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Antisense oligonucleotides: treatment strategies and cellular internalization

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REVIEW

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The clinical application of antisense oligonucleotides (ASOs) is becoming more of a reality as several drugs have been approved for the treatment of human disorders and many others are in various phases in development and clinical trials. ASOs are short DNA/RNA oligos which are heavily modified to increase their stability in biological fluids and retain the properties of creating RNA-RNA and DNA-RNA duplexes that knock-down or correct genetic expression. This review outlines several strategies that ASOs utilize for the treatment of various congenital diseases and syndromes that develop with aging. In addition, we discuss some of the mechanisms for specific non-targeted ASO internalization within cells.

Keywords: antisense oligonucleotide; Stabilin, endocytosis; clearance; splicing; phosphorothioate

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Introduction

One of the growing fields of interest pertaining to nucleic acid-based gene silencing/modification is the application of antisense oligonucleotides (ASOs). ASOs are chemically modified nucleic acid sequences that serve as highly selective sequence pairs to specific regions of mRNA and regulate the translation of genetic material into functional proteins. ASOs bind complementary RNA by Watson-Crick base pairing and can suppress gene expression via a number of distinct antisense mechanisms. ASOs which contain stretches of >5 DNA nucleotides activate the RNase H antisense mechanism. RNaseH1 is a ubiquitously expressed endonuclease which selectively cleaves the RNA strand of a DNA/RNA heteroduplex [1]. ASOs of other chemical compositions can bind RNA to modulate splicing, arrest translation or sequester endogenous microRNAs [2] (Fig. 1).

The phosphodiester (PO) backbone of DNA and RNA is susceptible to nuclease mediated degradation which limits application of unmodified oligonucleotides as therapeutics. In contrast, chemical modifications of the backbone enhance the metabolic stability of oligonucleotides which enables their use for therapeutic applications. One of the most widely used modifications in oligonucleotide therapeutics is the phosphorothioate modification [3]. The phosphorothioate (PS) modification replaces a non-bridging oxygen atoms in the PO linkage with a sulfur atom. This increases ASO stability in biological fluids and also enhances their protein binding and cell uptake properties [4]. ASO stability is essential for the molecules to not only remain intact while in circulation, but also to be biologically active and internalized into the cell for

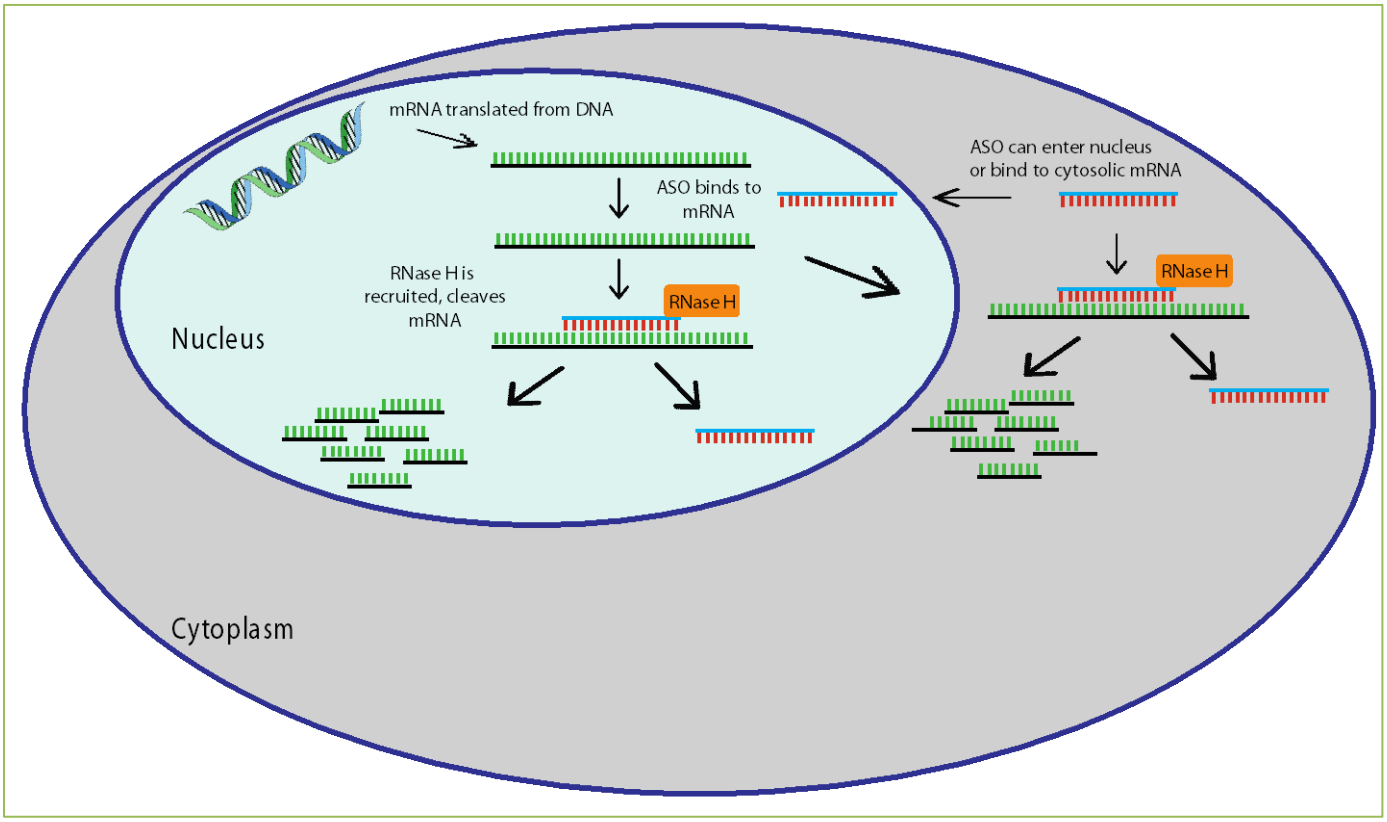

Figure 1. RNase H mediated destruction of targeted mRNA. ASOs are internalized in the cells by several mechanisms (clathrin/caveolin-mediated endocytosis, micropinocytosis, etc.) in which a small subset of the ASOs escape into the cytoplasm and/or nucleus. Hybridization of the ASO with complementary hnRNA or mRNA induces RNase H cleavage of the RNA while leaving the ASO intact for subsequent hybridizations.

gene silencing. However, this mechanism may only work if the ASO successfully navigates past the cell plasma/endosomal membranes to target the specific mRNA of interest. The mechanism for ASO internalization is not well understood, though it is thought that efficient uptake involves specific protein receptors [5, 6]. At the present time, it is believed that clathrin-mediated endocytic receptor(s) allows ASO internalization at concentrations in the nM-μM range [6-8].

Recent works focused on increasing the understanding of these drugs have enabled great strides in the therapeutic application. The FDA approved ASO therapeutic uses such as treatment for familial hypocholesteremia, with many others in phase III clinical trials [9-11]. New designs are constantly being developed to increase effectiveness of these drugs, as well as new approaches to different diseases, while at the same time decreasing potential off-target side-effects [6, 12]. The focus of this review is to highlight the development of ASOs in clinical drug development for a variety of diseases, as well as examine recent research to answer fundamental questions of cellular ASO internalization and systemic clearance.

Backbone Modifications for single stranded ASOs

One key aspect of ASO stability and functionality is derived from the modifications on the phosphate backbone of the nucleotide sequence. Second generation ASOs are comprised of a central gap region of PS DNA flanked on either ends with 2'-methoxyethyl RNA (MOE) nucleotides. The MOE nucleotides enhance binding affinity for complementary RNA and further enhance metabolic stability which results in longer effectiveness in the clinic [3, 13]. Other ASO backbone structures such as phosphodiester (PO) or methylphosphonates (MP) have been explored [14], but the PS backbone modifications to have high stability both *in-vitro* and *in-vivo* systems [6], in addition to *in-vivo* internalization [15] for single stranded ASOs.

MP oligonucleotides, which have reduced charge, have higher nuclease stability than PS-ASOs [16]. However, the lack of charge on the MP molecule reduces protein binding and cellular uptake [17-19]. Novel techniques are being utilized to increase the activity of ASOs, with a majority of clinical drugs having a PS-2'-methoxyethyl (2'-MOE) modification of the ribose sugar which increases the T_m of RNA by 2^{o} C for each modification, while also aiding to reduce access to

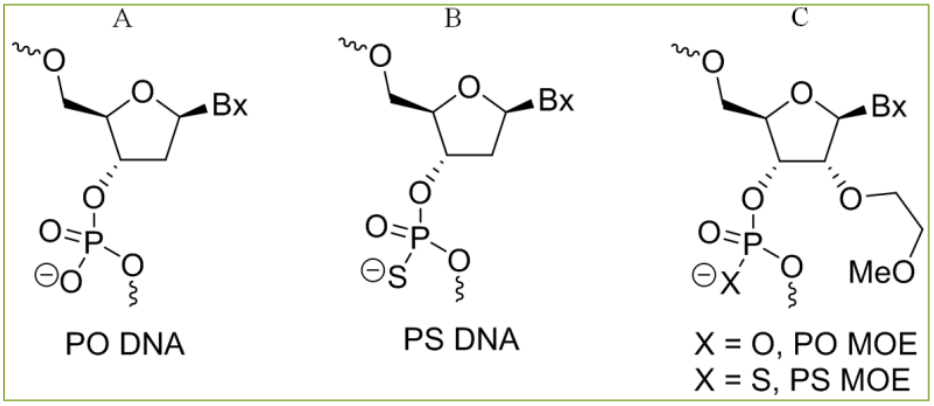

Figure 2. Backbone structures of ASO modifications. A. Unmodified phosphodiester backbone. B. Generation 1 phosphorothioate (PS-ASO) modification. C. Generation 2 2'-methoxyethyl phosphorothioate (2' MOE-PS-ASO) modification.

the linkage of the backbone [2] (Fig. 2). It is important to note that while PS-ASOs are currently being utilized based on their increased activity, the PS modifications are relatively easy to synthesize compared to other phosphate modifications [19]. Currently, over 35 ASOs are in clinical development, and of these, at least a majority contain PS backbone structures, and many newer chemical structures contain 2'-MOE modifications as well [2].

Diseases Targeted by ASOs

Duchenne's muscular dystrophy

Duchenne's muscular dystrophy (DMD) is a rare but debilitating X-linked disease causing muscle wasting by mutations arising from the DMD gene, which codes for dystrophin. This protein has been determined to connect the sub-sarcolemmal cytoskeleton to the extra-cellular matrix (ECM). Dystrophin, along with the dystrophin associated glycoprotein complex (DGC) work to stabilize muscle fibers during muscle contraction from the binding of the cytoskeleton to the ECM. When this protein is lost due to the DMD gene mutations, it leads to muscle degeneration, inflammation, and overall progression of DMD [20]. This disease is the most common fatal neuromuscular disorder found in children, which affects 1 in 3,500 males [20]. Patients are traditionally treated by the age of 5 to prevent premature death when diagnosed at an early age. Although if left untreated (without preventative measures), children often suffer from cardiac or respiratory failure leading to death before the age of twenty [20-22]. The disease is caused by genetic missense or nonsense mutations in the open reading frame of the DMD gene, dystrophin [22].

Antisense oligonucleotides may utilize multiple splicing mechanisms for when a gene is mistranslated including exon skipping, cryptic splicing restoration, or even changing levels of alternate gene splicing [23]. One of the early ASOs used in DMD treatment, PRO-051, or Drisapersen, utilized exon splicing modulation for exon 51 (of the 79 exons present) of Dystrophin to force skip this exon, allowing for rescue of the ORF [24]. Although it proceeded into stage III of clinical trials, it failed to be awarded FDA approval due to the high doses required for effective treatment and long-term toxicity side-effects [25]. Drisapersen was a modified 2'O-Me RNA modification. A second drug was developed with a morpholino backbone [2], which utilizes excision of the pre-mRNA splicing of dystrophin's exon 51 [26] based on the same rescue of the ORF as Drisapersen. The mechanism of a morpholino modified ASO allows for hybridization binding to exon 51, which when bound does not allow the splicing machinery to recognize it, allowing for the ORF to remain intact, while removing the mutated region [23] (Fig. 3A). Currently, this drug is in stage III clinical trials and has demonstrated lower toxicity than its 2' O-Me counterpart. Although further testing is required before FDA approval, there seems to be hope that this morpholino-based ASO will help in future treatments of DMD, based on the improvements from the previous 2' O-Me drug.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA), not to be confused with spinal muscular dystrophy, is currently the leading cause of genetic infant mortality. This disease causes motor neurons in the central nervous system to decay and lose function [27]. Loss of neuromuscular junctions causes muscles to weaken and atrophy over time. The loss of muscle function can have devastating secondary impacts from sleeplessness to scoliosis, joint contractures, and respiratory failure. This genetic disorder arises from mutations on exon 7 of the SMN1 gene, which codes for the survival motor neuron proteins, with 95-98% of patients displaying a homozygous trait of this gene [27]. SMN proteins localize to the cytosol

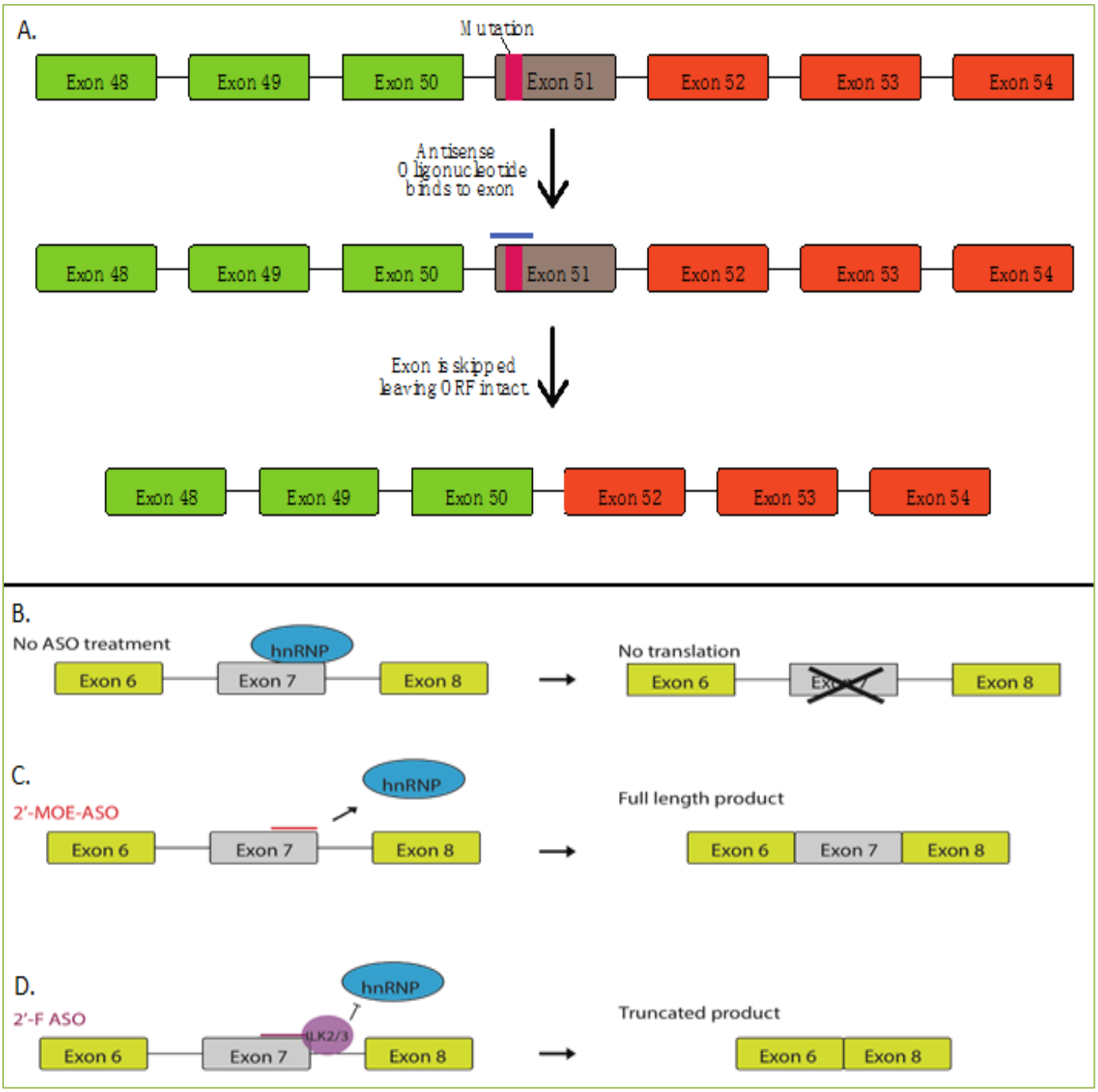

Figure 3. Exon skipping as a mechanism for "gene" correction. A. The ASO mechanism for the treatment of Duchenne's Muscular Dystrophy (DMD) in which the ASO binds to exon 51 containing the mutation and force skips the exon in transcription producing a slightly shorter but active gene product. B. In cases of Spinal Muscular Atrophy (SMA), heterogenous nuclear ribonuclear proteins (hnRNPs) bind to exon 7 and prevent further hnRNA modification and subsequent translation. C. SMA treatment with a 2'-methoxyethyl (2'-MOE) modified ASO (ASO-10-27) displaces hnRNPs and allows translation of the full-length gene product. D. SMA treatment with a 2'-Fluoro (2'-F) modified ASO recruits Interleukin Enhancer 2 and 3 proteins which displace hnRNPs and allow for the translation of a shorter mRNA producing a smaller protein product that is still partially active. Figure adapted and modified from *Aartsma-Rus et al*. [23] and *Rigo et al.* [35]

and nucleus, where they are involved in a multiprotein complex. These proteins are involved in RNP complex formation, and have been shown to accumulate in Cajal bodies [28, 29]. SMN2 is also involved in this disease, however, the copy number increases in the presence of SMN1 mutations, leading to phenotype modifications.

Currently, there is no specific treatment for the disease

itself, as most therapies are for treating the symptoms [30]. One potential therapy, proposed a decade ago, was to utilize quinizoline derivatives as a potential mechanism for increasing SMN2 promoter activity to induce SMN splicing patterns to relieve the mutated gene [31]. A study, compiling multiple compounds that increased SMN promotor activity identified these derivatives as a potential target with a 2.5 fold increase in activity [32]. Although this mechanism did produce improvement of motor function in mouse models, a work-stop was issued and the drug company has canceled clinical phase II testing [33, 34].

Modulating splicing of mRNA can also be accomplishes using ASOs. This class utilizes pre-messenger RNA as a source for correcting an aberrant mRNA product. When untreated with ASOs heterogenous nuclear ribonuclearproteins (hnRNPs) are recruited to the exon to silence the targeted region [35]. One example for treatment to inhibit hnRNP activation of pre-mRNA in SMA is ASO-10-27, which works to provide corrected SMN expression via a gene splicing mechanism similar to that of the DMD example [23, 36] (Fig. 3B-D). ASO-10-27 is a generation 2 drug that is used to boost functional SMN production by careful splice modification of SMN2, based on its paralogous gene similarity to SMN1 [33, 36]. The key mechanism for this ASO is when bound, it does not allow for hnRNP to bind to the targeted exon 7 by blocking the binding site, allowing for the gene to remain intact. Interestingly, a 2'-fluoro modified ASO, recruits interleukin enhancer-binding 2 and 3 complex (ILF2/3) to bind to the active site causing the exon to be skipped [35]. Currently, the ASO-10-27 MOE modified drug demonstrates marked improvement in clinical trials from the mechanism of exon 7 splicing, as this therapy is currently in clinical phase III trials. However, it is worth nothing that other chemical modifications are being studied for future use as a new mechanism for treatment of SMA along with a wide variety of other potential disease targets.

Factor XI for Thrombosis

Thrombosis (blood clotting) is a major health issue that may arise from adverse drug reactions, post-surgical outcomes, stroke, myocardial infarction, or spontaneously by ill-defined mechanisms. Factor XI is a serine protease enzyme that is converted to Factor XIa and directly involved in the coagulation cascade [37]. This enzyme has been targeted as a potential upstream inhibitor for thrombosis by ASO-based gene knock-down therapy utilizing a second generation ASO (FXI-ASO), which has a 2'-O-MOE RNA modification coinciding with the PS modification allowing for this ASO to stably be incorporated into the cell for targeting factor XI mRNA [37]. The rationale for targeting this specific factor, was the lack of potential bleeding events, while also lowering the rate of blood clotting. FXI-ASO was tested in animals and showed no disruption of homeostasis during knock-down of Factor XI protein activity by over 90% [38]. FXI-ASO is able to bind to mRNA of factor XI in the liver, specifically in the hepatocytes which is the primary site of factor XI formation [39]. The hepatic inhibition leads to a downstream reduction of plasma factor XI levels. Currently, this drug is in phase II of clinical trials, as it has been subject to a human open-label phase study, by which no treatment disguise or placebo is used [40]. This study showed that at higher doses, there were no reported toxicity events, and all noted side-effects were negligible, as the patients responded to positive anti-coagulation without increased bleeding risk, [40] which is novel to most anti-coagulant based treatments. At this time, more work needs to be done since ASO targeting of hepatic factor XI production has shown promising results for the future treatment of patients with high risk of suffering from thrombosis.

Hyperlipoproteinemia/Apolipoproteinemia

Hyperlipoproteinemia, also known as hypercholesterolemia, is a condition in which increased amounts of serum low density lipoprotein (LDL) cholesterol accumulates in the blood stream. This is traditionally a precursor for cardiovascular diseases, as well as plaque formation based on the age at which onset occurs [41]. There are a variety of factors that contribute to a patient diagnosed with this disease, though there is a genetic predisposition, known as familial hypercholesterolemia (FH). FH is thought to be most commonly associated with a mutation in the LDL receptor (LDLR) gene in patients expressing either heterozygous, with one allele being mutated, or homozygous genes, with both alleles mutated [42]. Although the LDLR mutation is very common in FH diagnoses, there are also reported cases indicating that a mutation in apolipoprotein B (ApoB) can also cause FH, with phenotypic similarities that make diagnosis virtually indistinguishable from other causes. FH caused by dysfunctional ApoB is found in approximately 1 in 500 people in Western cultures [43]. A common form of treatment for FH is to prescribe statins, which effectively reduce cholesterol synthesis by HMG-CoA reductase, although most patients require additional drug therapy and lifestyle changes [42].

With the advances in ASO technology, a RNase H1 active second generation 2'-O-MOE PS-ASO (Mipomersen) was developed that complements the coding region of ApoB mRNA located at position 3249-3268 of the mRNA [44]. Following subcutaneous injection, Mipomersen is distributed across a wide variety of tissues including the liver, and it has been determined through clinical studies that it effectively

reduces plasma LDL cholesterol levels. Additionally, it has been shown that Mipomersen does not have deleterious effects on high density lipoproteins (HDL), which are important for lipid transport to liver and other steroidogenic organs [44]. Due to the success of Mipomersen by both low toxicity reports and high apolipoprotein B inhibition, it has been approved by the FDA for clinical use for FH treatment [9]. Currently, with more mutations being discovered that lead to LDL Cholesterol accumulation, additional ASO-based drugs are currently being developed in the clinical pipeline. A few of these are: Ionis-APO(a)$_{Rx}$ which is a second generation (2'-O-MOE) drug that is currently in clinical trials for treatment of the protein apolipoprotein A, which is implicated in cardiovascular disease and aortic valve stenosis [45] and Apolipoprotein C-III and its role in plasma triglyceride levels. Currently, improvements and testing for 2'-O-MOE ASO Ionis 304801 (Apo-C3$_{Rx}$) is in refinement to reduce plasma triglyceride levels by binding to the the ApoC3 mRNA to reduce apolipoprotein C-III production [10]. This drug is currently in clinical phase III trials.

Cellular uptake of Phosphorothioate Modified ASOs

The understanding of uptake and clearance of PS-ASOs is essential to calibrate the dose and pharmacology of therapeutic ASOs. Cellular endocytosis of ASOs is of high interest as a high number of targeted proteins are synthesized by the liver [5, 10, 24]. PS-ASOs, as mentioned previously have a higher stability based on the chemical modifications, allowing them to remain intact for cellular uptake. The rationale for understanding the pharmacokinetics of ASOs is to correlate effective dose with clearance rates to optimize effectiveness while reducing potentially harmful side-effects. The chemical modifications allow for these drugs to have high stability, but they are still susceptible to lysosomal degradation, which allows them to be cleared from the body after administration [6].

Phosphorothioate Modified ASOs and the Extra-cellular Matrix

The extra-cellular matrix is an area that could hold the key for understanding the mechanism for PS-ASO binding and cellular internalization. Prevoius work has looked at the role of proteins such as fibroblast growth factor receptors (FGFR), as a poteintal mechanism of binding for PS-ASOs based on the polyanionic charge of other molecules (heparin sulfate and suramin ligands) that bind to these receptors [13, 46]. It was also of interest based on the high expression of the FGFRs in the liver, which is a major site of accumulation, activation, and degradation of ASO molecules [6]. Previous work that has looked at these proteins as well as many other heparin binding proteins, such as lamelin and fibronectin have been identified has poteintal targets for ASO binding based on the role of ASOs binding to the ECM, as well as their role in NF-kappaB nuclear transcription regulatory factor, as a site specific cellular adhesion blocker [47]. Although this reasoning has shown binding of the ASOs to the proteins, such as FGFR1 as a target for onset obesity by allele specific single nucleotide polymorphisms [48, 49], a clear mechanism of internalization from these proteins has remained elusive. Currently, there are two potential mechanisms that have begun to explain the internalization of these molecules; scavenger receptor A, and the stabilin receptors [5, 6]. Although these two protein receptors are good candidates to help address the question of internalization of ASOs, there are still many proteins in the ECM that could play a significant role in how these drugs are not only binding to cells, but being internalized as well.

Scavenger Receptor A

Scavenger receptors are a group of LDL recognition and internalization receptors. Roughly two decades ago, Bijsterbosch and coworkers detailed how a subset of scavenger receptor proteins were implicated in internalization of PS-ASOs into the endothelial cells of the liver. During the time of this publication there had been three types of receptor proteins to be analyzed; class A and B in mammalian systems, and class C which came from *Drosophila* [5]. Scavenger receptor A (SR-A) are trimer proteins that bind and internalize LDL, preferentially modified by acetylation (*in-vitro*) or by oxidization (*in-vivo*) [50]. The class A receptors (SR-A), specifically types AI and AII were well-known which allowed for a comprehensive investigation for these targets. Their work led to the conclusion that class A receptors were the primary internalization mechanism for PS-ASO uptake in the endothelial cells of the liver [5]. Further *in-vitro* and *in-vivo* studies were performed utilizing SR-A receptors for internalization, using co-administration of polyanionic compounds such as dextran sulfate [51]. These compounds were shown to be competitor polyanionic compounds that decreased PS-ASO saturation in tissue [51]. Following that claim, another group at Ionis Pharaceuticals published work that disputed the claim that SR-As were the primary scavenger receptors for PS-ASO internalization [52]. That work investigated the same PS-ASO sequence as previously reported by use of a SR-A murine knockout which demonstrated no discernable difference of PS-ASO internalization between the WT and SR-A KO mice leading them to speculate that SR-A has no substantial effect on the pharmacoactivity rates of the PS-ASO [52]. There was approximately a 50% decrease of high-affinity uptake mechanisms between the WT and KO cell lines, however, the authors speculated that other high affinity mechanisms

could be responsible for at least 50% of the remaining amount internalized. Currently, no new information regarding SR-A as a systemic clearance receptor for ASOs is available.

Stabilin-1 and Stabilin-2

Given the understanding of negatively charged PS-ASO interactions with heparin binding domains, it was of interest to determine if the class H scavenger receptors, Stabilin-1 and -2, played a role in PS-ASO systemic clearance and internalization. Both receptors bind to similar ligands such as heparin [53], advanced glycation end-products [54], and low density lipoproteins [55]. These proteins represent a good model based on their inability to have a strong binding affinity for phosphodiester-containing nucleic acids [56]. Stabilin receptors are type I receptors that contain seven Fas-1 domains, 20 epidermal growth factor (EGF) domains found in 4 clusters, an X-link domain, and a single transmembrane domain. Stabilin-1 and Stabilin-2 receptors are expressed in many tissues, particularly in key areas of PS-ASO accumulation in the body such as the liver, kidney, spleen adrenal cortex, macrophages, and lymph nodes [57, 58]. One key difference between both Stabilin receptors is that Stabilin-2 is expressed as two isoforms; the full-length 315-HARE and the 190-HARE which is derived from the 315-HARE by proteolytic cleavage and retains full protein functionality [59]. Another important distinction is that both receptors have distinct ligands (hyaluronan and several chondroitin sulfates for Stabilin-2 and Lactogen and SPARC for Stabilin-1) and different affinities for shared ligands (heparin, modified LDLs, etc).

Recent work has indicated that these receptors are mechanistically significant in PS-ASO cellular endocytosis and clearance [6]. This work has shown that by incorporation of 2'-O-MOE modifications with a PS backbone, ASOs are internalized at a much higher rate in recombinant cells over-expressing either Stabilin-1 or each of the Stabilin-2 isoforms than in the parental cell line which does not express either receptor. The content of PS-ASO in liver and spleen of Stabilin-2 knock-out mice is much lower than in the WT controls 3 days post-injection [6]. Using competition assays, similar chemically modified ASOs are internalized by these receptors, however, the 2'MOE second generation ASO has the highest rate of internalization by Stabilin receptors. PS-ASOs are able to bind to the 190-HARE by ionic interaction, allowing for internalization into the cell [6]. Once internalized via clathrin mediated endocytosis, the ASOs are transported via the endosomal pathway for degradation in the lysosome [6]. The increased rate of PS-ASO internalization in cell culture experiments also correlated to increased knock-down activity of the target gene of interest suggesting that once internalized, the ASO is escaping the endosomal pathway, leading to increased genetic silencing. Although recent work demonstrates how Stabilin receptors are implicated in the internalization of PS-ASO, a detailed understanding of the mechanism for ASO escape from endosomes for RNase H mediated mRNA degradation is lacking.

Conclusion

Recent and continued work of chemical modifications has allowed for progress of ASO drugs for suppressing gene expression for therapeutic purposes. Currently, over 35 of these drugs are either in clinical trials, or have been approved by the FDA for use against a variety of diseases. Although much work has been done to increase the selectivity and potency of these drugs, while decreasing side effects and toxicity, some of the basic mechanisms for PS-ASO internalization and activity against their specific targets is just beginning to be understood. More mechanisitic work in the basic science is required to determine the trafficking and activation of productive ASOs in primary cells. Despite these obstacles, the field of antisense oligonucleotide therapy has proven to be a promising one for current and future treatment of genetic disorders.

Conflicting interests

The authors have declared that no conflict of interests exist.

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Author Contributions

CMM and ENH wrote the paper, CMM designed figures.

Abbreviations

ASO: antisense oligonucleotide; DMD: Duchenne's muscular dystrophy; FH: familial hypercholesterolemia; hnRNA: heteronuclear ribonucleic acid; MOE: methoxyethyl; MP: methylphosphonate; PO: phosphodiester; PS: phosophorothioate; SMA: spinal muscular atrophy; SMN: survival motor neuron; SR-A: scavenger receptor class A.

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