

Silencing disease genes in the laboratory and the clinic

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Abstract

Synthetic nucleic acids are commonly used laboratory tools for modulating gene expression and have the potential to be widely used in the clinic. Progress towards nucleic acid drugs, however, has been slow and many challenges remain to be overcome before their full impact on patient care can be understood. Antisense oligonucleotides (ASOs) and small interfering RNAs (siRNAs) are the two most widely used strategies for silencing gene expression. We first describe these two approaches and contrast their relative strengths and weaknesses for laboratory applications. We then review the choices faced during development of clinical candidates and the current state of clinical trials. Attitudes towards clinical development of nucleic acid silencing strategies have repeatedly swung from optimism to depression during the past 20 years. Our goal is to provide the information needed to design robust studies with oligonucleotides, making use of the strengths of each oligonucleotide technology.

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Keywords: antisense oligonucleotides; siRNA; mRNA; gene silencing; therapeutics

Received 14 July 2011; Revised 23 August 2011; Accepted 30 August 2011

No conflicts of interest were declared.

Overview

The ability to use synthetic agents to control gene expression facilitates many aspects of biological research and would have a transformative impact on the treatment of many diseases. Oligonucleotides are one promising class of synthetic agents. Such compounds can be designed to recognize any species of cellular DNA or RNA and, in theory, have the potential to modulate gene expression and affect the course of almost any disease.

This concept is not new. In 1978, Zamecnik first reported that a synthetic oligonucleotide (at that time, a rare and difficult to obtain type of compound) complementary to Rous sarcoma virus 35S RNA acted as an efficient inhibitor of protein expression [1–3]. In spite of the obvious promise of this approach, progress has been slow because of the need to overcome many technical hurdles. Now, fuelled by advances in antisense technology and the emergence of RNA interference, the modulation of gene expression by nucleic acids has become a routine tool for laboratory research. For patient care, the field has seen repeated disappointments that often mask the underlying steady progress being made.

The concept is simple: a target RNA is chosen based on a hypothesis about its physiological significance; a complementary oligonucleotide is synthesized; gene expression is assayed; and phenotypes are examined. Reality is more complex. Here we describe strategies

for using nucleic acids to control gene expression, with a focus on translating the technology into the clinic.

Basic principles of oligonucleotide-mediated gene silencing

Single-stranded antisense oligonucleotides (ASOs) and RNA interference (RNAi) share their fundamental principle: an oligonucleotide binds a target RNA through Watson–Crick base pairing. An ASO must survive and function as a single strand (Figure 1A). In contrast, during RNAi, a small RNA duplex associates with the RNA-induced silencing complex (RISC), one strand (the passenger strand) is lost, and the remaining strand (the guide strand) cooperates with RISC to bind complementary RNA. In contrast to ASOs, the guide strand is always associated with a complementary strand or a protein (Figure 1B). This difference between the two approaches leads to different strengths and weaknesses that affect drug development.

Optimization strategies for antisense oligonucleotides: chemical modification

Unmodified single-stranded DNA or RNA oligonucleotides are too unstable to use in cells. The first type of optimization to be developed was therefore the

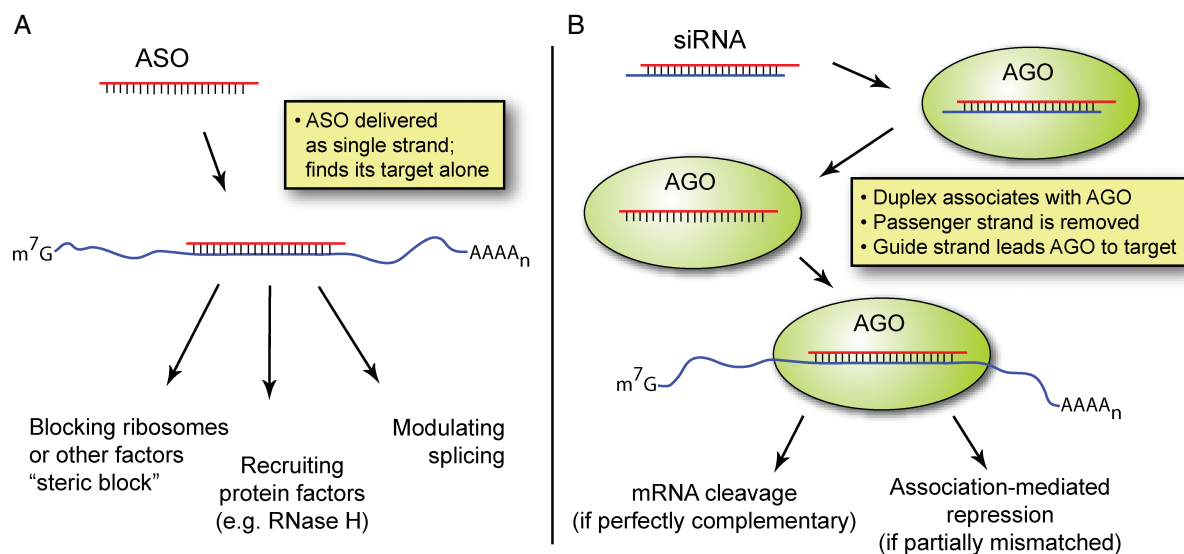


Figure 1. Comparison of the ASO and siRNA mechanisms. (A) ASOs must be stable as single-stranded oligonucleotides and find their target alone. (B) siRNAs are delivered as duplexes and then taken up by Argonaute (AGO), part of the RNA-induced silencing complex. Thus, the 'antisense oligonucleotide' that guides AGO to complementary mRNA is always present in the cell as a duplex or as part of a protein complex. Note that RISC also engages in complex biology with endogenous small RNAs [71].

use of chemical modifications to increase the nuclease resistance. The earliest major breakthrough was the introduction of phosphorothioate (PS) linkages in place of the phosphodiester bond [4] (Figure 2A). This modification greatly improved stability towards digestion by nucleases. PS linkages also improved binding to serum proteins *in vivo*, increasing half-life and permitting greater delivery of active compound to tissues [5,6]. ASOs that only contain PS modifications were capable of producing antisense effects inside cells, but potencies were not always high nor were reliable results routine [7].

Another obstacle was inadequate affinity for intended target sequences leading to low potencies. Poor potencies force the use of high concentrations of oligonucleotides, which can lead to 'off-target' effects—unintended phenotypes that are unrelated to inhibition of the intended target gene. Off-target effects can be due to recognition of other genes by binding to sequences that are similar to the intended target. Oligonucleotides can also bind directly to proteins and affect their function. While direct binding to proteins is generally considered an unwanted off-target effect in the gene silencing field, it has spawned a field of its own (see, for example, ref 8).

Chemical modifications can improve potency and selectivity by increasing the binding affinity of oligonucleotides for their complementary sequences. Widely used modifications include 2'-*O*-methyl (2'-*O*-Me) [9], 2'-fluoro (2'-F) [10–13], and 2'-*O*-methoxyethyl (2'-MOE) [14,15] RNA (Figure 2B). Even more affinity can be gained using oligonucleotides modified with locked nucleic acid (LNA), which contains a methylene bridge between the 2' and 4' position of the ribose [16,17]. This bridge 'locks' the ribose ring in a conformation that is ideal for binding, leading to high affinity for complementary sequences [18]. Related bridged

nucleic acid (BNA) compounds have been developed and share these favourable properties [19–27]. Their high affinity has permitted the development of far shorter oligonucleotides than previously thought possible which nonetheless retain high potency [28].

The chemistry for introducing 2'-*O*-Me, 2'-MOE, 2'-F, or LNA into oligonucleotides is compatible with DNA or RNA synthesis, allowing chimeras with DNA or RNA bases to be easily obtained. This compatibility allows the properties of chemically modified oligonucleotides to be fine-tuned for specific applications—a major advantage for development that makes LNAs and other BNAs convenient tools for many applications.

Amplifying the effectiveness of ASOs: RNase H

The RNA strand of DNA/RNA hybrids is cleaved by the enzyme RNase H, an enzyme that exists in both the nucleus and the cytoplasm of eukaryotic cells [29]. This catalytic cleavage can be exploited by synthetic oligonucleotides to increase potency [30,31]. 'Gapmer' oligonucleotides contain two to five chemically modified nucleotides (eg LNA or 2'-MOE) on each terminus flanking a central eight to ten base 'gap' of DNA [13,32]. The chemically modified oligonucleotides increase nuclease resistance and affinity for target sequences, while the DNA gap permits the formation of a DNA/RNA hybrid that can be a good substrate for RNase H. Most of the ASOs currently in clinical development are gapmers of this type. It is also possible to use chemically modified oligonucleotides that mimic the DNA structure and can recruit RNase H, yet bind strongly to complementary RNA. 2'-Fluoroarabinonucleic acid (2'-F-ANA, Figure 2B) is the best example of this type of oligonucleotide [33,34].

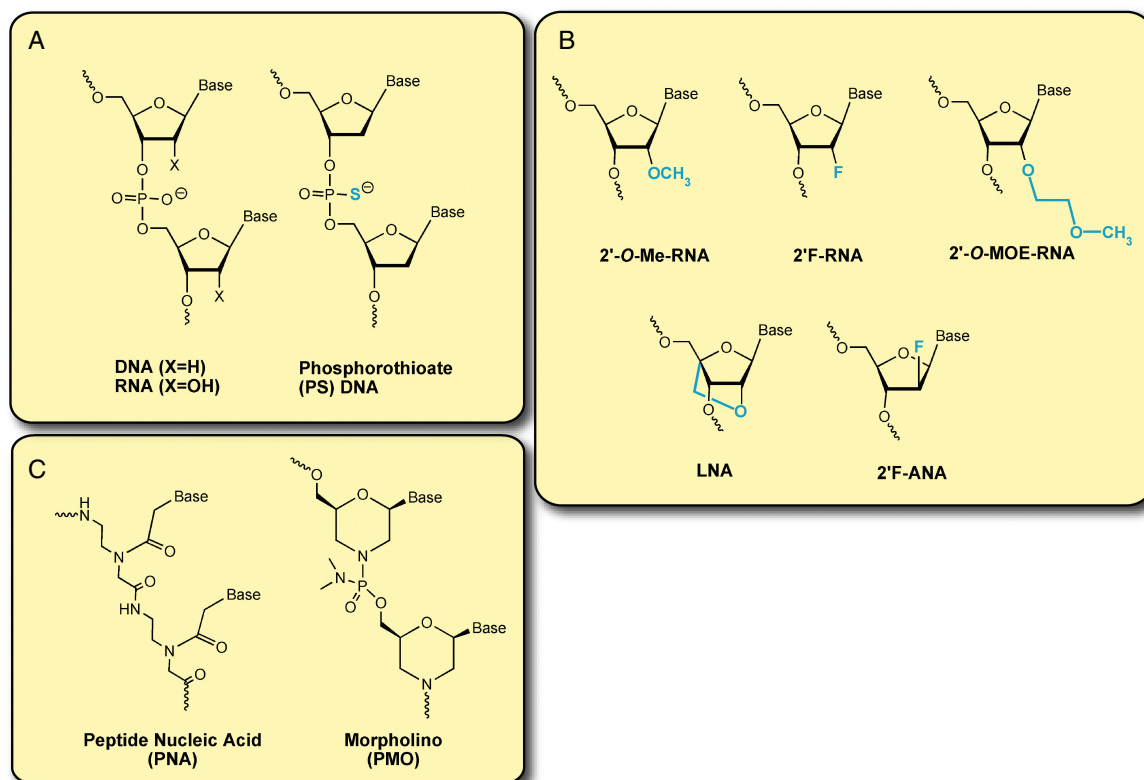


Figure 2. Structures of some common chemically modified nucleotides. (A) Replacement of a non-bridging phosphate oxygen with sulphur gives the phosphorothioate (PS) linkage, which dramatically increases nuclease stability. (B) Various sugar modifications are compatible with unmodified DNA/RNA synthesis and increase binding affinity and nuclease stability. (C) PNA and PMO modifications have a neutral backbone structure that is dramatically different from the sugar-phosphate backbone of natural oligonucleotides.

Blocking translation

ASOs that lack a contiguous stretch of DNA (or DNA-like nucleotides) can also bind RNA and block gene expression. These oligomers are known as 'steric blockers' because they act by blocking the ribosome rather than facilitating cleavage of RNA.

Besides the sugar-modified nucleotides discussed above, steric blockers can be made from oligomers that are quite different from DNA or RNA (Figure 2C). Peptide nucleic acid (PNA) is an oligonucleotide mimic whose bases are linked by amide bonds and whose synthesis shares many features with peptide synthesis [35]. Because the amide backbone is uncharged, binding is characterized by high rates of association and high affinity [36,37]. Phosphorodiamidate morpholino oligomers (commonly called PMOs or 'morpholinos') are another uncharged DNA analogue [38]. PMOs do not bind complementary targets with the high affinities that characterize PNA binding, but have proven to be effective agents inside cells. PMOs are widely used as silencing agents in zebrafish [39,40].

Modulating splicing

mRNA is initially transcribed as a pre-mRNA containing intervening sequences or introns. These introns

must be spliced out to form the mature mRNA. Up to 95% of multi-exon genes are subject to alternative splicing in which the pre-mRNA is spliced differently to form multiple variants of the mature mRNA [41]. Oligomers that target sequences within the pre-mRNA can affect splicing and increase the production of desired isoforms [42–48]. In this case, cleavage of the target RNA is not desired; these strategies use oligonucleotides that do not recruit RNase H, including LNA or 2'-modified oligonucleotides or uncharged analogues such as PNA or PMO oligomers.

An impressive variety of genes and conditions can be treated by splice-switching oligonucleotides [42,43]. The most advanced target is the dystrophin gene, a large (2.6 Mb) gene containing 97 exons that encodes a protein essential for healthy muscle cells. Mutations in the dystrophin gene cause muscular dystrophy. In Duchenne muscular dystrophy (DMD), the most severe form of muscular dystrophy, mutations cause a frame shift resulting in no functional dystrophin protein. However, oligonucleotides have been used to remove the mutant exon and restore the proper reading frame, producing a truncated but partially functional dystrophin protein and potentially leading to milder symptoms [46–48]. Researchers have also successfully modulated the splicing of several other therapeutic targets, including β -globin (for β -thalassaemia) [44,49,50], SMN2 (for spinal muscular atrophy) [45], HER2 or Bcl-x (for cancer) [51,52], and others [42,43].

Targeting miRNAs

MicroRNAs (miRNAs) are endogenous small RNAs that can regulate normal physiological processes and affect disease [53–58]. miRNAs act through the RNAi pathway and recognize target mRNAs through complex patterns of recognition that are incompletely understood. An increasing number of miRNAs have been implicated in physiological processes that affect disease, and interfering with these miRNAs might increase the expression of their target genes and provide therapeutic lead compounds.

ASOs that are complementary to miRNAs can block their function [59–61]. For example, miR-122 is an abundant liver-specific miRNA implicated in a variety of diseases including cancer and hepatitis C [62]. Oligonucleotides complementary to miR-122 have been shown to alter liver metabolism [63,64] and block hepatitis C virus replication [65,66]. Various chemistries have been shown to be active as inhibitors of miRNA function, including PNA [67], LNA [65–68], 2'-O-Me [69], 2'-MOE [64], and morpholino [70].

siRNAs

Over the past decade, double-stranded short interfering RNAs (siRNAs) have become widely used tools for silencing gene expression. When a duplex RNA enters cells, it binds the protein machinery of the RNA-induced silencing complex (RISC) [71]. Synthetic RNAs used for gene silencing are usually 19–22 bp duplexes. This length is sufficient to form a stable duplex and be recognized by RISC, but short enough to avoid most of the strong interferon response provoked by duplexes greater than 30 bp in length.

Since publication of the first report of gene silencing in mammalian cells in 2001 [72], siRNAs have been the subject of thousands of experimental studies aimed at examining function. While antisense oligonucleotides continue to be used for gene silencing, the robust nature of siRNAs and the relative ease of identifying active siRNAs have made them a favoured silencing tool for many laboratories.

Chemical modifications and duplex RNAs

Unmodified duplex RNA is surprisingly stable and chemical modification of siRNAs is usually not essential for silencing gene expression in cultured cells (see Table 1). *In vivo*, however, unmodified siRNAs are not highly active and chemical modification can significantly improve their properties [73]. Chemically modified siRNAs can feature improved nuclease stability and an associated increase in the duration of action [74–76]. Unmodified RNA is also rapidly cleared [77] and chemical modification, complexation with carrier

Table 1. ASOs and siRNAs use different methods to overcome similar obstacles. ASOs depend almost exclusively on chemical modification to optimize their properties. On the other hand, because siRNAs are always found as duplexes or associated with proteins, chemical modification of siRNAs is not always required for initial cell culture experiments

| Goal | Principal methods for achieving the goal | |
|---|---|--|
| | ASOs | siRNA |
| Nuclease stability | <ul style="list-style-type: none"> • Chemical modification | <ul style="list-style-type: none"> • Duplex structure • Protein association • Chemical modification |
| Binding affinity, disruption of existing target structure | <ul style="list-style-type: none"> • Chemical modification | <ul style="list-style-type: none"> • Seed sequence structural preorganization mediated by Argonaute |
| Overcoming immune stimulation | <ul style="list-style-type: none"> • Chemical modification | <ul style="list-style-type: none"> • Chemical modification |
| Specificity of knockdown | <ul style="list-style-type: none"> • Careful sequence selection | <ul style="list-style-type: none"> • Careful sequence selection • Chemical modification |
| Cellular uptake | <ul style="list-style-type: none"> • Chemical modification • Conjugation • Formulation | <ul style="list-style-type: none"> • Conjugation • Formulation |

agent, and local delivery to a disease target can help to achieve improved *in vivo* results.

Several patterns of chemical modification show increased potency across several sequences [76,78,79]. The introduction of double-stranded RNA into cells can activate the innate immune system [80–82]; this immune activation may be able to be harnessed for therapeutic benefit [83–87] but can also lead to dose-limiting toxicity when siRNAs are delivered systemically [88]. Chemical modifications can be used to reduce the immunogenicity of siRNAs [79,88,89]. Chemical modification becomes particularly important as an siRNA progresses towards clinical use [90,91].

The architecture of an siRNA duplex can also be modified. For example, longer RNA duplexes are recognized by Dicer, a component of the RISC machinery, and may have higher potency than their 21-mer counterparts [92,93].

Identifying a useful ASO or siRNA for gene silencing

The first step in any project is to identify a biology problem or disease state where manipulation of gene expression might produce a useful phenotype or physiological effect. The second step is to identify one or more target genes to manipulate. Efficient agents may already have been identified in the literature. If

no such data exist, commercial suppliers may offer duplex RNAs that have been validated for inhibition of the target gene. If commercial RNAs are unavailable, are too expensive, or are inadequate, investigators can experimentally identify ASOs or siRNAs themselves.

There are algorithms for siRNA design [94–96], but none is perfect and it will probably be necessary to test several duplex RNAs to find sequences that are sufficiently active and selective. Various groups have proposed computational [97–99] or experimental [99–104] methods for identifying potent ASOs. Aspects of mRNA secondary structure can be roughly predicted, but algorithms such as the popular mFOLD do not take into account factors such as tertiary structure or the involvement of RNA binding proteins, and so they are of limited utility in practice [105]. Ultimately, if a group is serious about finding a potent ASO, they should test as many oligonucleotides as time and budget permit [106,107].

Control experiments: you can never have too many

Regardless of whether ASOs or siRNAs are used for gene silencing, it is essential to use proper controls [108]. Understanding off-target effects can help investigators choose controls appropriately. ASOs and siRNAs are large synthetic molecules and can act in ways independent of Watson–Crick base pairing. For ASOs, this includes non-specific binding to proteins both in serum and inside cells. Phosphorothioate backbone oligonucleotides are particularly liable to bind proteins—this is the source of their slower clearance from serum, but also the source of much of their toxicity [109]. Oligonucleotides can fold into complex secondary structures and bind proteins in a sequence-specific manner related to shape rather than pairing [110].

For siRNAs, cells can recognize double-stranded RNA and activate the innate immune system [80–82] and this immunostimulatory activity has been misinterpreted as RNA interference effects [111,112]. Finally, high concentrations of siRNA can saturate the RNAi machinery, leading to a global perturbation of miRNA-mediated regulation [113–115].

An ASO or siRNA will always have partial complementarity to non-target transcripts, and this can cause unintended gene repression and misleading phenotypes [116,117]. For siRNAs, one of the most common off-target effects occurs through 7–8 nucleotide complementarity at the 5'-end of the guide strand (the so-called 'seed region') to sites in the 3'-untranslated region of other genes. This type of base pairing is a prerequisite for miRNA-mediated gene repression, and partially complementary siRNA duplexes can enter the miRNA pathway and repress non-target transcripts [117,118]. Careful use of chemistry and duplex design can help to alleviate this type of off-target effect. For example,

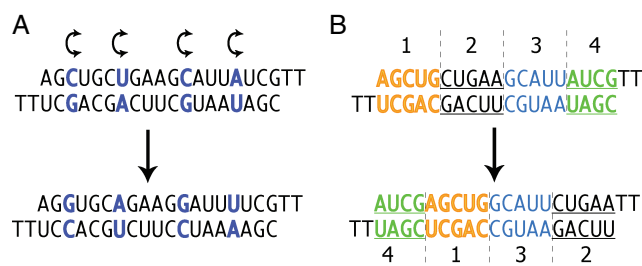


Figure 3. Typical mismatched and scrambled controls. (A) A duplex with several pairs of bases exchanged (from one strand to the other) should maintain very similar properties to the parent duplex but lose affinity for its target mRNA. (B) A scrambled control can be made by moving blocks of bases with respect to the parent oligomer.

including two 2'-O-Me-RNA nucleotides at the 5'-end of the siRNA reduces its ability to enter the miRNA pathway [117,119]. siRNA designs that ensure loading of the correct guide strand into RISC can also help to reduce off-target effects [120–126].

It is impossible to avoid some level of off-target effects in oligonucleotide-mediated gene silencing—the goal is to minimize them by careful use of chemistry and to use multiple approaches with non-overlapping off-target effects so that an observed phenotype can be confidently ascribed to recognition of the desired target. For example, an investigator may be testing the hypothesis that inhibition of a protein will lead to decreased cell proliferation. How can one build a case that the observed protein knockdown and decreased cell proliferation are not indirect effects? Several types of controls are recommended for any oligonucleotide experiment:

- When multiple ASOs or siRNAs target the same gene, they should have the same on-target effect but different off-target effects. Thus, possession of multiple ASOs or siRNAs that are complementary to the target mRNA and produce the same phenotype provides one piece of evidence supporting a relationship between recognition of the target mRNA, gene silencing, and phenotype.
- Experiments should include negative control compounds containing mismatched bases relative to the target mRNA or having blocks of bases moved relative to the sequence of the parent oligomer (Figure 3). Sequences that are closely related to the oligonucleotide of interest are more likely to have similar immunogenic or other off-target effects, making them better controls than totally unrelated sequences. An ideal mismatched or scrambled control does not significantly perturb the levels of a gene of interest.
- Protein and RNA levels of the target gene should both be tested where possible. If using siRNA or an RNase H competent ASO, both protein and RNA should decrease. A different result

should raise suspicion about the mechanism of the silencing observed.

- The dose–response of any knockdown should be tested and experiments should be carried out at the lowest possible concentration.
- For gapmers or siRNAs, a technique called rapid amplification of 5'-cDNA ends (5'-RACE) can be used to verify that the targeted transcript is being cleaved at the predicted site [127–130]. While valuable, it is important to note that 5'-RACE merely detects cleavage; it is not quantitative and is not an indication of efficiency.
- Finally, when possible, the ultimate control for gene silencing experiments is a functional rescue by an exogenous copy of the gene of interest containing silent mutations at the oligonucleotide's target site. Nevertheless, these experiments can be quite challenging, depending on the biological system being studied.

Off-target effects are a significant concern for laboratory use and clinical development of nucleic acid acids. Unintended phenotypes are, however, a concern for the development of any other type of drug including small molecules and proteins. The solution for minimizing off-target effects for nucleic acids is the same as for other classes of drug—iterative testing and rational optimization.

Introducing ASOs or siRNAs into cultured cells

The most common method for promoting uptake of ASOs and siRNAs in cell culture involves the use of cationic lipids to transfect nucleic acids. Mixing cationic lipid with negatively charged nucleic acids yields a complex that can cross cell membranes and release active oligonucleotide into the cytoplasm of cells.

Many different cationic lipids are available and activity depends on the cell line used. Even for a given cell line, the preferred lipid may vary depending on whether an ASO or siRNA is being transfected. Not all cell lines can be transfected using cationic lipid, and if literature precedent is unavailable, it may be necessary to experimentally test different cationic lipids to find one that can successfully transfect a cell line of interest. It is also possible to electroporate oligonucleotides into cells [131–133]. This method can be highly effective and useful for cell lines that cannot be readily transfected by lipid, but requires specialized equipment and expertise.

Recently, it has become apparent that active ASOs can freely enter some cell lines without the need to add lipid [134,135]. The transfection protocol is thus simplified and any off-target effects due to exposure of cells to lipid are avoided. The method can also be used with cell lines that are not compatible with lipid-mediated transfection. However, higher concentrations

of ASO are needed relative to the amounts used in lipid-mediated transfections.

Delivery of ASOs and siRNAs *in vivo*

As gene silencing technologies move from cultured cells into animal models and ultimately clinical application, the challenge of delivery increases. Delivering oligonucleotides in whole organisms requires crossing many barriers [136,137]. Degradation by serum nucleases, clearance by the kidney, or inappropriate biodistribution can prevent the oligonucleotide from ever reaching its target organ. The oligonucleotide must pass through the blood vessel wall and navigate the interstitial space and extracellular matrix. Finally, if the oligonucleotide succeeds in reaching the appropriate cell membrane, it will usually be taken up into an endosome, from which it must escape to be active.

ASOs are usually delivered in saline and rely on chemical modifications to enable uptake. Their phosphorothioate backbone binds to serum proteins, slowing excretion by the kidney [109]. The aromatic nucleobases also interact with other hydrophobic molecules in serum and on cell surfaces. Many types of cells *in vivo* express surface receptors that actively take up oligonucleotides; these are often lost when cells are cultured, which explains why lipid seems more important for delivering ASOs in culture than *in vivo* [138].

Delivery is even more challenging for duplex RNAs than single-stranded oligonucleotides. In an siRNA, all of the aromatic nucleobases are on the inside, leaving only heavily hydrated phosphates on the outside of the duplex. This hydrated surface interacts poorly with cell surfaces and is rapidly excreted in the urine. Thus, researchers have invested heavily in the development of delivery vehicles for siRNAs [137,139–142]. The predominant technologies for delivering siRNAs involve complexing the RNA with cationic and neutral lipids [139,143,144], although encouraging results have also been obtained using peptide transduction domains [145] and cationic polymers [130]. Including PEGylated lipids in the formulation prolongs the circulating half-life of the particles [146]. Conjugation of cholesterol to one strand of the siRNA gave effective knockdown in the liver of mice [147], but the quantities of material required (50 mg/kg) were several orders of magnitude higher than current lipid-based formulations (as low as 0.01 mg/kg [148]).

Lipid-based formulations are ideal for targeting the liver, since lipid nanoparticles are readily taken up by liver cells. For targeting other organs following systemic administration, researchers have conjugated various ligands to the siRNA itself or included them as part of a formulation. Promising strategies include the use of aptamers [149], antibodies/fragments [150], vitamins [151], and other targeting ligands [130,152].

siRNA or ASO?

For cell culture experiments, siRNAs will often be the better choice. It is relatively simpler to discover potent siRNAs and it may be easier to obtain siRNAs since unmodified RNA works with high potency. ASOs, on the other hand, must contain chemical modifications to be active inside cells. For experiments where the goal is to develop compounds for testing in animals or investigate therapeutic development, the choice is more complex.

Several studies have directly compared the activity of various ASOs and siRNAs [105,153–157]. These studies can be misleading if one of the compounds contains suboptimal chemistry or sequence selection (eg first-generation PS-DNA ASOs); both potent and effective antisense oligonucleotides or siRNAs can generally be found if researchers are willing to invest in optimizing each technology [158]. An ideal target sequence for an ASO is not necessarily ideal for an siRNA, and vice versa.

In vivo, the choice of ASO versus siRNA is unsettled and will continue to evolve over the next decade. The challenges involved in delivering oligonucleotides to a given target tissue should be considered before choosing between them. For example, in animal models of Huntington's disease, antisense oligonucleotides or siRNAs have been infused directly into the central nervous system. In the case of single-stranded oligonucleotides, researchers observed wide distribution throughout the mouse CNS including deep-brain penetration [159]. In contrast, others found that siRNA infused into the monkey brain penetrated into brain tissue only up to about 12 mm from the site of infusion [159].

ASOs and siRNAs share important similarities as drug candidates. Both platforms are intended to modulate gene expression. Both are nucleic acids and contain an antisense strand intended to recognize a target mRNA. They also have important differences. ASOs have one strand, while siRNAs have two, a basic fact that may lower cost and simplify delivery. On the other hand, siRNAs have proven to be a more robust technology in cell culture in the hands of most users. It is not clear whether this will be true *in vivo*, but the possibility that siRNAs might have superior potency for at least some applications is a major driving force for their continued development.

Clinical progress

The clinical progress of oligonucleotide drugs has been slow because realizing the potential for ASOs and duplex RNAs requires inventing a new model for pharmaceutical development that allows large, highly charged molecules to be synthesized economically, distribute to target tissues, enter cells, and function within acceptable limits for toxicity. Oligonucleotides

are unlike traditional small molecule drugs (<500–700 molecular weight) and much effort has been required to understand their properties and optimize them. Antibody therapeutics provide a useful comparison. This class of molecules is now a major source of new drugs, but they also required many years to develop. Oligonucleotides may eventually have similar success. One ASO has been approved by the FDA and at least 22 oligonucleotide drugs are in phase II or III clinical trials (Table 2). Many more are earlier in the process of clinical development [91,158,160,161].

ASO-mediated gene inhibition in the clinic

ASOs began clinical development in the 1990s with first-generation compounds consisting of phosphorothioate DNA. One programme from ISIS Pharmaceuticals succeeded, leading to FDA approval of fomivirsen for treatment of CMV retinitis [162,163]. Development was facilitated by the location of the disease target and mode of administration. Fomivirsen is administered directly into the eye, reducing the amount of material needed and decreasing concerns about systemic side effects. One lesson for future work from these studies was that local delivery of oligonucleotides can simplify clinical studies and contribute to efficient trial design.

Other trials, however, were not successful. Drugs from Genta, Hybridon, and ISIS Pharmaceuticals failed in phase III clinical trials. Reasons that contribute to the lack of success include (i) incomplete understanding of the biological target and the consequences of its repression; (ii) the use of relatively inefficient first-generation chemically-modified PS-DNA oligonucleotides; and (iii) targeting disease tissues that are not prime locations for oligonucleotide biodistribution.

More recent trials have begun to revive optimism. Gapmer designs with optimized chemistry have led to improved potencies [28,164]. Biodistribution of oligonucleotides is better understood and some of the more promising trials involve inhibiting the expression of genes in the liver, an organ known to accumulate ASO [165]. The importance of convenient markers of activity has been recognized. Such markers allow an ASO-mediated down-regulation of target gene expression to be demonstrated early in clinical trials, permitting resources to be devoted to the most promising drug candidates.

An example of the new wave of more promising ASOs is mipomersen, an ASO from ISIS Pharmaceuticals designed to inhibit the expression of ApoB (Figure 4) [166]. Mipomersen is a gapmer containing phosphorothioate-modified DNA and 2'-O-MOE-RNA. Data from animal models show a robust and prolonged repression of ApoB expression [167]. In patients, the desired physiological response upon systemic administration was demonstrated by monitoring serum LDL-cholesterol: all primary, secondary,

Table 2. Selected oligonucleotide drug candidates in advanced clinical trials (phase II or higher). Besides references listed in the table, information was taken from company websites and refs 158 and 160

| Drug | Company | Phase | Target gene and disease | Notes and references |
|--|----------------------------|-------|---|---|
| mRNA-targeted antisense oligonucleotide drug candidates | | | | |
| Mipomersen | ISIS/Genzyme | III | ApoB for hypercholesterolaemia | PS-MOE gapmer, intravenous delivery; met all endpoints in four phase III trials [166] |
| OGX-011 (Custirsen) | ISIS/Teva/OncoGenex | III | Clusterin for prostate, NSCLC and breast cancer | PS-MOE gapmer [184] |
| AP 12009 (Trabedersen) | Antisense Pharma | III | TGF- β 2 for brain cancer | PS-DNA |
| GS-101 (Aganirsen) | Gene Signal | III | Insulin receptor substrate-1 for corneal neovascularization | PS-DNA, topical delivery (eye drops) |
| LOR-2040 | Lorus | II | Ribonucleotide reductase for cancer | PS-DNA |
| ASM8 | Pharmaxis | II | Multiple targets for allergic asthma | Two PS-DNA ASOs, delivered by inhalation |
| Archexin | Rexahn | II | AKT-1 for cancer | PS-DNA |
| LY2181308 | ISIS/Lilly | II | Survivin for cancer | PS-MOE gapmer |
| ISIS-EIF4 _{Rx} | ISIS/Lilly | II | eIF4E for cancer | PS-MOE gapmer |
| OGX-427 | ISIS/OncoGenex | II | Hsp27 for cancer | PS-MOE gapmer |
| Alicaforfen | ISIS/Atlantic | II | ICAM-1 for ulcerative colitis | Available when requested by physicians for patients with inflammatory bowel disease |
| AEG35156 | Aegera | II | XIAP for cancer | PS 2'-O-Me gapmer |
| Splice-switching oligonucleotide drug candidates | | | | |
| PRO051 (GSK2402968) | Prosenza/GSK | III | Dystrophin (exon 51 skipping) for DMD | 20-mer PS, fully-2'-O-Me ASO [46] |
| AVI-4658 | AVI BioPharma | II | Dystrophin (exon 51 skipping) for DMD | 30-mer morpholino [48] |
| PRO044 | Prosenza | I/II | Dystrophin (exon 44 skipping) for DMD | PS, fully-2'-O-Me ASO |
| Anti-miR oligonucleotide drug candidate | | | | |
| Miravirsen (SPC3649) | Santaris | II | miR-122 for hepatitis C virus | LNA-modified 15-mer anti-miR oligonucleotide, delivered intravenously [66] |
| siRNA drug candidates | | | | |
| ALN-RSV01 | Alnylam/Cubist/Kyowa Kirin | II | RSV (viral nucleocapsid) | Unmodified siRNA, intranasal or inhaled |
| PF-655 | Quark/Pfizer/Silence | II | RTP801 for wet AMD and diabetic macular oedema | Chemically modified, intraocular |
| QPI-1002 | Quark/Novartis/Silence | II | p53 for acute renal failure | Chemically modified, intravenous |
| Excellair | ZaBeCor | II | SYK kinase for asthma | Inhaled |
| Other advanced oligonucleotide drug candidates | | | | |
| IMO-2055 | Idera/Merck | II | TLR-9 activation for cancer treatment | CpG-rich oligonucleotide causes activation of TLR-9 [185] |
| GRN163L (Imetelstat) | Geron | II | Telomerase inhibitor for cancer treatment | 13-mer N 3' thiophosphoramidate oligonucleotide (lipid conjugate); inhibits telomerase by direct binding, not an antisense effect [186,187] |

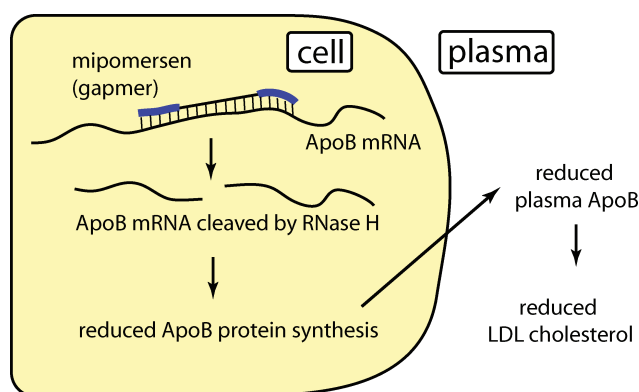


Figure 4. Mechanism of action of mipomersen.

and tertiary endpoints have been met in four separate phase III clinical trials [166]. Some toxic effects have been noted, and while these have been relatively mild, they may (at least initially) limit the patient population to patients who are at the most severe risk for atherosclerosis [168].

Eleven other traditional antisense oligonucleotides are in advanced clinical trials (Table 2). Targets relevant to cancer are the most highly represented, but there are also ASOs in trials against asthma, corneal neovascularisation, and ulcerative colitis. Many of these ASOs contain optimized chemistry and are taking advantage of the lessons learned over the past two decades in terms of delivery.

Modulating splicing in the clinic

Three splice-switching ASOs are in phase II or III clinical trials, all of them for treatment of Duchenne muscular dystrophy (DMD) (Figure 5). Prosensa has developed 2'-O-Me phosphorothioate oligonucleotides [46], while AVI BioPharma has favoured the development of morpholino oligomers [48]. Both drugs show promise in clinical development, but two particular challenges face splice-switching oligomers against DMD—the first is that of delivery. For significant clinical benefit, the ASOs would need to enter muscle tissue [144], including heart tissue. Currently, all of the splice-switching ASOs are delivered naked and are not efficiently taken up into heart muscle cells (cardiomyocytes) in particular. Delivery is aided to some degree by the weakened, 'leaky' nature of dystrophic muscle cells, but as the drug begins to take effect and the muscle cells recover, they are not such easy targets for further drug uptake. One possible solution is to use a targeted delivery system such as a cell-penetrating peptide [169,170]. Peptide-conjugated PMOs (PPMOs) take advantage of an active cell-internalization process and are taken up far more efficiently than unconjugated PMOs [171–177]. Even at lower doses, they distribute to more muscle cells body-wide, including healthy muscle cells and cardiomyocytes.

The second challenge is that DMD is caused by a large family of mutations. Exon 51 skipping would

in principle be helpful for ~13% of DMD patients, including those with deletions of exons 50, 52, 45–50, 48–50 or 49–50. Exon 44 skipping could help another ~6% of DMD patients. Further splice-switching ASOs could be developed that would help other patients, but the populations become increasingly small. It is unclear whether the regulatory process could one day be adjusted to approve, for example, personalized dystrophin-targeted splice-switching ASOs as a class [170]. Extensive clinical trials for a sequence with a small target population might be prohibitively expensive.

Clinical trials for ASOs targeting microRNA

The first anti-miR oligonucleotide to be tested in humans is being developed by Santaris Pharmaceuticals. Miravirsin is a 15-mer phosphorothioate oligonucleotide containing eight LNA modifications. It is complementary to miR-122 and designed to inhibit replication of hepatitis C virus (HCV) [65,66] (Figure 6). Since miravirsin was the first anti-miR to be tested in humans, no one could predict the effect of inhibiting a miRNA, thus simultaneously de-repressing a family of mRNA targets in humans. Accordingly, phase I testing started conservatively at 0.2 mg/kg delivered intravenously or subcutaneously. However, the drug was so well tolerated at the planned endpoint dose of 6 mg/kg that the trial was extended to a 12 mg/kg upper dose.

While miravirsin is designed to treat HCV, inhibition of miR-122 also lowers plasma cholesterol (Figure 6). Researchers at Santaris made use of this fact to demonstrate dose-dependent pharmacology in their phase I trial in spite of the fact that the trial enrolled healthy volunteers. Phase II clinical trials on HCV patients began in September 2010.

siRNAs in the clinic

A decade after the first siRNA experiments [72,178], a dozen siRNA drugs are in clinical development [91]. The four most advanced are in phase II trials (Table 2). As with ASOs, some of the earliest drugs to enter trials were very simple 'first-generation' siRNAs containing no chemical modifications. Two of these drugs, both targeting the VEGF pathway in the eye, had reached advanced clinical trials (phase II and phase III) but were withdrawn [91]. The therapeutic siRNA field is still young; however, many valuable lessons have been learned from 20 years of clinical work with ASOs that can now be applied to siRNA clinical development [179].

While cationic lipid-based formulations are clearly dominant in terms of potency, they deliver siRNAs into the endosome, the part of the cell where innate immune receptors are most intensely displayed. This means that

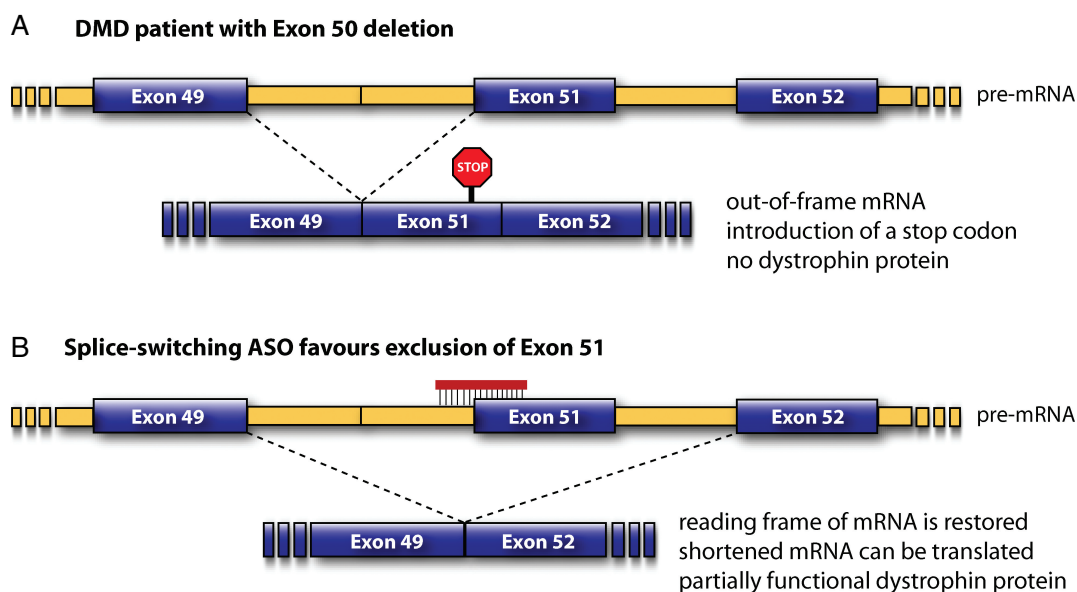


Figure 5. Mode of action of drug candidates PRO051 and AVI-4568. (A) This DMD patient is missing dystrophin exon 50. Splicing of the pre-mRNA gives mature mRNA that is out-of-frame and so no functional dystrophin can be produced. (B) In the presence of a splice-switching ASO that favours exclusion of exon 51, the cell splices exon 49 to exon 52, which restores the reading frame and causes translation of a shorter but partially functional dystrophin protein.

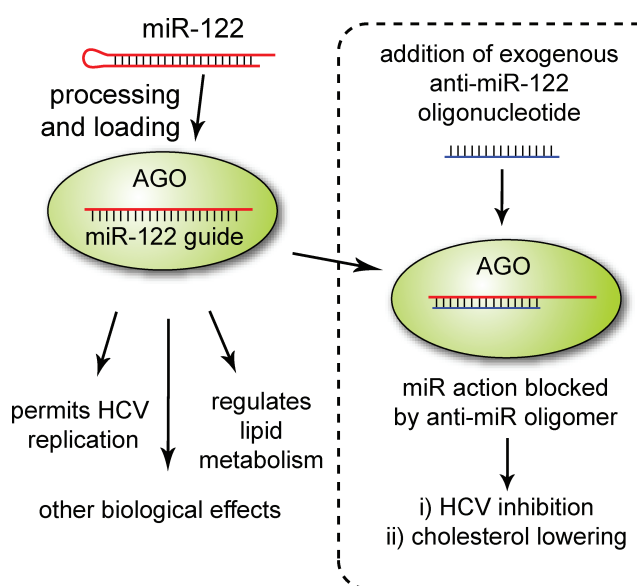


Figure 6. miR-122 is a liver-specific miRNA that regulates multiple pathways. Therapeutic inhibition of miR-122 by miravirsen blocks HCV replication and lowers plasma cholesterol.

siRNAs delivered by cationic lipid-based formulations are particularly vulnerable to immune stimulation. As such, a phase I drug candidate targeting ApoB from Tekmira Pharmaceuticals (<http://www.tekmirapharm.com>) was withdrawn from clinical trials after one patient at the highest dose level showed severe flu-like symptoms typical of an immune response. Tekmira halted the trial in autumn 2009 and optimized both the lipid delivery agent and the siRNA cargo, decreasing the immunogenicity of both, before returning to clinical testing of the improved candidate.

Conclusions and future prospects

The field of oligonucleotide therapeutics has often swung from irrational optimism to irrational despair. In the laboratory as well, gene knockdown experiments have fallen in and out of favour with researchers. In reality, oligonucleotides are useful tools with strengths and weaknesses. Different oligonucleotide technologies have different strengths, and many of the pendulum swings in the field have caused a switch from one oligonucleotide technology to another [180–183]. If researchers approach the oligonucleotide toolbox with careful experimentation and the broadest possible understanding, we believe that they will continue to find useful tools for both laboratory experiments and therapeutic development.

Acknowledgment

This work was supported by the National Institutes of Health (NIGMS 77253 and 73042 to DRC), the Robert A Welch Foundation (I-1244 to DRC), and the Natural Sciences and Engineering Research Council of Canada (postdoctoral fellowship to JKW).

Author contribution statement

JKW and DRC wrote the manuscript.

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