RNA Targeting Therapeutics: Molecular Mechanisms of Antisense Oligonucleotides as a Therapeutic Platform

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Abstract

Dramatic advances in understanding of the roles RNA plays in normal health and disease have greatly expanded over the past 10 years and have made it clear that scientists are only beginning to comprehend the biology of RNAs. It is likely that RNA will become an increasingly important target for therapeutic intervention; therefore, it is important to develop strategies for therapeutically modulating RNA function. Antisense oligonucleotides are perhaps the most direct therapeutic strategy to approach RNA. Antisense oligonucleotides are designed to bind to the target RNA by well-characterized Watson-Crick base pairing, and once bound to the target RNA, modulate its function through a variety of postbinding events. This review focuses on the molecular mechanisms by which antisense oligonucleotides can be designed to modulate RNA function in mammalian cells and how synthetic oligonucleotides behave in the body.

INTRODUCTION: AN RNA WORLD

The past 10 years have witnessed an explosion in knowledge regarding the diverse functional roles that RNAs play in regulating cellular processes and have provided many surprises (1). RNA has long been recognized as playing center stage in the central dogma of molecular biology, that is, messenger RNAs, transfer RNAs, and ribosomal RNAs. Shortly after the role of RNAs in protein biosynthesis was determined, it was discovered that most messenger RNAs derive from much larger precursors in which the coding region is interrupted by large blocks of RNAs that must undergo splicing reactions to remove intervening sequences. Surprisingly, a family of small RNAs, small nuclear RNAs (snRNAs), were found to play key roles in the splicing process by binding to the splice donor and acceptor sites. Recently, a new class of small RNAs has been identified, microRNAs, which control translation of targeted mRNAs, essentially acting as naturally occurring antisense oligonucleotides (2, 3). Thus, the roles of RNA in translating information from the genetic code to protein products have greatly expanded. These discoveries were followed by the identification of additional classes of small RNAs and a diverse group of large noncoding RNAs, whose functions are poorly characterized (4–7). Recent estimates suggest that as much as 90% of the genome is transcribed, resulting in production of an extremely diverse population of cellular RNAs, including overlapping transcripts in both the sense and antisense orientation, highlighting that we still have much to learn about RNAs (4, 8).

Another fairly recent advance is a greater appreciation that genetic changes can affect the function of RNA such that RNA is the initiator of pathology, independent of protein-coding activity (RNA-mediated toxicity) (9, 10). Perhaps the best-characterized examples of RNA-mediated disease are those diseases that feature an expansion of two- to six-nucleotide repetitive sequences in noncoding regions of the transcript. Examples include the neuromuscular diseases myotonic dystrophies (types 1 and 2), spinocerebellar ataxia 8, Huntington's disease–like 2, and fragile X-associated tremor ataxia syndrome. Among these diseases, type 1 myotonic dystrophy is best characterized from a mechanistic standpoint. Type 1 myotonic dystrophy is caused by expansion of a trinucleotide repeat cytotide-thymidine-guanidine (CTG) repeat in the 3′-untranslatated region of the gene that encodes dystrophia myotonica-protein kinase, resulting in a large (80 to 3000) trinucleotide repeat in the mRNA. Dystrophia myotonica-protein kinase transcripts containing this large CUG repeat are primarily retained in the nucleus and form nuclear foci with splicing factors such as muscleblind, depleting the nucleoplasm of the factors (9, 10). Other examples of how RNAs can directly contribute to disease include deregulation of microRNA expression and genetic changes that result in the alteration of microRNA binding sites in a protein-coding transcript (11–14). Given the increased understanding of potential roles of RNAs in the maintenance of normal health and in contribution to disease, directly targeting RNAs is an increasingly compelling therapeutic strategy.

Since its inception, the modern pharmaceutical industry has focused almost exclusively on proteins as drug targets, either by design or happenstance (15). Rationally designing or identifying traditional small-molecule drugs that selectively interact with nucleic acids, carbohydrates, or lipids in a safe and efficacious manner has been challenging. There are a small number of cytotoxic chemotherapeutic agents that primarily interact with DNA, and aminoglycoside and macrolide antibiotics that bind to bacterial ribosomal RNAs. However, these examples are the exception rather than the rule, and additional therapeutic platforms are needed to expand the list of druggable macromolecules. As described in detail in this review, we proffer that antisense oligonucleotides represent a promising drug platform that has the potential to target, in a selective manner, all RNAs in a cell, thus dramatically expanding the druggable universe and in the process producing medicines that will have a major impact on patients' lives.

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The authors recognize that antisense oligonucleotides are not a new concept as a therapeutic platform (16). However, there has been significant progress over the past few years, both in preclinical or research settings and in the clinic. First, results from clinical trials clearly demonstrate that it is possible to safely practice antisense pharmacology in humans at commercially attractive doses (17, 18). Second, antisense oligonucleotides that work through a variety of mechanisms have been identified and have progressed into clinical trials, thereby expanding the application of the technology (19). Finally, new oligonucleotide chemistries and formulations for delivery have been identified that promise to further enhance the properties of antisense drugs by increasing potency, safety, and broader tissue distribution. Several recent reviews and whole volumes have been dedicated to antisense technology, to which the reader is referred (20–25). We focus this review on molecular aspects of the technology, highlighting areas where significant advances have occurred and identifying some key questions and issues for future research.

ANTISENSE MECHANISMS

There has been some confusion in the scientific and lay literature regarding the definition of the types of molecules that should be considered antisense oligonucleotides. For the purposes of this review, we define antisense oligonucleotides as those oligonucleotides that are 8 to 50 nucleotides in length that, in toto or in part, bind to RNA through Watson-Crick base pairing (**Figure 1**) and upon binding to RNA, modulate the function of the targeted RNA. This definition includes a wide variety of oligonucleotide designs that modulate RNAs thorough a diverse set of postbinding mechanisms, including RNA interference. These mechanisms can be broadly categorized as (*a*) those that involve binding to the RNA and interference with its function without promoting RNA degradation and (*b*) those that promote degradation of the RNA either through endogenous enzymes, such as RNase H, or Argonaute 2 (RNA interference), or cleavage mechanisms designed into the oligonucleotide (**Figure 2**, **Table 1**). According to this definition, antisense oligonucleotides do not encompass oligonucleotides that form triple helix structures with DNA, double-stranded DNA structures that serve as transcription factor decoys, or oligonucleotides designed to bind to proteins, often referred to as aptamers. One could think of oligonucleotides as the drug family, antisense as the genus, and different mechanisms as the species.

The Receptor

The receptor for antisense-based drugs is RNA. Antisense oligonucleotides bind to the targeted RNA by the well-characterized base pairing mechanism first defined by Watson and Crick (**Figure 1**). This mechanism includes specific hydrogen bonding interactions between bases on the drug and the target RNA strand, as well as hydrophobic interactions resulting from base shape complementarity and coaxial base stacking. RNA targets in mammalian cells are a diverse set of molecules that range in size from less than 20 nucleotides in length on one extreme to hundreds of thousands or perhaps over 1 million nucleotides in length on the other. RNA folds into complex three-dimensional structures equal in complexity to proteins (26). Akin to proteins, RNAs have a primary structure (the sequence) and a secondary structure (the folding, or how the sequence interacts with itself). The binding interactions of RNA secondary structures tend to be dominated by Watson-Crick base pairing interactions, which drive the folding of RNAs into self-complementary stem-like structures. Imperfections in the duplex structure lead to unique shapes that can interact with other portions of RNA to form more complex tertiary structures in a manner similar to protein folding. In general, there is less potential for hydrophobic interactions in RNA structures than in proteins, which likely results in many RNA structures being quite

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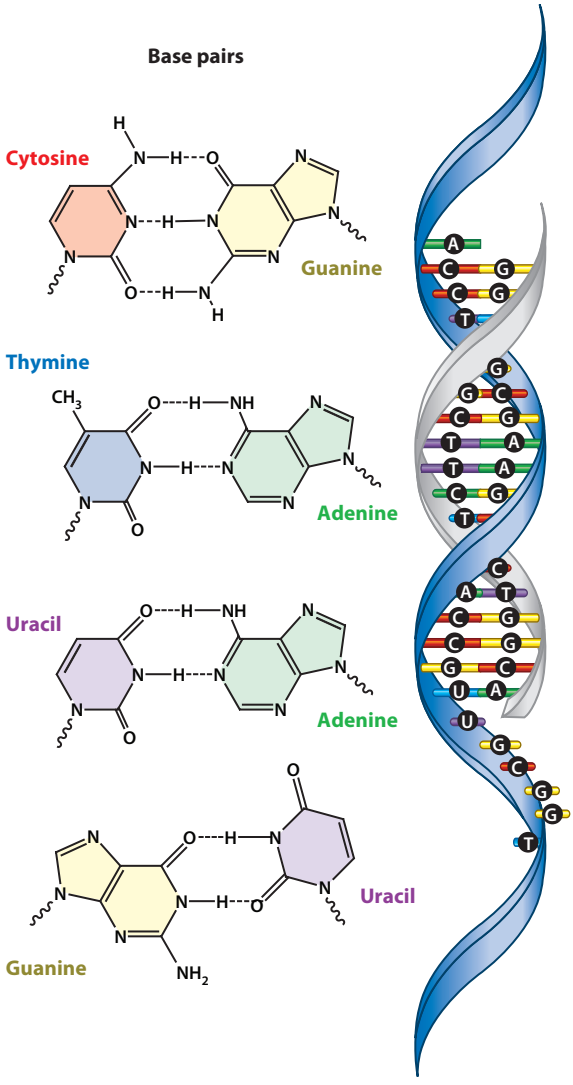

Figure 1

Base pairing interactions formed during Watson-Crick hybridization. The hydrogen bonds formed as a result of Watson-Crick hybridization are shown on the left side of the figure. Antisense oligonucleotide (*gray*) bound to a target RNA (*blue*) is shown in the right side of the figure.

dynamic and having several low-energy structures represented at equilibrium. Ribosomal RNA is a notable exception, being highly ordered and having many hydrophobic interactions (27).

How antisense oligonucleotides find their receptor (RNA) in a cell is largely unknown. A cell may contain over a hundred million RNA molecules, with the number of unique RNA transcripts ranging from one copy per cell for some mRNAs, to greater than 1 million copies per cell for snRNAs, to 10 million or more copies per cell for ribosomal RNAs. Given the complexity of the RNA transcriptome and the potential for partial interactions of the oligonucleotide with numerous RNAs, it is remarkable that an antisense oligonucleotide binds to a given target RNA with an apparently high degree of fidelity. Numerous experiments have demonstrated that antisense

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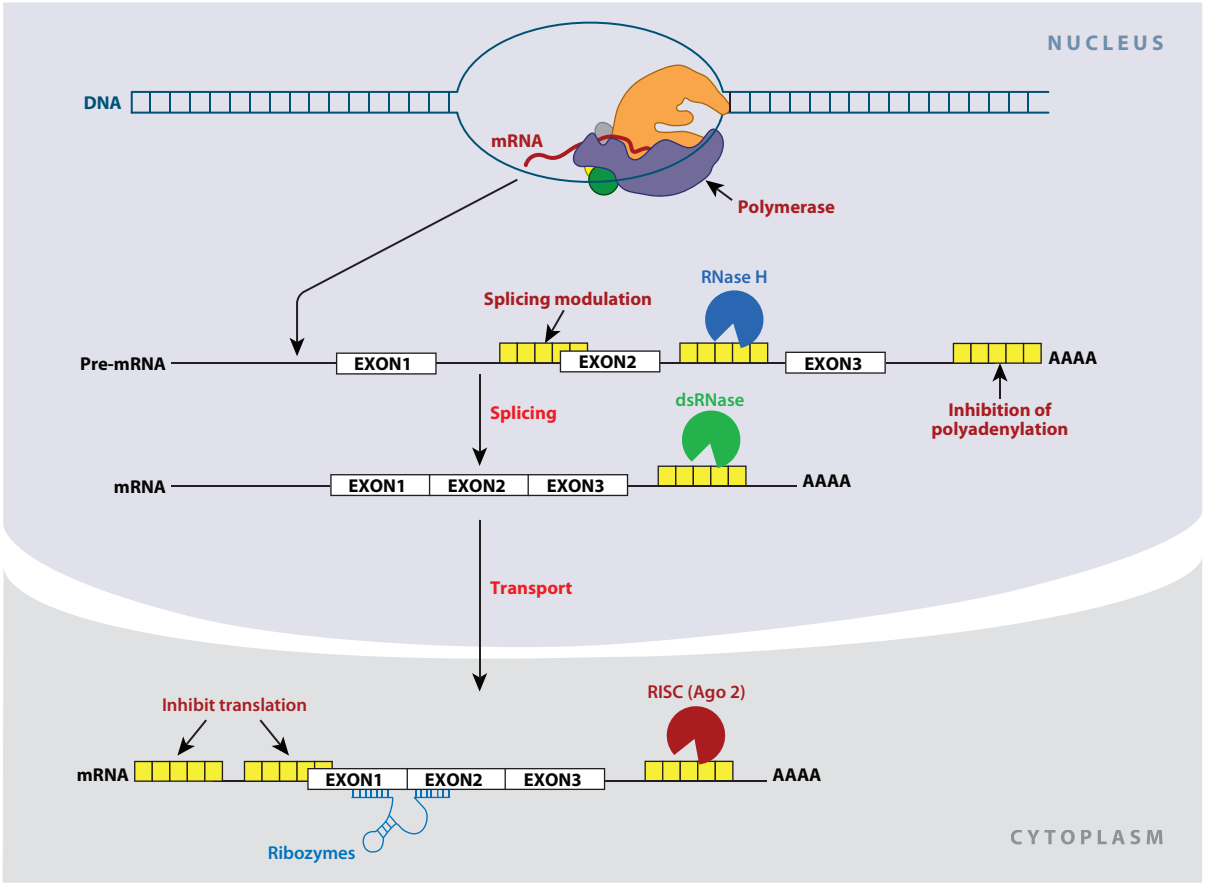

Figure 2

Different antisense mechanisms: different steps in the maturation of an mRNA where antisense oligonucleotides are known to interact and block the function of the mRNA. Different antisense mechanisms shown include the nondegradative mechanisms (e.g., modulation of RNA splicing, modulation of polyadenylation, inhibition of translation) and mechanisms that promote degradation of the RNA [e.g., RNase H, RNA interference (Ago 2), ribozymes, and double-stranded RNases (dsRNase)].

oligonucleotides exhibit exquisite specificity, capable of distinguishing a single nucleotide mismatch (28–31). Although we cannot rule out the possibility that interaction with the target RNA occurs by simple diffusion of the oligonucleotide in cytoplasm or nucleoplasm and random interaction with the target RNA, this seems unlikely given the specificity of the oligonucleotides. A hint may be provided by studying the natural antisense mechanisms, such as microRNAs, described in more detail below. In this case the oligonucleotide binds to a protein forming a heterodimeric complex, which in turn binds to the target RNA directed by the sequence of the oligonucleotide loaded into the protein complex. The oligonucleotide in the complex presumably has fewer nonproductive interactions with proteins and other nucleic acids. The protein-RNA complex may also localize in the cell where mRNAs are present. Another intriguing observation is the finding that RNA binding proteins, such as hnRNP A1 and YB1, facilitate nucleic acid hybridization up to 1000-fold (32, 33). Thus, it is possible that hnRNPs or other proteins enhance hybridization of the oligonucleotide to the RNA. Clearly, more work is needed to better understand how antisense oligonucleotides bind to the targeted RNA in cells.

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Table 1 Different antisense mechanisms

Mechanism	Example	Reference
Noncleavage	Translation arrest	(211, 212)
	Inhibition of translation initiation	(63)
	Inhibition of exon inclusion	(81)
	Promotion of exon inclusion	(213, 214)
	Inhibition of polyadenylation	(88)
	RNA antagonist	(68, 72, 215)
	RNA agonist	(54, 216)
	Disruption of RNA structure	(92, 217)
Cleavage	RNase H	(218, 219)
	Ago 2 (RNA interference)	(40, 41)
	MicroRNA (P-body)	(50, 54, 220)
	U1RNA adapters	(55)
	Ribozymes	(221, 222)
	DNAzymes	(57)
	Chemical facilitated	(60, 223)
Unknown	Promoter-targeted oligonucleotides	(95, 96, 224)

Because the nature of the binding interaction with its receptor is well known, it is easy to design an antisense oligonucleotide that will bind to the targeted RNA. However, owing to both the complex and dynamic secondary and tertiary structures that RNA forms and occupancy of RNA by proteins, not all sites on the receptor are accessible to oligonucleotide drugs. Recent advances in predicting oligonucleotide binding sites on the RNA receptor have enhanced the probability of identifying active oligonucleotides versus random selection but still do not adequately predict for activity. Therefore, screening for active antisense oligonucleotides in cell culture cannot be eliminated (34–36). Fortunately, with the availability of large volume oligonucleotide synthesizers, high-throughput RNA quantitation methodologies and robotic sample handling, screening large numbers of oligonucleotides can be easily accomplished.

All antisense mechanisms have in common the binding of the oligonucleotide to the targeted RNA. What happens after the oligonucleotide binds to the RNA is dictated by the chemistry, design of the oligonucleotide, where on the RNA the oligonucleotide binds, where in the cell the oligonucleotide binds to the RNA, and auxiliary factors associated with the RNA. Different types of antisense mechanisms can be broadly classified as cleavage-dependent mechanisms and occupancy-only mechanisms (**Table 1**).

RNA Cleavage Mechanisms

Oligonucleotides that work through an RNase H–dependent cleavage mechanism are the best-understood class of antisense oligonucleotides, accounting for the majority of drugs in development (**Table 2**). RNase H is a family of enzymes present in all mammalian cells that mediates the cleavage of the RNA in an RNA-DNA heteroduplex (37). RNase H recognizes an RNA-DNA heteroduplex, cleaving the RNA strand, resulting in a 5′-phosphate on the product and release of the intact DNA strand (oligonucleotide). Human cells contain two types of RNase H: RNase H1 and RNase H2. Human RNase H1 is active as a single peptide, whereas RNase H2 is a heterotrimeric enzyme (37). Both enzymes are thought to play a role in DNA replication and repair, but additional

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biological functions are likely for both. RNase H1 is the enzyme responsible for mediating the target RNA cleavage directed by antisense oligonucleotides containing five or more consecutive DNA nucleotides (38). Human RNase H1 binds to the RNA-DNA heteroduplex through an RNA binding domain located on the N terminus of the protein, with cleavage of the RNA occurring 7 to 10 nucleotides from the 5′-end of the RNA (approximately one helical turn). The RNase H mechanism has proven to be a robust antisense mechanism and is broadly exploited as both a research tool and a potential human therapeutic (**Table 2**).

Oligonucleotides that work through an RNA interference mechanism are another example of an antisense mechanism that induces cleavage of the target RNA through an endogenous enzyme (39). In this case, the enzyme responsible for cleavage of the RNA in human cells is Argonaute 2 (Ago 2), which cleaves the RNA through an RNase H–like enzyme mechanism (40, 41). Oligonucleotides that work through the RNA interference (siRNA) mechanism appear to mimic endogenous small RNAs present in cells, which naturally regulate expression of the targeted gene. The mechanisms by which siRNA oligonucleotides modulate expression of the target gene have been fairly well characterized, although the source and role of the naturally occurring siRNAs in mammalian systems have not. In *Drosophila* cells, the enzyme Dicer cleaves long double-stranded RNA substrates into 21 to 23 nucleotide products. These products are transferred to a protein complex termed the RISC loading complex, which in turn forms a pre-RISC complex with duplexed oligonucleotides (42). The pre-RISC complex contains Ago 2 protein and other unknown factors. The two strands are separated such that one of the strands loads into the Argonaute protein and forms a haloenzyme. The *Drosophila* Ago 2 protein is capable of binding duplex RNA substrate directly, cleaving, and releasing the sense strand (43). Exogenously delivered siRNAs appear to enter the pathway downstream of Dicer because inhibition of Dicer expression does not impact activity (44). Mammals differ from *Drosophila* in having only one Argonaute protein (Ago 2) capable of cleaving the target RNA; this protein is most closely related to the *Drosophila* Ago 1 protein. The mammalian Ago 2 protein does not bind to duplex RNA but is capable of binding single-stranded RNA (45, 46). Mammalian Ago 2 also differs from *Drosophila* Ago 2 in that the mammalian enzyme exhibits burst kinetics without release of product in the purified state, which is similar to the *Drosophila* Ago 1 enzyme (47).

Cellular factors that promote product release from the Ago 2 complex have not been identified. Interestingly, the PIWI domain of Argonaute proteins forms an RNase H–like structure. Ago 2 is the only Argonaute family member in human cells to have the critical active site residues that coordinate a divalent cation required for enzyme activity (48). These mechanistic studies clearly demonstrate that oligonucleotides that promote cleavage of a targeted RNA through the RNA interference mechanism do so through an antisense mechanism very similar to RNase H–dependent oligonucleotides. However, a key difference is that the oligonucleotide is bound to the cleavage enzyme prior to interacting with the target RNA for the RNA interference mechanism. In contrast, RNase H–dependent oligonucleotides bind to the target RNA prior to interaction with the enzyme, although the evidence supporting these steps occurring in the order outlined in cells or tissues is largely circumstantial. Another antisense mechanism that results in loss of RNA mimics the natural pathway through which microRNAs regulate expression of gene products (49, 50). For microRNA-mediated target RNA reduction, the oligonucleotide only requires hybridization of six to eight nucleotides in the 5′-end of the oligonucleotide, the so-called seed region, to a sequence that occurs predominantly in the 3′-untranslated region of an mRNA. Because microRNAs bind to target mRNAs through only six to eight nucleotides, a single microRNA may regulate hundreds of different mRNAs (51).

All four human Argonaute proteins have been associated with endogenous microRNAs, but Ago 1, 3, and 4 lack the critical amino acids needed to form the RNase H catalytic domain, thus these

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Table 2 Examples of antisense drugs in clinical trials or marketed

Drug	Indication	Target	Mechanism	Chemistry[a]	Route	Status
Fomiversen	Cytomegalovirus	IE2 gene	RNase H	PS ODN	Intravitreal	Approved
Oblimersen	Oncology	Bcl-2	RNase H	PS ODN	Systemic	Phase 3
Mipomersen	Cardiovascular	Apolipoprotein B	RNase H	2′-MOE chimera	Systemic	Phase 3
Trabedersen	Oncology-glioblastoma	Transforming growth factor-β	RNase H	PS ODN	Intratumoral	Phase 3
GS-101	Corneal neovascularization	Insulin receptor substrate-1	RNase H	PS ODN	Topical	Phase 3
LOR-2040	Oncology	Ribonucleotide reductase	RNase H	PS ODN	Systemic	Phase 2
Archexin	Oncology	AKT-1	RNase H	PS ODN	Systemic	Phase 2
TPI ASM8	Asthma	CCR3 and IL-5 receptors (two oligos)	RNase H	PS ODN	Inhaled	Phase 2
Alicaforsen	Colitis	Intercellular adhesion molecule-1	RNase H	PS ODN	Enema	Phase 2
Custirsen	Oncology	Clusterin	RNase H	2′-MOE chimera	Systemic	Phase 2
LY2181308	Oncology	Survivin	RNase H	2′-MOE chimera	Systemic	Phase 2
AEG35156	Oncology	X-IAP	RNase H	2′-*O*-Me chimera	Systemic	Phase 2
TV/ATL1102	Multiple sclerosis	CD49D	RNase H	2′-MOE chimera	Systemic	Phase 2
ISIS 1113715	Diabetes, type 2	Protein tyrosine phosphatase-1B	RNase H	2′-MOE chimera	Systemic	Phase 2
Monarsen	Myasthenia gravis	Acetylcholine esterase	RNase H	2′-*O*-Me chimera	Oral	Phase 2
ALNRSV-01	Respiratory syncytial virus	Nucleocapsid N gene	siRNA	dsRNA (unmodified)	Inhaled	Phase 2
PF-4523655	Age-related macular degeneration	DNA-damage-inducible transcript 4 (REDD-1, RTP801)	siRNA	dsRNA (modified)	Intravitreal	Phase 2
AVI-4126	Restenosis	c-Myc	Translation inhibition	Morpholino	Drug-eluting stent	Phase 2
PRO-051	Duchene muscular dystrophy	Dystrophin	Splicing modulation	2′-*O*-Me	Systemic	Phase 2

GRN163L	Oncology	Telomerase	RNA binding	Lipid-conjugated phosphoramidate	Systemic	Phase 2
OGX-427	Oncology	Heat shock protein 27	RNase H	2′-MOE chimera	Systemic	Phase 1
LY2275796	Oncology	eIF-4E	RNase H	2′-MOE chimera	Systemic	Phase 1
AIR 645	Asthma	Interleukin 4 receptor alpha	RNase H	2′-MOE chimera	Inhaled	Phase 1
ISIS-CRP$_{Rx}$	Cardiovascular/ inflammation	C-reactive protein	RNase H	2′-MOE chimera	Systemic	Phase 1
OMJP-GCGR$_{Rx}$	Diabetes	Glucagon receptor	RNase H	2′-MOE chimera	Systemic	Phase 1
ISIS-SGLT2$_{Rx}$	Diabetes	Sodium-dependent glucose transporter 2	RNase H	2′-MOE chimera	Systemic	Phase 1
iCO-007	Macular degeneration	C-Raf kinase	RNase H	2′-MOE chimera	Intravitreal	Phase 1
SPC2996	Oncology	Bcl-2	RNase H	LNA chimera	Systemic	Phase 1/2
EZN2968	Oncology	Hypoxia inducing factor 1-α	RNase H	LNA chimera	Systemic	Phase 1/2
EZN3042	Oncology	Survivin	RNase H	LNA Chimera	Systemic	Phase 1
QPI-1102	Acute kidney injury	p53	siRNA	dsRNA	Systemic	Phase 1
ALN-VSP	Oncology	Vascular endothelial growth factor and kinesin family member 11	siRNA	Modified dsRNA in liposome formulation	Systemic	Phase 1
CALAA-01	Oncology	Ribonucleotide reductase	siRNA	dsRNA in nanoparticulate formulation	Systemic	Phase 1
AVI-4658	Duchene muscular dystrophy	Dystrophin	Splicing	Morpholino	Systemic	Phase 1
SPC3649	Hepatatis C virus	microRNA 122	RNA blocking	LNA chimera	Systemic	Phase 1

[a]2′-MOE chimera, 2′methoxyethyl-DNA chimeric oligonucleotides with phosphorothioate linkages; 2′-*O*-Me chimera, 2′-*O*-methyl–DNA chimeric oligonucleotide with phosphorothioate linkages; LNA chimera, locked nucleic acid–DNA chimera with phosphorothioate linkages.

proteins are not capable of directly degrading the bound mRNA. Although the details have not been worked out, the Ago-microRNA complex bound to the target RNA (ternary complex) somehow directs the transcript to sites in cells called P bodies, which are enriched in RNA degradation factors such as deadenylation enzymes, decapping enzymes, and exonucleases (52, 53). In contrast to RNase H or siRNA oligonucleotides, in which the desired goal is to promote selective degradation of a single transcript, oligonucleotides that work through a microRNA-dependent pathway result in the degradation of numerous transcripts. This approach is being explored as a means of replacing microRNAs that are dysregulated in cancer and other diseases (54).

A novel approach for selectively decreasing the expression of a target RNA was recently described (55). The authors designed a bifunctional oligonucleotide containing a sequence that targeted the U1 snRNA splicing factor and a sequence complementary to the target RNA sequence, thus tethering U1 snRNPs to the targeted RNA. When the target site was 3′ to a site near the conserved polyadenylation sequence, the complex inhibited polyadenylation, resulting in degradation of the transcript. Although the authors addressed some of the concerns regarding depletion of U1 snRNA with the synthetic oligonucleotide, concerns about the long-term safety and specificity of this approach remain. In addition to the nucleases mentioned above, numerous other nucleases and natural RNA-degrading pathways, such as nonsense-mediated decay, that are present in cells could potentially be harnessed to promote selective degradation of RNAs (56).

Other approaches that have been developed to promote selective cleavage of RNA include ribozymes and DNAzymes in which the oligonucleotide possesses inherent catalytic activity (57–59). Although ribozymes and DNAzymes have shown promise in cell culture experiments, they have not proven to be as effective in vivo as some other approaches and have largely been abandoned as a therapeutic strategy for targeting RNA. Approaches to attach so-called chemical warheads such as metallonucleases to oligonucleotides as a means to promote selective RNA degradation have shown encouraging activity in cell-free assays (60, 61). These approaches have proven to be difficult synthetically and currently do not appear to offer an advantage over simpler approaches that take advantage of endogenous nucleases present in cells.

Non-RNA–Degrading Mechanisms

Blocking translation of an mRNA by an oligonucleotide is the prototypical antisense mechanism and is often referred to as translation or hybridization arrest. Such oligonucleotides are usually designed to bind to, or are adjacent to, the translation initiation region of an mRNA and are thought to block either the scanning of the 40S subunit on the transcript, assembly of the 40S and 60S ribosomal subunits, or movement of the ribosome down the transcript once assembly has occurred. Although evidence suggests that oligonucleotides utilize such mechanisms in cell free assays, there is limited evidence that oligonucleotides designed to work through a translation arrest mechanism actually do so in cell culture or in vivo. Translation arrest is not widely used as a therapeutic strategy (**Table 2**), although it is utilized as a research tool for studies of zebra fish (62). Factors that limit its potential use as a therapeutic approach include that it is more difficult to document the pharmacological activity of the oligonucleotide owing to variability in the availability of quality reagents for protein detection (because translation arrest does not decrease mRNA levels) and the limited sequence space on the RNA in which this mechanism is effective. Despite these limitations, oligonucleotides have been identified that work through translation arrest to selectively inhibit the expression of the targeted gene product. A variant of the translation arrest mechanism is use of oligonucleotides to block interaction of the 40S ribosome subunit to the mRNA by binding to the transcript adjacent to the 5′-cap (63). Such oligonucleotides likely prevent formation of the

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preinitiation complex on the mRNA transcript through blocking interactions of eIF4E or other 5′-cap–binding proteins with the transcript.

As discussed above, microRNAs are small noncoding RNAs present in eukaryotes that in essence function as naturally occurring antisense oligonucleotides. In addition to promoting degradation of the targeted RNA transcript, microRNAs can also block protein translation without affecting the level of the targeted mRNA transcript (3). The mechanisms by which microRNAs regulate translation are still the subject of debate (64–67). Insights into their mechanism are provided by the finding that the Mid domain on Argonaute proteins resembles eIF4E, binding the m7G cap structure found at the 5′-end of mRNA transcripts. Argonaute proteins that bind the cap structure compete with eIF4E for binding to the m7G cap present on the 5′-end of mRNAs (65).

Another approach to modulating microRNA function in tissues is to antagonize the microRNA. This approach has been successfully applied both in cell culture and in vivo (68–71). In this case, a single-strand synthetic oligonucleotide is designed to bind to the 21- to 23-nucleotide microRNA and upon binding to the microRNA blocks its ability to interact with mRNAs. Although initial reports suggested that the oligonucleotide binding to the microRNA promoted its degradation, this conclusion is questionable because the oligonucleotide binding to the microRNA interferes with its detection. Recent work clearly demonstrates that an oligonucleotide can interfere with microRNA function without promoting its degradation (72). Additional work is needed to clarify the mechanism by which microRNA-targeting oligonucleotides interfere with microRNA function. As our knowledge of the contribution of microRNAs to disease increases, it is likely that microRNA agonists and antagonists will become important therapeutic strategies.

Telomerase is another ribonucleoprotein complex in which a segment of the RNA is exposed and enables specific interaction of the complex with another nucleic acid. Telomerase binds to telomere sequences at the end of chromosomes and is involved in maintaining telomere length. Telomerase activity is present in cancer cells but not most normal somatic cells and may provide a survival advantage to the cancer by maintaining telomere length (73). Antisense oligonucleotides designed to bind to the exposed RNA in telomerase (complementary to the telomere sequence) have been shown to inhibit telomerase activity, promote telomere shortening, and inhibit proliferation of cancer cells (74, 75).

Two additional antisense mechanisms that rely on binding to the target RNA, but not its degradation, interfere in RNA intermediary metabolism. Most mRNAs undergo a complex series of processing steps that include splicing, polyadenylation, and addition of the 7mG 5′-cap structure (1). It has been estimated that approximately 90% of the mRNA transcripts exhibit alternatively spliced variants in human cells (76). The alternatively spliced transcripts may result in proteins with similar biological properties or can exhibit very distinct biological functions [e.g., BCL-X_L and BCL-X_S (77)]. In addition, many mRNAs exhibit alternate polyadenylation sites, which can result in inclusion or loss of RNA regulatory elements in the 3′-UTR of the transcript (78, 79). As a result of these possible alternative maturation steps, a given gene may encode multiple related transcripts that can have different biological functions or be subject to different regulatory controls. Multiple examples of genetic diseases caused by aberrant RNA intermediary metabolism exist; such disease settings present opportunities for antisense oligonucleotide–based therapeutics (9). These RNA processing events occur in the cell nucleus, where single-stranded oligonucleotides readily accumulate following introduction into the cytoplasm, which further enhances the probability of success (80). Numerous studies have demonstrated that antisense oligonucleotides are capable of binding to the pre-mRNA and modulating RNA splicing both in vitro and in vivo by masking splicing enhancer and repressor sequences (81–87). This includes exon skipping and inclusion of an alternatively spliced exon. In addition to modulating RNA splicing, it has been demonstrated

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that antisense oligonucleotides can modulate poly A site selection in a transcript (88). These results support the contention that antisense oligonucleotides represent a unique therapeutic strategy for treatment of diseases of RNA intermediary metabolism.

As previously discussed, RNA forms a variety of secondary and tertiary structures that are important for normal function and gain of function in pathologies (9, 10, 89, 90). Antisense oligonucleotides can be utilized to bind to a target RNA and disrupt RNA structures, interfering with the regulatory role provided by the structure. A well-characterized example is the conserved stem-loop transactivator response RNA structure present in the 5′-end of HIV transcripts. This RNA structure binds the HIV protein Tat, which is an important step in viral replication. Antisense oligonucleotides have been used to bind to the HIV RNA, disrupting the transactivator response RNA stem-loop structure, such that Tat protein no longer binds to it (91, 92). Another potential example is the recent demonstration that antisense oligonucleotides designed to bind to the trinucleotide CAG repeat in the first exon of the huntingtin mRNA selectively inhibit expression of mutated huntingtin versus wild-type protein (93). Expansion of the CAG repeat from 12 to 39 repeats to greater than 45 repeats is responsible for the toxic gain of function in the protein. It was proposed that oligonucleotides may disrupt a hairpin structure in the mutant huntingtin RNA formed by the expanded CAG repeat. These results were extended to ataxin-3, which has a CAG repeat in the coding region and causes spinocerebellar ataxia type 3 when the CAG repeat expands. A similar strategy has been successfully applied to an expanded CUG repeat that causes myotonic dystrophy (94). The oligonucleotide disrupts the interaction of muscleblind to the CUG repeat, reversing the splicing defects and defective chloride channel conductance observed in a mouse model of myotonic dystrophy. Both of these studies demonstrate the potential of antisense oligonucleotide as a therapeutic for triplet repeat disease.

A recent surprising finding was that oligonucleotides designed to bind to promoter sequences inhibited the expression of the targeted gene. This observation has been made with peptide nucleic acids, single-stranded oligonucleotides, and double-stranded oligonucleotides targeting the promoter sequence (95, 96). Remarkably, oligonucleotides targeting promoter sequences are also capable of activating gene expression (97). The mechanisms by which oligonucleotides targeting promoter sites activate or inhibit gene transcription are not well understood but appear to involve targeting of short RNA transcripts that are found at promoter sites in either the antisense or sense orientation (98–100). These short transcripts affect histone methylation patterns at the promoter site through a poorly characterized mechanism that results in changes in transcriptional rates. It is not known whether the oligonucleotide directly promotes a loss in the RNA transcript or binds to the RNA and blocks its function. Although it has not been definitively proven that these oligonucleotides work via an antisense mechanism as defined above, the prevailing body of data suggest that they do. Additional studies are necessary to better characterize the mechanism by which these oligonucleotides exert their action and determine how viable they are as therapeutic strategy.

The above discussion focuses on antisense mechanisms currently being exploited in the laboratory and as potential therapeutics. As knowledge of RNA regulatory pathways increases and oligonucleotide chemistry advances, it is likely that additional antisense mechanisms will be developed.

Comparisons of Different Antisense Mechanisms

Multiple articles have touted the attributes of one antisense mechanism while being critical of other mechanisms. In our experience, all the antisense mechanisms described above can be designed to function very effectively in cell culture assays. For example, a comparison of a series of optimized

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RNase H oligonucleotides to siRNA oligonucleotides in cultured cells revealed that they have similar potencies, maximal efficacies, and sequence specificities (30). Others have reported that siRNA oligonucleotides were more potent in cell culture than RNase H oligonucleotides (101, 102). In these instances, the RNase H oligonucleotides used were not optimally designed, incorporating low-affinity phosphorothioate-modified oligodeoxynucleotides (first-generation modification) and/or designs in which the oligonucleotide is not stable to nuclease degradation in cell culture. However, with recent advances in siRNA designs, including chemical modifications, it is possible to identify and use very potent siRNAs in cell culture assays; in many cases siRNA oligonucleotides may be more potent than oligonucleotides that work through other antisense mechanisms (103, 104).

RNase H oligonucleotides and those that mediate RNA splicing (85, 105) and prevention of ribosome assembly (63) have similar potencies in cell culture. The latter two examples are instructive because they demonstrate that catalysis or degradation of the mRNA is not required for the potency of oligonucleotides because in both of these cases, oligonucleotides that do not promote degradation of the RNA transcript were as potent as, or in the latter case more potent than, an oligonucleotide that promotes degradation. A likely explanation is that the number of oligonucleotides delivered to cells is in vast excess compared to the level of the transcript being targeted (106). These studies suggest that the rate-limiting step in antisense activity is gaining access to the target RNA rather than what happens after the oligonucleotide binds to the target RNA. We suggest that the main reason that optimized siRNA oligonucleotides tend to be more potent in cell culture is that the RISC complex may facilitate binding of oligonucleotides to the target RNA or, alternatively, decrease the number of nonproductive interactions that an oligonucleotide may undergo, rather than because of increased potency in catalytically cleaving the target RNA. Ago 2 does not appear to be as efficient an enzyme as RNase H (47).

In vivo, single-stranded oligonucleotides containing phosphorothioate modifications (**Figure 3**) have a pharmacokinetic advantage over uncharged oligonucleotides or double-stranded siRNA oligonucleotides, in that they readily distribute to tissues and are taken up into cells without the need for formulations. Such oligonucleotides can be designed to work through the RNase H mechanism, various translation inhibition mechanisms, both types of RNA splicing modulation, inhibition of polyadenylation, and microRNA antagonists. In contrast, results obtained with double-stranded oligonucleotides, which work through the RNA interference mechanism, require formulations or lipophilic conjugates for effective delivery to tissues, increasing complexity and overall costs (20, 107). With the development of more efficient formulations, this may be less of an issue in the future. Additionally, as discussed below, oligonucleotides that work through the RNA interference pathway may have a disadvantage with respect to sequence specificity and the potential to produce undesirable side effects owing to competition with endogenous microRNAs for RISC components (44, 108–110), although preliminary results suggest that siRNA oligonucleotides may not compete with endogenous microRNAs (111). With time, these issues with siRNA oligonucleotides may be minimized through better oligonucleotide design and chemical modifications.

MEDICINAL CHEMISTRY OF ANTISENSE OLIGONUCLEOTIDES

Unmodified DNA and RNA are inherently unstable molecules in biological systems, based on the action of ubiquitously expressed nucleases that cleave the phospohodiester linkage. This instability precludes the use of unstabilized nucleic acids as drugs because they are degraded before they have a chance to reach their target receptor. In addition to their susceptibility to attack by nucleases, the pharmacokinetics of RNA and DNA make them unacceptable systemic therapeutics because they are weakly bound to plasma proteins and rapidly filtered by the kidney and excreted into the

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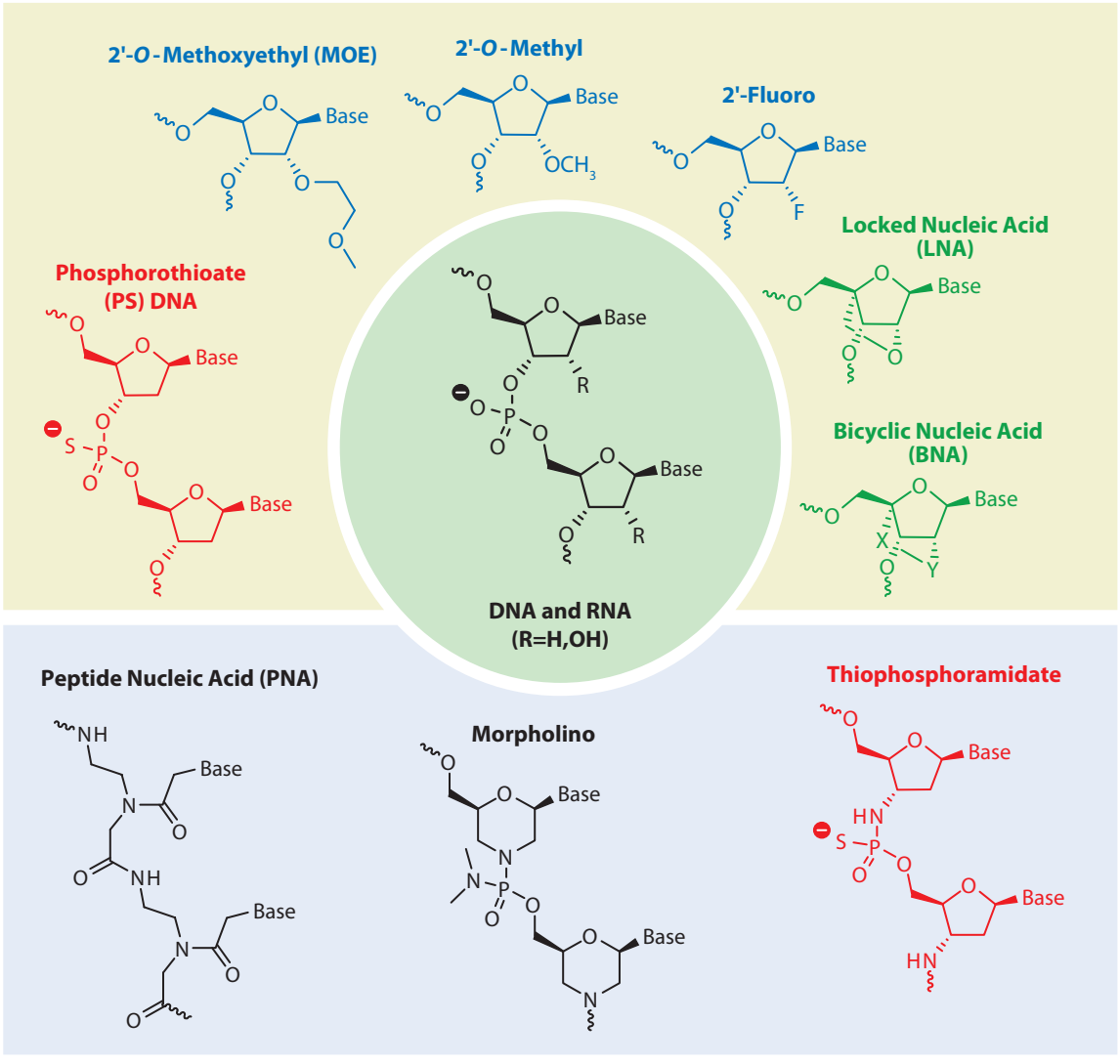

Figure 3

Examples of chemical modifications used in antisense oligonucleotides.

urine. Because intracellular RNA targets are likely to be highly structured, an antisense drug must be able to compete with those structures. DNA has a lower affinity for RNA than RNA does for itself, which presents a thermodynamic challenge that must be overcome. Increasing the intrinsic affinity for a complementary nucleic acid target is the most straightforward way to address this challenge.

Thus, both the intrinsic binding affinity and pharmacokinetics of natural oligonucleotides are insufficient for their use as systemic drugs. Oligonucleotides, akin to small-molecule drugs, are amenable to medicinal chemistry efforts that can improve their attributes. Cells use this strategy to stabilize endogenous nucleic acids such as ribosomal and transfer RNAs, and thus cues for the medicinal chemist can be taken from naturally occurring nucleoside modifications (112).

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Antisense drugs are intermediate in size between protein-based biologicals and conventional small-molecule drugs. They range in length from approximately 8 to over 50 nucleotides. Duplex drugs consisting of two strands (such as siRNA drugs) contain approximately 40 total nucleotides in two molecules held together by Watson-Crick base pairing. Most antisense oligonucleotides have a length of approximately 20 bases, which yields a molecular weight of approximately 7000 atomic mass units. Owing to the phosphate backbone, the same 20-mer oligonucleotide has a formal negative charge of −19, although it is likely that in solution at physiologic salt concentrations not all of those residues are anionic at any given time.

To be broadly useful in antisense approaches, modifications must retain, and preferably enhance, the ability to recognize their target RNAs by Watson-Crick base pairing. This property is commonly measured by the melting temperature (T_m) of an oligonucleotide that contains the modification of interest and is duplexed with a complementary strand of the RNA. For many drugs, binding to the receptor is sufficient to affect the desired pharmacology. However, for antisense approaches this is not always the case. The most widely used antisense drugs utilize cleavage of the target RNA to achieve their desired effects, using endogenous nucleases such as RNase H or Ago 2. Many studies have shown that modifications that are not substrates for these nucleases are poor antisense drugs, despite being tight binders to their complementary RNA, which is presumably their receptor. It is therefore crucial to examine the effects of modifications on these terminating events and to understand these effects when optimizing oligonucleotide designs.

As discussed above, resistance to nucleases must be engineered into oligonucleotides for them to be useful drugs. Nuclease stability is necessary but not sufficient for achieving a favorable pharmacokinetic profile. The drug must also distribute to and persist at the site of action for a reasonable period of time. As discussed in the pharmacokinetic section, protein binding is an important attribute that facilitates distribution to tissues.

Because oligonucleotide drugs are fairly large (molecular mass of ~7000 Da) molecules composed of monomeric building blocks, the cost of monomers is a key driver in the ultimate cost of the active pharmaceutical ingredient. Therefore, monomers with complex syntheses and those for which no cheap source of starting materials exists are less viable as antisense drugs, in particular if the goal is to compete in the marketplace with other treatments.

Backbone Modifications

Because of the inherent instability of the phosphodiester linkage to nucleases, the oligonucleotide backbone presents an obvious first target for improvement with chemical modification. As a result, extensive medicinal chemistry research has focused on efforts to find backbone modifications that increased nuclease resistance and maintained or improved affinity and specificity to the target RNA.

Phosphorothioate (PS)-containing oligonucleotides (**Figure 3**) were one of the earliest and remain one of the most widely used backbone modifications for antisense drugs. PS-containing oligonucleotides differ from natural nucleic acids in that one of the nonbridging phosphate oxygen atoms is replaced with a sulfur atom. The substitution of sulfur for oxygen in the phosphate ester confers several properties onto oligonucleotides that are crucial for their use as systemic antisense drugs (113). Foremost, the PS linkage greatly increases stability to nucleolytic degradation (114), such that after systemic administration to an animal, PS oligonucleotides possess sufficient stability in plasma, tissues, and cells to avoid metabolism prior to reaching the target RNA. Second, PS oligodeoxynucleotides are able to efficiently elicit RNase H cleavage of the target RNA, which is critical in the mechanism of action of many antisense drugs. Additionally, the PS modification confers a substantial pharmacokinetic benefit by increasing the binding to plasma proteins, which

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prevents rapid renal excretion and facilitates binding to other acceptor sites that facilitate uptake to tissues.

Although often maligned (115–118), PS remains the most successful modification to date in oligonucleotide therapeutics. Moreover, so-called first-generation antisense drugs (PS oligodeoxynucleotides) have advanced through various stages of clinical trials, with one, fomivirsen, achieving regulatory approval (119) (**Table 2**). The action of PS-modified oligonucleotides can involve all the different antisense mechanisms (**Table 1**). Furthermore, most of the current drugs in clinical trials (**Table 2**) incorporate PS modifications. It is difficult to envision a modification that will completely displace PS from antisense drugs and exhibit its broad utility. Another example of a backbone modification is the N3′→P5′ phosphoramidate oligodeoxynucleotides (**Figure 3**) in which the 3′-oxygen in the deoxyribose ring is substituted with a 3′-amino atom (120). Phosphoramidates exhibit high affinity toward the complementary RNA and high nuclease resistance (121) but do not activate RNase H. Thiophosphoramidate analogs were also prepared and found to be more acid stable when incorporated into oligonucleotides. Introduction of sulfur in place of one of the nonbridging oxygen atoms did not alter the RNA binding properties of these compounds (122). A palmitoyl-conjugated thiophosphoramidate-modified oligonucleotide, GRN163L, has shown promise as an antitelomerase agent (75).

Other backbone modifications of oligonucleotides have been less successful. Methylphosphonates have one of the nonbridging oxygen atoms replaced by a methyl group and are neutral in charge. Early progress in the standard solid phase synthesis of these molecules made possible a rigorous evaluation of the biophysical and biochemical properties of methylphosphonate-modified oligonucleotides. Although it provides high nuclease resistance, this modification does not support RNase H activity. Additionally, high methylphosphonate content in an oligomer leads to loss of affinity toward its complementary RNA and to poor solubility. Interestingly, removal of the negative charge did not improve the cellular uptake and pharmacokinetics relative to PS-modified oligonucleotides. These shortcomings have limited the utility of this modification. Many other attempts to modify the backbone have been attempted. Substitution of the phosphodiester with groups such as amides (123, 124), hydroxylamines (125), and acetals (126, 127) has succeeded in replacing the phosphorus atom and maintaining or increasing binding affinity, but improvements over the phosphorothioate backbone have not been demonstrated yet.

In addition to modifications of the phosphodiester, replacements of the sugar phosphate backbone with an isostere have been devised. One of these led to the phosphorodiamidate morpholino oligonucleotides (**Figure 3**), which have a morpholine ring as a replacement for the furanose, with a phosphorodiamidate linkage connecting the morpholine nitrogen atom with the hydroxyl group of the 3′-side residue. Because of the phosphorodiamidate linkage, morpholino oligonucleotides are neutral. These modifications are similar in affinity to DNA-DNA duplexes and are nuclease stable. However, they do not activate RNase H and are primarily used in translation arrest or other steric blocking mechanisms, such as alteration of splicing, where they have shown activity in animal models (83, 128). Morpholino oligonucleotides have been reviewed in detail (129) and have shown promise for modulating splicing and translation arrest (128, 129, 131).

Peptide nucleic acids (**Figure 3**) (PNAs) are a radically different class of oligonucleotide analogs that contain a peptide replacement for the sugar phosphate backbone, yet maintain the ability to Watson-Crick base pair with complementary RNA and DNA (132). First introduced by Nielsen and coworkers (133), PNAs are highly resistant to degradation by nucleases and proteases and exhibit high affinity when paired with RNA and DNA. PNAs do not activate RNase H and, as such, have been used primarily in translation inhibition (134) and splicing modulation antisense mechanisms (83, 105, 130). Despite their uncharged nature, PNAs do not readily cross cell membranes, so transfection methods are required for their use with cultured cells. These limitations

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have been addressed by the conjugation of short peptides (130, 135, 136) and the use of charged amino acids (137) in the PNA backbone, which increase solubility. Importantly, the short peptides employed were also able to improve tissue distribution. As an example, PNAs designed to modulate splicing, reducing expression of the targeted protein, were conjugated to 8-mer lysine peptides (E. E. Swayze et al., unpublished studies). Whereas little or no activity was observed in kidney and liver (the organs with the highest concentration of PNA), activity was observed in adipose tissue, with an alteration of splice products observed concomitant with a dose-dependent downregulation of the targeted protein. As in liver and kidney, a relatively high drug concentration was required to produce the pharmacological effect. This result suggests that both pharmacokinetic and potency hurdles remain for PNA oligonucleotide drugs and introduces concerns as to whether the modification is too stable in tissues.

Heterocycle Modifications

Modification of the heterocycle has focused mainly on increasing binding affinity for complementary nucleic acids (138–140). This may be accomplished through increasing stacking interactions or by increasing the strength of the Watson-Crick base pairs. The C5 position has been a common place for chemical manipulation on pyrimidine heterocycles because substitution at this position increases affinity without affecting the ability to serve as an RNase H substrate. Substituting the C5 hydrogen of deoxycytidine with a methyl group improves T_m of the DNA-RNA duplex by approximately 0.5°C per substitution (141) owing to enhanced base stacking on the 5′-side nucleobase. Further enhancing the stacking interactions with additional aromatic or π-rich surfaces prompted a substitution of the C5 position with a propynyl group (142). Enhanced duplex stability ($\Delta T_m \sim +2$°C per modification) and potent antisense effects were achieved in vitro using T- and C-rich phosphorothioate oligodeoxynucleotides poly-substituted with 5-propynyl nucleosides (143, 144). The presence of C5 propynyl pyrimidine bases did not interfere with RNase H cleavage kinetics but were found to be more toxic than unmodified counterparts when tested in vitro and in vivo (145). Given the lack of improvement in in vivo activity, coupled with the severe in vivo toxicity, it is unlikely that C5 propynyl–modified oligonucleotides will be useful in oligonucleotide drugs.

Sugar Modifications

To date, modifications to the 2′-position of the sugar moiety have provided the most value in enhancing the drug-like properties of oligonucleotides. Organization of the sugar into an RNA-like 3′-*endo* pucker or northern conformation in the furanose pseudorotation cycle (146) increases binding affinity. Furthermore, the proximity of the 2′-substituent to the 3′-phosphate in an oligonucleotide generally causes 2′-modified oligonucleotides to increase nuclease resistance. Unfortunately, essentially all 2′-modifications greatly reduce or completely inhibit the ability of RNase H to cleave the RNA strand opposite the modification, which restricts the use of 2′-modifications for antisense purposes. This limitation has been minimized by use of a gapmer strategy, where regions of 2′-modified residues flank a central DNA region of the oligonucleotide. The 2′-modified wings thus increase affinity and nuclease resistance, whereas the central gap region allows RNase H-mediated cleavage of the target RNA. Similar limitations are currently in the process of being elucidated for the use of 2′-modified nucleosides in siRNA duplexes, which utilize the RNAi machinery. Considerably more flexibility is afforded for use in oligonucleotide drugs that do not require a terminating mechanism, such as those that alter splicing of mRNA.

The increase in affinity observed with 2′-modifications is energetically driven by the electronegative substituent at the 2′-position. As such, the 2′-fluoro (**Figure 3**) modification imparts

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the highest binding affinity ($\Delta T_m \sim 2°C$ per modification, relative to the DNA) for the target RNA of the 2′-class of modifications (141). The 2′-fluoro modification has also been employed in the design of duplex siRNA oligonucleotides (147, 148). This substitution has allowed for the complete elimination of RNAs from siRNAs, providing duplexes with increased stability and potency that act via activation of the RNAi pathway (103).

2′-*O*-alkyl groups improve binding affinity to a lesser degree than do the 2′-fluoro nucleosides but impart a substantial degree of nuclease resistance to the resulting oligonucleotide. Antisense oligonucleotides with gapmer designs employing 2′-*O*-methyl nucleosides have been well characterized, and these designs have advanced to human clinical trials (149). The use of 2′-*O*-methyl nucleosides (**Figure 3**) in siRNA oligonucleotides also holds promise. The steric requirements of the RNAi machinery for recognition of the antisense strand appear to restrict the broad use of bulkier modifications, whereas 2′-*O*-methyl is often well tolerated (150). Minimal use of 2′-*O*-methyl nucleosides has been employed to stabilize siRNAs for successful in vivo proof-of-concept experiments (107, 151) and to minimize off-target effects due to limiting their ability to serve as microRNA agonists (152).

The 2′-*O*-methoxyethyl (MOE) (**Figure 3**) modification is currently the most advanced of the 2′-modified series and has entered clinical trials for multiple indications (**Table 2**). MOE increases T_m by about +2°C per modification versus RNAs, relative to DNAs, and greatly increases resistance to nucleases. It also appears to reduce certain nonspecific protein binding, which can reduce toxicities. MOE oligonucleotides have unique structural features evident from structural studies that help explain the properties of MOE oligonucleotides (153). MOE substitution at the 2′-position induces a C3′-*endo* (northern) conformation of the sugar and assumes a *gauche* orientation that traps water in a shell of hydration that includes the adjacent phosphate residue. This further increases rigidity of the C3′-*endo* sugar conformation, and the organization of the oligonucleotide into an A-form geometry is thought to largely account for the increased affinity for the RNA. The increased nuclease resistance is most likely due to steric hindrance imparted by the MOE substituent combined with the shell of hydration created by the bound water. These attributes have translated from the test tube to human, resulting in numerous MOE-modified antisense drugs entering the clinic (**Table 2**).

As with traditional small-molecule drug optimization efforts, the rewards for correctly constraining a ligand are large entropic gains in binding affinity. The sugar modification showing the largest known improvement in binding affinity is a bicyclic system with the 4′-carbon tethered to the 2′-hydroxyl group, called 2′,4′ bicyclic nucleic acid by Imanishi (154) and locked nucleic acid (LNA) by Wengel (155, 156), who published shortly thereafter (**Figure 3**). This modification can also be thought of as a constrained analog of 2′-*O*-methyl RNA, where the 2′-substituent is tethered to the 4′-*C* atom. This enforces a northern sugar pucker, which is essentially identical to that adopted by A-form RNAs and has been confirmed by structural analysis (157, 158). LNA shows dramatically improved hybridization properties relative to a DNA-RNA duplex and improves nuclease resistance. LNA-modified oligonucleotides, akin to MOE, have been exploited for numerous antisense mechanisms. Uniform LNA oligonucleotides do not support RNase H (159), thus a gapmer strategy must also be employed with LNA-modified oligonucleotides to support the RNase H mechanism. LNA-modified antisense drugs, if optimally designed, exhibit better potency than other 2′-modifications but also appear to have increased toxicological liabilities in the gapmer design that supports RNase H activity (160). Several analogs of LNA have been reported, some of which have improved activity and/or toxicity profiles in animals (161, 162). These studies further highlight the promise that this class of molecules holds for improving the potency of antisense drugs in the near future.

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Conjugation Strategies

Conjugation of oligonucleotides to ligands that can bind to various acceptor sites would be expected to alter the pharmacokinetic properties of the oligonucleotide, and many attempts have been made to increase the tissue and cellular uptake of oligonucleotide drugs via conjugation of various ligands. Cholesterol conjugates of oligonucleotide are one of the most studied classes of conjugates. Cholesterol conjugation has been shown to increase the exposure of the liver, with a concomitant reduction in exposure to kidney (69, 107, 163). Cholesterol-conjugated oligonucleotides have been used for multiple antisense mechanisms including RNase H, siRNA, and microRNA antagonists. However, cholesterol conjugation raises concerns regarding toxicity, with increased toxicity of cholesterol-conjugated oligonucleotides reported (164). In addition to cholesterol, other lipid groups can alter distribution of oligonucleotides in animals (163, 165). Cationic peptides have also been used to enhance intracellular delivery of oligonucleotides (87, 130, 136, 166). Although early data is promising for enhancing antisense delivery inside cells, their long-term safety needs to be defined.

PHARMACOKINETICS OF OLIGONUCLEOTIDES

Antisense oligonucleotides must reach the targeted RNA inside the cytoplasm or nucleoplasm to be effective. To reach the RNA, the oligonucleotide must traverse intact from the site of application to the cell surface, across the plasma membrane to the cytoplasm, and then to the target RNA. This is a significant challenge for a molecule that has a molecular mass between 4,500 and 18,000 Da and that contains numerous solvent-exposed negative charges. Several strategies are available to deliver the antisense oligonucleotide to the target RNA inside cells. The most widely used strategy is to administer chemically modified oligonucleotides to the organism, either locally or systemically, exploiting poorly described natural cellular uptake pathways that exist in cells in tissues. Oligonucleotides can be modified by appending lipophilic substituents, ligands for cell surface receptors, or cell-penetrating peptides to enhance their delivery to the cytoplasm. Numerous formulations are being explored as a means to enhance delivery of synthetic oligonucleotides to cells. A discussion of formulations used for nucleic acid delivery is beyond the scope of this review and has been presented in several recent reviews (22, 167, 168). A brief discussion of different conjugation strategies is provided in the medicinal chemistry section above and in a recent review (169). We focus this discussion on what is known regarding the mechanisms by which unformulated oligonucleotides accumulate in tissues and ultimately cross the plasma membrane. Readers who seek background information may consult recent reviews on the general pharmacokinetics of antisense oligonucleotides (170, 171).

Following parenteral (subcutaneous or intravenous) administration, oligonucleotides transiently circulate bound to plasma proteins. This interaction with plasma proteins prevents the oligonucleotides from being filtered through the glomerulus and excreted in the urine. The affinity for plasma proteins is dictated by the chemistry of the oligonucleotide, with single-stranded, but not double-stranded, PS-modified oligonucleotides exhibiting high affinity for plasma proteins (163, 172, 173). Uncharged oligonucleotides, such as PNAs morpholino, or oligonucleotides containing phosphodiester linkages tend to exhibit low plasma protein binding and are rapidly excreted in the urine (174–176). Thus, maintaining plasma protein binding is important in facilitating delivery to tissues. In addition to the PS modification, another way plasma protein binding can be modulated is by attaching lipophilic substituents, such as cholesterol, to the oligonucleotide (177, 178). A key question is whether plasma proteins play a direct role in the delivery of oligonucleotides to cells or act passively, essentially serving as a buffer to prevent the rapid renal excretion

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of oligonucleotides by increasing the size such that it is too large to be filtered by the glomerulus. Evidence for the former includes, first, the observation that PS oligonucleotides exhibit marked differences in their affinities for different plasma proteins (173). Second, the main plasma proteins that bind oligonucleotides interact with proteins expressed on the surface of cells; these include albumin binding to megalin (LRP2) in the kidney and α2 macroglobulin binding to low-density lipoprotein receptor-related protein 1 (LRP-1, CD91). Third, there is heterogeneity in the cell types that accumulate oligonucleotides, and this heterogeneity appears to be cell lineage dependent, suggesting that cellular accumulation is mediated by proteins expressed on the cells rather than by blood flow (174, 179). It cannot rule out that other cell-type-specific processes are responsible for differential accumulation of PS-modified oligonucleotides such as rates of pinocytosis.

A series of experiments was performed to directly address whether plasma proteins play an active or passive role in the delivery of PS-modified oligonucleotides to tissues (180). The results suggest that binding to high-affinity plasma proteins results in greater delivery of the oligonucleotide to the liver but unexpectedly do not result in better antisense activity. Dosing schedules and dosages that produce greater binding to low-affinity plasma protein or free oligonucleotide resulted in better efficacy even though less drug accumulated in the liver. These studies also suggest that there may be at least two distinct pathways in which oligonucleotides accumulate in cells: one delivering the oligonucleotide to a productive compartment containing the target RNA and a second delivering it to a nonproductive compartment, which can be competed for by excess oligonucleotide. The nonproductive cell uptake pathway appears to be more efficient because >80% of the oligonucleotide can be competed for without loss of antisense activity (180).

Recent studies of cultured hepatocytes and a mouse hepatocellular carcinoma cell line confirm that oligonucleotides enter these cells by at least two pathways: the productive and nonproductive pathways noted above (E. Koller and C. F. Bennett, manuscript in preparation). Studies of these cells demonstrate that, in contrast to most cultured cells (106, 181), these hepatocellular carcinoma cells accumulate single-stranded, but not double-stranded, PS-modified oligonucleotides into the cell, such that they are available to interact with the target mRNA. The nonproductive pathway results in accumulation of oligonucleotide in lysosomal structures and, similar to the in vivo observations, accounts for the bulk of oligonucleotide transported into the cell. The productive uptake pathway appears to be a novel vesicular transport pathway that is clathrin and caveolin independent but dependent on the adapter protein AP2M1. Chloroquine, which prevents acidification of late endosomes, blocks the effect of the antisense oligonucleotide without affecting the bulk accumulation of oligonucleotide into cells, providing further evidence that the productive uptake pathway accounts for a minority of the bulk oligonucleotide in cells. Antisense activity is also partially inhibited by brefeldin A, an antibiotic that blocks anterograde transport of materials from endoplasmic reticulum vesicles to the Golgi apparatus.

These results suggest that single-stranded antisense oligonucleotides are transported into cells by at least two distinct vesicular pathways, one of which delivers oligonucleotide to lysosomes, whereas the second results in release of oligonucleotide from vesicles into the cytoplasm (**Figure 4**). Once in the cytoplasm, the oligonucleotide can readily shuttle between the cytoplasm and the nucleus (182). The mechanisms by which oligonucleotides escape from endosomal vesicles are not well understood. There may be specific channels, which we call oligoportins, that allow passage of oligonucleotides. There is evidence that renal cells express a cation-regulated nucleic acid channel (183, 184). This nucleic acid channel is composed of two subunits, a 45-kDa pore-forming cation-binding protein and cytosolic malate dehydrogenase, which functions as a regulatory subunit (185). It remains to be determined if this or related channels are responsible for the release of single-stranded oligonucleotides from endosomal vesicles.

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Single-stranded and double-stranded oligonucleotides with a lipophilic conjugate such as cholesterol also interact with plasma proteins, but in this case they bind to plasma lipoproteins such as low-density or high-density lipoprotein (177, 186). Oligonucleotides that do not contain the lipophilic conjugate fail to interact with plasma lipoproteins (186). The lipoprotein appears to facilitate delivery of the oligonucleotide to lipoprotein-binding proteins expressed on the surface of cells such as the low-density lipoprotein receptor, high-density lipoprotein receptor, or scavenger receptors (178, 186). Internalization of cholesterol-conjugated double-stranded RNA does not appear to be mediated by endocytosis of the lipoprotein receptors, although binding to the receptors appears to be required. The double-stranded RNA appears to be transported into the cell by the transmembrane channel SID-1, which was first identified as responsible for the RNA spreading in *Caenorhabditis elegans* (178, 187). SID-1 does not appear to be involved in the cellular uptake of single-stranded unconjugated PS-modified oligonucleotides (E. Koller and C. F. Bennett, manuscript in preparation).

These studies clearly demonstrate that mammals are capable of accumulating systemically and locally administered single-stranded oligonucleotides and lipophilic-conjugated single- and double-stranded oligonucleotides inside the same subcellular compartment as the target RNA. The mechanisms for accumulation within cells are complex but appear to involve novel endosomal transport mechanisms. Additional work is needed to provide greater insights into these pathways because such information will enhance medicinal chemistry and formulation efforts to broaden the potential of antisense technology.

TOXICOLOGY OF ANTISENSE OLIGONUCLEOTIDES

Antisense oligonucleotides, like all drugs, exhibit dose-dependent toxicities. The best-characterized classes of antisense drugs with respect to such toxicities [owing to the large number of such drugs that have entered clinical trials (**Table 2**)] are first-generation PS oligodeoxynucleotides and second-generation 2′-MOE-modified antisense oligonucleotides. The preclinical and clinical toxicity of PS-modified first- and second-generation oligonucleotides has been reviewed extensively (188, 189). The toxicological properties of other types of antisense oligonucleotides are just beginning to be understood (25, 111, 160, 176, 190).

The observed or potential toxicities for antisense oligonucleotides can be classified as hybridization dependent or hybridization independent (**Table 3**). Hybridization-dependent toxicities can be attributed to exaggerated pharmacological effects and hybridization to nontarget RNAs. The former class of toxicities are akin to those of other drugs and can be avoided or minimized by proper selection of the target RNA and careful characterization of the pharmacology and toxicology of antisense inhibitors in preclinical models. Off-target hybridization-dependent effects can be minimized by performing careful bioinformatics analyses to identify targets with perfect mismatches or one to three mismatches. In general, an oligonucleotide 20 nucleotides in length will match with 100% complementarity to the targeted RNA transcript and not to any other unintended transcript but will exhibit partial complementarity to several genes. The number of genes to which the oligonucleotide is predicted to hybridize directly relates to the number of mismatches allowed. We generally limit our analysis to three mismatches because we have found that a greater number of mismatches than this results in almost complete loss of activity (28, 191). The effects on predicted off-target genes can be determined experimentally to validate the specificity of the antisense drug. Oligonucleotides that work via the RNA interference mechanism are more problematic because they have the ability to function as microRNAs in which only six to eight nucleotides (in the seed region) are required for activity and can result in hundreds if not thousands of potential off-target interactions (108, 190). The potential for seed-matched off-target

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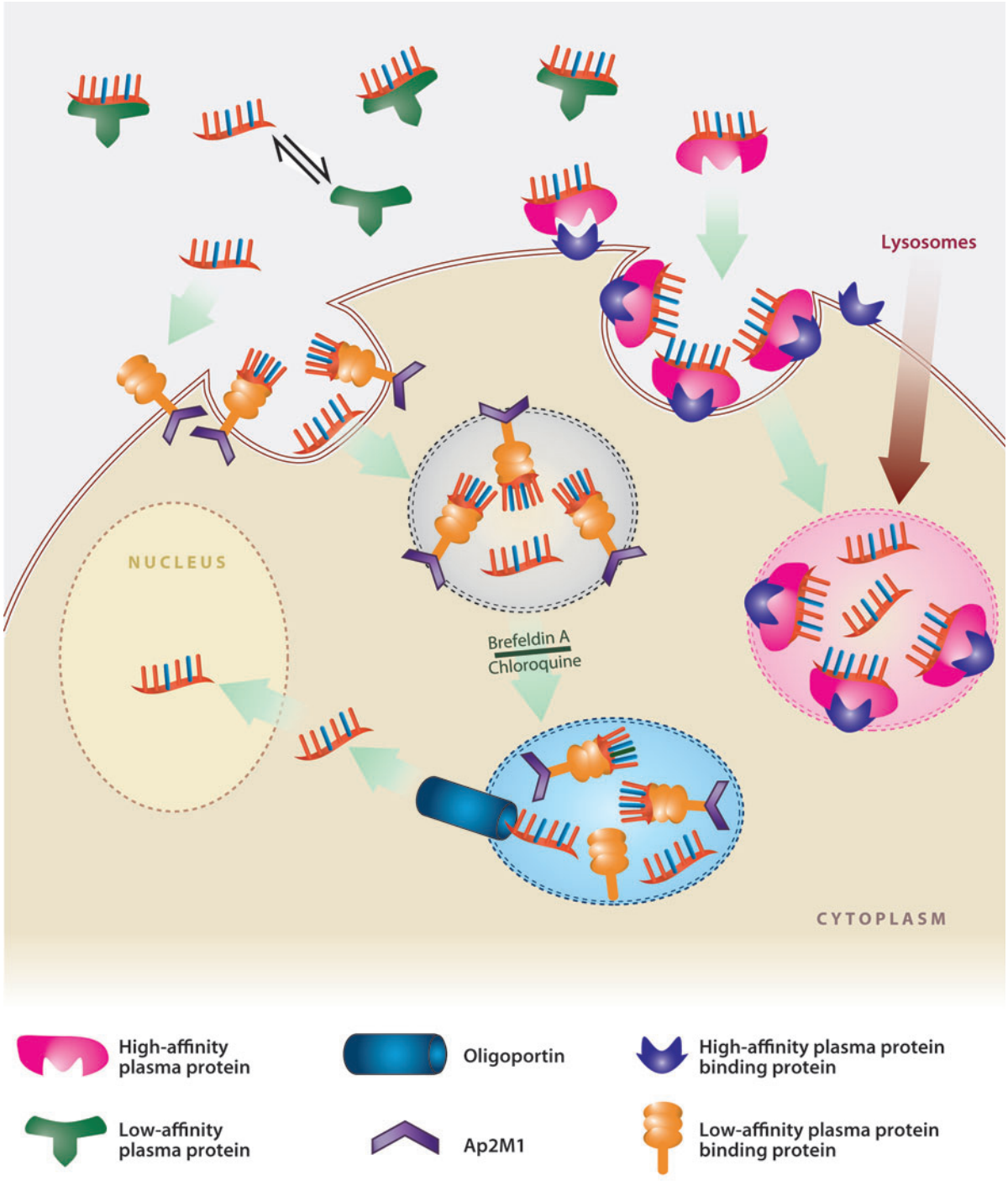

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Table 3 Examples of adverse effects described for antisense nucleotides

	Hybridization Dependent	Hybridization Independent
Preclinical	On target toxicity: Exaggerated pharmacology Off target toxicity: Perfect match to nontarget gene Mismatch to nontarget gene MicroRNA (RNAi mechanism only)	Plasma protein interaction: Increase in APTT Complement activation Tissue/cell interaction: Proinflammatory effects Decrease in platelets Kidney: proximal tubule cell effects at high doses Liver: increase in liver enzymes
Clinical	None described to date	Plasma protein interaction: Increase in APTT Tissue/cell interaction: Injection site reactions Constitutional symptoms (fever, chills, arthalgia, headache, etc.) Decrease in platelets

effects can be reduced through chemical modifications of the oligonucleotide (152). Taking all these precautions into account dramatically increases the probability of successfully identifying a selective antisense drug, but these precautions may not prevent off-target hybridization events, especially with such a large part of the genome being transcribed.

A second source of potential toxicities can be mediated through interactions of the oligonucleotide with proteins—so-called aptameric effects. These effects can be sequence dependent, such as interaction with Toll-like receptors (192, 193), or sequence independent. Generally, this class of toxicity depends on the chemistry of the oligonucleotides and the proteins with which each chemical class interacts, the net result being a common set of toxicities such as effects on coagulation (194), complement activation (195), and immune cell activation (196, 197). These effects are dose dependent and tend to occur at high doses of the oligonucleotides resulting in an adequate therapeutic index (188, 198). Once these toxicities have been characterized for a chemical class, the data dramatically enhance the efficiency of the technology because they define the class of toxicities that need to be monitored and narrow the dose ranges and duration of exposure to be explored in preclinical studies. Clinically, the primary tolerability issues for first- and second-generation antisense drugs have proven to be nonhybridization-dependent effects such as prolongation of activated partial thromboplastin time, injection site reactions, and constitutional symptoms such as fever, chills, and headache. In addition, decreases in platelet count have been occasionally described for some but not all phosphorothioate-modified oligonucleotides (189). Second-generation antisense drugs have proven to be better tolerated than first-generation antisense drugs and are well

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Figure 4

Proposed mechanism by which single-stranded, phosphorothioate-modified antisense oligonucleotides accumulate in hepatocytes. Briefly, it is proposed that oligonucleotides circulate in plasma bound to high-affinity plasma proteins and low-affinity, high-capacity plasma proteins. Oligonucleotides bound to high-affinity plasma proteins are taken up into cells by endocytosis, resulting in accumulation of the oligonucleotides in lysosomes. Oligonucleotides bound to low-affinity plasma proteins dissociate from the plasma protein where they bind to proteins expressed on the cell surface. Proteins are internalized by a clathrin-independent vesicular pathway and ultimately released from the vesicles where they can bind to RNA in the cytoplasm and nucleus. Release from the vesicles is proposed to be mediated by a fictional protein complex or channel that we call oligoportin. This second, productive uptake pathway is inhibited by inhibitors of the adapter protein Ap2M1, chloroquine, and Brefeldin A.

tolerated overall. The safety profile for other chemical classes of oligonucleotides remains to be determined because the drugs are still in early clinical testing.

PHARMACOLOGY OF ANTISENSE OLIGONUCLEOTIDES

Because antisense oligonucleotides can be designed to target any RNA, the pharmacological effects of antisense-based drugs are very diverse. Both cellular (host) RNAs and viral RNAs have been targeted with antisense oligonucleotides, with examples of both antiviral and host-targeted antisense drugs advancing into clinical trials and in one case to the market (**Table 2**). Use of antisense oligonucleotides targeting prokaryotes has not been well characterized. Preliminary studies have suggested that antisense oligonucletoides may have use in targeting prokaryotic RNAs (199–202), but these initial observations have not been extended. Furthermore, in many cases the studies were performed in bacteria strains with compromised cell walls to enhance uptake of the oligonucleotide, having been shown to be inactive in bacteria with normal cell walls. Although they may have utility as research reagents for prokaryaotes, it is questionable whether antisense oligonucleotides would prove to be commercially successful as antibacterial agents, given their likely narrow spectrum of activity, cost constraints, and technical challenges for enhancing delivery and uptake by bacteria at sites of infection in a safe manner.

Some common features of antisense drugs are worth discussing. First, antisense drugs do not affect a protein that is already expressed; instead they prevent the expression of the protein or, in the case of the microRNA antagonist and splicing-modulating oligonucleotides, increase expression. As a result, the onset of action for antisense drugs is variable, depending on the protein half-life. It is generally possible to detect changes in RNA levels 3 to 4 hours after administration of an oligonucleotide to cultured cells and 12 to 24 hours in animal tissues (30, 107, 203). The time for detectable effects on the target protein is highly variable and can range from several hours to more than a week for stable proteins. In most circumstances antisense drugs are transfected into cultured cells using cationic lipids, cationic dendrimers, or electroporation. Cationic lipids appear to be the most efficient and most broadly exploited delivery method for cultured cells. Typical doses of antisense oligonucleotides used in the presence of cationic lipids in cell culture experiments range from 0.1 to 100 nM. Doses used in rodents for first-generation PS oligodeoxynucleotides range from 10 to 75 mg/kg/day, whereas doses for second-generation 2′-*O*-methoxyethyl- and LNA-modified oligonucleotides range from 5 to 50 mg/kg/week, depending on the tissue being targeted. For systemic administration, most published reports for double-stranded siRNAs utilize some form of lipid formulation and administer the drug in doses ranging from 1 to 10 mg/kg (107). In general, both second-generation antisense oligonucleotides and double-stranded siRNA oligonucleotides produce effects for 10 to 15 days after single administration, allowing weekly, biweekly, or possibly monthly dosing.

As of this writing, more than 35 antisense oligonucleotides are in development (**Table 2**). The most advanced class of antisense drugs are those that work by the RNase H mechanism of action (**Table 2**). The pharmacological properties of both first- and second-generation RNase H oligonucleotides have been consistent from preclinical studies to studies of humans (17, 18, 204–208). For example, mipomersen, an antisense drug that targets Apolipoprotein B (Apo B) in the liver, produces a dose-dependent reduction in Apo B levels in plasma with doses ranging from 50 to 400 mg per week (18, 208). The degree of Apo B reductions and pharmacokinetics have proven consistent across different patient populations and in the presence of statins, suggesting that intersubject variability should not be a major issue for this class of antisense oligonucleotides (208). Results from clinical studies of mipomersen and several other antisense drugs are encouraging and conclusively demonstrate that antisense oligonucleotides can modulate target gene expression

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in humans. Although this is promising, additional studies are required to demonstrate that the antisense drugs currently in development produce a robust enough clinical benefit and have safety profiles to support registration and widespread use in patients.

CONCLUSIONS

The development of antisense oligonucleotide technology has progressed steadily over the past 20 years. Although there have been some disappointments in the clinic with some of the early drugs entering clinical trials for each mechanistic class of antisense oligonucleotides, these failures have been instructive in identifying the attributes and limitations of the technology. In particular, studies have been very helpful in defining those target tissues where antisense technology works well and those that are going to be more challenging to demonstrate robust activity. As a result, many of the drugs currently in clinical trials focus on target tissues that have proven to be sensitive to antisense drugs, enhancing their probability of being successful (**Figure 2**). The development of antisense technology parallels the development of other platform technologies such as monoclonal antibodies (209, 210). This is not to say that all of the drugs currently in development will be successful. The success rate for more established drug platforms such as small molecules is typically approximately 10% of drugs starting clinical testing. There is no reason to suspect that the success rate for antisense oligonucleotides entering clinical trials will be markedly different. However, if several of the drugs currently in clinical trials are ultimately succesful, it will be an important milestone for the technology and patients.

Although much progress has been made in the field, and most of the technology-disruptive questions and issues have been addressed, many additional questions remain to be answered regarding antisense oligonucleotide drugs. A major focus of biology over the next 10 years will be to gain a better understanding of the function of the RNA transcriptome and, as part of the process, to identify additional therapeutic targets for antisense oligonucleotoide drugs. It remains to be determined if certain types of RNA targets will prove to be more efficiently approachable with antisense oligonucletoides than others, although data generated to date suggest that this is unlikely to be the case. As already discussed in this review, there is a large gap in our understanding of how oligonucleotides distribute to tissues, are taken up by cells, and find their targeted RNAs without use of formulations. Better understanding of these steps will greatly facilitate approaches to increase the potency and broaden the tissues in which antisense pharmacology can be practiced, either through medicinal chemistry or formulations.

Not discussed in this article, but also a major opportunity for the technology, is how to demonstrate that formulations and other delivery technologies can enhance the performance and/or patient convienence of antisense oligonucleotide drugs, such as oral delivery. A big unknown for the field is whether the technology has long-term toxicology issues. Patients have been treated with several RNase H antisense oligonucleotide drugs for over two years, providing some comfort that this class of antisense drugs will prove to be safe for chronic diseases. However, the number of patients treated for this length of time is relatively small, so it remains to be seen if issues will arise with longer-term therapy. As already mentioned, antisense oligonucleotides that utilize the RNA intereference mechanism have an additional potential liability, in that they may either compete with endogenous microRNAs for processing or incorporation into Argonaute proteins and also exhibit promiscuous off-target effects by entering microRNA pathways. It remains to be seen whether these potential liablilities manifest themselves in producing toxicities in preclinical studies or in humans and, if so, whether they can be diminished or eliminated. Finally, it would be naive to expect that antisense oligonucleotide drugs would be a commerically viable therapy for all types of diseases. As we gain more clinical and commercial experience with different

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antisense approaches, the types of diseases and molecular targets that are commerically attractive will become better defined.

Although many questions remain unanswered, the results to date are very encouraging; antisense oligonucleotide drugs should join small molecules and protein-based therapeutics as the third drug discovery platform.

DISCLOSURE STATEMENT

The authors are employees and shareholders of Isis Pharmaceuticals, Inc., a biotechnology company focused on RNA targeting therapeutics.

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