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Liver as a target for oligonucleotide therapeutics

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Summary

Oligonucleotide-based therapeutics are an emerging class of drugs that hold the promise for silencing "un-druggable" targets, thus creating unique opportunities for innovative medicines. As opposed to gene therapy, oligonucleotides are considered to be more akin to small molecule therapeutics because they are small, completely synthetic in origin, do not integrate into the host genome, and have a defined duration of therapeutic activity after which effects recover to baseline. They offer a high degree of specificity at the genetic level, thereby reducing off-target effects. At the same time, they provide a strategy for targeting any gene in the genome, including transcripts that produce mutated proteins.

Oligonucleotide-based therapeutics include short interfering RNA (siRNA), that degrade target mRNA through RISC mediated RNAi; anti-miRs, that target miRNAs; miRNA mimics, that regulate target mRNA; antisense oligonucleotides, that may be working through RNAseH mediated mRNA decay; mRNA upregulation, by targeting long non-coding RNAs; and oligonucleotides induced alternative splicing [1]. All these approaches require some minimal degree of homology at the nucleic acid sequence level for them to be functional. The different mechanisms of action and their relevant activity are outlined in Fig. 1. Besides homology, RNA secondary structure has also been exploited in the case of ribozymes and aptamers, which act by binding to nucleic acids or proteins, respectively. While there have been many reports of gene knockdown and gene modulation in cell lines and mice with all these methods, very few have advanced to clinical stages. The main obstacle to date has been the safe and effective intracellular delivery of these compounds in higher species, including humans. Indeed, their action requires direct interaction with DNA/RNA within the target cell so even when one solves the issues of tissue and cellular access, intracellular/intranuclear location represents yet another barrier to overcome. To date, hepatic delivery of oligonucleotides has been the area with greatest progress, and thus we have focused on liver-targeted therapeutics that have shown promise at the preclinical and/or clinical level.

The liver is the largest internal organ in the body, playing a central role in metabolism, detoxification, synthesis, and secretion of major plasma proteins (carrier proteins, coagulation factors, complement components, hormones, and apolipoproteins), and iron homeostasis. It is therefore not surprising that a large number of disease targets reside in the liver where they are susceptible to modulation by oligonucleotide therapies.

Clinical-stage oligonucleotide therapies addressing liver targets

The number of oligonucleotide therapies targeting liver expressed targets in clinical trials is growing rapidly. The first trials involved antisense MOE/Gapmers; however, they have now been joined by siRNA, locked nucleic acid (LNAs), and morpholinos. MOE/Gapmers are antisense oligonucleotides with phosphorothioate backbone linkages. They have a stretch of nucleotides with deoxy sugars and the remaining nucleotides containing an O′-methyl O′-ethyl substitution at the 2′ position (MOE). siRNA are double-stranded RNA molecules that vary in length from 18 to 30 bp, for therapeutic purposes they are typically chemically modified, e.g., with 2′O-methyl nucleotides to increase stability and to limit their immunogenicity. LNAs are modified RNA nucleotides with an extra bridge connecting the 2′ oxygen and 4′ carbon in the ribose sugar that increases the melting temperature of the molecule. Morpholinos are synthetic molecules with standard nucleic acid bases bound by morphine rings instead of deoxyribose, and linked through phosphoramidite groups. They act by creating steric hindrance after binding to the target site in a molecule.

This area of metabolic disease has seen the greatest advances with multiple compounds in clinical trials in the US and/or Europe. Gene targets for hyperlipidemia, hypercholesterolemia, and diabetes have all been well validated as loss of function targets via human genetic studies. Several oligonucleotide therapeutics targeting liver expressed gene targets have shown promise in recent clinical trials (Table 1). Specifically, in the area of hypercholesterolemia, several programs are progressing through clinical trials.

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Abbreviations: *ALAS1*, δ-aminolevulinate synthase 1; *ANGPTL3*, angiopoietin-like 3; FVII, factor VII; FXI, factor XI; *GCGR*, glucagon receptor; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HoFH, homozygous familial hypercholesterolemia; *KSP*, kinesin spindle protein; LDL, low density lipoprotein; LNA, locked nucleic acid; miR, microRNA; MOE, 2′-O-methoxyethyl; MTP, microsomal triglyceride transfer protein; *PCSK9*, proprotein convertase subtilisin/kexin type 9; *PLK1*, Polo like kinase 1; RISC, RNA induced silencing complex; SAA, serum amyloid A; siRNA, short interfering RNA; *TMPRSS6*, transmembrane protease serine 6; *TTR*, transthyretin; UTR, untranslated region; *VEGF*, vascular endothelial growth factor.

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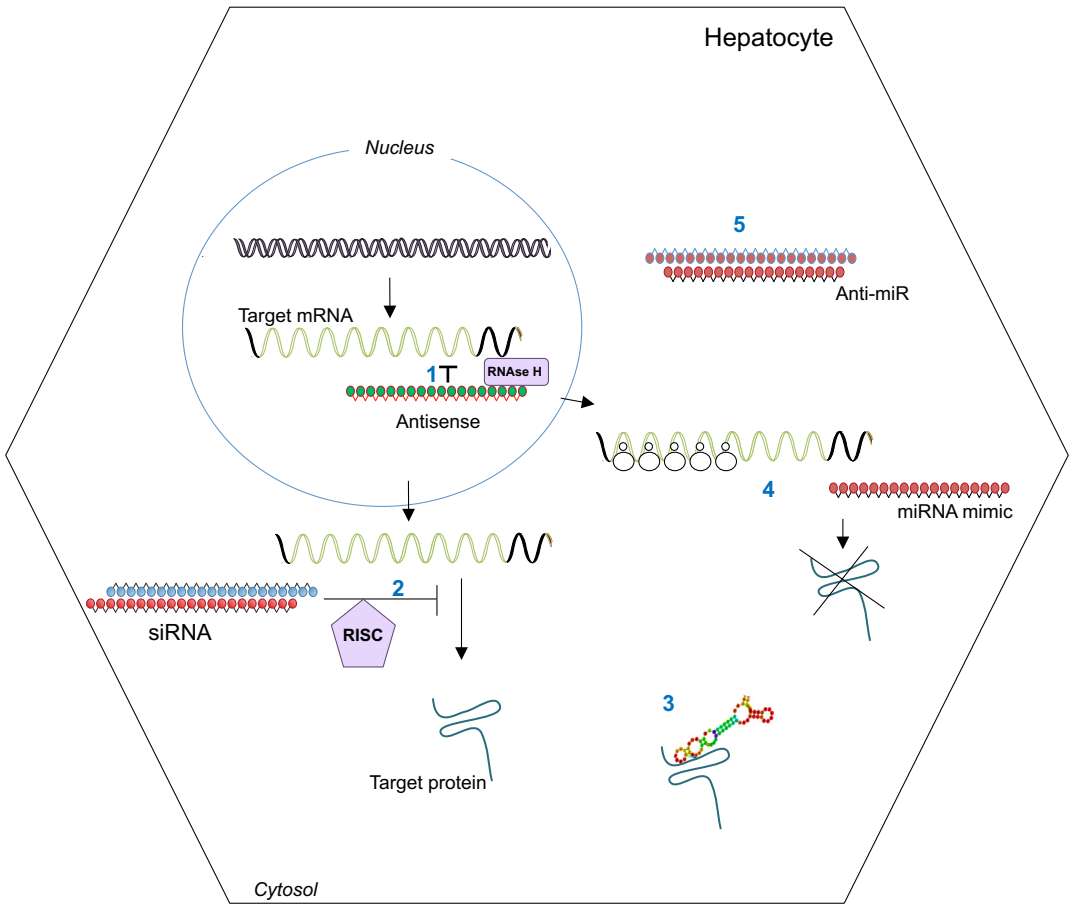

Fig. 1. Proposed mechanism of action for oligonucleotide based therapeutics. (1) RNase H mediated mRNA decay by antisense molecules in the nucleus; (2) siRNA mediated target mRNA decay exploiting the naturally occurring RNA induced silencing complex (RISC); (3) inhibitory binding of oligonucleotide based aptamer to target protein; (4) regulation of mRNA translation mediated by binding of miRNA mimics to miRNA binding sites in target mRNA; (5) anti-miR binding to miRNAs in the cytoplasm, rendering them ineffective for binding their targets.

Apolipoprotein B (ApoB) is one of the primary components of circulating atherogenic lipids. Human genetic studies have shown that mutations in ApoB, which reduce their affinity for the Low Density Lipoprotein receptor (LDLR), cause marked hypercholesterolemia. Complete loss of function in animal models, however, results in lower total and low density lipoprotein cholesterol (LDL-C). While being a genetically validated target, loss of ApoB has also been associated with on-target liabilities, such as liver steatosis [2].

Currently, the most clinically advanced oligonucleotide therapeutic against a liver gene is an antisense molecule (mipomersen) targeting ApoB (NCT01414881). In phase 3 trials, mipomersen was recently shown to lower LDL-C in heterozygous and homozygous familial hypercholesterolemia (FH) patients. In heterozygous FH, mipomersen treatment resulted in a −28% *vs.* +5% (p <0.001) reduction of LDL-C with almost half of the patients (~45%) achieving a target LDL-C of <2.6 mmol/L (100 mg/dl) [3]. Of note, however, 10.8% of the patients receiving mipomersen withdrew from treatment due to adverse effects such as injection site reactions and liver enzyme elevations, as compared to no patients in the placebo arm. Mipomersen has been approved by the FDA, but not the EMA, as a treatment to reduce LDL and total cholesterol in patients with HoFH. A phase 1 trial with an ApoB siRNA formulated in a first generation lipid nanoparticle (LNP) [4] molecule was carried out by Tekmira Pharmaceuticals; the trial was halted during dose escalation in 2009 (NCT00927459).

Proprotein convertase subtilisin kexin 9 (PCSK9) was originally identified in a human genetic study which linked it to severely elevated LDL-C. More specifically, loss-of-function mutations in PCSK9 in humans resulted in lower LDL-C levels and protection from cardiovascular disease [5]. Consistent with this observation, studies in animal models demonstrated that loss of PCSK9, a protease that physiologically downregulates the LDL receptor (LDLR), leads to an increase in LDLR levels on the hepatocyte, resulting in increased LDL-C clearance. The RNAi therapeutic ALN-PCS, targeting PCSK9, has been evaluated in a phase 1 clinical study (NCT01437059) [4,6,7]. It was safe, well-tolerated and showed dose-dependent reduction in PCSK9 and LDL-C. A similar phase 1 single ascending dose of a LNA targeting PCSK9 by ISIS/BMS was halted during dose escalation due to safety concerns [8].

Apolipoprotein C-III (ApoC3) is a component of very low density lipoprotein (VLDL), and is thought to be an active inhibitor of lipoprotein and hepatic lipases, both of which are involved in the processing and uptake of triglyceride-rich particles. Mutations in ApoC3 in humans result in lower circulating triglycerides, and a lowering risk for the development of cardiovascular disease [9]. Phase 1 studies of an antisense molecule targeting ApoC3 (ISIS Pharmaceuticals, NCT01529424) have shown promise in

lowering serum triglycerides. Preclinical data with siRNA targeting ApoC3 have also shown the expected impact on triglycerides in hyperlipidemic models [10].

Lipoprotein(a), (Lp(a)) levels are associated with an increased risk of atherosclerosis, coronary heart disease, heart attack and stroke [11]. ISIS-APOA$_{Rx}$ is an antisense drug designed to reduce Lp(a) in the circulation, and has recently entered phase 1 clinical trials.

Additional phase 1 programs from ISIS Pharmaceuticals in the area of diabetes have been reported (but have yet to be published), and include antisense compounds targeting protein tyrosine phosphatase 1B (PTP1B) (NCT00330330) and glucagon receptor (GCGR) (NCT005197270). Each of these targets has some degree of validation in animal models or human genetic studies for the modulation of diabetes and/or obesity [12–14]. Beyond metabolic targets, a number of other programs address liver-expressed genes of pathogenic interest. C-reactive protein (CRP) is an acute-phase reactant involved in response to inflammation. CRP levels are known to be elevated in cardiovascular disease and possibly diabetes [15]. ISIS-CRPrx is an antisense molecule targeting CRP. The drug was well-tolerated in a phase I trial and is currently in phase 2 trials in atrial fibrillation and rheumatoid arthritis (NCT01710852).

ISIS-FXI is another antisense molecule currently in phase 2 (NCT01713361). It inhibits the production of factor XI (FXI) and is predicted to have anti-thrombotic activity. The preclinical data in cynomolgus monkeys and phase 1 data in healthy volunteers showed dose-dependent decreases in FXI [16]. The ongoing phase 2 trial is a comparator-controlled study in patients undergoing knee replacement surgery/total knee arthroplasty. An aptamer against factor IXa, a liver produced coagulation factor, was tested in humans for anti-thrombotic activity by Regado Biosciences. RB006 demonstrated the ability to inhibit plasma factor IX activity in a dose dependent manner in the phase 1 trial (NCT00932100) [17].

Diseases of protein aggregation are also considered prime candidates for intervention by oligonucleotide therapies. Elimination of the amyloidogenic protein by silencing of the corresponding transcript can enable clearance of protein aggregates such as amyloid-like deposits. One such disease, transthyretin (TTR)-mediated amyloidosis (ATTR) is a life-threatening disorder caused by the deposition of hepatocyte-derived mutant TTR in peripheral nerves and heart [18]. Therapies under development for ATTR have shown promise in pre-clinical models, where treatment has resulted in clear disease regression. Both siRNA and antisense approaches are being investigated in clinical studies for the treatment of ATTR, including ALN-TTR02 from Alnylam (NCT01617967) and ISIS-TTR$_{Rx}$ (NCT01737398) from ISIS [19,20]. In addition to ALN-TTR02, Alnylam is developing ALN-TTRsc (NCT01814839), a subcutaneously administered RNAi therapeutic that has also recently entered clinical development. ALN-TTRsc is a chemically modified siRNA conjugated to an N-acetylgalactosamine moiety that helps in liver targeting of the drug via hepatocyte asialoglycoprotein receptor mediated uptake and allows for subcutaneous administration.

Pre-clinical stage oligonucleotide therapies addressing liver targets

Along with the oligonucleotides in different stages of clinical trials, there are many other candidates being pursued for different diseases (Table 2). While several of these diseases are caused by mutations in a single gene, others are not monogenic but may be mitigated by modulating a single gene product in the liver. The liver diseases can be inherited or acquired during the life-span of an individual.

Inherited diseases

Alpha-1 antitrypsin (AAT) is an abundant liver-secreted plasma protein. The primary biological function of this serpin is to inactivate proteases like neutrophil elastase. The Glu-342-Lys point mutation in AAT leads to misfolded protein that polymerizes and accumulates in the hepatocyte endoplasmic reticulum, leading to impaired liver function, fibrosis, cirrhosis, and possibly liver failure or HCC [21]. An oligonucleotide approach targeting AAT mRNA should be an efficient and safe way to halt pathogenic protein production. Consistent with this expectation, both RNAi therapeutic (ALN-AAT [22]) and antisense (ISIS-AAT [23]) approaches have shown promising results in preclinical models.

Angiopoietin like 3 (ANGPTL3) is a member of the angiopoietin family of secreted proteins. Its major function appears to be an inhibitor of both endothelial and lipoprotein lipases. Homozygous loss of function ANGPTL3 mutations in humans result in profound lowering of total cholesterol, LDL-C, triglycerides and high density lipoprotein cholesterol (HDL-C). Gain of function ANGPTL3 mutations in humans result in hyperlipidemia [24]. ALN-ANG, a liver targeted RNAi therapeutic directed towards ANGPTL3, has demonstrated the expected lowering of both total cholesterol and triglycerides in pre-clinical models [25]. Antisense compounds targeting fibroblast growth factor receptor 4 (FGFR4) for obesity and diacylglycerol acyltransferase-2 (DGAT2) for non-alcoholic fatty liver disease (NASH) are being explored in preclinical studies. An oligonucleotide targeting miR33, being developed by Regulus Therapeutics, was shown to increase plasma HDL and lower VLDL triglycerides in preclinical studies [26,27].

Hepatic porphyrias are a group of rare disorders of the heme synthesis pathway. Mutations in several enzymes in this pathway can cause a build-up of pathway intermediates which are toxic. The first enzyme in the pathway, ALAS1, is highly regulated and is induced in response to low levels of heme in the liver. This induction leads to flux through the pathway and an increase of the toxic intermediates, 5-aminolevulinic acid (ALA) and porphobilinogen (PBG). Inhibition of ALAS1 induction in acute intermittent porphyria is a promising strategy that should result in pathway downregulation and prevention in the buildup of ALA and PBG. ALN-AS1, a subcutaneously administered RNAi therapeutic targeting ALAS1, is being developed to treat this ultra-orphan disease (http://www.alnylam.com/Programs-and-Pipeline/index.php).

Hemophilia A and B as well as several other rare bleeding disorders are caused by loss of function mutations in various clotting factors, resulting in reduced thrombin generation and excessive bleeding. There is genetic evidence suggesting that coinheritance of prothrombotic mutations, which enhance thrombin production, in hemophilia leads to a less severe bleeding phenotype [28]. ALN-AT3, a subcutaneously administered RNAi therapeutic targeting antithrombin, leads to correction of thrombin generation in hemophilia mice [29]. In contradistinction, an antisense targeting Factor VII (ISIS-FVII$_{Rx}$) is being developed by Isis as a treatment for thrombosis in a strategy similar to

that described for FXI above (http://www.isispharm.com/Pipeline/index.htm).

Strategies to increase hepcidin levels are also being explored. TMPRSS6 is a negative-regulator of hepatocyte hepcidin expression. ALN-TMP, an RNAi therapeutic [30] being developed by Alnylam, and an antisense compound from ISIS [31,32] both downregulate TMPRSS6 expression and increase hepcidin secretion leading to improvement in iron overload in models of hemochromatosis and β-thalassemia intermedia. Targeting TMPRSS6 may also find application in other dyserythrpoeitic anemias where iron overload is a feature.

Acquired diseases

The liver is also the central player in iron homeostasis, and offers targets in the hepcidin pathway for intervention under conditions of both iron overload and anemia. Upon sensing high iron levels, hepatocytes synthesize and secrete hepcidin. Hepcidin binds to ferroportin and reduces the iron efflux by macrophages and enterocytes [33]. In cases of chronic anemia, reduction in hepcidin can lead to an increase in circulating iron and improved erythropoiesis. Silencing hepcidin, either directly, or by targeting a positive regulator of hepcidin, such as transferrin receptor II or hemojuvelin, has shown promise in preclinical models. These strategies are being deployed to treat anemia of inflammation by both Isis/Xenon and Alnylam [34] (http://www.xenon-pharma.com/product-candidates/anemia/).

A known outcome for most chronic liver diseases is hepatocellular carcinoma. Owing to their preferential distribution in the liver, oligonucleotide therapeutics have therefore been used against liver cancer. ALN-VSP, an RNAi therapeutic containing two encapsulated siRNAs targeting kinesin spindle protein (KSP) and vascular endothelial growth factor-A (VEGF), is being developed for both primary and secondary liver cancer. In early trials, ALN-VSP has been shown to be generally well tolerated [35]. Of particular note, a complete response (defined per Response Evaluation Criteria in Solid Tumors) was observed in one patient with endometrial cancer and multiple hepatic metastases that stopped treatment after 26 months. In addition, prolonged disease stabilization for 1–1.5 years was seen in patients with hepatic and extrahepatic metastases and, in some patients, there was a substantial decrease in tumor blood flow on DCE-MRI, consistent with an anti-VEGF effect. Moreover, a 5′ RACE method was utilized on patient tumor biopsy samples to show that RNAi based cleavage of VEGF was occurring in patients [22]. In addition to siRNA targets, microRNA mimic strategies for miR-34 (NCT01829971) and miR-7 have also been proposed as approaches for hepatocellular cancer (HCC) [36]. TKM-PLK1-001 is another siRNA based approach, targeting Polo like kinase I, a major player in cell cycle in phase I trial for solid tumors (NCT01262235). Early results from the ongoing phase I show that TKM-PLK1 is generally well-tolerated and 4 patients showed clinical benefit and achieved stable disease.

Amongst infectious agents, hepatitis B and C are the commonest risk factors for liver cancer. Oligonucleotide therapies that decrease these viruses would have a direct impact on liver infection, and reduce the risk of HCC. Hepatitis C virus (HCV) requires miR-122 for its propagation ([37]); hence inhibition of miR-122, using a complementary oligonucleotide termed an anti-miR, is an attractive strategy. Santaris Pharmaceuticals is testing an LNA-anti-miR (miraversen) for miR122 in a phase 2a clinical trial.

Table 1. Oligonucleotide therapeutics in clinical trials.

Disease	Targeted gene	Targeting agent	Pursued by	Latest status
Acromegaly	Growth hormone receptor	antisense	ISIS/Antisense Therapeutics Limited	Ph 2 recruiting
Hepatitis C virus	*miR-122*	LNA-antimir	Santaris	Ph 2 ongoing
Hypercholesterolemia	*ApoB-100*	antisense	ISIS/Genzyme	Approved by FDA
Hypercholesterolemia	*ApoB-100*	siRNA	Tekmira	Ph 1 terminated
Hypercholesterolemia	*ApoB-100*	LNA	Santaris	Ph 1 completed
Hypertriglyceridemia	*ApoCIII*	antisense	ISIS	Ph 2 recruiting
Hypercholesterolemia	*PCSK9*	siRNA	Alnylam/Medicines Company	Ph 1 completed
Hypercholesterolemia	*PCSK9*	LNA	ISIS/BMS	Ph 1 halted
Inflammatory disorders	*CRP*	antisense	ISIS	Ph 2 recruiting
Liver cancer	*KSP/VEGF*	siRNA	Alnylam	Ph 1 completed
Liver cancer	*PLK1*	siRNA	Tekmira/Protiva	Ph 1 ongoing
Liver cancer	*miR-34*	miRNA mimic	miRNA therapeutics	Ph 1 recruiting
Obesity	*FGFR4*	antisense	ISIS/Verva Pharmaceuticals	Ph 1 ongoing
Thrombosis	Factor IXa	aptamer	Regado Biosciences	Ph 2a completed
Thrombosis	Factor XI	antisense	ISIS	Ph 2 recruiting
TTR amyloidosis	*TTR*	antisense	ISIS/GSK	Ph 3 recruiting
TTR amyloidosis	*TTR*	siRNA	Alnylam	Ph 2 recruiting
Type 2 diabetes	*GCCR*	antisense	ISIS	Ph 1 ongoing
Type 2 diabetes	*PTP-1B*	antisense	ISIS	Ph 1 completed
Cushing's syndrome	*GCGR*	antisense	ISIS	Ph 1 ongoing

Table 2. Preclinical oligonucleotide based therapeutics.

Disease	Targeted gene	Targeting agent	Pursued by	Latest status
AAT-LivD	*AAT*	siRNA or antisense	Alnylam	Presented, active
AAT-LivD	*AAT*	siRNA or antisense	ISIS/GSK	Presented, active
Alcohol dependence	*ALDH2*	siRNA	Tekmira	Announced on website
Anemia of inflammation/ CKD	HAMP pathway	siRNA	Alnylam	Presented, inactive
Atherosclerosis	*miR-33*	anti-miR	Regulus/AstraZeneca	Presented, active
Atherosclerosis/high Lp(a)	*ApoAI*	antisense	ISIS	Announced on website
Liver cancer	*miR-7*	miRNA-mimic for EGFR	MiReven/Silence	Presented, inactive
Cardiometabolic diseases	*miR-378*	miR mimic	Miragen Therapeutics	Announced on website
Liver cancer	*miR-21*	anti-miR (ss)	Regulus/Sanofi	Presented, active
Liver cancer	*Myc*	siRNA	Dicerna	Presented, active
Hepatitis C virus	*miR-122*	anti-miR (ss)	Regulus/GSK	Presented, active
Hepatitis C virus	5'UTR	ribozyme	Sirna/Eli Lilly	Presented, inactive
Hepatitis C virus	5'UTR and NS5B	shRNA against 3 regions	Tacere Therapeutics	Presented, active
Hepatitis C virus	NS3	aptamer	Umehara et al[39]	Publication
Hemochromatosis	*TMPRSS6*	siRNA	Alnylam	Presented, active
Hemophilia A, B	Antithrombin III	siRNA	Alnylam	Presented, active
Hypertriglyceridemia	*ApoCIII*	siRNA	Alnylam	Presented, inactive
Hyperlipidemia	*ANGPLT3*	siRNA or antisense	Alnylam	Presented, inactive
Hyperlipidemia	*MTP*	antisense	ISIS	Publication-Preclin
NASH	*DGAT2*	antisense	ISIS	Presented, active
Porphyria	*ALAS1*	siRNA	Alnylam	Presented, active
Rare bleeding disorders	Antithrombin III	siRNA	Alnylam	Presented, active
SAA-amyloidosis	Serum amyloid A	siRNA or antisense	Alnylam, ISIS	Preclinical
Thrombosis	Factor VII	antisense	ISIS	Presented, inactive

The results show that a 4-week monotherapy with the anti-miR reduces HCV RNA by 2–3 logs [38]. In this study, 4 of 9 patients at the high dose group of 7 mg/kg showed a decrease in HCV levels below the limit of detection.

Conclusions

In 1998, the intravitreally-administered antisense molecule, fomivirsen, for the treatment of cytomegalovirus retinitis, was the first oligonucleotide drug to be approved by the FDA (NCT00002356). The first human oligonucleotide therapeutics trials involving systemic delivery were published in 1998. In this clinical trial, an antisense oligodeoxynucleotide against ICAM-1 was delivered via 13 intravenous doses over 26 days in steroid-treated Crohn's disease patients (NCT00048113). The drug was found to be generally safe and well tolerated but did not show efficacy in a dose-responsive manner. Since that time, tremendous progress has been made, culminating in the recent approval of the first systemically delivered oligonucleotide, mipomersen, for the treatment of hypercholesterolemia. Improvements in oligonucleotide delivery, increased stability of the molecules, and decreased toxicity have led to the generation of several new classes of oligonucleotide based therapies. With well over a dozen different targets being pursued using a variety of oligonucleotide therapeutic compounds (siRNA, antisense, anti-miRNA, miRNA mimics) the future has never looked brighter for these disease-modifying modalities.

Key Points

- Oligonucleotide-based therapies enable targeting and knockdown of targets that were previously considered undruggable
- The development of oligonucleotide therapeutics targeting hepatocyte-expressed genes is progressing rapidly
- There is currently a large and growing pipeline of therapeutics, with the first systemic oligonucleotide therapy recently approved by the FDA

Conflict of interest

All authors are employees of Alnylam Pharmaceuticals.

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