1. Introduction

Consecutive dysregulation of lipoprotein metabolism is the greatest contributor to the development and progression of atherosclerosis, which leads to coronary artery disease (CAD). Abnormal elevation of plasma low-density lipoprotein (LDL) and triglyceride (TG)-rich lipoproteins as well as the dysfunction of anti-atherogenic high-density lipoprotein (HDL) are both recognized as essential components of the pathogenesis of atherosclerosis and are classified as dyslipidemia. This review describes the arc of development of antisense oligonucleotides for the treatment of dyslipidemia. Chemically-armed antisense candidates can act on various kinds of transcripts, including mRNA and miRNA, via several different endogenous antisense mechanisms, and have exhibited potent systemic anti-dyslipidemic effects. Here, we present specific cutting-edge technologies that have recently been brought into antisense strategies, and describe how they have improved the potency of antisense drugs in regard to pharmacokinetics and pharmacodynamics. In addition, we discuss perspectives for the use of armed antisense oligonucleotides as new clinical options for dyslipidemia, in the light of outcomes of recent clinical trials and safety concerns indicated by several clinical and preclinical studies.

Key words: Antisense drug, Chemical modification, Lipid lowering drug, Molecular targeting, Dyslipidemia

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Without this genetic background\(^4\)\(^5\)\(^6\). For patients with familial hypercholesterolemia (FH), the necessity of earlier identification of their disease and lifelong intense LDL cholesterol management is greater than in hypercholesterolemic patients without this genetic background. FH is an autosomal dominant-type genetic disorder caused by specific gene mutations relevant to LDL metabolism. FH shows severe hyper-LDL cholesterolemia and premature CAD. Although the stronger class of statins has largely helped to attenuate severe blood LDL cholesterol, statins are not always effective and may not provide sufficient LDL reduction particularly for homozygous FH (HoFH) patients and severe heterozygous FH patients (HeFH). Therefore, alternative or additional drugs are required for these patients.

There is extensive evidence that elevated TG and low HDL cholesterol levels are both independent risk factors for CAD\(^7\). In addition, an extremely high blood TG level increases the risk of pancreatitis. Furthermore, current lipid-lowering drug interventions do not achieve sufficient efficacy in patients with severe hypertriglyceridemia accompanied by low HDL cholesterolemia having such diseases as familial combined hyperlipidemia (FCHL), familial chylomicronemia syndrome (FCS) and familial partial lipodystrophy (FPL).

Recently developed chemically-armed antisense oligonucleotides (AONs) are potent enough to provide a therapeutic option even for patients with severe inherited dyslipidemia. In fact, numerous molecular
targets responsible for severe dyslipidemia have been identified and some AONs targeting these molecules have shown great therapeutic potential against dyslipidemia in animal model studies. In addition, some ongoing clinical trials are evaluating AONs in patients with severe inherited dyslipidemia and interim reports on the lipid-controlling effects of AONs have just been published (Table 1). In this review, we provide general and extensive detailed information on recent advances in antisense drug development platforms as well as individual clinical candidates for the treatment of dyslipidemia.

2. Chemical Modifications for AONs

AONs are synthetic short single-stranded nucleic acid oligomers (typically 5-25 nucleotides-long) designed to form hybrids with target transcripts that have complementary sequences. The recognition of target RNAs by AONs is highly accurate and binding is tight due to their specific Watson-Crick-type base-pairing interaction. It was only recently that therapeutic AONs exhibited perceptible systemic activity without delivery vehicles and achieved excellent outcomes in clinical trials when furnished with chemically-armed nucleic acid building blocks. The key to success in improvement of the in vivo potency of AONs was the introduction of chemical modifications into the AON structure that makes AONs more stable in a biological context and give them higher binding affinity to target RNAs. There are three motifs comprising the AON architecture: phosphate backbone, ribose and nucleobase (Fig. 1)\(^8\), all of which are potentially chemically modifiable, and numerous chemical modifications have been introduced into the motifs over the past four decades.

The first innovation was phosphorothioate internucleotide modification technology, which drastically avoids unintended nuclease digestion of AONs under biological conditions and improves their pharmacokinetics\(^9\). Ionis Pharmaceuticals, a leading company developing antisense drugs, produced the first FDA-approved clinical antisense drug, Vitravene®, based on this technology in 1998. The second generation of AONs was also developed by Ionis Pharmaceuticals, achieved by introducing an affinity-enhancing modification into a nucleic acid building block called MOE (2’-O-methoxyethyl RNA)\(^10\). They demonstrated that the complementary characteristics of MOE on a ribose moiety and phosphorothioate backbone modification further strengthened the potency of AONs, enabling systemic application. The second generation technology eventually led to the development of Kynamro\(^\text{®}\), a FDA-approved anti-apolipoprotein B (ApoB) AON for homozygous FH, in 2013 (discussed below).

Our group first succeeded in developing a novel ribose modification, 2’,4’-bridged nucleic acid (2’,4’-
RNase H-mediated Mechanism

It is known that AONs can control gene expression by multiple intrinsic mechanisms, such as promoting the degradation of transcripts, modifying RNA processing, and perturbing RNA-protein interaction patterns. Hybridization-mediated destabilization of transcripts promoted by “RNase H” is one of the best-studied mechanisms of action of AONs. Kynamro® and most of the current clinical candidates for dyslipidemia come under this mechanistic class. RNase H is a ubiquitously expressed endoribonuclease that preferentially binds to the DNA-RNA hetero-duplex over RNA-RNA and DNA-DNA homo-duplexes. After an AON binds to the target RNA, RNase H selectively hydrolyzes the RNA strand of the AON-RNA duplex, and RNase H1 is more likely to be responsible for this mechanism than RNase H2. The AON is expected to be recycled after the target RNA is cleaved by RNase H1 for the next catalytic reaction. As RNase H1 is found in both the nucleus and cytoplasm, both organelles are potential sites of action of an AON that utilizes the RNase H1 mechanism. Putative molecular targets for an AON are therefore regarded as not only cytosolic mature mRNA, but also pre-mRNA and non-coding RNAs typically in the nucleus. We recently demonstrated that an AON designed to bind to part of an exon region undergoes cleavage of both mature mRNA and its pre-mRNA. In addition, these pre-mRNA and mature mRNA fragments, which are produced by RNase H1 in the nucleus, are rapidly processed by a nucleus exoribonuclease XRN2. A similar mechanistic study by others found that mature mRNA fragments formed by RNase H are further processed by cytoplasmic exoribonuclease XRN2.

Fig. 2. RNase H mediated functional mechanism of ASO and degradative pathway of cleavage products.
modified nucleic acids, such as MOE, 2',4'-BNA/LNA and their analogues, with fully phosphorothioate backbone modifications\textsuperscript{23, 32, 33}. This strategy is now widely appreciated, as seen in many of the clinical candidates that support the RNase H1 mechanism (Fig. 3). Later, we will discuss several candidates for dyslipidemia that are currently being tested in clinical trials.

3.2. MicroRNA-targeting Antisense Drugs

More than 5,000 human microRNAs (miRNAs) have so far been identified and most mRNAs have been shown to have miRNA target sites on their

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{gapmer_structure.png}
\caption{Gapmer structure and some sugar-modified nucleotide analogs with phosphorothioate backbone.}
\end{figure}
3’-untranslated (UTR) region, indicating that mRNA translation is under the strict spatiotemporal control of miRNAs. Therefore, dysregulation of the biogenesis and function of individual or families of miRNAs causes many types of human diseases, including cardiovascular and metabolic diseases.

miRNA is an endogenous, short (typically ~22 nucleotides-long) non-coding RNA that works as a guide for RNA silencing machinery to the 3’-UTR of the target mRNA. Most miRNAs are generated from much longer hairpin transcripts by the function of RNase III-like protein machineries, Drosha and Dicer. Argonaute family proteins (AGO) are then responsible for the further maturation of miRNAs in cytoplasm and behave as a core scaffold for miRNA-induced RNA silencing complex (miRISC). Each miRNA has a “seed” sequence (~7 RNA stretch) on its own 5′ flank to recognize a set of target mRNAs possessing seed-match regions and form full-match Watson-Crick base-pairs, triggering miRNA-induced RNA silencing. The major miRNA-induced RNA silencing mechanisms include the removal of the 3’-polyA tail and 5’-cap structure of mRNAs, followed by translation repression and mRNA decay. Despite the ability of miRNAs to elicit direct endonucleolytic cleavage of the target mRNAs, animal systems rarely utilize this mechanism.

There are several strategies to suppress or supply miRNA activity. In this context, utilizing synthetic oligonucleotides that block target mRNA binding to parent mRNAs or that guide AGO machineries to mRNAs through a reliable Watson-Crick interaction can be powerful strategies to perturb miRNA function even in vivo. The former strategy, termed “antimiR”, is one that represses miRNA activity, while the latter, termed “miRNA mimic”, is used in miRNA replacement therapy. In general, chemical modification is more favorable to antimiR AON than miR mimic because a chemically over-armed miR mimic is more likely to fail to be an inherent substrate for AGO and related factors comprising miRISC while the antagonizing of the presented seed region of miRNA by antimiR is a process relatively free from precise recognition by enzymes.

### 3.3. Pharmacokinetics of Chemically Modified AONs

The recently demonstrated strong systemic antagonism of AONs without any encapsulation is primarily due to their preferable pharmacokinetics achieved by chemical modification. Phosphorothioate chemistry has made the largest contribution to the improvement of the pharmacokinetics of oligonucleotides that were previously rapidly degraded and showed almost no pharmacological effects in biological systems. Phosphorothioate modification provides AONs with high-protein binding ability and a nuclease resistant property, which helps them to be distributed to the target organs, tissues, and cells as intact as possible. Once AONs reach their target sites, high affinity modification plays a critical role in potency. Significant reduction in systemic activity generally happens if even one of two modifications is lacking.

The preferred route of administration of chemically-armed AONs for systemic application is parenteral injection, including intravenous and subcutaneous injection. After injection, AONs are rapidly transferred to the systemic circulation (~minutes) and are mostly eliminated from blood to peripheral tissues in a few hours. AONs typically show broad biodistribution and the organs with the highest concentrations are likely to be the liver and kidney. In these organs, AONs have long half-lives and prolonged knockdown activity (2–4 weeks). These aspects have driven researchers to develop AONs primarily for the treatment of liver-related disorders. Interestingly, however, Hung et al. recently showed that chemically-armed AONs can target mRNAs that are expressed not only in the liver and kidney but in, literally, any organs, tissues or cells except for part of the brain, which would prompt broader therapeutic application of AONs. However, the molecular background of the cellular internalization process of AONs largely remains to be elucidated.

### 4. Development of Clinical AONs for Dyslipidemia

Regarding AONs for dyslipidemia, three are currently under clinical testing in humans and one, Kynamro®, has been approved by the US Food and Drug Administration (FDA). The molecular targets of these AONs are apolipoprotein C-III (ApoCIII) mRNA, lipoprotein (a) or Lp(a) mRNA and angiopoietin like-3 protein (ANGPTL3) mRNA, all of which are expressed mainly in the liver and for which selective inhibitors using other strategies have not been previously developed. All of these candidate AONs were originally discovered and developed by Ionis Pharmaceuticals and basically have MOE modification in combination with phosphorothioate backbone modification. The AONs, IONIS-APO(a)-LRX and IONIS-ANGPTL3-LRx, both contain liver-targeting (Ligand-conjugated Antisense Technology, LICA) technology to achieve much lower and less frequent dosing of the AON.

In this section, we will consider recent progress that has been made in clinical trials on these AONs. We will also mention some interesting pre-clinical and
experimental phase trial reports, including one by us.

4.1. Apolipoprotein B-100; Kynamro® (Mipomersen)

Gene defects in Apolipoprotein B-100 (ApoB-100) were found in patients presenting FH-like symptoms, but having normal LDL receptor (LDLR) activity. Loss-of-function mutations in ApoB-100 may cause reduction in affinity between LDL and LDLR protein, resulting in a lower elimination rate of LDL from the blood and an elevation of plasma LDL-cholesterol51. On the other hand, mutations that reduce the production of ApoB-100 are responsible for reduced plasma ApoB-100 levels and LDL-cholesterol concentrations52. These observations have fueled the development of ApoB-targeting AONs53-55).

Kynamro® injection contains an AON inhibitor of ApoB-100 mRNA and is the first FDA-approved systemic AON for homozygous FH (HoFH). Having the generic name mipomersen, it has shown excellent systemic AON for homozygous FH (HoFH). Having the generic name mipomersen, it has shown excellent LDL-cholesterol reduction potential in HoFH patients in a number of clinical trials. Raal et al. have reported the results of a phase 3 study undertaken in seven different countries, in which 51 HoFH patients who were already taking the maximum dose of lipid-lowering drugs were enrolled and randomly assigned to subcutaneous injection of mipomersen at a dose of 200 mg/week or placebo for 26 weeks. This study demonstrated the significant LDL-cholesterol lowering effect of mipomersen (−24.7%) over placebo (−3.3%), though the rate of adverse events observed, which included injection-site reaction, flu-like symptoms, increase in transaminases and steatosis, could not be ignored56).

Santos et al. recently reported the interim results of an on-going long-term efficacy and safety study on mipomersen57. It enrolled FH patients who had been receiving lipid-lowering drugs and changes in efficacy and safety parameters during treatment with 200 mg/week of mipomersen had been continuously monitored for 104 weeks. The mean changes in LDL-cholesterol concentration from baseline were consistently large, between −27 and −28%, from week 26 to 104. Although an increase in liver transaminases and hepatic steatosis associated with the administration of mipomersen were also observed in this study, as in the case of other phase 3 trials, these adverse effects did not progress or increase in frequency over an extended period of time. These findings are important not only to the broader application of mipomersen, but also provide a useful guide for the development of next generation AON drugs. However, it should be noted that 55% of the enrolled patients dropped out in the middle of the trial due to the severe adverse events such as injection-site reaction, influenza-like symptoms and liver problems. We should also note that it is unknown whether or not mipomersen reduces the risk of CAD.

4.2. Apolipoprotein (a)

Apolipoprotein (a) (Apo(a)), which is bound to ApoB-100 via a disulfide bond, leads to the formation of Lipoprotein (a) (Lp(a)), a cholesterol-rich LDL-like particle. Elevated Lp(a) has been recognized to be one of the risk factors of CAD and stroke58, 59). The physiological action of Lp(a) further supports its atherogenic effect. Lp(a) potentially exerts atherogenic effects on vascular surfaces because its composition is similar to that of LDL. Lp(a) is also known to carry oxidized phospholipids, which are pro-inflammatory agents60).

In addition, due to the structural similarity between Apo(a) and plasminogen, Apo(a) can act as an intrinsic antagonist and inhibit activation of plasminogen and fibrinolysis61). Lowering Lp(a) levels by apheresis was shown to be effective in preventing cardiac events62).

Ionis Pharmaceuticals has developed LICA-unconjugated IONIS-APO(a)-Ls, and LICA-conjugated IONIS-APO(a)-Ls, both of which possess a MOE-based chemical modification as well as a phosphorothioate modification and target Apo(a) mRNA. Phase 2 and Phase 1/2a trials on IONIS-APO(a)Ls and LICA-conjugated IONIS-APO(a)-Ls, respectively, are currently on-going in subjects with elevated Lp(a). Although safety and efficacy information have not yet officially been published, the company recently reported outstanding interim results for both trials as well as a pre-clinical study on a LICA-unconjugated AON in transgenic mouse models63). (http://www. ionispharma.com/pipeline/). It is noteworthy that Ionis Pharmaceuticals achieved a mean reduction of 92% in Lp(a) in a Phase1/2a study evaluating LICA-conjugated IONIS-APO(a)-Ls. Overall results indicated that the potency of IONIS-APO(a)-Ls was 30 times stronger than LICA-unconjugated IONIS-APO(a)Ls. They also reported that none of the 159 subjects receiving the injection showed injection-site reactions or flu-like symptoms.

4.3. PCSK9

Proprotein Convertase Subtilisin/Kexin 9 (PCSK9) was identified as the third gene of FH in 200364, 65). A number of genetic and intervention studies have found a positive correlation between plasma PCSK9 protein levels and LDL-cholesterol concentrations66). PCSK9 is expressed as a zymogen mainly in the liver, intestine and kidney and secreted as a 63-kDa processed mature form of PCSK9. The
secreted PCSK9 is thought to be directly involved in LDLR maintenance where circulating PCSK9 binds to LDLR using an extracellular epidermal growth factor-like repeat A (EGFA) domain of LDLR and stimulates internalization of LDLR within lysosomes to diminish elimination of plasma lipoproteins in the liver. Since PCSK9 is secreted in the blood, priority has been given to the development of monoclonal antibody-based antagonists, some of which have so far shown great cholesterol-lowering effects in human subjects\textsuperscript{65, 68}. On the other hand, Graham \textit{et al.} demonstrated a positive effect of a MOE AON-targeting PCSK9 mRNA in high fat-fed mice\textsuperscript{69}. However, possibly due to the insufficient binding affinity of the MOE modification targeting PCSK9 mRNA, quite a high dose (100 mg/kg/week) was required to achieve an adequate reduction in PCSK9.

Teams from Santaris Pharma (currently Roche group) have improved the potency of anti-PCSK9 AONs by utilizing a 2’,4’-BNA/LNA modification, a higher-affinity modification\textsuperscript{70, 71}. A 2’,4’-BNA/LNA-modified AON achieved an 85% reduction in liver PCSK9 mRNA and serum PCSK9 protein and a 50% reduction in serum LDL-cholesterol concentration in monkeys in their 4-week study (20 mg/kg on day 0 and subsequently 5 mg/kg/week as maintenance dose). These results were the impetus for the clinical development of the 2’,4’-BNA/LNA-based AON and a Phase 1 study in healthy volunteers was commenced in May 2011, though it was terminated in October 2011 due to an insufficient therapeutic window for chronic use\textsuperscript{72}.

Our group has been developing an anti-PCSK9 AON possessing two different high-affinity modifications, 2’,4’-BNA/LNA and 2’,4’-BNA\textsuperscript{NC}, with phosphorothioate chemistry\textsuperscript{73}. We demonstrated that 20 mg/kg/week of 2’,4’-BNA/LNA-AON for 6 weeks achieved a greater than 30% reduction in serum LDL-cholesterol and a slight increase in liver transaminases. However, a 2’,4’-BNA\textsuperscript{NC}-based AON did not have this effect on liver transaminases but did show an earlier LDL-cholesterol lowering action. Based on these results, we are moving forward on a pre-clinical study for evaluating candidates.

4.4. Apolipoprotein C\textit{III} (ApoC\textit{III})

Hypertriglyceridemia is recognized as a major independent risk factor for CVD\textsuperscript{74, 75}, and severe hypertriglyceridemia is associated with fatal pancreatitis\textsuperscript{76, 77}. A number of clinical research and pre-clinical studies with genetically-engineered animal models have shown that elevated ApoC\textit{III} is associated with high plasma TG levels\textsuperscript{78-83}. ApoC\textit{III} is a glycoprotein synthesized mainly in liver and secreted in blood as a component of TG-rich lipoproteins, such as chylomicron and VLDL, and their remnants, as well as HDL particles\textsuperscript{82, 83}. ApoC\textit{III} primarily attenuates lipolysis of TG-rich lipoproteins by inhibiting lipoprotein lipase (LPL) activity on capillaries\textsuperscript{84}. It is also known to delay clearance of TG-rich lipoproteins and their remnants by undermining interaction of apolipoprotein B or E on lipoproteins with LDL receptors (LDLR)\textsuperscript{85, 86}. ApoC\textit{III} may also play a role in the activity of hepatic lipase\textsuperscript{87} and assembly and secretion of TG-rich lipoproteins\textsuperscript{88}. Therefore, ApoC\textit{III} plays a key role directly and indirectly in the pathogenesis of atherosclerosis and could be a potential therapeutic target for hypertriglyceridemia\textsuperscript{89-91}.

Ionis Pharmaceuticals is a leading company in the development of anti-ApoC\textit{III} AONs. While the number of published studies showing clinical outcomes has been limited, an early phase study in healthy volunteers demonstrated a potent dose-dependent reduction in plasma ApoC\textit{III} protein levels and TG concentrations\textsuperscript{92}. Gudet \textit{et al.} reported that the Ionis investigational drug volanesorsen showed efficacy in three patients with Familial Chylomicronemia Syndrome (FCS). Before initiating dosing, patients had TG concentrations ranging from 1406 to 2083 mg/dL and 13 weeks of dosing achieved a 56-86% reduction in TG reduction as well as a 71-90% reduction in ApoC\textit{III} protein in blood, resulting in plasma TG concentrations of less than 500 mg/dL in all patients\textsuperscript{93}. In a Phase II trial conducted by Ionis Pharmaceuticals in patients with type 2 diabetes, in addition to a 69% reduction in TG, a 1.22% reduction in Hba1c was achieved (http://isispharm.com/). These results led to two Phase III trials. In 2014, the Phase III APPROACH trial for evaluation of volanesorsen was started in patients with PCS. This trial is a randomized double-blind, placebo-controlled, 12 month study in approximately 50 PCS patients with TG levels of 750 mg/dL or above. Volanesorsen was given weekly at a dose of 300 mg/week and the primary endpoint is the percent reduction in fasting TG levels after three months of dosing. In 2015, the BROADEN trial started enrolling patients with familial partial lipodystrophy. Additional clinical efficacy data and safety information for volanesorsen will hopefully be reported soon.

We have demonstrated a strong antagonistic effect for an anti-ApoC\textit{III} AON having 2’,4’-BNA/LNA or a 2’,4’-BNA\textit{AM} chemistry series\textsuperscript{94, 95}. A 2’,4’-BNA/LNA-modified AON reduced hepatic ApoC\textit{III} mRNA by 80% after multiple doses over 16 days. Serum total TG reduction of 87% was recorded on day 16 and lipoprotein profiling revealed that this reduction was derived mainly from reduction in the
4.5. ANGPTL3

Numerous genetic analyses have shown that genetic defects in or at close proximity to angiotensin-like 3protein (ANGPTL3)-encoding loci are associated with high plasma lipid concentrations and subjects with elevated plasma ANGPTL3 are likely to exhibit plasma TG elevation accompanied by high LDL cholesterol concentrations96-99). It has been suggested that a primary molecular mechanism by which ANGPTL3 influences elevation of plasma TG is inhibition of lipoprotein lipase activity (LPL)100). Biochemical studies indicate that ANGPTL3 inhibits LPL activity not only by antagonizing the lipolytic activity of LPL, but also by stimulating removal of LPL from the cell surface, typically mediated by FURIN and PCSK6101).

Ionis Pharmaceuticals originally developed the LICA-unconjugated AON IONIS-ANGPTL3Rx and reported a Phase 1 study on it in 2015. In this study, IONIS-ANGPTL3Rx achieved significant reductions in ANGPTL3, TG and LDL cholesterol with mean reductions of 84%, 49% and 28%, respectively. These results prompted them to develop a LICA-conjugated version of a MOE-based AON with phosphorothioate chemistry, IONIS-ANGPTL3-LRx, and in December 2015, Phase 1/2 studies evaluating IONIS-ANGPTL3-LRx in subjects with elevated TGs and hypercholesterolemia started. The publication of their results should further support the potential utility of this drug.

4.6. miR-33a/b

To the best of our knowledge, miRNA-targeting AONs for the treatment of dyslipidemia are still under pre-clinical development; however, some studies have indicated the great potential of anti-miR in modification of dyslipidemic states102-109). In this regard, recent results for inhibition of miR-33a/b using anti-miRs having different types of modifications (2'-F RNA/MOE109) or LNA (67) with phosphorothioate backbone modification) have consistently indicated a positive effect on plasma HDL-cholesterol levels. miRNA-33a/b are both intronic miRNAs encoded in the same genetic loci with sterol response element binding proteins 2/1 (SREBP 2/1), respectively, and co-transcribed with them. There are miR-33a/b target genes relevant to cholesterol efflux, including ATP-binding cassette transporters (ABCA1 and ABCG1), and also those involved in fatty acid homeostasis and insulin signaling110, 111).

Najafi et al. administered unencapsulated LNA-modified anti-miR-33a to western diet-fed mice through the tail vein at a dosage of 20 mg/kg/injection for 3 consecutive days. Mice were sacrificed 48 hours after the last injection and serum was analyzed. Moderate but significant increases in plasma HDL cholesterol and hepatic ABCA1 mRNA were observed with no indication of drug-induced toxicity107). Aiming to see if these observations of the therapeutic potential of an anti-miR-33 strategy in mice could be extrapolated to humans, Rayner et al., demonstrated further proof of concept of the anti-miR-33 therapy in African green monkeys with a 2'-F RNA/MOE-modified anti-miR-33a/b AON106, 109). Animals were subjected to multiple subcutaneous injections of anti-miR-33a/b at a dose of 5 mg/kg/injection over twelve weeks. Consistent with the murine study, an increase in hepatic ABCA1 expression and a sustained increase in plasma HDL-cholesterol concentration were observed in the monkeys. Moreover, the authors found specifically, that in this non-human primate model, miR-33a/b inhibition significantly reduced plasma very-low-density-lipoprotein (VLDL) levels as a result of an increase in the expression of genes related to fatty acid oxidation (CROT, CPT1A, HADHB and PRAKK1) and a reduction in fatty acid synthesis genes (SREBF1, FASN, ACLY and ACACA).

Regarding the effectiveness of anti-miR-33 therapy against atherosclerosis progression, Marquart et al. showed that LNA-based anti-miR-33 had no effect in 1.25% of cholesterol containing western diet-fed Ldlr−/− mice, while Rotllan et al. showed that 2'-F RNA/MOE-modified anti-miR-33 had a positive effect on atherosclerosis progression in less cholesterol-loaded western diet-fed Ldlr−/− mice112, 113). Although these results seem to be incompatible, the results of a number of previous statin studies indicate that experimental settings, for example the type of animal model or pharmaceutical modifiers used, greatly affect the study outcome114, 115) and therefore, experiments need to be carefully planned and conducted to obtain efficacy data for antimiR-33 therapy that can be extrapolated to humans.

5. Understanding Mechanisms of Cellular Uptake and Intracellular Disposition of AONs for Further Improvement of Potency

As mentioned earlier, there is only a small amount of knowledge as to why naked AONs can be taken up by cells in vivo10). Although there have been
studies on the molecules of putative endocytotic pathways that AONs would take, a major shortcoming that has delayed the elucidation of a mechanism for their uptake is, that to a large extent, the activity of AONs in cultured cells does not reflect their potency in vivo. In this context, Stein et al. recently developed a method called “gymnosis”, in which high concentrations of AONs (typically >10 µM) are slowly taken up by cultured cells without the use of transfection agents. This method has been shown to maintain consistency between the in vitro and in vivo activity of AONs, indicating a common physiological mechanism between in vitro and in vivo systems.

Our group independently developed a novel in vitro system called CEM in which AONs are rapidly taken up by various cell lines simply by adding a 9 mM CaCl2-containing culture medium. With it, an excellent positive correlation has been demonstrated between AON activity in a cell culture and mice. CEM has enabled us to conduct more accurate cell-based high-throughput screening of clinical candidates and facilitated the further elucidation of the cellular uptake mechanisms of chemically-modified AONs.

An active targeting strategy involving attachment of small molecular ligands to chemically-armed AONs has been gaining attention. A highly important strategy in dyslipidemia therapy is to use trivalent N-acetylgalactosamine (GalNAc)-tethered AONs. GalNAc is a carbohydrate ligand for asialoglycoprotein receptors (ASGPR), which are abundant on the surface of hepatocytes. Mouse studies have revealed that AONs to which these ligands are attached are 5- to 10-fold more potent than unconjugated congeners. In our own research, we recently developed a simplified version of the GalNAc structure and demonstrated that its very high in vivo activity was maintained. Greater flexibility in synthesis led us to observe that conjugation of pentameric GalNAc provided better in vivo potency than the conventional trivalent GalNAc. As mentioned above, Ionis Pharmaceuticals is a leading company in the clinical application of ligand-conjugated AONs and has started achieving outstanding clinical outcomes.

6. Safety Concerns

A key lesson that has been learned from the results of a number of past clinical trials and pre-clinical experiments evaluating chemically-modified AONs is that toxicity rates in human subjects are relatively high. Hepatic and renal toxicity are the most common adverse events observed in animal and human trials. However, such toxicity never appeared in cultured cell systems and therefore, the mechanism of onset is still unclear. Extensive efforts have been recently devoted to predicting and understanding these accompanying toxicities, with some recent studies suggesting that hepatotoxicity can be ascribed to hybridization-dependent off-target toxicity, while others have suggested it can be attributed to hybridization independent toxicity. Thus, the observations so far seem to be controversial, and multiple pathways may be involved in AON toxicity. However, some studies have found that slight structural modification of AONs can potentially reduce their hepatotoxicity. Considering these findings together, to overcome the potential safety issues accompanying AON drugs, we should focus more on having better in vitro assay systems that predict in vivo toxicity as well as a better understanding of toxicity mechanisms, in addition to developing better alternative chemical modifications for AONs.

7. Conclusion

The advantage of using an antisense strategy as a novel therapeutic modality for the treatment of dyslipidemia is that it is supported by a number of technologies enabling in vivo application of AONs as well as rapid and systematic identification of etiological molecules, which include next-generation high-throughput DNA sequencing technology. Theoretically, because primary sequences of transcripts of etiological or disease-related molecules are the only information required for the generation of AON-based antagonists, once superior platform technologies for AON modification are in place, they could produce a number of clinical AON inhibitors. In fact, many researchers who first identified new etiological molecules have already used antisense inhibitors for their first knock-down experiments in vitro and in vivo. In the near future, AONs may provide a good therapeutic option for dyslipidemia patients.

8. COI

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