Pharmacology of Antisense Drugs

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Annu. Rev. Pharmacol. Toxicol. 2017. 57:81–105

First published online as a Review in Advance on October 10, 2016

The *Annual Review of Pharmacology and Toxicology* is online at pharmtox.annualreviews.org

This article's doi: 10.1146/annurev-pharmtox-010716-104846

Keywords

antisense oligonucleotides, eteplirsen, mipomersen, nusinersen, oligonucleotide, patisiran, RNA, RNA interference, RNA splicing, RNase H

Abstract

Recent studies have led to a greater appreciation of the diverse roles RNAs play in maintaining normal cellular function and how they contribute to disease pathology, broadening the number of potential therapeutic targets. Antisense oligonucleotides are the most direct means to target RNA in a selective manner and have become an established platform technology for drug discovery. There are multiple molecular mechanisms by which antisense oligonucleotides can be used to modulate RNAs in cells, including promoting the degradation of the targeted RNA or modulating RNA function without degradation. Antisense drugs utilizing various antisense mechanisms are demonstrating therapeutic potential for the treatment of a broad variety of diseases. This review focuses on some of the advances that have taken place in translating antisense technology from the bench to the clinic.

INTRODUCTION

Antisense technology complements small molecules and protein-based drug discovery platforms by targeting RNA rather than proteins. Targeting RNA as a therapeutic strategy greatly expands the numbers and types of targets that can be approached therapeutically. Antisense drugs can be designed to bind to RNAs encoding difficult-to-target proteins, such as scaffold proteins and transcription factors, noncoding RNAs, and RNAs that are directly toxic to cells. Furthermore, the technology is well positioned to leverage advances in human genetics and genomics to create drugs for the treatment of both monogenic and polygenic diseases. The approval of mipomersen for the treatment of familial hypercholesterolemia validates antisense technology for the treatment of systemic diseases (1). In addition, results from multiple clinical trials demonstrate clearly that it is possible to practice antisense pharmacology safely in humans using a variety of different mechanisms. Antisense drugs currently in clinical development target diverse tissues both systemically and locally (**Table 1**). Advances in oligonucleotide chemistry promise to further enhance the properties of antisense drugs by increasing potency, safety, and broader tissue distribution. Several recent reviews have been dedicated to antisense technology, to which the reader is referred (2–5). This review focuses on the recent progress advancing antisense drugs into clinical trials and ultimately to the patient.

ANTISENSE MECHANISMS

For the purposes of this review, we define antisense oligonucleotides (ASOs) as oligonucleotides that bind to RNA through Watson-Crick base pairing and, upon binding, modulate the function of the targeted RNA. This definition includes a wide variety of oligonucleotide designs that modulate RNA through a diverse set of postbinding mechanisms, discussed in detail in previous reviews (3, 6). Briefly, these mechanisms can be categorized broadly as (*a*) those that bind to RNA and interfere with its function without promoting RNA degradation, such as translation arrest or modulating RNA processing, and (*b*) those that promote degradation of the RNA through endogenous enzymes, such as RNase H or argonaute-2 [RNA interference (RNAi)] (**Figure 1).** Recently, researchers have shown that ASOs can also be used to increase protein production, either through antagonizing microRNAs, which normally suppress protein production, or through masking upstream open reading frames (7–9).

MEDICINAL CHEMISTRY OF ANTISENSE OLIGONUCLEOTIDES

Unmodified DNA and RNA are inherently unstable in biological systems, based on the action of ubiquitously expressed nucleases. To be broadly useful as antisense drugs, modifications must retain—and preferably enhance—the ability to recognize their target RNA by Watson-Crick base pairing, increase resistance to nucleolytic degradation, distribute to tissues, and localize in cells in the same compartment as the targeted RNA. Several different chemical approaches can be used to achieve these goals.

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. A phosphorothioate (PS) backbone (**Figure 2**), in which one of the nonbridging phosphate oxygen atoms is replaced with a sulfur atom, is the most widely used antisense modification. The PS linkage greatly

Table 1 Examples of antisense drugs marketed or in clinical trials

Drug	Target	Indication	Mechanism	Chemistry	Delivery route	Status	Reference or NCT ID[a]
Cardiovascular diseases							
Mipomersen	Apolipoprotein B-100	Familial hypercholesterolemia	RNase H	2′MOE gapmer	Subcutaneous	Marketed	1, 84, 86
Volanesorsen	Apolipoprotein CIII	Familial chylomicronemia syndrome, familial partial lipodystrophy	RNase H	2′MOE gapmer	Subcutaneous	Phase III	88, 90, 91
IONIS-FXI$_{Rx}$, BAY 2306001	Factor XI	Thrombosis	RNase H	2′MOE gapmer	Subcutaneous	Phase II	92
IONIS-APO(a)-L$_{Rx}$	Apo(a)	Very high Lp(a)	RNase H	GalNAc-conjugated 2′MOE gapmer	Subcutaneous	Phase II	135
IONIS-ANGPTL3-L$_{Rx}$	Angiopoietin 3	Mixed dyslipidemias	RNase H	GalNAc-conjugated 2′MOE gapmer	Subcutaneous	Phase II	NCT02709850
Revusiran	Transthyretin	Familial amyloid cardiomyopathy	siRNA	GalNAc-conjugated siRNA	Subcutaneous	Phase II	NCT02319005
ALN-PCS$_{sc}$	PCSK9	Hypercholesterolemia	RNAi	GalNAc-conjugated siRNA	Subcutaneous	Phase I	NCT02597127
Metabolic and endocrine diseases							
IONIS-GCCR$_{Rx}$	Glucocorticoid receptor	Type 2 diabetes	RNase H	2′MOE gapmer	Subcutaneous	Phase II	NCT01968265
IONIS-GCGR$_{Rx}$	Glucagon receptor	Type 2 diabetes	RNase H	2′MOE gapmer	Subcutaneous	Phase II	NCT02583919
IONIS-PTP1B$_{Rx}$	Protein tyrosine phosphatase-1B	Type 2 diabetes	RNase H	2′MOE gapmer	Subcutaneous	Phase II	NCT00455598
IONIS-FGFR4$_{Rx}$	Fibroblast growth factor receptor 4	Obesity	RNase H	2′MOE gapmer	Subcutaneous	Phase II	NCT02476019
ATL 1103	Growth hormone receptor	Acromegaly	RNase H	2′MOE gapmer	Subcutaneous	Phase II	
IONIS-DGAT2$_{Rx}$	Diacylglycerol *O*-acyltransferase 2	Nonalcoholic steatohepatitis	RNase H	2′MOE gapmer	Subcutaneous	Phase I	

(*Continued*)

Table 1 *(Continued)*

Drug	Target	Indication	Mechanism	Chemistry	Delivery route	Status	Reference or NCT ID[a]
Neurological and neuromuscular diseases							
Eteplirsen	Dystrophin	Duchenne muscular dystrophy exon 51	Splicing modulation	Morpholino	Intravenous	Approved	111, 113
Drisapersen	Dystrophin	Duchenne muscular dystrophy exon 51	Splicing modulation	Uniform 2′-*O*-methyl	Subcutaneous	Phase III	110, 136
IONIS-TTR$_{Rx}$	Transthyretin	Familial amyloid polyneuropathy and cardiomyopathy	RNase H	2′MOE gapmer	Subcutaneous	Phase III	29
Patisiran	Transthyretin	Familial amyloid polyneuropathy	siRNA	Liposomal formulation	Intravenous	Phase III	121, 122
Nusinersen	Survival motor neuron 2	Spinal muscular atrophy	Splicing modulation	Uniform 2′MOE	Intrathecal	Phase III	27, 127
SRP-4053	Dystrophin	Duchenne muscular dystrophy exon 53	Splicing modulation	Morpholino	Intravenous	Phase II	NCT02310906
BMN-045	Dystrophin	Duchenne muscular dystrophy exon 45	Splicing modulation	Uniform 2′-*O*-methyl	Subcutaneous	Phase II	NCT01826474
IONIS-DMPK-2.5$_{Rx}$	Dystrophia myotonica protein kinase	Myotonic dystrophy type 1	RNase H	2′-cEt and 2′MOE gapmer	Subcutaneous	Phase II	NCT02312011
IONIS-HTT$_{Rx}$	Huntingtin	Huntington's disease	RNase H	2′MOE gapmer	Intrathecal	Phase II	NCT02519036
BIIB067/IONIS-SOD1$_{Rx}$	Superoxide dismutase 1 (SOD1)	Familial ALS due to mutations in SOD1	RNase H	2′MOE gapmer	Intrathecal	Phase I	NCT02623699
SRP-4053	Dystrophin	Duchenne muscular dystrophy exon 53	Splicing modulation	Morpholino	Intravenous	Phase II	NCT02310906
SRP-4045	Dystrophin	Duchenne muscular dystrophy exon 45	Splicing modulation	Morpholino	Intravenous	Phase I	NCT02500381
DS-5141b	Dystrophin	Duchenne muscular dystrophy exon 45	Splicing modulation	ENA	Subcutaneous	Phase I	NCT02667483

Cancers							
Custirsen	Clusterin	Prostate cancer, NSCLC	RNase H	2′MOE gapmer	Intravenous	Phase III	137, 138
Apatorsen	Heat shock protein 27	Prostate cancer, NSCLC, and bladder cancer	RNase H	2′MOE chimera	Intravenous	Phase II	139
AZD9150, IONIS-STAT3-2.5$_{Rx}$	STAT3	Various cancers	RNase H	cEt gapmer	Intravenous	Phase II	102
IONIS-AR-2.5$_{Rx}$	Androgen receptor	Prostate cancer	RNase H	cEt gapmer	Intravenous	Phase II	NCT02144051
MRX34	MicroRNA 34 mimic	Various cancers	MicroRNA mimic	Lipid nanoparticle formulation of dsRNA	Intravenous	Phase I	NCT01829971
BP1001	Growth factor receptor–bound protein 2 (Grb-2)	Leukemia	Undisclosed	Liposome-formulated ASO	Intravenous	Phase I	NCT01159028
MRG106	MicroRNA 155	Various cancers	MicroRNA antagonist	Chimeric LNA	Subcutaneous	Phase I	NCT02580552
DCR-MYC	C-Myc	Various cancers	siRNA	Lipid nanoparticle–formulated siRNA	Intravenous	Phase I/II	NCT02314052
Inflammatory diseases							
Alicaforsen	Intercellular adhesion molecule 1 (CD54)	Pouchitis	RNase H	P = S, ODN	Enema	Phase III	96, 98
Mongersen	SMAD7	Crohn's disease	RNase H	P = S, ODN	Oral	Phase III	94
ATL 1102	Very late antigen 4 (CD49D)	Multiple sclerosis	RNase H	2′MOE gapmer	Subcutaneous	Phase II	140
SB010	GATA3	Asthma	DNAzyme	Undisclosed	Aerosol	Phase II	141
IONIS-PKK$_{Rx}$	Kallikrein B1	Hereditary angioedema	RNase H	2′MOE gapmer	Subcutaneous	Phase I	
ALN-CC5	Complement factor 5	Complement-related diseases	siRNA	GalNAc-conjugated siRNA	Subcutaneous	Phase I	NCT0235243
Infectious diseases							
Miravirsen	MicroRNA-122	Hepatitis C virus	Anti-miR	LNA chimera	Subcutaneous	Phase II	132, 133
RG-101	MicroRNA-122	Hepatitis C virus	Anti-miR	GalNAc-conjugated cEt/2′MOE mixmer	Subcutaneous	Phase II	134
ARC-520	Hepatitis B virus	Hepatitis B virus	siRNA	Nanoparticle formulation of siRNA	Intravenous	Phase II	142

(*Continued*)

Table 1 (*Continued*)

Drug	Target	Indication	Mechanism	Chemistry	Delivery route	Status	Reference or NCT ID[a]
ARB-1467	Hepatitis B virus	Hepatitis B virus	siRNA	Liposome formulation of siRNA	Intravenous	Phase II	
IONIS-HBV-L_{Rx}	Hepatitis B virus	Hepatitis B virus	RNase H	GalNAc-conjugated 2′MOE gapmer	Subcutaneous	Phase I	NCT02647281
Miscellaneous							
QPI-1002	P53	Kidney transplant, acute kidney injury	siRNA	Chemically modified siRNA (undisclosed)	Intravenous	Phase III	NCT02610296
QPI-1007	Caspase 2	Nonarteritic anterior ischemic optic neuropathy, glaucoma	siRNA	Chemically modified siRNA (undisclosed)	Intravitreal	Phase III	NCT02341560
PF-655	Undisclosed	Diabetic macular edema, wet AMD	siRNA	Chemically modified siRNA (undisclosed)	Intravitreal	Phase II	NCT01445899
RG-012	MicroRNA 21	Alport syndrome	Anti-miR	Not reported	Subcutaneous	Phase I	
Fitusiran	Antithrombin	Hemophilia	siRNA	GalNAc-conjugated siRNA	Subcutaneous	Phase I	NCT02035605
ALN-AS1	Aminolevulinic acid synthase 1	Porphyria	siRNA	GalNAc-conjugated siRNA	Subcutaneous	Phase I	NCT02452372
QR-010	Cystic fibrosis transmembrane conductance regulator	Cystic fibrosis	Splicing modulation	Undisclosed	Inhaled	Phase I	NCT02564354
ISTH0036	Transforming growth factor-β2	Glaucoma	RNase H	LNA gapmer	Intravitreal	Phase I	NCT02406833
MRG-201	MicroRNA 29b mimic	Fibrosis	MicroRNA mimic	Undisclosed	Intradermal	Phase I	NCT02603224

Abbreviations: 2′MOE, 2′-*O*-methoxyethyl; ALS, amyotrophic lateral sclerosis; AMD, age-related macular degeneration; anti-miR, anti-microRNA; Apo(a), apolipoprotein (a); ASO, antisense oligonucleotide; cEt, constrained ethyl; dsRNA, double-stranded RNA; ENA, ethylene-bridged nucleic acid; GalNAc, *N*-acetylgalactosamine; LNA, locked nucleic acid; Lp(a), lipoprotein (a); NSCLC, non-small-cell lung cancer; ODN, oligodeoxynucleotide; P = S, phosphorothioate; RNAi, RNA interference; siRNA, small interfering RNA.
[a]NCT ID, **http://clinicaltrials.gov** identifier.

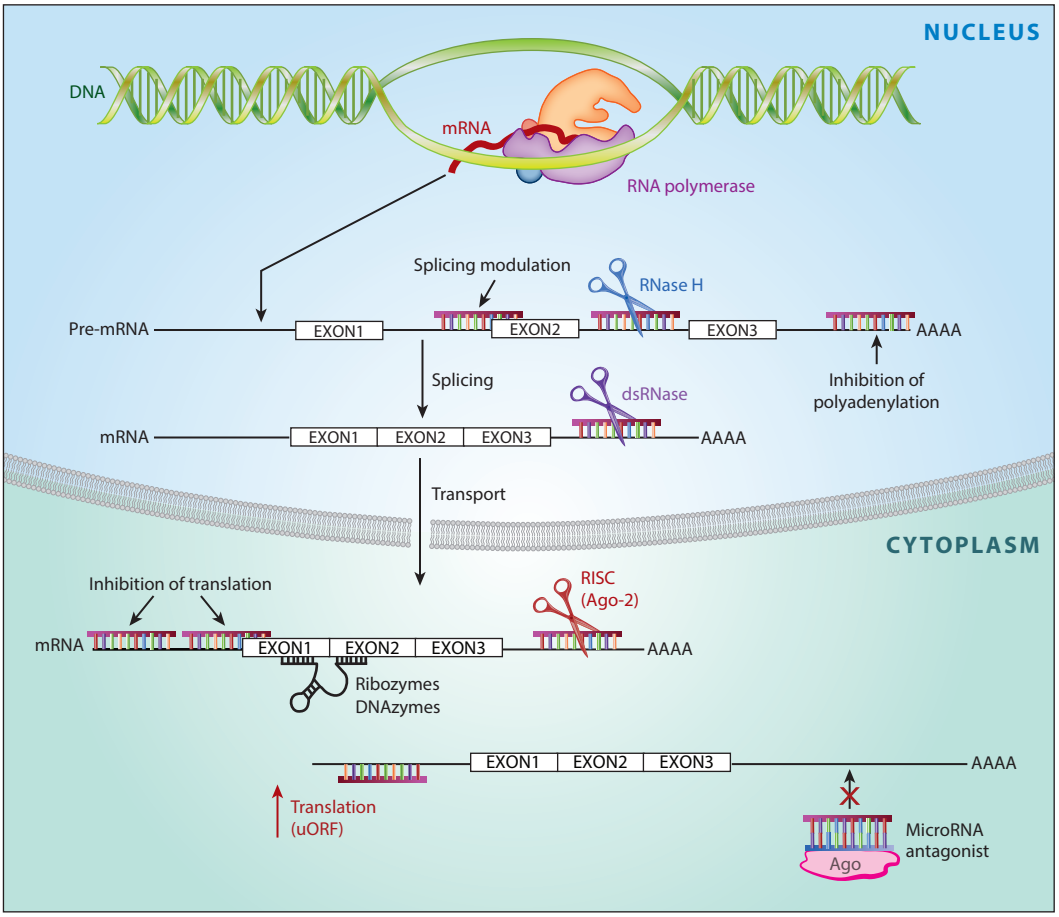

Figure 1

Different antisense mechanisms. Different steps in the maturation of an mRNA at which antisense oligonucleotides are known to interact and perturb the function of the RNA are shown. Mechanisms shown include the nondegradative mechanisms (e.g., modulation of RNA splicing, polyadenylation, inhibition of translation, enhancing translation, blocking microRNA function) and those that promote degradation of the RNA (e.g., RNase H, siRNA/RNA interference, and ribozymes). Abbreviations: Ago-2, argonaute-2; dsRNase, double-stranded ribonuclease; RISC, RNA-induced silencing complex; siRNA, small interfering RNA; uORF, upstream open reading frame.

increases resistance to nucleolytic degradation (10), such that following systemic administration, PS oligonucleotides possess sufficient stability in plasma, tissues, and cells to reach the target RNA. PS oligodeoxynucleotides (ODNs), which are commonly referred to as first-generation ASOs, are able to elicit RNase H cleavage of the target RNA efficiently, 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 prevents rapid renal excretion and facilitates uptake to tissues.

Another example of a backbone modification is the N3′→P5′ thiophosphoramidate ODN (**Figure 2**), in which the 3′ oxygen in the deoxyribose ring is substituted with a 3′ amino atom. Thiophosphoroamidates and phosphoramidates exhibit high affinity toward complementary RNA and high nuclease resistance (11) but do not support RNase H.

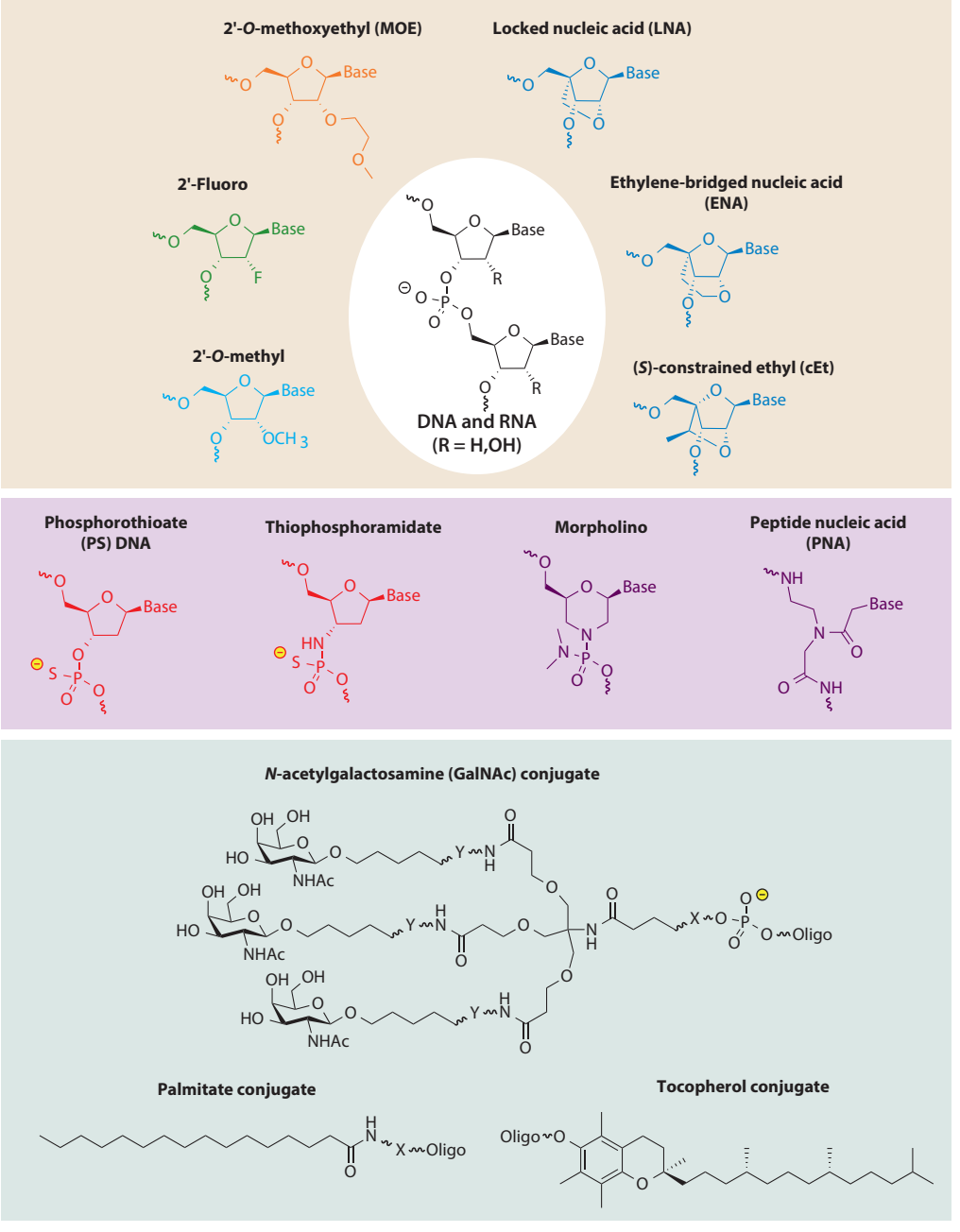

Figure 2

Examples of chemical modifications used in antisense oligonucleotides.

In addition to modifications of the phosphodiester backbone linkage, replacements of the sugar-phosphate backbone with an isostere have been devised. One of these led to phosphorodiamidate morpholino oligonucleotides (**Figure 2**). Because of the phosphorodiamidate linkage, morpholino oligonucleotides are neutral. These modifications are similar in affinity to DNA and are nuclease stable. They do not support an RNase H mechanism and are used primarily in translation arrest or other steric blocking mechanisms.

Heterocycle Modifications

Heterocycle (the bases) modifications have focused mainly on increasing binding affinity for complementary nucleic acids (12). Substituting the C5 hydrogen of deoxycytidine with a methyl group improves the T_m of the DNA-RNA duplex by about 0.5°C per substitution (13) owing to enhanced base stacking on the 5′ side nucleobase. This modification supports the RNase H mechanism and is used in several antisense drugs to decrease their immune stimulatory potential (14). Further enhancing the stacking interactions with additional aromatic or π-rich surfaces prompts a substitution of the C5 position with a propynyl group, resulting in a further increase in duplex stability, but also increased toxicity (15, 16).

Sugar Modifications

To date, modifications to the 2′ position of the sugar moiety have provided the greatest value in enhancing the drug-like properties of oligonucleotides. Oligonucleotides containing 2′ sugar modifications are often referred to as second-generation ASOs. Organization of the sugar into an RNA-like conformation increases binding affinity. Unfortunately, essentially all 2′ modifications greatly reduce or completely inhibit RNase H cleavage of the RNA strand opposite the modification. This limitation has been minimized by use of a chimeric strategy (gapmer), in which regions of 2′-modified residues flank a central DNA region of the oligonucleotide (17). Limitations also exist for the use of 2′-modified nucleosides in small interfering RNA (siRNA) duplexes, which utilize the RNAi machinery. Considerably more flexibility is afforded for use of this modification in oligonucleotide drugs that do not require an enzyme-mediated terminating mechanism, such as those that alter splicing of mRNA.

The increase in affinity observed with 2′ modifications is driven energetically by the electronegative substituent at the 2′ position. As such, the 2′-fluoro (**Figure 2**) modification imparts increased binding affinity ($\Delta T_m \sim 2$°C per modification, relative to DNA) for target RNA (13). 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. ASOs with gapmer designs employing 2′-*O*-methyl nucleosides have advanced to human clinical trials (18), as have uniformly modified 2′-*O*-methyl oligonucleotides that modulate RNA splicing (19). The use of 2′-*O*-methyl nucleosides (**Figure 2**) in siRNA oligonucleotides also holds promise. Minimal use of 2′-*O*-methyl nucleosides has been employed to stabilize siRNAs for successful in vivo proof-of-concept experiments (20, 21) and to minimize off-target effects by limiting their ability to serve as microRNA agonists (22). The combination of 2′-*O*-methyl and 2′-fluoro substitution has allowed for the complete elimination of RNA from siRNAs, providing duplexes with increased stability and potency that activate the RNAi pathway (23). Similar siRNA designs employing extensive 2′-fluoro and 2′-*O*-methyl modification have advanced to clinical trials (24).

The 2′-*O*-methoxyethyl (2′MOE; **Figure 2**) modification is currently the most advanced of the 2′-modified series (3). 2′MOE increases T_m by about 2°C per modification versus RNA,

relative to DNA, and greatly increases resistance to nucleases (13). It also appears to reduce certain nonspecific protein binding, which can reduce toxicities (25). 2′MOE oligonucleotides are being used broadly to support a variety of different antisense mechanisms (7, 26–30). The utility of the second-generation, 2′MOE-modified oligonucleotides has translated from the test tube to humans, resulting in numerous 2′MOE-modified antisense drugs entering the clinic (**Table 1**).

As with traditional, small-molecule, drug optimization efforts, the rewards for constraining a ligand correctly are large 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 a 2′,4′ bridged nucleic acid by Obika et al. (31) and locked nucleic acid (LNA) by Wengel (32) (**Figure 2**). LNAs show dramatically improved hybridization properties and increased nuclease resistance. Uniform LNA oligonucleotides do not support RNase H (33), so a gapmer strategy must also be employed with LNA-modified oligonucleotides to support the RNase H mechanism. Multiple structural analogs of LNAs have been prepared (34), with some exhibiting improved properties (35, 36). Although LNA-containing gapmer oligonucleotides can clearly improve potency relative to 2′-modified designs, they also appear to have increased toxicological liabilities (37, 38). Attempts to reduce this liability have led to the synthesis and in vivo evaluation of many analogs of LNA, including bridge heteroatom substitutions (39), stereoisomers (40), alkyl substitutions (41), and replacements of the bicyclic ring with more rigid hexitol-derived systems (42, 43). Oligonucleotides containing the (*S*)-constrained ethyl (cEt) modification have exhibited promising profiles (44, 45) and have advanced to human clinical trials for several indications. These studies further highlight the promise this class of molecules holds for improving the potency of antisense drugs in the near future.

Conjugation Strategies

Conjugation of oligonucleotides to ligands that can bind to various cell surface acceptor sites would be expected to alter the pharmacokinetic properties of the oligonucleotide, and many attempts have been made to increase tissue and cellular uptake of oligonucleotide drugs via conjugation of various ligands. Cholesterol conjugates of oligonucleotides are one of the most studied classes of conjugates; they increase the exposure to the liver, with a concomitant reduction in exposure to the kidney (20, 46, 47). Cholesterol-conjugated oligonucleotides have been used for multiple antisense mechanisms, including RNase H, siRNA, and microRNA antagonists. In addition to cholesterol, other lipid groups such as fatty acids and tocopherol can alter the distribution of oligonucleotides in animals, increasing potency, in particular for liver-expressed targets (46, 48–50).

A major advance in the targeted delivery of oligonucleotides to hepatocytes was made with the use of multivalent *N*-acetylgalactosamine (GalNAc) conjugates. Trivalent GalNAc conjugates to siRNAs (24) and single-stranded traditional ASOs (51) were shown to elicit efficient and potent activity for liver targets in animals. These observations have been translated to multiple clinically relevant targets, and potent gene inhibition with GalNAc siRNAs (24), microRNA inhibitors, and RNase H ASOs have been demonstrated in primates and human clinical trials. An optimized GalNAc conjugate of a second-generation 2′-MOE ASO that targets apolipoprotein (a) mRNA was shown to be 30-fold more potent in humans relative to the parent oligonucleotide of the same sequence (52). These advances are anticipated to translate to an improved therapeutic index, reduced therapy costs, and improved patient convenience such as monthly or longer dosing schedules for hepatic therapeutic targets. Whether the success of conjugate-mediated targeted delivery of oligonucleotides to the liver can be expanded to other tissues is unknown, but it is an exciting and promising area of future research in oligonucleotide conjugation.

PHARMACOKINETICS OF OLIGONUCLEOTIDES

The pharmacokinetic properties of oligonucleotides are driven largely by the chemistry of the backbone and thus are largely sequence independent within a chemical class (53, 54). Tissue bioavailability is assisted by plasma protein binding that limits glomerular filtration and ultimately urinary excretion of oligonucleotides. As discussed above, PS-modified ASOs exhibit increased plasma stability and protein binding and, ultimately, tissue bioavailability compared to unmodified ASOs. Systemic biodistribution of PS-modified oligonucleotides is broad, and typically, the organs with the highest concentrations are the liver and kidney, followed by the bone marrow, adipocytes, and lymph nodes (**Figure 3**). Cell uptake is mediated predominantly by endocytosis (53, 55). Both their size and charge prevent ASO distribution across the blood-brain barrier. However, modified single-strand oligonucleotides administered by intrathecal injection into the cerebrospinal fluid (CSF) distribute broadly in central nervous system (CNS) tissues (28, 56). The majority of intracellular oligonucleotide distribution following systemic or local administration occurs rapidly in just a few hours and is facilitated by rapid endocytotic uptake mechanisms.

Following subcutaneous (SC) administration, ASOs are absorbed rapidly from the injection site into the circulation, with peak plasma concentrations reached by 3–4 h for second-generation

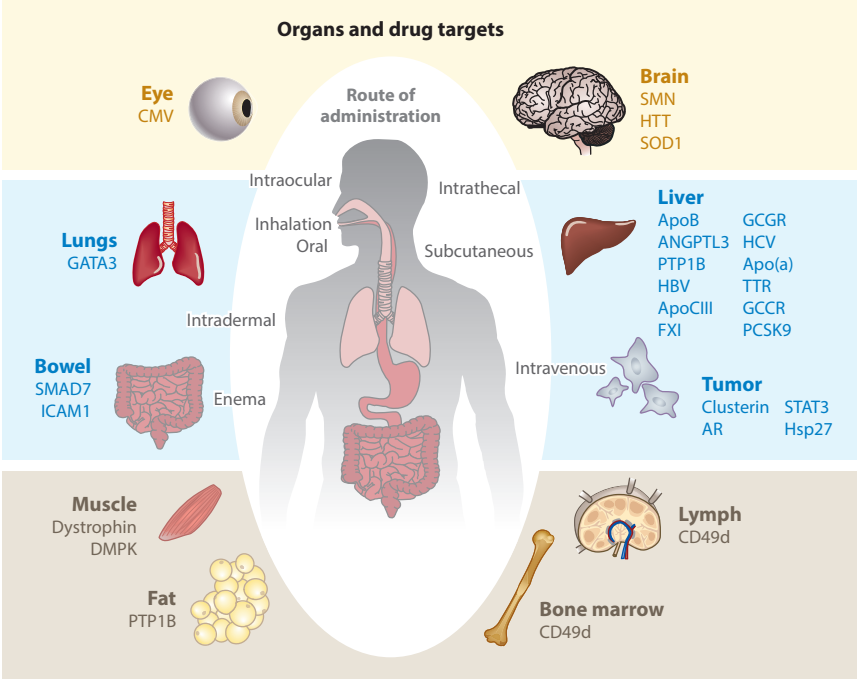

Figure 3

Examples of tissues in which antisense drugs have been shown to work and routes by which these drugs can be administered. Abbreviations: ANGPTL3, angiopoietin like 3; Apo, apolipoprotein; AR, androgen receptor; CD49d, integrin alpha 4; CMV, cytomegalovirus; DMPK, dystrophin myotonica protein kinase; FXI, factor XI; GATA3, GATA binding protein 3; GCCR, glucocorticoid receptor; GCGR, glucagon receptor; HBV, hepatitis B virus; HCV, hepatitis C virus; Hsp27, heat shock protein 27; HTT, huntingtin; ICAM1, intercellular adhesion molecule 1; PCSK9, proprotein convertase subtilisin/kexin type-9; PTP1B, protein tyrosine phosphatase 1B; SMAD7, Mothers against decapentaplegic homolog 7; SMN, survival motor neuron; SOD1, superoxide dismutase 1; STAT3, signal transducer and activator of transcription 3; TTR, transthyretin protein.

ASOs (26, 54). Following either intravenous (IV) or SC administration, plasma concentrations decline rapidly from peak concentrations in a multiexponential fashion, which is characterized by a dominant initial rapid distribution phase wherein drug transfers to tissues in minutes to a few hours, followed by a slower terminal elimination phase from tissues (half-life of up to several weeks). The major systemic tissues of distribution include the liver, kidney, bone marrow, adipocytes (cell body but not lipid fraction), and lymph nodes (54, 57, 58). Absorption into the systemic circulation following oral, intravitreal, or pulmonary dosing is low, with less than 1% of the administered dose generally absorbed (59–61). Increases in oral absorption into the systemic circulation have been achieved when ASOs are administered together with permeation enhancers that alter paracellular pathways reversibly in the small intestine (62).

Ultimate clearance and elimination of first-generation PS-modified ODNs is facilitated by endo- and exonuclease metabolism that results in small-molecular-weight fragments of the parent ODNs that lose their ability to bind plasma proteins avidly and are filtered and eliminated ultimately in urine (53, 54). Second-generation ASOs with various sugar modifications are stabilized against these enzymes and are thus metabolized slowly over weeks, with clearance half-lives generally on the order of 2–4 weeks to afford once-weekly or less frequent dosing.

Oligonucleotides that lack charge or are more weakly bound to plasma proteins, such as peptide nucleic acids, morpholinos, and unmodified and unformulated siRNAs, exhibit more rapid clearance from blood (63–65). These compounds and their metabolites are filtered and excreted readily, resulting in low or negligible tissue uptake. Thus, appropriate and balanced plasma protein binding appears to be required for broad and significant systemic delivery to tissues and cells.

TOXICOLOGY OF ANTISENSE OLIGONUCLEOTIDES

ASOs, like all drugs, exhibit dose-dependent toxicities. The best-characterized classes of antisense drugs, owing to the large number of such drugs that have entered clinical trials (**Table 1**), are first-generation PS-modified ODNs and 2′MOE-modified ASOs (66, 67). Researchers are just beginning to understand the toxicological properties of other types of ASOs (38, 63, 68–70).

The potential toxicities for ASOs can be classified as hybridization dependent or hybridization independent. Hybridization-dependent toxicities can be attributed to exaggerated pharmacological effects and hybridization to non-target RNAs. This class of toxicities is 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 the antisense inhibitors in preclinical models. Off-target hybridization-dependent effects can be diminished by performing careful bioinformatics analyses to identify targets with perfect matches or a few mismatches. Shorter oligonucleotides with high-affinity modifications have a greater potential to interact with non-targeted transcripts and, if RNase H active, promote degradation of the off-target transcript (69). Oligonucleotides that work via the RNAi mechanism have the ability to function as microRNAs, in which only 6–8 nucleotides (in the seed region) are required for activity and can result in hundreds if not thousands of potential off-target interactions (71–73). The potential for seed-matched off-target effects can be reduced through chemical modifications (22). Taking all these precautions into account increases the probability of identifying a selective antisense drug successfully.

A second source of potential toxicities can be mediated through interactions of the oligonucleotide with proteins. These effects can be sequence dependent, such as interaction with Toll-like receptors (14), or sequence independent. Generally, this latter class of toxicity depends on the chemistry of the oligonucleotides and the proteins with which the chemical class interacts. Examples of side effects that are largely sequence independent are effects on coagulation (74) and complement activation (70, 75), whereas immune cell activation can be both sequence dependent

and independent (76, 77). Understanding these toxicities provides an opportunity to screen for antisense drugs that have better tolerability. 2′MOE-modified antisense drugs have proved to be better tolerated than PS ODNs and are well tolerated overall (25, 66, 78, 79). Oligonucleotides containing bicyclic sugar modifications such as LNAs can be hepatotoxic in rodents, in part owing to the increased potential for off-target hybridization (38, 69). Some specific bicyclic sugars such as cEt modifications appear to be better tolerated than LNAs (43). The safety profile for other chemical classes of oligonucleotides has not been well described in the literature because they are in earlier stages of clinical testing.

PHARMACOLOGY OF ANTISENSE OLIGONUCLEOTIDES

Over the past 25 years, numerous antisense drugs have entered clinical trials for the treatment of a broad variety of diseases (2, 80–82). Fomivirsen is a first-generation antisense drug targeting cytomegalovirus that was approved for the treatment of cytomegalovirus retinitis, at the time a common opportunistic infection in HIV patients (83). As discussed above, second-generation antisense drugs containing 2′MOE modifications exhibit increased potency, better tolerability, and longer duration of action compared to first-generation PS-modified ODNs. Many second-generation drugs are in development (**Table 1**) and are showing encouraging activity in the clinic (**Figure 3**), as are several siRNA drugs in development. A comprehensive review of antisense drugs that have entered development is beyond the scope of this review. Rather, we feature a few examples of antisense drugs in several different therapeutic areas.

Cardiovascular Diseases

Mipomersen is the first second-generation ASO approved by the US Food and Drug Administration (FDA) as an adjunct therapy for homozygous familial hypercholesterolemia (HoFH). It reduces apolipoprotein B (apoB) mRNA levels, thus lowering the plasma levels of apoB-containing particles [e.g., low-density lipoprotein cholesterol (LDL-C)]. The preclinical and clinical pharmacological properties of mipomersen have been reviewed previously (26, 84). A recent post hoc subanalysis of a Phase III randomized controlled trial found that mipomersen reduced levels of apoB and LDL-C effectively in pediatric patients (12–18 years old) with HoFH on maximally tolerated statins (85). Similar reductions in measured atherogenic lipoproteins were observed between the pediatric patients compared to the adult cohort. Mipomersen was also documented recently to reduce levels of all apoB-containing atherogenic lipoproteins including lipoprotein (a) [Lp(a)], an independent, causal, genetic risk factor for cardiovascular disease. In an analysis of pooled data from four Phase III studies (86), mipomersen was found to reduce Lp(a) levels significantly in patients with a wide variety of lipid abnormalities. Finally, treatment with mipomersen up to 400 mg for 4 weeks did not show any effect on cardiac repolarization (87). These results suggest a lack of effect on QT interval with mipomersen and possibly other 2′MOE ASOs because of their similar physicochemical properties and pharmacokinetics.

Apolipoprotein C III (apoCIII) plays a critical role in the metabolism of triglyceride-rich lipoproteins, and decreased expression is associated with a lower risk of cardiovascular disease (88, 89). A Phase I, randomized, double-blind, placebo-controlled clinical trial of volanesorsen, a second-generation antisense drug designed to reduce apoCIII mRNA levels, demonstrated a robust dose-dependent and prolonged reduction in plasma apoCIII and triglyceride levels in healthy volunteers (88). A subsequent, open-label Phase II study was conducted in three patients with familial chylomicronemia syndrome (FCS) (90), demonstrating a dramatic decrease in plasma apoCIII protein (from 71% to 90%) and plasma triglyceride levels (from 56% to 86%). In

addition, a randomized, placebo-controlled Phase II study in patients with hypertriglyceridemia demonstrated the dose-dependent effects of the drug as a single agent and as an add-on therapy (91). Plasma high-density lipoprotein cholesterol levels increased and very low-density lipoprotein cholesterol levels decreased, both in a dose-dependent manner. The drug is currently being investigated in placebo-controlled Phase III clinical trials for the treatment of FCS and familial partial lipodystrophy.

Evidence suggests that severe factor XI deficiency provides protection against deep-vein thrombosis and therefore cardiovascular morbidity and mortality. Reducing factor XI levels in patients undergoing total knee arthroplasty with IONIS-FXI$_{Rx}$ was shown to be an effective method in preventing venous thromboembolism (VTE) and appeared to be safe with respect to the risk of bleeding. In an open-label, parallel-group study, patients taking the drug were shown to have a lower risk of VTE and clinically relevant bleeding compared to those who took enoxaparin (92). IONIS-FXI$_{Rx}$ lowered factor XI levels in a dose-dependent manner without lowering the other components of the intrinsic pathway, which highlights the specificity of the drug. The drug has the potential to be more effective than conventional anti-thrombotics. A Phase II study with IONIS-FXI$_{Rx}$/BAY 2306001 is ongoing to investigate the drug's effects in patients with end-stage renal disease on hemodialysis.

Inflammation and Autoimmune Diseases

ASO drugs have been and are currently being evaluated for multiple inflammatory diseases, with drugs under investigation for the treatment of inflammatory bowel disease being most advanced. SMAD7 is an intracellular inhibitor that interferes with the receptor-dependent phosphorylation of other SMAD proteins, which are positive effectors of transforming growth factor-β1 signal transduction. Mongersen, a first-generation PS ODN that targets the SMAD7 mRNA, is delivered orally in tablet form using an external pH-dependent coating (93). The first clinical test of mongersen involved 15 patients with active steroid-resistant and/or -dependent Crohn's disease (93) and was followed by a randomized, placebo-controlled, dose-ranging Phase II trial in patients who had active moderate to severe disease (94). The same daily dose schedule was tested, but over a broader dose range and an extended treatment period of 14 days. Treatment was well tolerated, with 119 of 124 (96%) of the patients who received oral mongersen completing the 2-week treatment period. Notably, a significant proportion of the patients in the two higher dose groups (40 and 160 mg daily mongersen) achieved clinical remission. Based on these encouraging Phase II results, mongersen has advanced to a longer-term Phase III evaluation and is also being investigated in a placebo-controlled Phase III study for its effects on patients with moderate to severe ulcerative colitis.

Alicaforsen, a first-generation, PS-modified ASO to intercellular adhesion molecule 1 (CD54), has been tested for its effects by systemic delivery in patients with Crohn's disease (95) and by rectal enema in patients with mild to moderate active left-sided ulcerative colitis (or active unremitting pouchitis) (96–98). Based on encouraging data from placebo-controlled and open-label studies, the drug is currently being developed for the treatment of chronic refractory pouchitis (96, 99).

Oncology

Because of the high unmet medical need, cancer has been a major area of therapeutic investigation for antisense technology. Some of the earliest clinical studies for antisense drugs investigated their potential as treatments for various cancers (100). Despite early entry into the clinic, identifying robust antitumor effects in clinical trials has been challenging. Advances in antisense technology

have resulted in increased potency and better tolerability, which hopefully will translate to increased clinical benefit. Currently, one antisense drug is in Phase III clinical trials, and several are in earlier stages of development for the treatment of various malignancies (**Table 1**). Custirsen, a chimeric 2′MOE-modified antisense drug targeting clusterin, has shown promise in several Phase II studies and is currently being evaluated in Phase III clinical trials for the treatment of prostate and lung cancers (101).

Two drugs that take advantage of the increased potency of the cEt sugar modification are currently in clinical trials. AZD9150 is an ASO targeting signal transducer and activator of transcription 3 (*STAT3*), which is activated in several types of cancers (102). In the open-label Phase I trial, the drug has shown encouraging activity as a single agent in several cancer types, with responses in diffuse large B cell lymphoma being most notable (102). A second, cEt-modified oligonucleotide targeting the androgen receptor is in an open-label clinical trial as a possible treatment for prostate cancer (103). Several additional antisense drugs, including microRNA mimics and siRNAs, are in early-stage clinical studies (**Table 1**).

Neurology

Antisense drugs are being evaluated for multiple neurological diseases and are administered both systemically and into the CSF that surrounds the brain (**Table 1**). ASOs do not cross an intact blood-brain barrier efficiently; therefore, they must be introduced directly into the CSF or parenchyma to treat brain or spinal cord diseases. Broad distribution of second-generation, PS-modified ASOs into the spinal cord and brain tissues occurs following injection directly into the CSF (28, 56). In contrast to single-stranded oligonucleotides, double-stranded siRNAs have more limited distribution and activity in the CNS, with most siRNA drugs being delivered directly into the brain parenchyma, producing local suppression of the target gene (104, 105). Thus, neurological diseases can be approached using different antisense mechanisms and oligonucleotide designs, with single-stranded ASOs providing broad coverage of CNS structures and siRNAs used for local therapy.

Duchenne muscular dystrophy is a progressive, severely disabling, and ultimately lethal neuromuscular disease caused by point mutations, insertions, or chromosomal rearrangements in the dystrophin gene resulting in truncated protein or loss of transcript through nonsense-mediated decay (106). Several laboratories have used ASOs to promote skipping of specific dystrophin exons, resulting in a spliced dystrophin product with an extended open reading frame resulting in partial restoration of function (107, 108). Because multiple genomic alterations can result in Duchenne muscular dystrophy, no single oligonucleotide will address all forms of the disease. ASOs designed to promote skipping of exon 51 are the most advanced in clinical trials, with two drugs in regulatory review for marketing approval (**Table 1**).

Drisapersen is a uniform 2′-*O*-methyl PS-modified oligonucleotide designed to bind to a sequence within exon 51, promoting skipping of the exon (108), whereas eteplirsen is a uniformly modified morpholino drug designed to bind to a similar site as drisapersen in exon 51 of the dystrophin pre-mRNA (109). Drisapersen is administered as an SC injection, and eteplirsen is administered as an IV infusion (19, 110, 111). Despite encouraging clinical effects in early placebo-controlled Phase II studies (19, 110), drisapersen failed to demonstrate a benefit over placebo in larger placebo-controlled Phase III studies for the primary endpoint, the six-minute walk. Eteplirsen was evaluated initially in an open-label study in which six dose levels of the drug were delivered by weekly IV infusion. Overall, the drug was well tolerated, and variable increases in dystrophin expression were observed in muscle biopsies for the highest dose levels evaluated (112). A small controlled study followed in which subjects were randomized to receive

two dose levels of the drug or placebo weekly for 24 weeks ($n = 4$/group), and at week 25 placebo subjects were switched to receive either 30 mg/kg ($n = 2$) or 50 mg/kg ($n = 2$) eteplirsen (111). At week 24, there were no significant differences in six-minute-walk distances between placebo and drug-treated cohorts. By week 48, subjects treated with 50 mg/kg eteplirsen maintained a stable distance or increased distance walked in six minutes versus a decline in placebo patients switched to active drug (111). Long-term follow-up of subjects (36 months) who participated in the study showed the majority of patients continued to maintain ambulation (113). Immunohistochemical analysis of dystrophin expression in muscle tissue was variable and showed a modest increase in dystrophin staining for both drugs. Both drugs were submitted to the US FDA for market authorization. Eteplirsen received accelerated approval, conditional upon completion of additional studies, whereas drisapersen was rejected.

Additional antisense drugs are currently in development targeting other exons, which will broaden the patient population treated with antisense drugs (**Table 1**). These studies are still ongoing, and no data have been reported.

Myotonic dystrophy type 1 (DM1) is a multisystemic disease caused by a triplet repeat expansion (CTG) in the 3′ untranslated region of the dystrophin myotonica protein kinase (*DMPK*) gene (114). The resulting transcript, containing hundreds to thousands of CUG repeats, binds to RNA splicing factors such as muscleblind proteins, sequestering them in discrete nuclear foci, resulting in loss of activity (115). Researchers are exploring two different antisense mechanistic approaches as potential treatments for DM1, using nondegrading ASOs to displace muscleblind from the transcript (116, 117) or, alternatively, using RNase H–active ASOs to promote the degradation of the toxic RNA transcript, releasing muscleblind in the process (118, 119). IONIS-DMPK-2.5_{Rx} is a chimeric ASO containing both 2′MOE modifications and cEt modification in the wings with a central DNA gap to support the RNase H mechanism of action; it is currently in a randomized controlled study in DM1 patients (**Table 1**).

Transthyretin amyloidosis is a form of systemic amyloidosis caused by misfolded transthyretin protein (TTR), which deposits as toxic amyloid fibrils in multiple tissues, including peripheral nerves, the gastrointestinal tract, and the heart (120). A therapeutic strategy is to lower the amount of TTR substrate, decreasing the rate of fibril formation using ASOs. The main tissue source of circulating TTR is the liver, which is readily approachable using antisense technology. Three different antisense drugs are currently in development for the treatment of TTR amyloidosis, with two drugs in placebo-controlled Phase III clinical trials for the treatment of TTR familial polyneuropathy (**Table 1**). IONIS-TTR_{Rx} is a second-generation, RNase H–dependent antisense drug being developed for both familial amyloid polyneuropathy (FAP) and cardiomyopathy (29). The drug demonstrated dose-dependent reductions in plasma TTR levels in a Phase I normal volunteer study (29). The drug is currently in Phase III clinical trials. Patisiran is an siRNA formulated in a lipid nanoparticle and is currently in Phase III clinical trials for the treatment of FAP. The drug was tested in healthy human volunteers (121) and demonstrated dose-dependent reductions of plasma TTR when administered intravenously. Mild to moderate infusion-related reactions were observed that resolved spontaneously (mild) or with temporary interruption with or without additional glucocorticoids (moderate). In Phase II studies, confirmation of the dose-dependent reduction in plasma TTR in polyneuropathy patients was achieved with a similar safety profile (122). A GalNAc-conjugated siRNA and IONIS-TTR_{Rx} are also currently in development for the treatment of cardiomyopathy (**Table 1**) (123).

Spinal muscular atrophy (SMA) is a severe, progressive, motor neuron disease that usually occurs in infancy or childhood. The disease is caused by deletions or mutations in the survival of motor neuron 1 (*SMN1*) gene (124). Humans have a paralogous copy of the *SMN1* gene, *SMN2*, which differs from *SMN1* by a few nucleotides, one of which weakens the splice site for exon 7,

resulting in exon 7 deletion. The exon 7 deleted transcript produces a truncated, rapidly degraded protein. Nusinersen is a fully modified 2′MOE oligonucleotide designed to bind to a specific sequence in intron 7 of the SMN1 and 2 pre-mRNAs, enhancing exon 7 inclusion and increasing the production of SMN protein (125, 126). A Phase I open-label trial in children with type 2 and type 3 SMA was completed recently. The drug was found to be well tolerated, and the intrathecal bolus administration in children was demonstrated to be a feasible method for delivery (27, 127). A dose-dependent increase in motor function was observed following a single intrathecal dose (27). This study was followed with two multiple-dose Phase Ib/IIa studies—one in a population similar to that evaluated in the single-dose study and a second study in the more severe type 1 SMA population (128). Based on these encouraging findings from the single-dose study and the open-label, multiple-dose studies, two sham-controlled Phase III studies are in progress—one in childhood-onset SMA and a second in infantile-onset SMA. Based on a successful interim analysis of the Phase III infantile onset study, the study was stopped and an application for market authorization has been submitted to both US and European regulatory agencies.

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder resulting from an expanded CAG repeat in the huntingtin (*HTT*) gene, which causes a toxic gain of function due to an expanded polyglutamine tract in the resulting protein. ASOs designed to lower total HTT have been shown to provide a prolonged improvement in an HD mouse model and, importantly, to reduce HTT after intrathecal dosing in a primate (56). A similar ASO has progressed to a placebo-controlled clinical trial in HD patients (**Table 1**).

Infectious Diseases

Different antisense mechanisms can be used to inhibit viral replication—for example, by binding to viral mRNA to sterically block translation of the protein or degrade the viral RNA through an RNase mechanism, or by blocking host microRNAs that support viral replication. Several antisense therapies are currently undergoing clinical trials for various infectious diseases (**Table 1**).

MicroRNA-122 (miR-122) is highly abundant in the liver and is essential to the stability and propagation of hepatitis C virus (HCV) (129). It binds to a highly conserved 5′ untranslated region of the HCV genome, protecting it from degradation and host innate immune responses (129). In addition, miR-122 is also believed to play a role in inflammatory activity in the liver (130). Inhibiting miR-122 activity with the LNA-modified ASO miravirsen in chimpanzees infected with HCV resulted in inhibition of HCV replication (131). In a Phase IIa placebo-controlled study in 36 patients with chronic HCV, 5 weekly doses of miravirsen reduced levels of HCV RNA, an effect that was sustained beyond the administration period (132). Some patients had undetectable HCV RNA, which remained undetected through the end of the study or rebounded by the end of the study. A long-term retrospective analysis on the clinical trial saw no long-term safety problems among the HCV patients treated with miravirsen for up to 35 months (133). A Phase II study is currently ongoing in HCV patients who were null responders to pegylated interferon alpha and ribavirin to test miravirsen in combination with telaprevir and ribavirin

RG-101 is a GalNAc-conjugated oligonucleotide designed to inhibit miR-122 and HCV replication through a similar steric blocking mechanism as miravirsen. RG-101 utilizes cEt and 2′MOE-modified nucleotides. A Phase I clinical trial of RG-101 was designed for both healthy volunteers and HCV-infected patients (134). In healthy volunteers, a single dose of RG-101 increased serum alkaline phosphatase (a direct miR-122 target) and decreased serum cholesterol (an indirect miR-122 target), which is consistent with the intended pharmacology. Significant reductions in HCV RNA were observed in HCV patients receiving a single dose of 2 or 4 mg/kg of RG-101 versus placebo. The majority of patients dosed with RG-101 had HCV RNA levels below the limit of

quantification at study day 57, an effect that was sustained in most of them at study day 85. RG-101 was safe and well tolerated in healthy volunteers and HCV patients. The results from this clinical trial were very encouraging and support continued study of the drug.

CONCLUSIONS

The development of antisense-based therapeutics continues to progress, with three approved drugs and numerous drugs in late-stage development. In addition, more than 30 drugs are in development for a wide variety of disease indications, with many showing promise in early clinical trials and demonstrating broad therapeutic applications. Although the technology has seen some successes over the past 25 years, opportunities exist for further improvements. Oligonucleotide modifications have improved the potency and tolerability of the antisense drug and have enhanced drug distribution to tissues and ultimately to the targeted RNA inside cells. Further improvement of antisense drugs should be possible through better ASO designs, more comprehensive screening, and novel chemistries and perhaps formulations. Gaining more in-depth insights into the molecular mechanisms by which oligonucleotides traffic in the body and within cells and ultimately bind to the target RNA will provide a rational basis for further improving antisense drugs. Although we have considerable knowledge concerning the mechanisms by which oligonucleotides produce unwanted effects, additional investigations are warranted to further improve safety and tolerability, especially for new chemistries and ASO designs. Finally, identifying more convenient methods to administer antisense drugs is important to enhance commercial viability of the technology. Although there have been tremendous advances in antisense technology, more must be done to realize its full potential.

DISCLOSURE STATEMENT

All authors are employees of Ionis Pharmaceuticals, Inc., Carlsbad, CA.

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