ASO has high affinity and specificity, the high production cost and required routes for intravenous or subcutaneous administration limit its use in individuals with hyperlipidemia.

#### **siRNA**

The intravenous administration of single-chain siRNAs in lipid nanoparticles is a new therapeutic approach to inhibiting PCSK9 activity<sup>[103]</sup>. Studies in mice and rats have reported that siRNA-induced PCSK9 silencing decreased the PCSK9 mRNA levels by 50%–70% and the TC concentrations by 60%. Another study in non-human primates found that siRNA-mediated knockdown of PCSK9 was rapid, sustained, and reversible and that it resulted, on average, in a 56% reduction in the LDL-C levels. A Phase I clinical trial by Alnylam Pharmaceuticals (ALN-PCS) demonstrated that administration of their siRNA (ALN-PCSsc) resulted in a 70% reduction in plasma PCSK9 and a 40% reduction in LDL-C relative to baseline<sup>[104]</sup>. Another Phase I clinical trial of subcutaneously administered ALN-PCSsc has also been completed<sup>[59]</sup>. A Phase II trial of ALN-PCSsc is currently in progress<sup>[58]</sup>.

#### **Interfering with PCSK9 secretion**

Two specific mediators, sortilin<sup>[105]</sup> and Sec24a<sup>[106]</sup>, are known to be involved in PCSK9 secretion. Sortilin is important in lipoprotein metabolism as a transmembrane type I transport receptor, and it is not directly regulated by PCSK9. Conversely, sortilin, which co-localizes with PCSK9 in the trans-Golgi network, facilitates PCSK9 secretion from primary hepatocytes in the late secretory pathway. Sortilin is encoded by the gene SORT1 and is a high-affinity sorting receptor for PCSK9. Sortilin thus represents a good target for the treatment of hypercholesterolemia. Plasma PCSK9 is reduced in sortilin-deficient mice but is elevated following sortilin over-

expression in the liver. Moreover, a positive correlation exists between the levels of circulating PCSK9 and sortilin levels in healthy humans. One study found that the absence of Sec24a (also known as coat protein complex II adaptor protein) inhibited the early transport of PCSK9 from the ER to the *cis*-Golgi, leading to an increase in LDLR levels and a decrease in LDL-C levels. Moreover, SRT3025, a sirtuin 1 deacetylase activator, inhibited the development of atherosclerosis in ApoE<sup>-/-</sup> mice by decreasing PCSK9 secretion and increasing LDLR levels<sup>[107]</sup>.

The Q152H loss-of-function mutation in PCSK9 specifically blocked PCSK9 secretion by decreasing its intramolecular cleavage, leading to the accumulation of unprocessed proPCSK9 within the cell<sup>[108]</sup>.

#### **Challenges and future perspectives**

Despite the proven efficacy of PCSK9 mAbs, many challenges must be addressed in future clinical research. Special attention should be given to the side effects associated with PCSK9 mAbs. Cognitive decline may result from the deposition of soluble amyloid- $\beta$  (A $\beta$ ) on the walls of cerebral capillaries and arteries, a process that is potentiated by the formation of immune complexes such as those formed between mAbs and PCSK9<sup>[109]</sup>. Immune complexes can also elicit complement activation through the classic C1q-mediated antibody-dependent pathway, leading to abnormal glutamate release and neurotoxicity<sup>[110]</sup>.

#### **Side effects of PCSK9 mAbs**

##### **Neurocognitive adverse effects**

The incidence of neurocognitive adverse events in alirocumab-treated subjects was 0.8%, compared with 0.7% in the placebo-treated group. Alirocumab treatment resulted in memory impairment and confusion in 0.2% of patients, whereas the incidence of these side effects in the placebo groups did not reach 0.1%<sup>[63]</sup>. In other trials, treatment with evolocumab produced abnormally low LDL-C levels (<50 mg/dL) in <1% of patients; these trials reported side effects such as amnesia and mental impairment<sup>[111]</sup>.

##### **Other adverse events in clinical trials of PCSK9 mAbs**

In clinical trials of alirocumab, the incidences of other adverse events in the alirocumab group compared with the placebo group, respectively, were as follows: injection site reactions (7.2% vs 5.1%), nasopharyngitis (11.3% vs 11.1%), influenza (5.7% vs 4.6%), urinary tract infection (4.8% vs 4.6%), cough (2.5% vs 2.3%), myalgia (4.2% vs 3.4%), sinusitis (3.0% vs 2.7%), musculoskeletal pain (2.1% vs 1.6%), bronchitis (4.3% vs 3.8%), diarrhea (4.7% vs 4.4%), and upper respiratory tract infection (3.1% vs 2.4%)<sup>[63]</sup>. In clinical trials of evolocumab, the incidences of adverse events in the evolocumab group compared with the placebo group, respectively, were as follows: nasopharyngitis (10.5% vs 9.6%), upper respiratory tract infection (9.3% vs 6.3%), influenza (7.5% vs 6.3%), back pain (6.2% vs 5.6%), injection site reactions (5.7% vs 5.0%), cough (4.5% vs 3.6%), urinary tract infection (4.5% vs 3.6%), sinusitis (4.2% vs 3.0%), headache (4.0% vs 3.6%), myalgia (4.0% vs 3.0%), diz-

ziness (3.7% vs 2.6%), musculoskeletal pain (3.3% vs 3.0%), hypertension (3.2% vs 2.3%), diarrhea (3.0% vs 2.6%), and gastroenteritis (3.0% vs 2.0%). Overall, adverse events were reported more frequently in patients receiving alirocumab or evolocumab compared with placebo<sup>[112]</sup> (Table 2).

### Side effect profiles of nearly all antibodies

#### Hypersensitivity reactions

Allergic reactions, including urticaria, pruritus, and rash, as well as serious adverse events such as hypersensitivity vasculitis have been reported with alirocumab treatment. Allergic reactions were reported more frequently in patients treated with alirocumab compared with those treated with placebo (8.6% vs 7.8%, respectively). The percentage of patients who discontinued therapy because of allergic reactions was higher than that in the placebo group (0.6% vs 0.2%, respectively)<sup>[35]</sup>.

The incidence of allergic reactions was 5.1% in evolocumab-treated patients and 4.7% in placebo-treated patients. In general, hypersensitivity reactions (in the evolocumab vs placebo groups) involve rash (1.0% vs 0.5%), erythema (0.4% vs 0.2%), eczema (0.4% vs 0.2%), and urticaria (0.4% vs 0.1%)<sup>[57]</sup>.

#### Immunogenicity

Alirocumab and other therapeutic proteins carry the potential for immunogenicity. In 10 placebo-controlled studies, patients obtaining alirocumab who developed anti-drug antibodies (ADAs) were detected; and the alirocumab rate was 4.8%, whereas the control rate was 0.6%<sup>[35]</sup>. Subjects with ADAs had a higher incidence of injection site reactions (10.2%) compared with subjects lacking anti-drug antibodies (5.9%). Moreover, 1.2% of patients developed neutralizing antibodies (NABs),

all of which occurred in the alirocumab group. Most of these patients had only one positive neutralizing sample. Only 0.3% of patients had 2 or more NAB-positive samples. The long-term consequences of alirocumab immunogenicity require further investigation<sup>[35]</sup>. Additionally, 0.1% of subjects receiving evolocumab developed ADAs at least once over a series of trials. Neutralizing antibodies continue to be found in clinical trials, but their long-term effects remain unknown<sup>[111]</sup>.

MABs that are used to treat cardiovascular disease, such as abciximab and inclacumab, are associated with off-target effects, non-specific adverse events, on-target adverse events, and the production of ADAs<sup>[113-115]</sup>. In our previous research, we demonstrated that PCSK9 can influence neuronal apoptosis in two ways: it can maintain apoptosis at low levels and can limit increases in apoptosis. However, mAbs against PCSK9 are of limited use in the CNS to treat nervous system diseases because of their large molecular weight (>400–600 kDa)<sup>[116]</sup> and the neurotoxicity of the mAb-PCSK9 immune complex. The high cost of PCSK9 mAbs is also prohibitive. One elegant study found that adding a PCSK9 inhibitor to existing statin therapy in all eligible patients increased the annual prescription drug expenditure in the United States (US) by approximately \$125 billion over ezetimibe use alone (a 38% increase from \$329 billion in prescription drug expenditures) and increased US health care spending by approximately \$120 billion (a 4% increase from the \$2.8 trillion dollars in total US health care expenditures) in 2015<sup>[117]</sup>.

#### Future perspectives

Clinical trials have demonstrated that drugs targeting PCSK9 effectively reduce LDL-C levels. PCSK9 mAbs, including

**Table 2.** Side effect profiles of PCSK9 mAbs.

Side effect profiles	Symptoms	Description	
		Occurrence	
		Alirocumab vs placebo (%)	Evolocumab vs placebo (%)
Neurocognitive adverse effects	Amnesia mental impairment	0.8 vs 0.7	No
	Injection site reactions	7.2 vs 5.1	5.7 vs 5.0
	Nasopharyngitis	11.3 vs 11.1	10.5 vs 9.6
	Influenza	5.7 vs 4.6	7.5 vs 6.3
	Urinary tract infections	4.8 vs 4.6	4.5 vs 3.6
	Cough	2.5 vs 2.3	4.5 vs 3.6
	Myalgia	4.2 vs 3.4	4.0 vs 3.0
	Sinusitis	3.0 vs 2.7	4.2 vs 3.0
	Other adverse events in clinical trials of PCSK9 mAbs	Musculoskeletal pain	2.1 vs 1.6
Bronchitis		4.3 vs 3.8	
Diarrhea		4.7 vs 4.4	3.0 vs 2.6
Upper respiratory tract infections		3.1 vs 2.4	9.3 vs 6.3
Back pain			
Headache			6.2 vs 5.6
Dizziness			4.0 vs 3.6
Hypertension			3.7 vs 2.6
Gastroenteritis			3.2 vs 2.3
			3.0 vs 2.0

alirocumab and evolocumab, were approved for the market in 2015, and a Phase III trial of bococizumab was completed recently<sup>[68]</sup>. The development of other therapies, such as gene-silencing agents, small molecules (BBR and OA), mimetic peptides, adnectins, and inhibitors of PCSK9 secretion, are described in this review. A novel genome editing technology, CRISPR-Cas9, induced a high rate of PCSK9 mutagenesis of approximately 50%; this approach significantly decreased plasma PCSK9 protein levels, with a low incidence of off-target mutations<sup>[118]</sup>. The challenges associated with the development of drugs targeting PCSK9, including long-term safety concerns, effects on the CNS, and the cost-effectiveness of PCSK9 mAbs, are discussed at the end of this review. Despite the inhibitory effect of PCSK9 on neuronal apoptosis, the utility of PCSK9 mAbs in the CNS is limited because of their high molecular weight and the neurotoxicity of the PCSK9-mAb immune complex. Moreover, the cost of PCSK9 mAbs is prohibitive. In summary, PCSK9 inhibitors appear to have clinical value, but more work is needed to understand PCSK9 protein structure, to identify novel sites of drug action, to understand the mechanisms by which drugs target PCSK9, and to develop novel small molecule compounds that act as PCSK9 antagonists.

### Acknowledgements

This work was supported by grants from the National Natural Science Foundation of China (No 81200217, 81370376), the Natural Science Foundation of Hunan province (No 2015JJ4097), the Key Project of Science and Technology Department of Hunan Province (No 2015JC3081), the Key Project of the Educational Department of Hunan Province (No 15A137), the Visiting Scholar Foundation of Key Laboratory of Biorheological Science and Technology (Chongqing University), the Ministry of Education (No CQKLBST-2014-002, CQKLBST-2015-004), and the Construct Program of the Key Discipline in Hunan Province.

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