Designing RNase H1 "Gapmer" Antisense Oligonucleotides

Introduction

Antisense oligonucleotides (ASOs) have been used for over three decades to modulate the expression of precise RNAs both *in vivo* and *in vitro* [1]. ASOs can be designed to function by two mechanisms: by activating RNase H1 to cleave an RNA target, or by sterically blocking the access of regulatory proteins or nucleic acids to an RNA (Figure 1). The RNase H class of endonucleases act primarily in the nucleus, although it has been shown that RNase H1 is also active in the cytoplasm [2–4]. For RNase H1 degradative ASOs, the RNase H1 endonuclease specifically cleaves RNA only when it is hybridized as a heteroduplex with DNA (in this case, DNA residues which are a part of the ASO). Once cleavage of an RNA molecule occurs, the ASO can dissociate and be recycled multiple times to cleave new RNA molecules [5,6]. In contrast, a steric blocking ASO (SBO) is chemically modified so that it does not form a substrate for RNase H1 when hybridized to the RNA target, typically by using 2′-modified RNA residues throughout the ASO (excluding DNA). Instead, an SBO molecule will bind tightly to a single RNA molecule with no turn-over, obstructing the ability of other biomolecules to functionally bind at that site [7–11]. Here, the focus will be on strategies for designing RNase H1-mediated degradative ASOs.

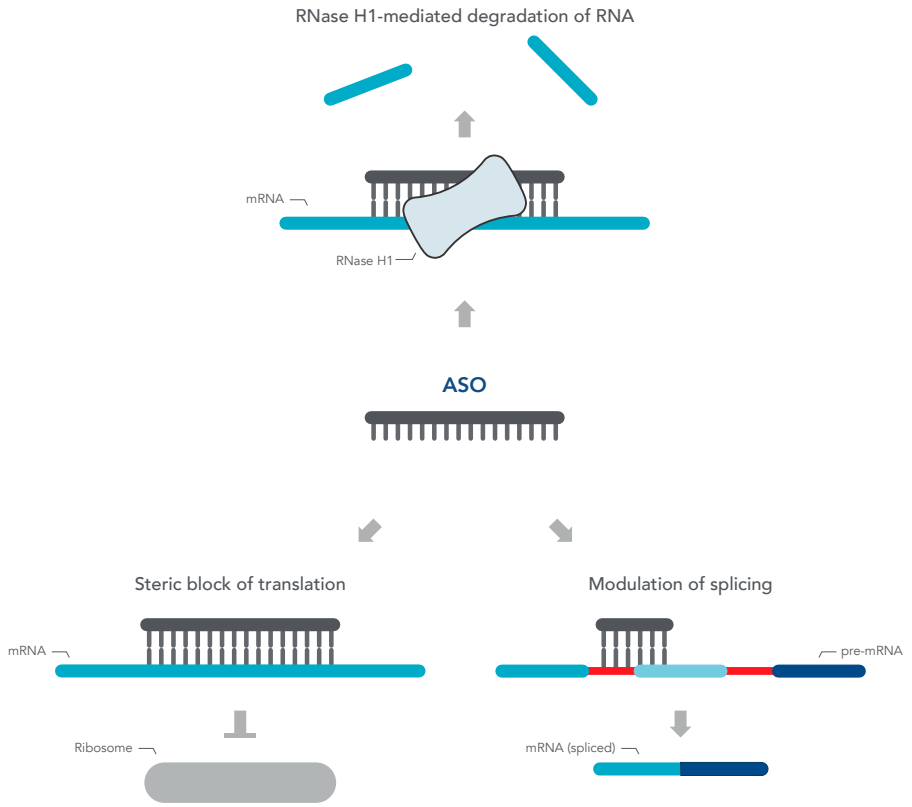

Figure 1. Antisense oligonucleotide (ASO) mechanisms of action. ASOs can modulate gene expression through RNase H1-mediated recognition of an RNA:DNA hybrid followed by degradation of the target RNA. Alternatively, ASOs can bind to an RNA functional domain to sterically block proteins or other nucleic acids from binding at that site.

Ideally, any researcher should be able to choose a select RNA target sequence of interest, order the synthesis of their designer ASO, introduce it into their system of choice, and measure the effects without toxicity or off-target activity. However, it is not always this simple in practice. The selection of optimal sequence and chemical modifications for the antisense oligonucleotide is crucial to the success of the experiment [1]. An ideal ASO candidate should:

- Be easy to synthesize and purify
- Not disrupt normal Watson-Crick base-pairing
- Activate RNase H1-degradation pathways
- Have a half-life appropriate for the application
- Be able to productively internalize into cells and localize to the desired intracellular compartments
- Have effective *in vivo* circulation time
- Not induce any unintended sequence-independent or off-target biologic effects
- Not be physiologically toxic

Considerations for selecting chemical modifications

Oligonucleotides are polymers comprised of nucleobases that are linked together by a sugar-phosphate backbone. Chemical modifications can be introduced into the phosphate backbone, the sugar moiety or the nucleobase itself, altering the biophysical properties of the oligonucleotide with the intent to enhance their utility as antisense drugs. Additionally, targeting ligands can be appended to an ASO to assist with tissue-specific delivery. Strategically incorporating chemical modifications into an ASO can help increase the nuclease resistance, modify the binding affinity to the target, improve productive intracellular uptake, increase *in vivo* circulation time and reduce immunostimulatory responses [1,10,12,13].

Natural phosphodiester oligonucleotides are quickly digested by nucleases both in serum and in the intracellular environment [14]. Although multiple endo- and exonucleases exist *in vivo* [15], it appears that the bulk of biologically significant nucleolytic activity in serum is derived from a 3′ exonuclease [16]. Within the cell, both 3′ and 5′ exonuclease activity is present [17,18]. While unmodified oligonucleotides have very brief intracellular half-lives, introducing chemical modifications into an oligonucleotide can extend the half-life on the order of days or weeks [13,15,19].

The binding affinity of an ASO to the target RNA can impact the efficacy and selectivity of the ASO [20]. Intracellular RNAs can be highly structured which may impede the accessibility of an RNA target site to an ASO [21–24]. This can be especially problematic as RNA:RNA interactions (i.e., inherent RNA secondary structure or RNA hybridized with other intracellular RNAs) have a higher affinity to bind than DNA:RNA interactions (i.e., an unmodified ASO with its RNA target). Increasing the binding affinity of the ASO with chemical modifications can help the ASO invade target RNA secondary structure and improve ASO potency [20]. Once cleavage of the target RNA occurs, the ASO molecule is capable of turning-over multiple times by disassociating from the cleaved target and hybridizing to a new RNA target [5]. If the binding affinity of an ASO to the cleaved RNA target is too high, the disassociation rate is reduced which slows the catalytic ability of the ASO and decreases the potency [6]. Thus, fine tuning the binding affinity of the ASO to optimize both the association and disassociation rate can be instrumental in identifying a potent RNase H1 ASO [6]. Having an ASO binding affinity that is higher than required for optimal RNase H1 cleavage of an on-target RNA (maximal potency) can also decrease ASO selectivity, as mismatches to off-targets are more tolerated [23,25–28]. In fact, if a mismatched sequence (off-target) reduces the ASO binding affinity so that it falls into

the optimal range for multiple ASO turn-over or if the mismatched site is in a more accessible region than the on-target site, then the mismatched site can have the same or even higher activity than the on-target site [27]. Therefore, it is prudent not to increase the ASO binding affinity beyond what is required for maximal potency, as this may decrease both on-target potency as well as increase off-target potency [26,28].

The way by which an ASO binds to proteins is a key determinate of the fate of the ASO. Chemical modifications can alter the serum, cell membrane, and intracellular protein binding profile of an ASO, affecting both trafficking and uptake [13,29–34]. DNA with a native phosphodiester backbone is rapidly cleared by the renal system without significant peripheral tissue absorption, limiting its utility for *in vivo* use [13]. Chemically modifying the DNA in a way that increases affinity towards serum proteins (e.g., phosphorothioate linkages, discussed below) is instrumental in increasing the ASO circulation time to a level that is appropriate for tissue exposure [32]. Chemical modifications can also increase the ASO binding affinity towards plasma membrane proteins which then facilitate ASO internalization into the cell [34]. Depending on which cell surface protein the ASO binds to will determine whether the subcellular distribution of the ASO is productive (i.e., conducive for ASO functionality) or non-productive [32,34]. Binding to intracellular proteins can further modulate ASO intracellular distribution and may also increase or decrease ASO activity [35–38]. While the ability to non-specifically bind proteins is clearly beneficial for productive delivery of ASOs, this characteristic can also introduce undesired toxicities. For example, some chemically modified ASOs have been reported to bind to paraspeckle proteins, mislocalizing the ASO to the nucleoli and causing nucleolar stress leading to apoptosis [39–41]. It has also been reported that promiscuous protein binding by some gapmer ASOs can result in hepatoxicity, which can be mitigated by strategically introducing chemically modified sugars, nucleobases or abasic nucleotides into the DNA "gap" to reduce protein binding [39,42,43]. Chemically modified ASOs can also induce acute neurotoxicity which is presumably a result of the ASO binding to proteins, such as cell surface receptors, and interfering with normal neuronal signaling [44,45]. ASOs can also induce a proinflammatory response by interacting with innate immunity proteins such as toll-like receptors (TLRs), triggering the expression of cytokines [46]. Importantly, unmodified CpG motifs in ASOs are potent stimulators of TLR9 [47–49]. Chemically modifying the "C" nucleobase to 5-methyl-dC in all CpG motifs in an ASO sequence (or avoiding these motifs altogether) has become a routine strategy to help mitigate an unwanted TLR9-dependent innate immune response *in vivo* [49,50]. However, non-CpG containing ASOs can still trigger a TLR9-independent proinflammatory response [49]. Finding a balance in chemical modification patterns that maximize the pros of protein binding (improving oligo trafficking and uptake) and minimize the cons of protein binding (toxicity and proinflammatory reactions) is key to designing a beneficial ASO.

Chemical modification	Common name	Class of modification	IDT product	Activates RNase H1	Binding affinity effects	Comments
Phosphorothioate	PS	Backbone	YES	YES	Decreases	Binds non-specifically to proteins, exists as two stereoisomers
2'-*O*-Methyl	2'OMe	Sugar	YES	NO	Increases	Natural RNA modification
2'-*O*-Methoxyethyl	2'MOE	Sugar	YES	NO	Increases	Reduces protein binding, decreased toxicity
Locked nucleic acids	Affinity Plus	Sugar	YES	NO	Increases	High binding affinity at the cost of increased toxicity
(S)-Constrained ethyl	cEt	Sugar	NO	NO	Increases	High binding affinity at the cost of increased toxicity
5-Methylcytosine	5-MedC	Base	YES	YES	Increases	Natural RNA modification; prevents activation of TLR9 in CpG motifs

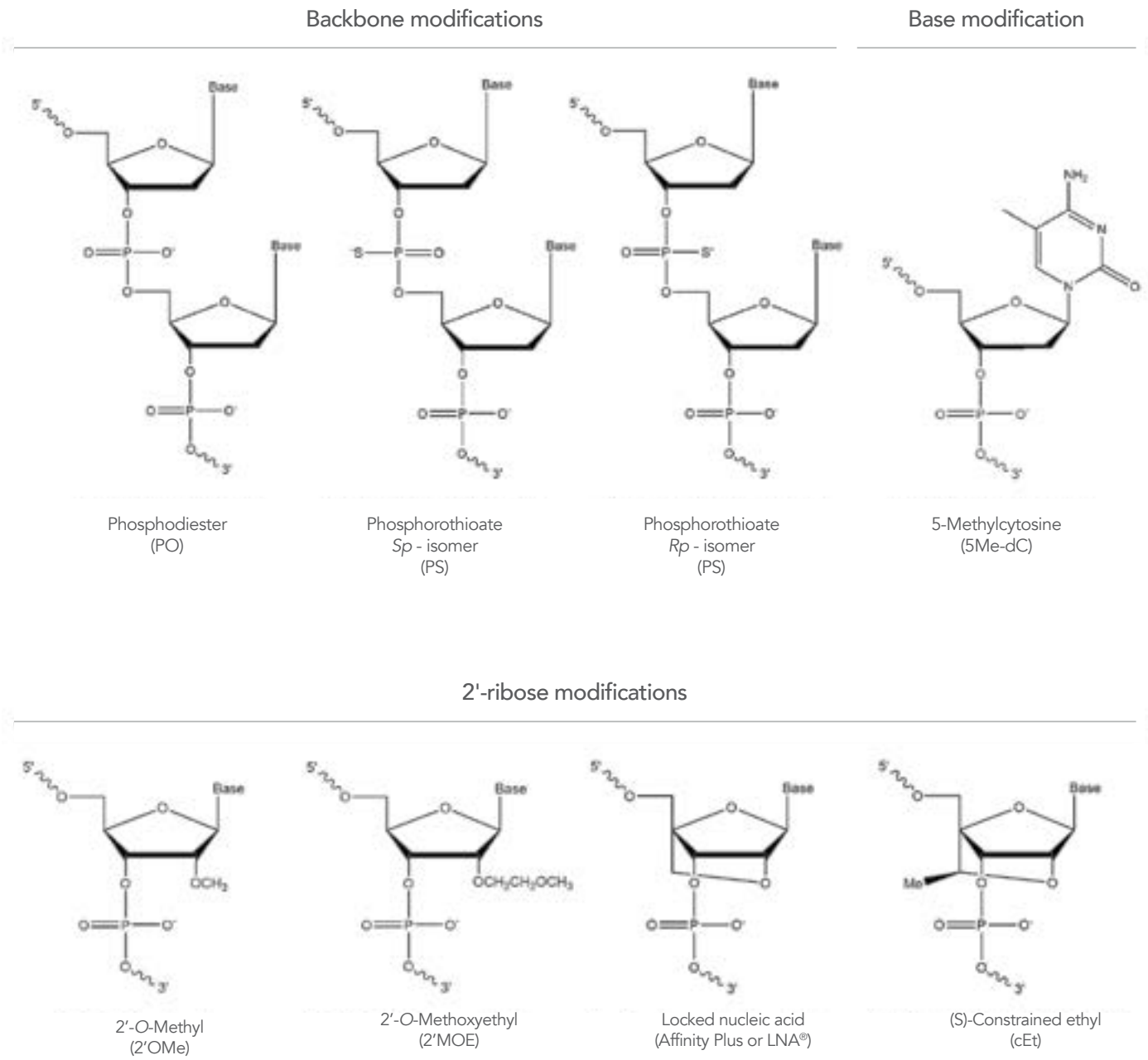

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Figure 2. Chemical modifications commonly used in antisense oligonucleotides.

Backbone modifications

The most widely used modification in ASOs is the phosphorothioate (PS) linkage (Figure 2), where a non-bridging oxygen on the phosphate backbone of a nucleic acid is substituted with sulfur (see Figure 2). DNA oligonucleotides containing PS linkages were the "first generation" ASOs as they are one of the few chemical modifications that will support RNase H1 activity [51]. One of the greatest benefits of incorporating PS linkages into an ASO backbone is that they improve nuclease resistance, which increases the ASO half-life and leads to higher bioavailability [30,52,53]. Disadvantageously, PS linkages additively weaken the binding affinity of an ASO to its RNA target with a ΔT_m (melting temperature) of ~–1°C to –0.5°C per linkage, which can effectively reduce the potency of the ASO [20,27,30,54–57]. Due to the polyanionic nature of the PS backbone, PS ASOs are known to bind non-specifically with proteins (see Considerations for selecting chemical modifications). ASO length can influence protein binding potential, with longer ASOs having increased binding affinity to proteins than shorter ASOs [38,58,59]. The extent of PS modification is also a determining factor for ASO protein binding, and it has been shown that a minimum of 10 PS linkages are required to support sufficient protein interactions [1,38,60]. Non-specific protein binding can improve cellular uptake and intracellular trafficking of an ASO, but it can also cause toxicity depending on the class of proteins bound [13,29,30,32,34,39,40,46,47,61,62]. For example, PS linkages can enhance binding to multiple serum proteins (e.g., albumin), which reduces renal filtration and increases the ASO circulation half-life time to 1–2 hours which is essential to support tissue adsorption [32]. PS modified ASOs can also bind to the extra-cellular exposed domains of several cell membrane proteins to facilitate ASO internalization [34]. Once internalized, a PS ASO can interact with ~80 different intracellular proteins found in the cytoplasm, nucleoplasm and the nucleolus, impacting the intracellular distribution of the ASO [29,32,36,37,62–65]. Providing the ability to bind proteins in a manner that is optimal for ASO uptake and productive intracellular distribution makes the PS moiety the most important determinant of ASO fate after all routes of administration [32]. PS ASOs can also bind to proteins which result in toxicological effects; for example, they can bind to complement Factor H which can activate the alternative complement cascade and cause inflammation, or they can bind to immune receptors which can result in a toxic proinflammatory response [1,66]. PS linkages have two stereoisomers, designated *R*p and *S*p, which can have different pharmacologic impacts on the ASO. The *Rp* diastereomer has been characterized to have improved RNase H1 activation and increased binding affinity towards an RNA target when compared to its *Sp* counterpart [67]. Alternatively, the *Sp* diastereomer confers better nuclease resistance than the *Rp* isomer [68,69]. During standard oligonucleotide synthesis, the *R*p and *S*p diastereomers are introduced at random in a ~1:1 mixture, creating a chiral center at each internucleotide linkage and resulting in 2^n (*n* = the number of linkages) possible individual molecules. For example, the 2013 FDA-approved drug mipomersen, developed to treat familial hypercholesteremia, is a 20mer ASO that contains 19 PS linkages [70]. In this drug alone there is the potential for 2^{19} (or 524,288) unique drug molecules, each with the potential for distinct pharmacologic properties. It has been reported that controlling the position of each stereoisomer (*R*p vs *S*p) during synthesis can improve ASO efficacy by increasing nuclease resistance and promoting RNase H1 cleavage of the target RNA [19,68]. Recently, Wave Life Sciences Ltd. demonstrated that strategically positioning stereopure PS isomers to maximize the beneficial properties of each isomer (e.g., placing an *S*p PS at the 3'end to block exonuclease degradation and using an "*SSR*" motif in the "gap" to increase RNase H1 activity) into a 2'MOE or a 2'MOE/2'OMe asymmetric wing gapmer ASO (see "Gapmer" designs) significantly improved potency over their stereorandom gapmer ASO counterparts in human ALS motor neurons [71]. Other reports suggest that there is no achievement advantage to producing stereopure PS ASOs with respect to ASO activity or toxicity [72]. In any case, controlling the chirality of an ASO to provide a better characterized and defined population of ASO molecules is an attractive strategy from a safety standpoint. Currently, stereopure PS ASOs are not commercially available, but strides are being made to simplify synthesis and purification of these oligonucleotides.

"Gapmer" designs

Although early work using DNA PS ASOs was very encouraging, it is now clear that some of the most exciting results were not from an antisense mechanism but were due to sequence independent biological effects caused by phosphorothioated DNA (described above) [61,73,74]. The stereorandom mixture of PS isomers in these "first generation" ASOs reduces the binding affinity of the ASO and are not completely resistant to nucleases. This results in the necessity for repeated administration at high doses to elicit a biological effect which can lead to undesired toxicity [12,61,75]. To overcome these limitations, chemical modifications with higher binding affinities and increased metabolic stability are required. Modifications to the 2′ ribose can satisfy these criteria; however, they can also negatively impact the ability of an ASO to trigger RNase H1 mediated degradation of RNA following hybrid formation [12,76]. This can be circumvented by using a chimeric "gapmer" design, where a stretch of bases that are substrates for RNase H1 (e.g., DNA with PS linkages) termed the "gap" are flanked by ribose modifications that increase the binding affinity and nuclease resistance to improve ASO potency [12]. The human RNase H1 enzyme has optimal RNA cleavage activity when the DNA gap in an ASO is 8–10 residues [1,77–79]. A common strategy for ASO gapmer design is a 5-10-5 design, whereby a stretch of 5 contiguous high binding affinity modifications surrounds a central stretch of 10 DNA PS (substrates for RNase H1) on both the 5′- and 3′-ends (see example below, where "M" = 2′ modified RNA, "D" = DNA and "*" = phosphorothioate linkage). Adjustments to the length and/or chemical modification placement in a gapmer ASO may be required for optimal ASO functionality to account for inherent differences in chemical modifications (e.g., binding affinity increases), sequence context (e.g., GC content), delivery modality (e.g., naked "gymnotic" delivery) and selectivity (e.g., SNP discrimination or reduction in off-target effects).

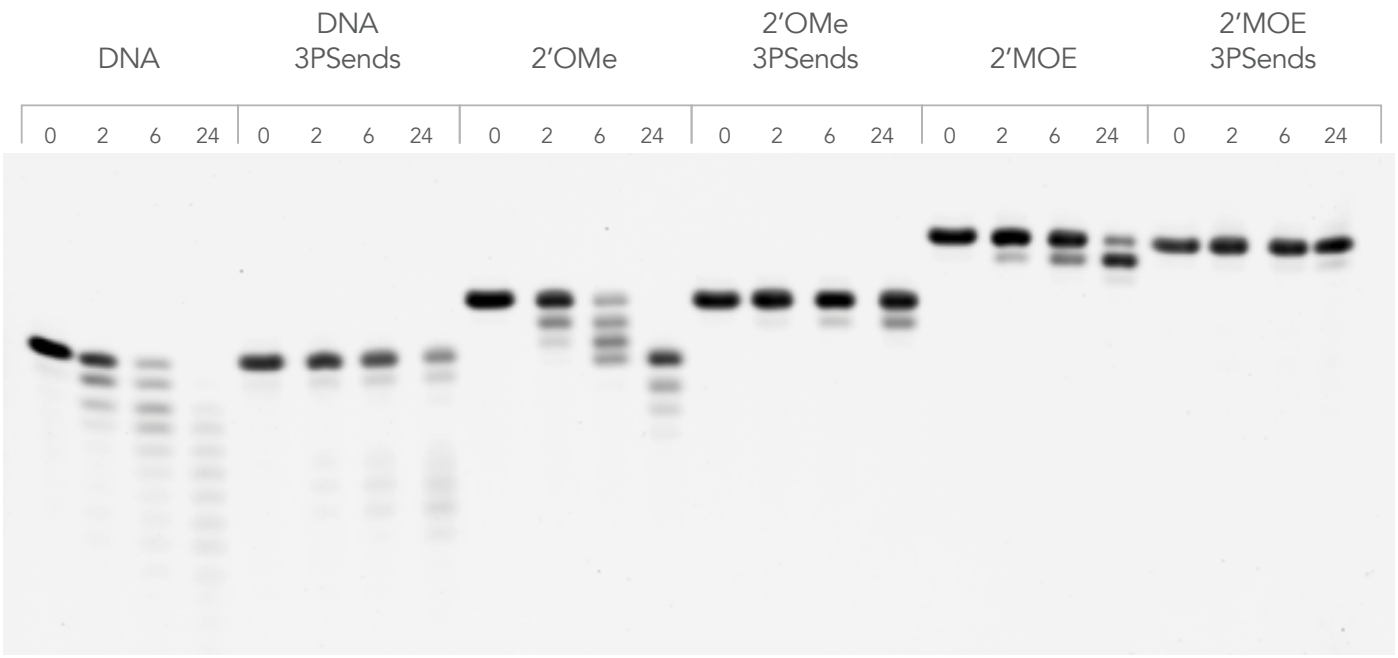
M*M*M*M*M*D*D*D*D*D*D*D*D*D*D*M*M*M*M*M

2′-Ribose modifications

A common strategy to enhance the pharmacological properties of a PS ASO is to chemically modify the 2′-hydroxyl (2′-OH) of the ribose, which is typically done on each end of an ASO to create a "gapmer" (see **"Gapmer" designs**). Modifying the 2′- position can increase the metabolic stability of an oligonucleotide by stabilizing it against nuclease attack in both serum and the intracellular environment, especially against endonucleases that may be present in the cell [78,80–82]. The proximity of the 2′-ribose modification to the 3′-phosphate on the backbone likely confers nuclease resistance by impeding the ability of a nuclease to cleave at the modified site [20]. Importantly, this class of modifications can influence the conformation of the ribose ring to favor the C3′-endo sugar pucker, resulting in an increase in RNA-binding affinity [83]. Incorporating 2′-ribose modifications into a PS ASO can also alter the protein-binding profile of the ASO and modulate its activity [38,63,64,84]. For example, bound proteins can enhance ASO activity by facilitating nuclear uptake and retention, or they can inhibit ASO activity by competing with RNase H1 binding, preventing RNase H1 cleavage, or mislocalizing the ASO into non-productive subcellular compartments [36–38,63,85]. There seems to be a correlation with the hydrophobicity of the 2′-ribose modification and the affinity by which a protein is bound, whereby the more hydrophobic modifications (e.g., locked nucleic acids and cEt, discussed below) tend to bind more tightly and promiscuously with proteins than more hydrophilic modifications (e.g., 2′MOE and 2′OMe, discussed below), resulting in as much as a 10-fold difference in protein binding affinity [37,38,63]. Optimally selecting and positioning the various 2′-ribose modifications in combination with a PS backbone in an ASO has been critical to the development of safe and efficacious restorative agents [1].

One of the earliest 2′-ribose modification used in antisense applications is 2′-*O*-methyl RNA (2′OMe), a natural RNA analogue that is widely used in a variety of nucleic acid reagents including RNA interference and CRISPR genome editing (Figure 2) [86,87]. Like other 2′-ribose modifications, 2′OMe increases the nuclease resistance of an ASO [80,81,88]. In the context of a phosphodiester backbone, 2′OMe are resistant to endonucleases but are still susceptible to degradation by exonucleases, highlighting the importance of combining this modification with PS linkages to increase the half-life [81]. The 2′OMe modification also increases the binding affinity to an RNA target with a ΔT_m of ~0.7–1.0°C per substitution when compared to DNA of the same sequence, off-setting the decrease in binding affinity contributed by PS linkages [27,54,78,80,81,87,89]. Introducing 2′OMe modifications into oligonucleotides is also an attractive strategy to help avoid undesired immune stimulation that can be triggered by exogenous nucleic acids [90].

One of the most widely utilized 2′-ribose modifications in antisense applications is 2′-*O*-methoxyethly (2′MOE) (Figure 2). This modification is extensively being used in ASO studies, and three RNase H1 gapmer ASOs have been FDA-approved to treat homozygous familial hypercholesterolemia (Mipomersen, 2013), hereditary transthyretin-mediated amyloidosis (Inotersen, 2018), and familial chylomicronemia syndrome (Volanesorsen, 2019) [1]. ASOs modified with 2′MOE have improved pharmacological properties relative to 2′OMe ASOs which can be attributed to the bulkiness of the methoxyethyl group [78,83]. For example, the location of the second oxygen atom limits rotation around the ethylene linker and assumes a gauche orientation, resulting in extensive hydration and steric hindrance near the 3′-phosphate and further protecting this bond from nuclease attack [57,83,91]. This increase in nuclease resistance is evident when comparing the exonuclease stability of a 2′MOE with a 2′OMe modified oligonucleotide after incubation in human serum or mouse liver cell extracts (Figure 3). The enhanced nuclease resistance of 2′MOE ASOs increases the tissue adsorption half-life from 48 hours to up to 4 weeks, allowing for a weekly to monthly drug administration schedule [1]. The constrained rotation of the methoxyethyl side chain also reduces conformational flexibility to a greater extent, favoring the C3′-endo sugar pucker and causing a greater increase in RNA-binding affinity relative to 2′OMe (ΔT_m of ~0.9–2°C per modification) [12,57,83]. In general, non-specific protein binding tends to be reduced with 2′MOE and 2′OMe modified ASOs when compared with other more hydrophobic 2′-functional groups (e.g., locked nucleic acid and cEt, discussed below), which may negatively impact antisense activity by affecting uptake efficiency and/or ASO intracellular localization [37,38,63]. Loss in plasma protein binding affinity of a PS ASO containing 2′MOE modifications can also impact *in vivo* biodistribution to favor delivery to the kidney over liver [92]. Advantageously, lowering non-specific protein binding affinity may be less inhibitory to the complement system and may mitigate proinflammatory responses, reducing overall ASO toxicities [1,84]. The pharmacologic improvements from the 2′MOE modification have resulted in substantial improvement in ASO activity, with a >20-fold increase in potency when compared with PS ASOs in cell culture [78]. 2′MOE ASOs have also shown robust pharmacokinetic and pharmacodynamic activity in animal models [92–99].



DNA	T C A A C A T C A G T C T G A T A A G C T A
DNA 3PSends	T*C*A*A C A T C A G T C T G A T A A G*C*T*A
2′OMe	U C A A C A U C A G U C U G A U A A G C U A
2′OMe 3PSends	U*C*A*A C A U C A G U C U G A U A A G*C*U*A
2′MOE	T C A A C A T C A G T C T G A T A A G C T A
2′MOE 3PSends	T*C*A*A C A T C A G T C T G A T A A G*C*T*A

Black: DNA Red: 2′OMe Blue: 2′MOE *: Phosphorothioate bond

Figure 3. Stability of chemically modified oligonucleotides against exonuclease degradation. Oligonucleotides were incubated at 10 µM in 80% human serum at 37°C and 5% CO_2 for the designated timepoints. Samples were run on a 7M urea 18% denaturing polyacrylamide gel and stained with GelStar™ Nucleic Acid Gel Stain (Lonza).

A more recent advancement in nucleic acid medicinal chemistry is the development of constrained nucleic acids. These nucleotides are covalently modified to lock the ribose into the C3′-endo sugar conformation which enhances base stacking and phosphate backbone pre-organization, creating chemical modifications that have the greatest increase in hybridization affinity to-date [100,101]. Locked nucleic acids (Affinity Plus or LNA® (Qiagen), see Figure 2) are conformationally constrained with a methylene bridge that links the 2′ oxygen and 4′ carbon on the ribose ring to dramatically increase the binding affinity to an unprecedented level (ΔT_m of ~2.0–9.6°C per modification with an RNA target) [27,79,102–109]. In fact, the substantial increase in binding affinity afforded by locked nucleic acids enables shorter ASOs (~15–16mers, typically as 3-10-3 gapmers) to function with high potency, outperforming their 2′MOE counterparts [79,110–112]. Similar to many other 2′-OH ribose modifications, locked nucleic acids also improve resistance to nucleases [79,113–115]. Locked nucleic acid oligonucleotides are resistant to endonucleases present in liver cell extracts, and they slow down degradation from 3′-exonucleases present in human serum when 1–3 residues are positioned at the 3′ end of a phosphodiester oligonucleotide (data not shown) [81]. Another well-characterized bicyclic sugar is (S)-constrained ethyl (cEt) (Figure 2), a methylated analogue of locked nucleic acids that demonstrates dramatic increases in RNA binding affinity, but to a lesser degree than their locked nucleic acids counterparts (ΔT_m of ~2.0°C per modification) [1,101,116]. cEt modified oligonucleotides have been reported to have enhanced nuclease resistance to snake venom phosphodiesterase (SVPD) when compared with locked

nucleic acid or 2'MOE oligonucleotides [101]. The hydrophobic constrained nucleic acids (both cEt and locked nucleic acids) have been reported to non-specifically bind proteins with higher affinity than the more hydrophilic 2'-ribose modifications (such as 2'OMe or 2'MOE) which can increase uptake efficiency, alter subcellular distribution, and modulate (increase or decrease) RNase H1 activity [34,37,38,63,64]. In fact, short locked nucleic acid ASOs are a very effective chemical modification strategy to use for efficient naked uptake (gymnosis) into cells, which may be an effect of their shortened length (more permeable) or their increased binding affinity to serum proteins [117]. The ability for efficient naked uptake of a locked nucleic acid ASO is beneficial when screening ASOs for potency in cell culture, as gymnotic delivery has been shown to predict *in vivo* ASO activity more accurately than when using transfection reagents [110,117]. As with other chemical modifications, there is always a risk of sequence-dependent (e.g., degradation of closely related sequences) and sequence-independent (e.g., non-specific protein binding) associated toxicities. Increased frequency and severity of cytotoxicity, hepatotoxicity and neurotoxicity are especially evident with constrained nucleic acid ASOs. Multiple reports have attributed the increased incidence of toxicity from these modifications to RNase H1-dependent sequence-specific off-target effects [118–122]. Other reports demonstrate that increased toxicological liability from constrained nucleic acid ASOs can also be a result of non-specific binding to proteins [39,43,123,124]. *In silico* screening methods to avoid off-targets to both mature RNAs and introns or avoid motifs associated with non-specific protein binding, as well as *in vitro* cellular assays to identify toxic ASO sequences is necessary and becoming commonplace in developing constrained nucleic acid restoratives [39,122,123,125–128].

Nucleobase modifications

In addition to backbone and ribose modifications used to enhance ASO activity, the nucleobase can also be chemically altered. While many nucleobase modifications have been identified, the most beneficial for ASO applications is at the C5 hydrogen of deoxycytidine (dC) (**Figure 2**). Substituting the hydrogen with a methyl group (5-methylcytosine or 5Me-dC) is an attractive strategy for ASOs as this modification does not interfere with RNase H activity and therefore can be included into the PS DNA "gap" of a gapmer ASO. Inclusion of 5Me-dC into a DNA oligonucleotide has been reported to increase the thermal stability by ~0.5°C per substitution when duplexed with an RNA target as a result of enhanced base stacking [54]. Importantly, substituting dC with 5Me-dC in CpG motifs in an ASO reduces immunostimulatory effects induced by toll-like receptor 9 (TLR9) recognition [49,50,129]. Mitigating immune responses is particularly critical for *in vivo* applications; therefore, substituting all dC bases in the ASO "gap" with 5Me-dC is now a routine design strategy to increase the effectiveness of the ASO. While other nucleobase modifications have been reported to reduce the hepatoxicity of gapmer ASOs, many of these modifications are still in the infancy of their characterization and are not widely commercially available [43].

Design considerations: ASOs

When designing ASOs, there are numerous design options and parameters to consider that can impact ASO activity. For most applications, the overall goals for ASO activity are to maximize on-target potency and productive uptake efficiency while minimizing off-target effects and toxicity potential. *In silico* analysis of the biophysical properties of both the ASO sequence and the target RNA and how they interact can help eliminate ASO sites that are predicted to be suboptimal and reduce the screening effort [1]. While selecting potent ASO sites isn't a perfect process, these guidelines will help assist with enriching for sites that achieve the overall goals with the caveat that some "good" ASO sites may also be eliminated.

Biomolecular interactions within an ASO sequence itself can impact ASO efficacy [130]. ASOs that form stable hairpins in physiological conditions can impede ASO potency by a variety of mechanisms (Lennox, internal observation) [131]. For example, a stable hairpin can maintain stem formation intracellularly and restrict the ability of the ASO to fully anneal to its target RNA in linear form. Østergaard *et al.* empirically demonstrated that an increase in hairpin stem stability in a molecular beacon ASO strongly correlates with decreased ASO potency [131]. Conversely, Lisowiec-Wąchnicka *et al.* reported that adding additional DNA bases to form hairpins on the 5'- or 3'-end of an ASO can maintain or often improve potency of locked nucleic acid ASO gapmers; however, the effect also correlated with hairpin stem stability with the least thermodynamically stable hairpins performing better [132]. Hairpin formation and stability can be of greater concern with oligonucleotides containing high binding affinity modifications. For example, locked nucleic acid:locked nucleic acid base pairs anneal with much stronger affinity than locked nucleic acid:RNA or locked nucleic acid:DNA base pairs [133]. Thus, if the hairpin stem forms with locked nucleic acid:locked nucleic acid base pairing, then the hairpin structure of the ASO will be thermodynamically preferred over the ASO annealing to cognate RNA sequences. Of note, if an ASO forms a natural hairpin, its reverse complement RNA target site may also form the reverse hairpin and restrict ASO access to the sequence. Additionally, it has also been demonstrated that hairpin-forming ASO gapmers bind proteins to a lesser degree [131]. While this could reduce the potential for ASO toxicity, it can also impact naked uptake efficiency that relies on non-specific protein binding (see **Considerations for selecting chemical modifications**). In fact, the potency of ASOs containing stable hairpins was even more negatively impacted when using gymnotic delivery (no knockdown) as opposed to cationic lipid delivery (IC_{50} = 20 nM), while the ASOs without hairpins were minimally impacted by delivery method in MHT cells [131]. Another biomolecular interaction to be cautious of when designing ASOs is the potential for ASO self-dimerization. This self-interaction can be thermodynamically favorable to the point that it interferes with antisense hybridization, especially when high binding affinity modifications anneal to each other (similar to hairpins). While there haven't been systematic studies published that demonstrate that ASO self-dimerization negatively affects ASO potency, this parameter should still be considered when designing ASOs.

The length of an ASO can also impact functionality and it is generally accepted that chemically modified ASO gapmers (e.g., Affinity Plus or 2'MOE) between 16–20 nucleotides (nts) long are an ideal range for optimal potency and selectivity [1]. When ASOs are longer, a "length penalty" has been observed in which uptake efficiency, potency, and selectivity is negatively impacted [110,134]. *In vitro* experiments have demonstrated that shorter gapmer ASOs (12–16 nt locked nucleic acids) are more favorable for efficient gymnotic uptake than longer gapmer ASOs [110]. This has also been observed *in vitro* with 2'MOE ASO gapmers, where every additional base addition over an 18mer incrementally decreases gymnotic delivery efficiency (Lennox, internal observation). Continuing to lengthen an ASO will eventually result in exceeding the "threshold affinity", whereby an ASO reaches the optimal binding affinity for maximal potency and any further increases in affinity can result in a decrease in potency (a reduced "off-rate") or decrease in selectivity (increased mismatch tolerance) [6,110]. Longer ASOs (>20–22 nt) are also more prone to self-dimerize or form hairpin structures that impede hybridization to their target RNA [1,135]. Conversely, shortening an ASO can also negatively impact ASO uptake efficiency, potency and selectivity. Cellular uptake is enhanced

with the addition of PS linkages, and there is a minimum requirement of 10 PS linkages in an ASO for productive uptake [1,38,60]. ASOs that don't meet this requirement (e.g., if they are too short) will not bind proteins with high enough affinity for cellular internalization. Potency can also be negatively affected with ASOs that are too short, as the binding affinity may not be high enough for efficient target binding and cleavage [6,20]. Selectivity to the transcriptome (all other RNAs expressed in the cell) is inversely correlated with length up to ~18–20 nt; i.e., the shorter the ASO, the more likely it will bind to another RNA in the cell (off-target) [1,26]. Hagedorn *et al.* examined transcriptome-specificity as a function of ASO length for ASOs ranging between 12–20 nt [26]. They demonstrated that almost none of the ~25,000 12 nt ASO sequences they analyzed are unique to their target RNA and are on-average predicted to have hundreds of perfectly matched off-targets. However, as the ASO is lengthened the transcriptome-specificity is increased. For example, about half of all ~25,000 16 nt ASO sequences analyzed were computationally predicted to be unique to their target RNA, while almost all of the ~25,000 20 nt ASOs were unique to the targeted RNA. Another study by Yasuhara *et al.* empirically demonstrated that lengthening a 14 nt locked nucleic acid gapmer ASO to 18 nts reduced off-target degradation [136]. In summary, it is found that chemically modified ASO gapmers that are 16–20 nt in length often fall in the ideal range for uptake efficiency, binding affinity and selectivity.

Another parameter to consider when designing ASOs is GC%, which represents the percentage of bases in an ASO that are either guanine (G) or cytosine (C). The GC content of an oligonucleotide is correlated with its melting temperature (T_m), as G:C base pairing is more thermodynamically stable than adenine:thymine (A:T) base pairing [137]. Therefore, increasing the number of G:C pairs in an ASO will also increase the hybridization affinity. This is a generalization, as the T_m of an oligonucleotide is impacted by the type and number of chemical modifications as well as nearest neighbor base stacking influences. While there is not a perfectly defined rule set for the ideal GC% in ASO design, there are estimations that can be made to enrich for ASO sequences that fall within the desired binding affinity range [138]. In general, when designing 16–20 nt ASO gapmers containing high binding affinity modifications (i.e., 5-10-5 2'MOE or 3-10-3 locked nucleic acid gapmers), 40–60% GC content typically encompasses the range of desired ASO binding affinities for optimal potency. Limiting the GC content in an ASO can mitigate the risk of the ASO forming strong secondary structure or self-dimers which can impact the functionality of the ASO. The 40-60% GC range also limits having an excess of Gs in the ASO sequence, which have been shown to cause increased cytotoxicity and/or cellular antiproliferative responses by a non-antisense mechanism [122,139–144]. This can be particularly problematic when G-tetrads (e.g., runs of 4 Gs) are present, as they can stack into square planar arrangements when stabilized by monovalent cations to form higher order G-quadruplex structures [145–147]. Both unstructured G-rich ASOs as well as G-quadruplexes have been reported to bind to certain surface or intracellular proteins (e.g., eEIFA, nucleolin, STAT3), which in turn can cause these undesired non-antisense mediated biologic effects [142,144,148–153].

Design considerations: Target RNAs

RNAs are complexly organized with multiple intramolecular and intermolecular interactions. An RNA sequence can fold back on itself to form highly structured intramolecular hairpins that can also form tertiary structures, or it can bind to other intracellular nucleic acids or proteins with high affinity [154–157]. One of the primary factors in determining ASO activity is accessibility to the target RNA, and any of these interactions can impede or obstruct ASO-RNA hybridization [1,22–24]. While intracellular RNA secondary structure is difficult to predict *in silico*, simulating RNA folding using computational algorithms such as UNAFold can indicate possible RNA structure formation and lead to improved ASO site selection (Lennox, internal observation) [158]. Chemical probing has become a methodology used to empirically define the secondary structure of select RNA transcripts, while methods have also been developed for high-throughput RNA structure analysis on a transcriptome-wide level [159–163]. Predicting how other intracellular nucleic acids bind to the target RNA can be more complex to predict *in silico*, and therefore it is often prudent to evaluate multiple sites to identify accessible regions amenable for optimal ASO activity. In theory, RNA binding proteins (RBPs) could also compete with ASO hybridization to an RNA by blocking access to the site. In practice, many RBPs have insufficient affinities to their target RNA to outcompete access with a gapmer ASO [1,23,85]. This is intuitive, as steric blocking ASOs are often a tool used to inhibit proteins binding to an RNA, as in the case of translation blockers, non-sense mediated decay (NMD) inhibitors or splice-shifting oligonucleotides (SSOs)[164,165]. However, Liu et al were able to achieve preferential knockdown of the pathogenic C9orf72 G_4C_2-repeat expansion transcript variant (V3) associated with amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) over a "beneficial" transcript variant (V2), which starts downstream of the hexanucleotide repeat and therefore synthesizes normal C9orf72 protein [71]. Even though the pathogenic (V3) and beneficial (V2) transcript variants harbor the same ASO site, the V2 transcript interacts with the splicing machinery at that site and blocked access to the gapmer ASO, demonstrating that in some instances RBPs can interfere with ASO hybridization to an RNA target [71].

Intracellular RNAs can have vastly different expression levels and rates of transcription, and whether these factors impact the ability of an ASO to degrade its target is a question that often arises. Miraglia *et al.* examined how different transcription rates and varying RNA copy numbers can affect the potency of gapmer ASOs for an exogenous (Ha-*ras*/luciferase reporter) and endogenous (TNF-α inducible ICAM-1) RNA target [166]. Their findings demonstrated that neither mRNA transcription rates nor variations in RNA transcript numbers affect ASO potency; however, they only assessed one RNA target for each system [1,166]. In a different approach, Michel *et al.* investigated the potency of ASOs that are predicted to target multiple genes (e.g., one ASO may target ~100, ~500, or ~2500 different genes) as a means to control for parameters such as ASO secondary structure, binding affinity, sequence context etc., and instead looked at whether transcript length, expression levels or the presence of multiple binding sites in a transcript can affect ASO degradation levels [167]. Their results indicate that higher expressed genes are slightly (but significantly) more likely to be knocked down by the same ASO when compared to moderately expressed genes (low expressed genes were omitted due to high variance between replicates) [167]. While this trend may seem counterintuitive, other factors such as possible condensate formation from lower abundance RNA transcripts may play a role in transcript accessibility [168,169]. Intuitively, transcripts containing multiple ASO binding sites were significantly more likely to be downregulated than transcripts with a single ASO binding site; this is consistent with previous studies that have demonstrated a ~5–10 fold gain in potency when an ASO targets repeat regions versus those targeting single sites [167,170,171]. Moreover, longer transcripts (unspliced mean length = 382,160 bp) were almost twice as likely to be downregulated than shorter transcripts (unspliced mean length = 28,689 bp) [167]. Of note, an important limitation of this study is that all but one of 6,704 ASO binding sites were located within introns, and splicing efficiency wasn't a controlled variable for the various pre-mRNAs which could influence the interpretation of the results [167]. It has been well documented that the efficacy of intron-targeting ASOs can be negatively impacted when the pre-mRNA processing kinetics are more rapid than the rate of heteroduplex formation

and RNase H1 recruitment, as now the ASO binding site is spliced out faster than the ASO cleavage rate [85,121]. These studies highlight that not only is the ASO sequence context important in identifying potent ASO sites, but that multiple attributes of the target RNA itself can also play a valuable role in ASO efficacy. Better characterization of these attributes and how they contribute to ASO accessibility and degradation rates will help improve ASO prediction models.

Hybridization-dependent off-target effects

Off-target effects (OTEs) occur when a gapmer ASO hybridizes to and modulates the expression, processing or functioning of an unintended RNA target. It is likely that the dominating mechanism for OTEs from gapmer ASOs is through degradation of non-targeted RNAs, and so here the selectivity of a gapmer ASO is defined as the rate of cleavage of the on-target RNA relative to the rate of cleavage of any off-target RNAs [26]. Designing ASOs that avoid OTEs has become achievable with the increased transcriptome coverage in genome annotation databases such as RefSeq or Ensembl, where ASO sequences can be searched for whole transcriptome sequence similarities using the BLASTn tool.

The challenge with avoiding OTEs is in the ability to predict how the number and position of mismatches between an ASO and an unintended RNA target affect the ASO selectivity. Many factors can contribute to how a mismatched ASO will behave, including the overall binding affinity of the ASO, where the mismatch is positioned within the ASO and the favorability of the mismatch [26]. As discussed in Considerations for selecting chemical modifications, there is a "sweet spot" for the binding affinity of a gapmer ASO whereby the binding affinity to the RNA target is high enough to achieve maximal potency, and exceeding this binding affinity doesn't further increase potency but can put the ASO at risk of tolerating mismatches [23,25–28,121,126]. The relationship between the binding affinity of the ASO to a mismatched target and the extent that off-target knockdown is achieved has been well studied [25,27,121,126]. For example, Lennox *et al.* demonstrated a positive correlation between binding affinity (T_m) of the mismatched ASO and the knockdown efficiency for both locked nucleic acid and 2'OMe gapmer ASOs that were microinjected into *Xenopus* single-celled embryos [27]. In this study, the binding affinity of the locked nucleic acid gapmer ASO was high enough to achieve 90% knockdown with the perfect match ASO, and still achieve ~50% knockdown even when 1–3 mismatches were present [27]. Using a different approach, Rukov *et al.* generated a luciferase plasmid pool that contained a target sequence with 7 degenerate nts (16,384 different plasmids) corresponding to the 7-nt gap of a 3-7-3 locked nucleic acid gapmer ASO and assessed knockdown levels using Library Sequencing (LibSeq) [25]. They reported that the majority of knockdown from OTEs came from targets containing only one mismatch, and that the knockdown levels positively correlated with increased binding affinity of the ASO to the mismatched target [25]. Mismatch promiscuity is also evident with how difficult it can be to design single nucleotide polymorphism (SNP) discriminating ASOs for allelic specific knockdown [172–174]. For example, hundreds of locked nucleic acid ASO gapmer designs may have to be evaluated to identify the perfect combination of sequence, length and chemical modifications to achieve SNP discrimination [173].

Mismatches between an ASO and an off-target RNA can alter the preferred heteroduplex structure required for RNase H recognition and cleavage and affect the binding affinity to an off-target RNA to varying degrees [26,174–176]. It has been shown that introducing chemical modifications into an ASO can modify the helical geometry of the heteroduplex structure, resulting in altered RNase H1 cleavage patterns and reduced cleavage kinetics [175,176]. The position and sequence context of a mismatch may also impact the heteroduplex structure to varying degrees, and as a result affect the tolerance (or intolerance) of RNase H1 to cleave a target in the presence of a mismatch [174]. However, the most important factor to consider when assessing off-target potential is how the mismatch(es) negatively impacts the binding affinity to a non-targeted RNA. Mismatched base pairs vary in their favorability with respect to each other (e.g., as a standalone base, G:T is more favorable than G:G), as well as to

their sequence context (e.g., sometimes G:G is more favorable than G:T based on neighboring bases) [26]. While the effects that mismatches have on the T_m of an ASO can be calculated *in silico* using tools such as OligoAnalyzer™, the binding affinity of a mismatched duplex can also be empirically measured with melting experiments and used to predict the potential for off-target degradation [28,126,177,178]. Although there is currently no consensus on the ideal placement of a mismatch to maximize discrimination (as discussed with SNP discriminating ASOs), incorporating both binding affinity reductions and key heteroduplex structural disruptions may in the future assist with *in silico* design of target specific ASOs.

It makes intuitive sense that limiting the number of hybridization-dependent OTEs from an ASO can translate into a more promising safety profile for the ASO [26]. In fact, it has been reported that there is a direct correlation between the number and type of unintended RNA targets that an ASO degrades *in vivo* and its hepatoxicity potential [119,120,126,128,179]. Dieckmann *et al*. investigated if there is a correlation between the T_m of a locked nucleic acid gapmer ASO and the potential for hepatotoxicity from off-target RNase H-dependent degradation [126]. Using an *in vitro* evaluation method in primary mouse and human hepatocytes, they found that there was a T_m threshold of 55°C that, when exceeded, was associated with increased *in vivo* hepatoxic potential [126]. Interestingly, they speculated that the ASO-induced apoptosis they observed is not a result of degrading a certain transcript (i.e., hitting essential to life genes), but rather from gross RNA degradation that triggers the RNA surveillance machinery that tracks intracellular RNA quality and induces apoptosis when a threshold of RNA cleavage is found [126]. Two other studies have recently demonstrated that the unintended degradation of pre-mRNAs with high affinity locked nucleic acid ASO gapmers was correlated with hepatoxicity in an RNase H1-dependent manner, highlighting the importance of including introns when evaluating ASOs for OTEs [119,120]. Regardless of the mechanism responsible for cytotoxicity, hybridization-based OTEs can be mitigated with careful design using stringent *in silico* off-target evaluation methods combined with judicious *in vitro* testing efforts and analytical methods (e.g., RNA-seq). It is important to remember that for an *in silico* predicted off-target to be problematic, the off-target RNA needs to be expressed in the appropriate cell type and the ASO site needs to be accessible similar to an on-target site [23]. Additionally, evaluating two ASO sequences that target the same RNA is crucial to help deconvolute phenotypes from on-target degradation versus phenotypes from off-target degradation [180].

In vivo delivery: Targeting ligands and fatty acid conjugates

The *in vivo* efficacy of an ASO is determined not only by the potency of the ASO, but also in its ability to follow the pathway from the site of injection to the targeted RNA. Post-injection, the ASO must travel to the intended tissue, be internalized into the desired cell type, escape the endosome and localize to the appropriate subcellular compartment where both the RNA and RNase H1 are present. Chemical modifications (e.g., a phosphorothioate backbone) can greatly facilitate this process by enabling an oligonucleotide to bind non-specifically and with appropriate affinity to proteins that keep it in circulation and assist with cellular internalization. Fortunately, ASOs are still biologically active even with extensive chemical modification (heavily modified small interfering RNAs (siRNAs) are often less active) and can be efficacious even when directly injected *in vivo* without the assistance of a delivery tool (i.e., naked, or gymnotic delivery). *In vivo* ASO delivery methods vary depending on the targeted tissue type. For systemic applications, the primary methods of administration are intravenous (IV) infusion or subcutaneous (SC) injection, which primarily deliver an ASO to the liver and kidney and to a lesser extent bone marrow, adipocytes and lymph nodes (reviewed in [13]). However, robust gene knockdown has also been reported in tissues such as lung, adrenal, gall bladder and prostate following SC administration of a cEt gapmer ASO targeting MALAT1 in mouse [13]. The biodistribution of an ASO after systemic administration is widespread and reaches most tissues to some extent, except for the central nervous system (CNS). The CNS can instead be accessed by administering an ASO intrathecally (IT) or intracerebroventricularly (ICV) into the cerebrospinal fluid (CSF), resulting in broad distribution into the spinal cord and brain with uptake in multiple cell types [13,181–184]. In fact, the FDA-approved antisense drug SPINRAZA® (nusinersen) used to treat spinal muscular atrophy (SMA) is an 18-nt 2′MOE PS splice shifting oligonucleotide (SSO) that is delivered unassisted IT into the CSF [185].

Oftentimes, the broad distribution of an ASO after systemic delivery is undesired as it requires a higher dose to achieve the necessary knockdown levels in targeted cell types and can cause unwanted effects and/or toxicities in non-targeted cell types. Conjugating ligands or fatty acids to an ASO is an attractive strategy to increase selectivity over which cell types the ASO is taken up *in vivo*. Using hydrophobic lipid-based conjugates is an appealing option to enhance oligonucleotide delivery, as they bind serum lipoproteins to improve bioavailability and can facilitate receptor-mediated membrane permeability. For example, cholesterol-conjugated oligonucleotides delivered systemically will adhere to high- and low-density lipoproteins (HDL and LDL) in serum and are subsequently internalized into cells via cholesterol binding receptors [186]. One of the earliest successes with using bioconjugated oligonucleotides involved attaching cholesterol to siRNAs to enhance uptake in the liver [187,188]. Like siRNAs, ASOs conjugated with cholesterol moieties have been shown to have reduced renal clearance, improved cellular uptake and increased accumulation in the liver [189–191]. Wada *et al*. demonstrated that hepatic accumulation and RNA knockdown levels of cholesterol-conjugated locked nucleic acid PS gapmer ASOs are influenced by the orientation of the cholesterol (3′-end show higher accumulation and knockdown) and type of linker, which may be a result of how the different design variations affect the binding profile to blood circulating proteins [190]. Interestingly, the ASO conjugated to the 3′-cholesterol with a TEG linker had the lowest hepatic accumulation yet highest knockdown level in mouse liver of any other linkers tested at either the 5′- or 3′-end, suggesting that a 3′-TEG-cholesterol ASO may have improved productive intracellular uptake [190]. Importantly, having a cleavable cholesterol, such as a phosphodiester bond between the cholesterol and ASO that is cleaved by nucleases once internalized, was key to improving ASO activity [190,191]. While cholesterol-conjugated oligonucleotides accumulate mostly in the liver, they can also be delivered to extrahepatic tissue such as intestine, kidney, adrenal, adipose, lung, muscle, ovary, heart and brain depending on which serum lipoprotein (e.g., HDL or LDL) the cholesterol molecule is associated [172,186,188,192–194]. While cholesterol-conjugated oligonucleotides have improved uptake in liver compared with unconjugated oligonucleotides, there are still multiple cell types that express cholesterol binding receptors which reduces the cell selectivity of this lipid conjugate.

When selecting ideal ligand-receptor candidates for receptor-mediated uptake of ASOs, several attributes should be considered. First and foremost, the ligand chemical structure should be amenable to attachment to an ASO. Additionally, the receptor should be specific to the targeted cell population and should be highly expressed with rapid internalization and recycling kinetics. Importantly, the ASO should be delivered to productive intracellular compartments and not interfere with RNase H1 function. These criteria were all met with the development of the triantennary N-acetyl galctosamine (GalNAc, GN3) conjugate, a high affinity ligand for the hepatocyte-specific asialoglycoprotein receptor (ASGPR) which is highly expressed on the plasma membrane of hepatocytes (~0.5–1.0 million copies per cell) and rapidly recycled every 15 minutes [12,195,196]. While systemically delivered, unconjugated ASOs containing a PS backbone accumulate mostly in the liver, they are preferentially taken up by nonparencymal liver cells than hepatocytes (representing 80% of the total liver mass [197–199]). The preferential uptake into NP liver cells can be redirected by conjugating GN3 onto the ASO which drastically shifts the suborgan distribution from NP liver cells to hepatocytes [196,199]. In a seminal study, Prakash *et al.* demonstrated that the potency of GN3-conjugated 2′MOE and cEt gapmer ASOs was increased ~10-fold in mouse liver compared with unconjugated ASOs, with enhanced duration of action [196]. In a follow-up study, Watanabe *et al.* directly compared the efficacy of GN3-locked nucleic acid ASOs targeting Apo-B mRNA in whole liver, hepatocytes and NP cells with unconjugated ASO or cholesterol- and α-tocopherol (Toc)-ASO conjugates [199]. They demonstrated that both the delivery and efficacy of GN3-ASOs were significantly increased only in hepatocytes (~7-fold and >10-fold, respectively), while the lipophilic ligand conjugated ASOs had increased delivery and efficacy in both hepatocytes (4–5 fold and >5 fold, respectively) and NP cells (3–4 fold and 5 fold, respectively) when compared with the unconjugated ASO [199]. Of note, both studies (as well as others) employed a GN3-conjugated ASO containing a phosphodiester bond between GN3 and ASO and demonstrated that the ASO was liberated from the GN3 once internalized in the cell which was a requirement for the improved efficacy [196,199,200]. Positioning the G3 conjugate at the 5′- versus the 3′- end of a 2′MOE gapmer also has different effects on ASO potency, with the 5′-G3 ASO showing higher potency *in vitro* (2-fold increase) and *in vivo* (1.5 fold increase) than the 3′-G3 ASO; this difference may be attributed to the increased rate of intracellular GN3 removal from the 5′-GN3 ASO compared with the 3′-GN3 ASO, which still retained various linker moieties after 72 hours [201]. While ASGPR is known to be highly expressed in hepatocytes, Kim *et al.* discovered that ASGPR expression is reduced in hepatocellular carcinoma (HCC) cells (e.g., ~50–60% reduced expression in mouse HCC) and is yet interestingly unchanged in non-cancer-related diseases such as fatty liver disease and chronic hepatitis [202]. They investigated whether the reduced expression of ASGPR in HCC cells were still capable of internalizing functional levels of GN3-cEt ASOs targeting *MyD88*, which is implicated in HCC; compared with an unconjugated cEt ASO, the GN3-cEt ASO had a 5–10 fold increase in potency in HCC tumors with significant reduction in tumor burden [202]. The addition of a G3 conjugate has been shown to increase the potency of both 2′MOE and cEt gapmer ASOs in humans by 15- to 30-fold for hepatocyte targets compared with their unconjugated counterparts, which enables lower dosing that can reduce side effects [1,203]. Another receptor-ligand combination has also been exploited for selective delivery of ASOs to pancreatic β-cells, which have previously been shown to be refractory to ASO uptake [204,205]. Ämmälä *et al.* conjugated a cEt (targeting MALAT1) or a cEt/2′MOE mixmer (targeting FOXO1) ASO to an engineered GLP1R peptide ligand (eGLP1) that binds with high affinity to the glucagon-like peptide-1 receptor (GLP1R) on pancreatic β-cells [205]. They demonstrated productive and selective ASO uptake in pancreatic β-cells in a mouse model with an IC_{50} of 0.007 μmol/kg for MALAT1 and 0.04 μmol/kg for FOXO1 (> 50-fold increase in potency) and no discernable knockdown in liver or other tissue [205]. The overall success with using the GN3-ASOs for hepatocyte-specific ASO delivery and eGLP1-ASOs for selective delivery to pancreatic β-cells exemplifies the importance of identifying other receptor-ligand candidates that meet the criteria for cell-specific efficient delivery of nucleic acids.

Identifying conjugates that enhance delivery of ASOs to extrahepatic tissue is an important endeavor for a multitude of diseases. Notably, systemic ASO delivery with enhanced uptake in skeletal and cardiac muscle would be advantageous for many muscle diseases such as Duchenne muscular dystrophy (DMD), myotonic dystrophies (DM1), myotubular myopathy (MTM) and spinal and bulbar muscular atrophy (SBMA)[206–209]. In order to be effective in muscle cells, a systemically delivered ASO is required to transport from the capillary across the continuous endothelium by transcytosis into the interstitium of muscle tissue, through the plasma membrane of the myoctye and into a productive subcellular compartment for RNA knockdown [210,211]. This journey for the ASO can be facilitated by hitchhiking onto plasma proteins that are efficiently transported across the continuous endothelium and into the interstitial space by caveolin-mediated transcytosis, such as albumin and lipoproteins [212–214]. Conjugation of hydrophobic moieties such as cholesterol, fatty acids or fat-soluble vitamins to an ASO has been shown to augment binding to circulating plasma proteins and improve delivery of the ASO to extrahepatic tissue [194,215]. Østergaard *et al.* evaluated the plasma protein binding profiles of an ASO conjugated to cholesterol, α-tocopherol or palmitate and found that all three ASO conjugates demonstrated significant increases in binding affinity to albumin, LDL and HDL [215]. The cEt gapmer ASO conjugated to palmitate via a hexylamino linker targeting dystrophia myotonica protein kinase (DMPK) was found have enhanced potency in cardiac and skeletal muscle of mice (~2–4 fold increase) and rats (~2–3 fold increase), with a modest increase in potency in monkey (~1–2 fold increase) when compared to the unconjugated parent cEt gapmer ASO [215]. When the cEt gapmer was conjugated to cholesterol via a TEG linker, there was a ~3.5–5 fold increase in potency to cardiac and skeletal muscle, but toxicities associated with the higher dose eliminated this conjugated ASO from further testing in other animal models [215]. The α-tocopherol-TEG conjugated cEt gapmer showed a ~2–3.5 fold increase in potency in mouse muscle tissue and ~1.3–2.5 fold increase in monkey muscle tissue compared with the unconjugated cEt gapmer ASO [215]. Interestingly, each hydrophobic conjugate (cholesterol, α-tocopherol and palmitate) had different binding affinity profiles to albumin, HDL, LDL and HRG (histidine-rich glycoprotein, a plasma protein that binds to PS ASOs) and each species had variable concentrations of these proteins in their plasma. These differences account for the interspecies variability in the level of tissue-specific potency enhancement for the conjugated ASOs, as the effectiveness of the hydrophobic conjugate correlated with the level of relevant plasma proteins in each species [215]. This has compelling implications when navigating an ASO-conjugate, as it may behave contradictorily in different animal models. In a parallel study, Prakash *et al.* screened a series of fatty acids with chain lengths ranging from C8–C22 conjugated on the 5′-end of a cEt gapmer ASO targeting MALAT1 and investigated how the level of fatty acid hydrophobicity impacted *in vivo* functionality [210]. All fatty acids tested were found to significantly enhance binding to albumin compared to the unconjugated ASO, with longer chains (C16–C22) having a 2–5 fold increase in albumin binding affinity compared with shorter chains (C8–C14); the correlation between increased carbon chain length and increased albumin binding is consistent with previous studies [210,216]. Enhanced albumin binding also positively correlated with enhanced ASO potency in mouse quadricep and heart, with fatty acid chain lengths between C16–C22 showing the greatest improvement [210]. Palmitate (C16) was shown to have >150-fold increase in albumin binding affinity, and when delivered SC in mice the 5′-palmitate-cEt MALAT1 ASO accumulated to a greater extent in heart (~2–4 fold increase), quadricep (~1.5-fold increase) and liver (~2-fold increase) compared with the unconjugated ASO; however, this only translated into higher ASO potency in heart (>6-fold) and quadricep (~3-fold), with no change in ASO effect in liver [210]. When targeting CD36, DMPK and Cav3 mRNAs *in vivo* in mouse, the palmitate-cEt ASOs improved potency 3–7 fold over the unconjugated ASO in skeletal and cardiac muscle [210]. These studies exemplify how beneficial it can be to attach a targeting ligand or fatty acid to an ASO to improve efficacy in certain tissue types, enabling the use of lower administered doses to achieve the desired knockdown.

Conclusions

Research in optimizing gapmer ASOs for RNA knockdown has been ongoing for over 30 years, and the technology has only recently begun to deliver on its promise as a viable strategy for gene regulation. In the past few years, gapmer ASOs have had several breakthroughs in the clinic resulting in 3 FDA approved gapmer ASOs (Mipomersen, Inotersen, and Volanesorsen), leading to a more widespread interest in developing ASOs for both genetic diseases and RNA viral infections. These successes would not have been possible without the perseverance of so many scientists working on characterizing the biophysical and biochemical properties of a wide variety of nucleic acid chemical modifications, determining how to improve the toxicology safety profile and mitigate OTEs to make sure that ASO drugs are safe and tolerable, and searching out tricks and tools to deliver them more effectively into the targeted disease-associated cells in the body. Efforts regarding each of these aspects are still ongoing in the search for the optimal gapmer ASO chemical modification strategy. Still, one of the biggest hurdles with ASO strategies is targeting certain organs or cell populations. While recent advancements with conjugated targeting ligands such as GalNAc (liver), GLP1R (pancreatic β-cells) and to a lesser extent palmitate (skeletal and cardiac muscle) are providing hope for having an arsenal of cell type-specific delivery tools, more progress is still required to have sufficient targeting to other cell types. Importantly, a revived interest in developing ASO strategies comes with an increase in knowledge gained by *in vitro* gapmer ASO screenings for site selection followed by additional studies, which will translate into how to more effectively design potent, selective, and non-toxic ASOs.

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