New drugs

Antisense oligonucleotides for the treatment of dyslipidaemia

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Antisense oligonucleotides (ASOs) are short synthetic analogues of natural nucleic acids designed to specifically bind to a target messenger RNA (mRNA) by Watson–Crick hybridization, inducing selective degradation of the mRNA or prohibiting translation of the selected mRNA into protein. Antisense technology has the ability to inhibit unique targets with high specificity and can be used to inhibit synthesis of a wide range of proteins that could influence lipoprotein levels and other targets. A number of different classes of antisense agents are under development. To date, mipomersen, a 2′-O-methoxyethyl phosphorothioate 20-mer ASO, is the most advanced ASO in clinical development. It is a second-generation ASO developed to inhibit the synthesis of apolipoprotein B (apoB)-100 in the liver. In Phase 3 clinical trials, mipomersen has been shown to significantly reduce plasma low-density lipoprotein cholesterol (LDL-c) as well as other atherogenic apoB-containing lipoproteins such as lipoprotein (a) [Lp(a)] and small-dense LDL particles. Although concerns have been raised because of an increase in intrahepatic triglyceride content, preliminary data from long-term studies suggest that with continued treatment, liver fat levels tend to stabilize or decline. Further studies are needed to evaluate potential clinical relevance of these changes. Proprotein convertase subtilisin/kexin-9 (PCSK9) is another promising novel target for lowering LDL-c by ASOs. Both second-generation ASOs and ASOs using locked nucleic acid technology have been developed to inhibit PCSK9 and are under clinical development. Other targets currently being addressed include apoC-III and apo(a) or Lp(a). By directly inhibiting the synthesis of specific proteins, ASO technology offers a promising new approach to influence the metabolism of lipids and to control lipoprotein levels. Its application to a wide variety of potential targets can be expected if these agents prove to be clinically safe and effective.

Keywords
Antisense oligonucleotides • Hypercholesterolaemia • ApoB • PCSK9 • Lp(a) • Mipomersen • Cardiovascular disease

Introduction

In both primary and secondary prevention trials, a reduction in low-density lipoprotein cholesterol (LDL-c) by statins has consistently reduced morbidity and mortality from cardiovascular disease (CVD). Consequently, statin therapy has become a crucial part in the management of patients at increased risk for CVD. Over the years, guidelines have suggested to increasingly lower LDL-c target levels to <100 mg/dL for most patients and <70 mg/dL for patients at very high risk for CVD.1 Unfortunately, neither statin monotherapy nor combination therapy of statins with additional lipid-lowering compounds has been successful in achieving these target levels in a significant portion of patients; especially not in the subset of patients with genetically determined very high baseline LDL-c levels, e.g. patients with familial hypercholesterolaemia (FH), and/or high-risk patients intolerant to statins. Thus, there is a clear medical need for additional and/or alternative therapeutic strategies to effectively lower LDL-c in subjects already on statins or in lieu of statins in those unable to take them. Statins lower LDL-c levels by increasing expression of hepatic LDL receptors (LDL-R), thereby leading to increased clearance of LDL-c from plasma. This review will focus on the promise of a novel approach to lowering LDL-c levels achieved by the use of antisense oligonucleotide (ASO) inhibition of apolipoprotein B (apoB) as well as proprotein convertase subtilisin/kexin-9 (PCSK9) synthesis, which is a mode of action distinct from all available agents.
Antisense technology

Antisense oligonucleotides are short, single-stranded, synthetic analogues of natural nucleic acids designed to specifically bind to a target messenger RNA (mRNA) in a sequence-specific manner through the Watson–Crick base-pair interactions (Figure 1). Depending on design and chemistry, sequence-specific hybridization of an ASO to a target mRNA may result in selective degradation by endogenous nucleases, or alternatively, inhibition of the processing and/or function of the mRNA by an occupancy-only mechanism. In both situations, inhibition of the synthesis of the target protein is achieved. Owing to the sequence-specific hybridization possible via this therapeutic option, highly specific target suppression can be achieved with proper ASO selection and validation.

Apolipoprotein B-100: a suitable therapeutic target for antisense

Apolipoprotein B has a central role in packaging and distribution of both dietary and endogenously produced cholesterol and triglycerides by lipoproteins (Figure 2). In humans characterized by profound elevations of apoB, such as FH, the prevalence of premature CVD is increased substantially. On the other side of the spectrum, in patients with familial hypobetalipoproteinaemia (FHBL), characterized by genetically determined very low apoB levels, the CVD rate is very low. In many of these subjects, the very low LDL-c levels are caused by truncation mutations in apoB, which leads to impaired hepatic secretion of full-length apoB, impaired hepatic very low-density lipoprotein (VLDL) secretion, and profound reductions in plasma apoB. Such subjects, with life-long, profound reductions in plasma apoB, show no signs of unexpected toxicity besides mild steatotic changes in the liver. The clinical relevance of the hepatosteatosis is considered low, since the incidence rate of liver complications in FHBL patients is only sporadic.

Apolipoprotein B-100 is an essential structural component of triglyceride-rich VLDL, which exports endogenous lipids from the liver. Since, apoB-100 remains tightly bound to its lipoprotein particle following secretion by the liver, and removal of its triglyceride content, a single apoB-100 molecule is present on all remnant lipoprotein metabolites of VLDL, including the atherogenic lipoproteins intermediate-density lipoprotein, LDL, and lipoprotein (a) [Lp(a)]. Since the liver is one of several tissue compartments that naturally accumulates second-generation ASOs demonstrating a robust exposure–response relationship for multiple gene targets, hepatic apoB-100 is an ideal target organ for the ASO technology. Mipomersen is the first agent available for human use to directly target apoB-100.

Design and chemistry of mipomersen second-generation antisense oligonucleotides

Mipomersen (ISIS 301012) is a second-generation antisense that complements a 20-base sequence within the coding region of the human apoB mRNA. It has been designed with two distinct functional domains. A central domain of 10 contiguous 2′-deoxy-d-ribose (2′-H) residues serves to support RNase H-mediated degradation of the target mRNA (Figure 3). And two flanking domains, referred to as an MOE ‘gap-mers’, serve to
protect the ASO from nuclease cleavage and to increase durability and potency. The safety and efficacy of this antisense drug has now been evaluated in both animal models, as well as clinical trials.

Extensive preclinical studies with mipomersen have shown it to reduce plasma apoB levels and LDL-c in a variety of species in a dose-dependent, and exposure-dependent, manner. The pharmacokinetics (PK) of this class of ASOs are remarkably similar across species and sequences. It is highly bound to plasma proteins and broadly distributed to many tissues, but with the highest concentrations in the liver and kidney. It is poorly distributed to the skeletal muscle and does not cross the blood–brain barrier. Its resistance to exonuclease degradation results in long terminal plasma half-lives, of \( \approx 30 \) days in monkeys and \( \approx 23–46 \) days in humans. Absorption is nearly complete following subcutaneous injection (the current mode of therapy in humans) with bioavailability ranging from 80 to 90% in monkeys. The majority of ASO metabolites are 8–14 nucleotides in length, which thereafter are released by the tissues and eliminated by renal filtration.

Extensive preclinical studies in a variety of murine models have demonstrated that a murine apoB-specific ASO produces profound reductions in circulating apoB levels and all associated apoB-containing lipoproteins. In recent studies in hypercholesterolaemic LDL-R-deficient mice, dose-dependent reductions in hepatic apoB mRNA and plasma LDL-c of 60–90% resulted in parallel reductions in atherosclerosis of 50–90% as well. In fact, the highest dose treatment resulted in less atherosclerosis than observed in saline-treated, chow-fed LDL-R mice. Because no changes were seen in intestinal cholesterol absorption, these data establish a direct link between inhibition of hepatic apoB synthesis and secretion and corresponding decreases in plasma LDL-c levels and consequently atherosclerosis. These profound reductions in hepatic apoB synthesis and plasma LDL-c levels were achieved without alterations in plasma aspartate aminotransferase or alanine aminotransferase (ALT) and without changes in body weight or lean body mass. Importantly, relative to saline-treated mice, hepatic triglyceride levels were not changed in these mice treated with the ASO to apoB. However, interpretation of these data must be qualified by the observation that many ASOs appear to reduce liver triglycerides in mice. More recent data show that apoB synthesis inhibition in fat fed animal models results in a slight increase in liver fat after 6 weeks of treatment which declines with continued administration of the drug. In fact, after 20 weeks of treatment, the total liver fat was decreased by 65% compared with controls. Data in both mice and monkeys suggest that these effects are secondary to compensatory transcriptional changes that result in decreased lipogenesis. In addition, increased expression of genes involved in hepatic fatty acid oxidation secondary to the changes in lipogenesis may have also occurred.

These data contrast with preclinical studies (and studies in humans as well) in which apoB secretion was inhibited by interfering with the microsomal transport protein (MTP), which led to
distinct hepatic steatosis. These preclinical data predicted that an ASO to apoB in humans would not be associated with hepatosteatosis. Though, as discussed below, mild-to-moderate steatosis has been observed in early clinical studies, particularly in those with the most profound reductions in plasma apoB, whereas preliminary data from long-term administration suggest that with continued treatment, liver fat levels tend to stabilize or decline.

**Clinical experience with mipomersen, the most widely studied antisense oligonucleotides to date**

**Phase 1**

The first-in-man study was a randomized, double-blind, placebo-controlled dose-escalation study in 36 healthy subjects with mild hypercholesterolaemia. Subjects were randomized at a ratio of 4:1 (active drug to placebo) and treated for 4 weeks at doses ranging from 50 to 400 mg either intravenously or subcutaneously. A dose-dependent and prolonged reduction in apoB and LDL-c was demonstrated in this study, with a maximum mean per cent change from the baseline of 50 and 35%, respectively, in the 200 mg dose group (n = 8) (Figure 4). In a second Phase 1 study, no interaction between mipomersen and simvastatin or ezetimibe was observed, which is primarily attributed to the distinct metabolism of mipomersen by nucleases instead of the cytochrome P450 enzymes. Indeed, mipomersen does not interact with the cytochrome P450 enzymes.

**Phase 2**

Three randomized, double-blind, placebo-controlled Phase 2 studies were designed to evaluate mipomersen in subjects with mild-to-moderate hypercholesterolaemia on lifestyle advice, or on top of statin therapy, and as an add-on therapy in subjects...
with heterozygous (He) FH. In addition, an open-label dose-escalation study was conducted in subjects with homozygous (Ho) FH on maximally tolerated lipid-lowering therapy. In these studies, mipomersen resulted in statistically significant reductions in LDL-c levels as a single agent and as an add-on to statins and other lipid-lowering agents in all hypercholesterolaemic phenotypes tested (Table 1). Reductions in other CVD risk factors, e.g. triglycerides and Lp(a), were also demonstrated. The PK–pharmacodynamic relationship was predictable across all subjects, both in the presence or absence of other lipid-lowering agents. The most common adverse events were injection site reactions (ISRs) and flu-like symptoms. Elevations in liver transaminase levels of >3 × the upper limit of normal, on two or more consecutive measures, occurred in a subset of subjects treated with mipomersen especially in the higher dose range. Hepatic steatosis was detected occasionally during the follow-up of patients with liver enzyme increases.

### Phase 3

Mipomersen has thus far been evaluated in four randomized, double-blind, placebo-controlled Phase 3 trials. Three of the four trials focused on patients with FH or severe hypercholesterolaemia and the fourth investigated the effects of mipomersen in patients at high risk for CVD. In each of the four trials, patients were randomized in a 2:1 ratio to receive 200 mg mipomersen or placebo by s.c. injection once per week for 26 weeks. The primary efficacy endpoint in each trial was percentage reduction in LDL-c from baseline to Week 28. Data are expected to be published shortly. Mipomersen administration at 200 mg for 26 weeks as an add-on to statins and other lipid-lowering drugs resulted in profound reductions in LDL-c in patients with HeFH as well as HoFH, severe hypercholesterolaemia, and in patients at high risk for CVD (Table 2). Significant reductions were also achieved for other atherogenic lipoprotein particles including triglycerides, Lp(a), and small dense LDL-c (smLDL). In these longer term studies, mipomersen had a satisfactory safety profile. Similar to Phase 1 and 2 clinical trials, the most common adverse events were ISRs, flu-like symptoms, and increases in ALT. In hypercholesterolaemic patients at increased CVD risk, ALT increases were associated with a mild increase in intrahepatic triglyceride (IHTG) content as measured by magnetic resonance imaging. In patients with HeFH and increased CVD risk, IHTG content increased from baseline with a median increase of 4.9% in the mipomersen subjects, compared with 0.4% for the placebo subjects. No adverse effects or clinically significant changes in other parameters such as blood pressure or plasma glucose have been noted.

### Adverse reactions and safety of mipomersen

Injection site reactions are typically characterized by mild painless erythema that occurs within 24 h after the injection. More than 90% of the patients experience ISRs, which, however, do not worsen on repeated dosing. Histological analysis has shown activated polymorphonuclear leucocytes and macrophages without evidence for necrosis, abscess formation, ulceration, or giant cell reactions. Two types of delayed responses have been observed: reappearance of the erythema and hyperpigmentation. Whereas hyperpigmentation may be a common response to skin injury, the pathophysiological mechanism for reappearance of the erythema is unknown. Of interest, similar ISRs have been observed in clinical studies with other ASO’s. Whereas thus far antibodies have not been observed, ISRs may require careful monitoring to ensure that prolonged administration of mipomersen does not lead to auto-immunity or treatment resistance.

Flu-like symptoms have been reported for some subjects in the clinical studies. The flu-like symptoms appear shortly after mipomersen administration, resolve within 1–2 days, and are generally limited to the first few weeks of treatment. Flu-like symptoms may be secondary to pro-inflammatory activation following antisense administration, although, importantly, no significant changes in high-sensitivity C-reactive protein levels during mipomersen therapy have been reported.
inhibitors increased IHTG content,26 safety concerns regarding may be the result of intrahepatic fat accumulation. e.g. the inhibition of apoB synthesis and secretion from the liver, or gested to result from a direct pharmacological effect of mipomersen, seen in preclinical studies. Transaminase increases have been sug-

Increases in ALT were common, particularly following treatment with higher doses of mipomersen. Alanine aminotransferase increases were not accompanied by increases in total bilirubin, alkaline phosphatase, and prothrombin time or by decreases in albumin. After discontinuation of treatment, transaminases returned to normal in all patients. The exact cause for transaminase elevations during mipomersen treatment is unclear, and as noted, was not seen in preclinical studies. Transaminase increases have been suggested to result from a direct pharmacological effect of mipomersen, e.g. the inhibition of apoB synthesis and secretion from the liver, or may be the result of intrahepatic fat accumulation.

Since previous attempts to inhibit VLDL production with MTP inhibitors increased IHTG content,26 safety concerns regarding mipomersen have focused on the liver. In mipomersen clinical studies, hepatic steatosis was detected during the follow-up of patients with elevated liver enzymes following active treatment. Unfortunately, baseline measurements of hepatic fat were not performed in most of these patients. To investigate the impact of mipomersen on IHTG content, we conducted a Phase 2 study in 21 patients with HeFH.27 After 13 weeks of treatment, 1 of 10 patients in the mipomersen-treated group developed mild hepatic steatosis, which was reversible following treatment discontinuation. Whereas our study showed the absence of a profound steatotic response of the liver following mipomersen treatment, this study did have limitations: a limited group size and a short treatment period resulting in moderate decreases in apoB (−22%). Recent results from Phase 3 clinical trials reported increases in IHTG content following more prolonged treatment with mipomersen. However, in line with preclinical studies, preliminary data from long-term administration studies suggest that with continued treatment, liver fat levels tend to stabilize or decline (data on file).

The clinical significance of steatotic changes is unclear. In comparison, in patients with FHBL, characterized by life-long hepatic steatosis, reports of fibrosis, or even cirrhosis, are extremely rare.25 These findings do, however, support prolonged safety monitoring of liver fat content in long-term studies of patients under treatment with mipomersen.

### Table 2 Mipomersen (200 mg) lipid-lowering profile in the 26-week dose cohorts of the Phase 3 clinical studies

<table>
<thead>
<tr>
<th>Study population</th>
<th>Hypercholesterolaemia (200 mg/week)</th>
<th>Familial hypercholesterolaemia (200 mg/week)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid parameter % change</td>
<td>Severe</td>
<td>High CVD risk</td>
</tr>
<tr>
<td>n</td>
<td>ApoB</td>
<td>LDL-C</td>
</tr>
<tr>
<td>39</td>
<td>−36</td>
<td>n.a.</td>
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<tr>
<td>105</td>
<td>−37</td>
<td>−36</td>
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<tr>
<td>34</td>
<td>−27</td>
<td>−25</td>
</tr>
<tr>
<td>82</td>
<td>−26</td>
<td>−19</td>
</tr>
</tbody>
</table>

Values presented are the mean % reduction from baseline 2 weeks after the last dose of a 13-week treatment period. n.a., data not available; n.c., no change.

*Triglycerides and Lp(a) are presented as the median % change from baseline.

## Proprotein convertase subtilisin/kexin-9: another ideal therapeutic target for antisense

Proprotein convertase subtilisin/kexin-9 encodes a 692-amino acid protein that is predominantly expressed in the liver, kidney, and intestine.28 Proprotein convertase subtilisin/kexin-9 is involved in the degradation of the hepatic LDL-R and thereby contributes to regulation of levels of circulating LDL-c. Gain-of-function mutations in PCSK9 cause hypercholesterolaemia and premature CVD, whereas a large number of loss-of-function mutations in PCSK9 have been identified causing low levels of LDL-c and decreased risk for CVD.29 Interestingly, two individuals with total PCSK9 deficiency have been identified showing extremely low levels of LDL-c (14–16 mg/dL) but no other adverse clinical consequences.30,31 These findings suggest that lowering PCSK9 is an attractive target for therapeutic intervention to lower LDL-c alone or in conjunction with statin therapy.

The LDL-R is responsible for transporting cholesterol into hepatocytes and subsequently is recycled to the cell surface to be available to bind LDL-c particles again. PCSK9 enhances intracellular degradation of the LDL-R, thereby preventing its return and consequently lowering the number of LDL-R at the hepatocyte cell surface. The LDL-R is responsible for maintaining the cholesterol pool in the endoplasmic reticulum. Depletion of a regulatory hepatic cholesterol pool results in activation of key transcription factors, the sterol regulatory-binding proteins, which lead to increased synthesis of the LDL-R. At the same time when the LDL-R expression is increased, there is a parallel increase in PCSK9 synthesis, most likely as a measure to prevent excessive uptake of cholesterol into cells.32 At present, no other functions of PCSK9 are known beyond the degradation of the LDL-R, although potential beneficial effects on liver regeneration have recently been reported.28 Since PCSK9 is highly expressed in the liver it is, like apoB, an ideal molecular target for the ASO technology.
Preclinical experience with PCSK9

A second-generation antisense targeting murine PCSK9 was tested in high-fat fed mice. Six weeks of treatment (twice weekly) resulted in a 92% decrease in PCSK9 mRNA and a two-fold increase in hepatic LDL-R protein levels with a concomitant reduction in LDL-c of 38%. This study was followed by a Phase 1 clinical trial that was prematurely terminated to continue with the development of a more potent short anti-PCSK9 oligonucleotide using the locked nucleic acid (LNA) technology. The use of LNA technology can lead to the design of shorter ASOs (12–13-mers) with increased potency compared with longer oligonucleotides. This 13-mer LNA gap mer has thus far been tested in vitro and in vivo. When the anti-PCSK9 LNA oligonucleotide was injected in mice, the level of PCSK9 mRNA was reduced by ~60%, an effect lasting more than 16 days. Hepatic LDL-R protein levels were significantly up-regulated by 2.5–3 folds for at least 8 days and ~2-fold for 16 days demonstrating a long-lasting consequence of inhibiting PCSK9 synthesis. A 14 gap-mer LNA oligonucleotide targeting non-human primate and human PCSK9 has recently been reported to induce a potent, specific, and long-lasting inhibition of PCSK9 and lowered plasma LDL-c by up to 74% in cynomolgus monkey (Straarup et al., data presented in Nantes, 2010). Several other PCSK9 inhibitors are currently under development, including other ASOs, small-interfering RNAs as well as monoclonal antibodies that bind to PCSK9 and prevent its interactions with the LDL-R.

Other antisense oligonucleotide targets for the treatment of hypercholesterolaemia: apoC-III and apo(a)

In addition to apoB and PCSK9, several other potential ASO targets for the treatment of hyperlipidaemia have been identified, e.g. apoC-III and Lp(a). Apolipoprotein C-III plays a central role in the regulation of plasma triglycerides and is expressed in the liver. Genetic studies in humans have associated lower levels of apoC-III with lower triglyceride levels possibly with improved health and extended longevity. In animal models, the administration of apoC-III ASOs resulted in reductions in plasma triglyceride levels of 40–60%. In addition, these ASOs appeared to reduce hepatic steatosis. An apoC-III antisense inhibitor is currently being tested in a Phase 1 study.

In addition, an extensive body of evidence, including a number of so-called Mendelian randomization studies, has now substantiated that an elevated Lp(a) level is a major and independent CVD risk factor. However, evidence that lowering Lp(a) levels is beneficial with respect to CVD prevention is not yet available, as therapeutic agents to specifically lower Lp(a) without major effects on other atherogenic lipoproteins are not available. Developing agents to lower Lp(a) levels is additionally complicated because details of the mode of synthesis and assembly of Lp(a), as well as its clearance, are not understood. Apolipoprotein (a) is made by the liver and is believed to be secreted into the space of Disse, where it covalently links to apoB-100 via a disulfide bond, to form the mature Lp(a). Early preclinical studies suggest that targeting liver expression of apo(a) with ASOs directed to KIV-2 repeats—which are expressed in multiple copies in the human apo(a) gene—may provide a highly effective approach to lower elevated Lp(a) levels in humans. The development of such ASOs to lower Lp(a) levels might then allow clinical tests of the importance of lowering Lp(a) levels for the therapy and prevention of CVD.

Summary

The antisense technology has the ability to inhibit unique targets with high specificity. Available antisense drugs distribute predominantly within the liver and kidney and lack interaction with the cytochrome P450 system. Therefore, this novel technology may be a valuable therapeutic modality for the treatment and prevention of CVD. Mipomersen, an apoB synthesis inhibitor, is the most advanced of ASOs for the treatment dyslipidaemia. It has shown good efficacy results, comprising reductions in LDL-c and all other atherogenic lipid particles, including smLDL and Lp(a). Assuming continued safety with this and other early agents in this class, we can anticipate the application of the antisense technology to a variety of targets for the therapy and prevention of CVD.

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