Oligonucleotide Therapies: The Past and the Present

Karin E. Lundin,* Olof Gissberg, and C.I. Edvard Smith

Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Karolinska University Hospital Huddinge, Stockholm, Sweden.

In this review we address the development of oligonucleotide (ON) medicines from a historical perspective by listing the landmark discoveries in this field. The various biological processes that have been targeted and the corresponding ON interventions found in the literature are discussed together with brief updates on some of the more recent developments. Most ON therapies act through antisense mechanisms and are directed against various RNA species, as exemplified by gapmers, steric block ONs, antagomirs, small interfering RNAs (siRNAs), micro-RNA mimics, and splice switching ONs. However, ONs binding to Toll-like receptors and those forming aptamers have completely different modes of action. Similar to other novel medicines, the path to success has been lined with numerous failures, where different therapeutic ONs did not stand the test of time. Since the first ON drug was approved for clinical use in 1998, the therapeutic landscape has changed considerably, but many challenges remain until the expectations for this new form of medicine are met. However, there is room for cautious optimism.

BRIEF HISTORY OF OLIGONUCLEOTIDE THERAPEUTICS

The early development of synthetic oligonucleotides

THE LANDMARK DISCOVERY of DNA as the hereditary material by Avery et al. in 1944,[1] followed by the insightful report on the helical structure of DNA,[2] paved the way for our current understanding and use of nucleic acids, including the development of oligonucleotide (ON) therapies. In this review we will briefly discuss some of the key discoveries in this field leading to the development of ON drugs. Although the history of ONs is intimately connected with that of basic research in molecular biology, we will focus on key areas of direct importance for nucleic acid medicines.

Two chemical modifications may be regarded as starting points for this field, namely, 2′-fluoro (2′-F) substitutions and phosphorothioate chemistry, since ONs with these modifications constitute versatile, synthetic analogs of the naturally occurring counterparts (Fig. 1). Thus, as described in one of the following sections, 2′-F substitutions are used in many synthetic ONs,[3,4] since they increase binding affinity to the complementary target and also provide some protective effects. Also, the phosphorothioate chemistry, which was developed by Fritz Eckstein,[5] remains a highly important modification for most of today's ON drugs, since it both confers protection against degradation and aids in the cellular uptake of ONs.[6,7] In the 1960s another 2′ modification, 2′-*O*-methyl (2′-*O*-Me), was also synthesized in a laboratory for the first time.[8] Although this nucleotide exists naturally in certain endogenous RNA species, it serves as an important ingredient in several synthetic, therapeutic ONs.

Oligonucleotide binding to Toll-like receptors

Another key discovery in the 1960s was the identification of poly(I:C) as an inducer of interferon in rabbits.[9] The biological basis for this observation was not understood at the time, and it took another 34 years before the Toll-like receptor 3 (TLR3) was shown to be the receptor for double-stranded RNA.[10] Related to these findings was the notion in 1984 that bacterial DNA has an antitumoral effect.[11] Eleven years later it was demonstrated

*Correspondence: Dr. Karin E. Lundin, Department of Laboratory Medicine, Clinical Research Center, Karolinska Institutet, Karolinska University Hospital Huddinge, 14186 Stockholm, Sweden. E-mail: karin.lundin@ki.se

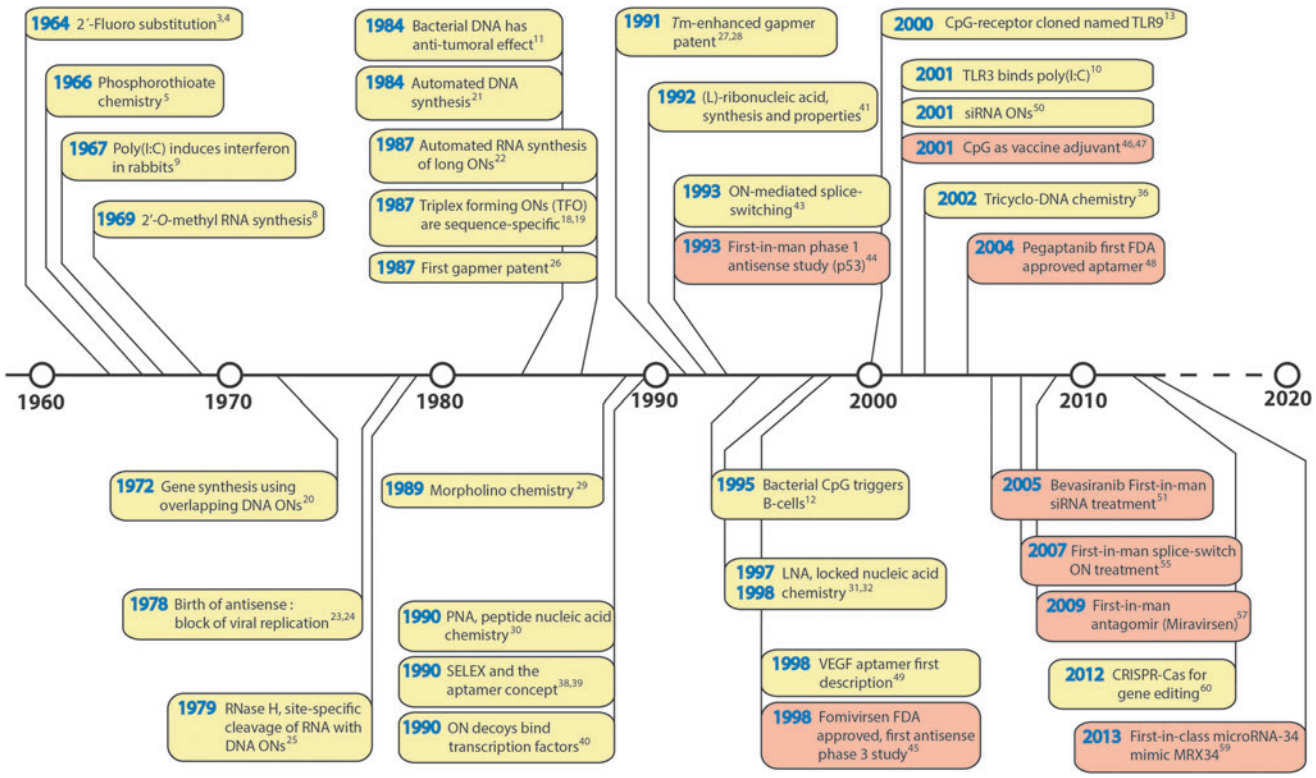

Figure 1. History of oligonucleotide therapeutics. Basic biology and chemistry with yellow background and clinical applications with coral red background. ON, oligonucleotide. The different chemical structures can be found in Supplementary Fig. S1 (Supplementary Data are available online at www.liebertpub.com/hum).

that bacterial CpG triggers the activation of B-lymphocytes,[12] and this was followed by the cloning of the corresponding receptor, named TLR9, in the year 2000.[13] While the TLR intracellular signaling pathways have been delineated,[14] it is interesting to note that short DNA ONs impair TLR3 signaling in both primates and humans, thereby constituting a potential tool for clinically interfering with this receptor-mediated activity.[15]

Hoogsteen binding, G-quadruplexes, and triplex-forming oligonucleotides

The above example with TLR activation clearly demonstrates that ONs can work by mechanisms completely different from that of Watson–Crick base pairing. However, with regard to hybridization there is yet another binding mode, namely, Hoogsteen binding.[16] Thus, a decade after James Watson and Francis Crick published their model of the DNA double helix, Karst Hoogsteen reported a crystal structure in which methylated A and T formed a base pair that had a different geometry from that described by Watson and Crick. This mode of hybridization was later found to take place both in G-quadruplexes and when triplex-binding ONs (TFOs) attach to a duplex. Intracellular G-quadruplexes have so far not been targeted by ONs in a therapeutic context, but G-quadruplex formation constitutes an integral part of at least one of the aptamers, which has entered into clinical trials.[17] In 1987, TFOs were first demonstrated to bind sequence-specifically, when the Dervan and Hélène laboratories independently reported site-specific cleavage of a DNA target.[18,19] TFOs have so far not been studied in the clinics.

Synthesis of long ONs and the birth of antisense

The early 1970s mark the beginning of more advanced methods for ON synthesis. In 1972, the Nobel laureate Gobind Khorana filled the entire 28th of December issue, corresponding to almost 300 pages, of the *Journal of Molecular Biology* with a technical feat. In the first of 13 articles, the strategy is provided for how to synthesize a DNA duplex corresponding in sequence to the major yeast alanine transfer RNA, by the use of overlapping DNA ONs.[20] Automated ON synthesis came later, and was developed in the mid-1980s.[21,22] Such procedures were developed over a period of several years and many investigators should be credited for these efforts, but owing to space limitations we can only refer to a few major reports. The 1970s is also the decade that witnessed what is generally considered as the birth of antisense therapy. Thus, in 1978 Paul Zamecnik

and Mary Stephenson reported in two back-to-back articles in the *Proceedings of the National Academy of Sciences U.S.A.* that the addition of a 13-mer oligodeoxyribonucleotide could inhibit Rous sarcoma virus in infected cell cultures.[23,24] The following year, a publication described that RNase H site-specifically cleaves the RNA strand in RNA–DNA heteroduplexes,[25] thereby demonstrating that antisense therapy can work not only by steric blocking, but also through an enzyme-mediated process.

In November 1987 the first antisense patent describing the gapmer concept was filed by Joseph Walder et al.,[26] and 4 years later a *T*m enhanced variant followed from ISIS pharmaceuticals.[27,28] Gapmers contain an internal region (gap), made from DNA. The gap is surrounded by a short stretch of synthetic nucleotides with strong hybridization properties that protect from exonuclease degradation as discussed below.

The second-generation synthetic ONs

In 1989, phosphorodiamidate morpholino oligomer (PMO) chemistry was developed,[29] followed next year by another synthetic chemistry, the peptide nucleic acid (PNA).[30] Another invention in the 1990s was the conformationally restricted locked nucleic acid (LNA) chemistry, which was simultaneously developed in Japan[31] and in Denmark.[32] These synthetic building blocks are highly versatile, with numerous applications both in biotechnology and in clinical practice.[33] One example is the Zorro-LNA type of Z-shaped ON, which simultaneously targets both strands of a DNA duplex.[34] Many additional synthetic chemistries exist, and we refer to other publications for a review.[7,35] However, tricyclo-DNA, which was developed in 2002,[36] has recently shown interesting treatment effects in an animal model for Duchenne muscular dystrophy (DMD) and is therefore worth mentioning in a therapeutic context.[37]

In 1990, two laboratories independently reported the efficient production of ON aptamers, that is, ONs that bind to a specific target molecule, often a protein, in the absence of hybridization.[38,39] The enrichment procedure was called systematic evolution of ligands by exponential enrichment (SELEX). In 1990, duplex ONs were also developed to serve as decoys by sequestering transcription factors.[40] In 1992, (L)-ribonucleic acids were synthesized and their properties studied.[41] The (L)-type ONs, also known as Spiegelmers, or mirror-image ONs, were recently reviewed.[42] The following year ON-mediated splice-switching was developed in the laboratory of Ryszard Kole.[43] For this therapeutic approach, RNase H activity is unwanted, since altered splicing, and not degradation, is the aim.

CpG therapy, aptamers, RNAi, and therapeutic splice switching

The clinical development of antisense ONs (AONs) commenced in the early 1990s with a phase I trial using AONs directed against *p53* transcripts in patients with either relapsed or refractory acute myelogenous leukemia or myelodysplastic syndrome.[44] The first AON going through a phase 3 study and to be FDA approved was Fomivirsen.[45] This was an AON directed against cytomegalovirus (CMV), and used in HIV patients with CMV retinitis, and was administered by intravitreous injections into the eye. Owing to the improved efficacy of current HIV therapies, CMV retinitis no longer constitutes a medical problem for this patient group, and the drug has been withdrawn.

In 2001, the first use of CpG ONs in the clinics was reported. This compound was coadministered, in a phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen, as an immunostimulatory phosphorothioate ON adjuvant.[46,47] Three years later pegaptanib (brand name: Macugen) became the first FDA-approved aptamer.[48] It was used as an antiangiogenic medicine for the treatment of neovascular (wet) age-related macular degeneration and delivered by intravitreous injections. This aptamer was first described in 1998,[49] and like many other aptamers it contains 2′-F substitutions, a modification developed already in the 1960s.[3,4]

In 2001, RNAi entered the ON arena through the development of siRNAs,[50] and already in 2005 bevasiranib became the first siRNA AON drug to be used in humans.[51,52] However, while the AON construct was in the form of an siRNA, its mode of action was unexpectedly mediated through a TLR3-dependent mechanism.[53] Since then, several siRNAs have entered into clinical trials and this development was recently reviewed.[54]

Splice switching, as a therapy, was first reported in humans in 2007.[55] This study, which was sponsored by Prosensa B.V, describes local, intramuscular injections into patients with DMD. It has been followed by the systemic delivery of both 2′-*O*-Me and PMO AONs, as reviewed elsewhere.[56] Micro-RNAs (miRs) constitute a target that only very recently has entered into clinical trials. The first one was initiated already in 2009,[57] with a full report appearing in 2013 in a study sponsored by the Danish company Santaris Pharma A/S, currently Roche Innovation Center Copenhagen.[58] In this trial, AON antagomirs directed against miR-122 were used as a treatment for hepatitis C infection. First-in-class micro-RNA ON treatment in the form of a miR-34 mimic entered phase I clinical trials in 2013.[59]

A recent major development in molecular biology is the CRISPR-Cas technology for genome editing, first published in 2012.[60] This enzyme–RNA complex is dependent on two short RNA sequences, which may also be engineered as a single RNA chimera, "dual-tracrRNA:crRNA," or guide RNA, serving as a very efficient tool for editing purposes, and such short RNA species can be synthesized as ONs.

From all the attempts to develop ONs into medicines, as depicted in Fig. 1, it is obvious that there are many entirely different approaches being used. The seven biological processes that have been targeted and the corresponding ON therapeutic interventions used are shown in Fig. 2. In addition to the historical overview, we will in the next sections also provide a brief update on some of the more recent developments involving ON medicines.

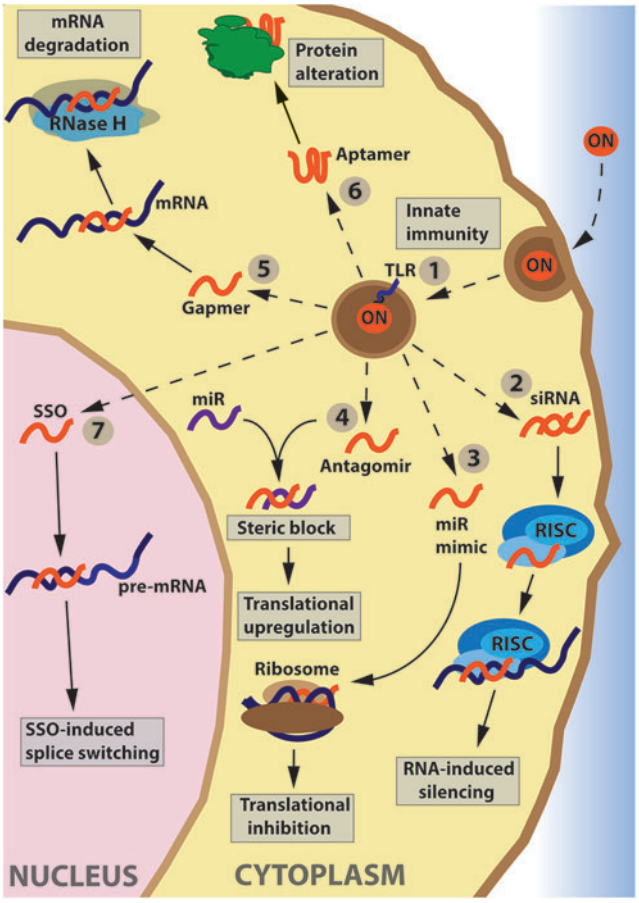

Figure 2. Schematic representation of seven mechanisms for ON medicines that have been used in the clinic. (1) Binding to Toll-like receptors (TLRs) in the endosome. (2) Small interfering RNA (siRNA). (3) Micro-RNA (miR) mimic. (4) Antagomir, sterically blocking endogenous miR. (5) Gapmer AON, inducing RNase H degradation (steric block ONs also exist). (6) Aptamer, binding alters protein surface. (7) Splice switching ON (SSO). Not depicted are anti-gene ONs, and ONs directed against nuclear regulatory RNA species, which are not yet used clinically.

AONs INDUCING RNA DEGRADATION

For many diseases a therapeutic strategy is to reduce the level of a gain-of-function RNA, which by itself is toxic, produces a dominant malfunctioned or toxic protein, or induces disease by disturbing the regulation of other genes. The two main strategies for AON-induced enzymatic degradation of specific RNAs are siRNAs and gapmer AONs. Today a number of such ON therapeutics have reached clinical trials, for example, for the treatment of cancer,[61] neurological diseases,[62] infections,[63] or other diseases that preferably can be treated by targeting the liver.[64]

Gapmer AONs utilize the intracellular enzyme RNase H, which degrades the RNA strand in an RNA–DNA hetero-duplex. To prevent rapid catalysis, such AONs are generally synthesized with a phosphorothioate backbone.[65] To increase affinity and protect the ONs from exonucleases, a number of chemically modified nucleic acid analogs have been inserted at each end of the ON to create what is called a gapmer. A gap with six to eight unmodified DNA nucleotides in the middle is mediating efficient induction of RNase H degradation. Very few base modifications are allowed within this DNA gap, in order not to disturb the catalysis.[66] In dominant diseases caused by a mutation in a single allele, RNase H degradation can be selectively targeted against the mutated mRNA through ONs binding to (1) the specific point mutation,[67] (2) structural differences between wild-type and mutant mRNA,[68–70] or (3) a single nucleotide polymorphism that is unique to the mutant RNA.[71,72]

siRNAs are short 20–24 bp dsRNA ONs with phosphorylated 5′ ends and hydroxylated 3′ ends with two overhanging nucleotides. These dsRNAs mediate degradation of the cognate mRNA target, when the correct antisense strand has been loaded into the RNA-induced silencing complex. In serum, unmodified siRNA is almost completely degraded in less than a minute. Consequently, researchers have for more than a decade incorporated chemically modified nucleotide analogs to improve stability and efficiency.[73] Internally segmented interfering RNAs (sisiRNA) harboring two short passenger strands constitute an interesting approach to increase the tolerance for insertion of different base modifications.[74] Thus, for example, LNA and 2′-*O*-Me nucleotides incorporated at optimal positions can increase nuclease resistance, enhance activity and specificity, and reduce potential immunogenicity of the delivered siRNA.[33] Even though different chemical modifications can stabilize and increase the biostability of synthetic ONs, siRNAs will always

display a relatively transient effect, as compared with intracellularly produced shRNAs. Thus, they are less likely to induce heterochromatin formation in target genes as has been reported for some shRNAs.[75]

Modified ss-siRNA with abasic substitutions have also been shown to discriminate between mutated and normal *Huntingtin* and *Ataxin* mRNA, inhibiting expression of the mutated RNA in an allele-specific manner. Inserted modifications and mismatched bases interfere with the RNA degradation and result in a miR-like effect on the expression.[76,77]

The absolute majority of registered clinical trials using antisense or siRNAs are for treatment of different forms of cancer. Beside this, the most common target organs are the liver and the eye. Martinez et al. recently wrote a very nice overview of different diseases that are targeted by, for example, siRNAs.[64]. Treating diseases through targeting of a specific gene expressed in the liver is appealing because of the easy access of this organ by systemic delivery. Nanoparticles and liposomal formulations accumulate in the liver, but specific uptake also occurs via, for example, binding to the asialoglycoprotein receptor. Already in 2004 a study by Westerlind et al. demonstrated that the trimeric form of the N-acetylgalactosamine (GalNAc) was the most optimal targeting moiety for uptake in hepatocytes via this receptor.[78] Chemistries used for solid-supported synthesis of ON conjugates for improved delivery and targeting of ON drugs have been reviewed,[79] and recently a simplified method to synthesize trimeric GalNAc ligands for siRNA delivery to the liver was reported.[80] Cellular uptake and intracellular trafficking of therapeutic ONs have also been frequently reviewed.[81] Interesting routes are the delivery of siRNA into the brain by systemic injection of targeted exosomes,[82,83] and uptake of siRNAs into the brain following intranasal administration of cell-penetrating peptide-modified nano-micelles.[84] Targeting specific organs by aptamers is also an interesting approach further discussed in the aptamer section.[85]

With the delivery to most organs mainly unsolved, there is so far no approved siRNA-based clinical treatment,[86,87] whereas two FDA-approved gapmers have reached the clinic for the treatment of CMV retinitis[88] and severe hypercholesterolaemia, respectively.[89]

ANTAGOMIRs AND MIR MIMICS

miRs are small noncoding RNAs that influence translation through the binding to imperfect, complementary sites on the target mRNA. Depending on the degree of homology, the miR can either induce degradation in an RNAi-like manner or, more often, sterically block mRNA translation (Fig. 2). These small endogenous RNAs regulate processes involved in, for example, proliferation, differentiation, and cell death. There exist tumor suppressor miRs responsible for the inhibition of oncogenes, as well as miRs that, when overexpressed, inhibit translation of tumor suppressor genes, thus designated onco-miRs. In many cancers the miR pattern is disturbed, and if no additional mutation in known oncogenes exist, a possible treatment can be to block the overexpressed miR with a complementary, synthetic ON, an antagomir. Alternatively, when miRs are missing they can be replaced by administrating synthetic miR-mimics. Antagomirs have been designed as potential therapeutics for the treatment of, for example, hepatitis C infection (anti-miR122),[90,91] breast cancer (anti-miR221),[92] brain tumors (anti-miR155),[93] neurodegenerative disorders,[94] and obesity.[95] Synthetic miR mimics, like the miR-34-mimicking ON, have already reached clinical studies for the treatment of unresectable liver cancer.[96]

SPLICE SWITCHING OLIGONUCLEOTIDES

Splicing is a very delicate process, and beside the highly conserved 5′ and 3′ splice sites in the pre-mRNA, it involves a number of splicing factors interacting with specific target sequences in both exons and introns. Inducing exon skipping, restoring a malfunctioning splicing pattern, and shifting the ratio between existing splice forms have emerged as possible means to treat a number of genetic diseases.

Since the first proof of principle with ON-mediated correction of the splicing of a mutated thalassemic, betaglobin intron,[43] synthetic antisense ONs have become interesting as possible splice-modulating compounds in a number of diseases, for example, DMD,[97] spinal muscular atrophy (SMA),[98] familial hypercholesterolemia,[99] and X-linked agammaglobulinemia (XLA),[100,101] The earliest reported clinical trials with splice-switching ONs (SSOs) have been as treatment of DMD (Fig. 1). Here the aim is to induce skipping of a mutated exon to restore the reading frame and convert the severe DMD phenotype to the milder Becker dystrophy.[102] For this disease two different chemistries have been tried, 2′-*O*-methyl-phosphorothioate RNA (2′-*O*-Me-PS) ONs (Drisapersen; Prosensa) and PMOs (Eteplirsen; Sarepta Therapeutics), as earlier reviewed.[56,103]

SSOs can act through either exon exclusion or exon inclusion mechanisms. Pseudo-exon exclusion

can be achieved by splice correcting ONs targeting the actual mutation site, a promising approach to restore splicing of, for example, the iron–sulfur cluster scaffold, pre-mRNA, or to treat hereditary myopathy with lactic acidosis.[104,105] Another strategy is to target an exon splice enhancer sequence in, for example, XLA,[100] or in homocystinuria.[106]

The efficiency of SSOs is highly dependent on the ON chemistry, as well as on the target tissue. Most studies make use of PMO or 2′-*O*-Me-PS ONs. Phosphorothioate ONs bind serum proteins, leading to reduced renal clearance and an increased circulation time.[6] The uncharged PMO backbone, on the other hand, allows conjugation to positively charged cell-penetrating peptides, which also increase cellular uptake.[107] Another interesting approach is the addition of a nuclear localization signal by linking a tri-methylated m_3G-cap, which increased the nuclear concentration and splice-correcting effect of an SSO.[108] Other modifications like insertion of intercalating ZEN residues have also been tried in order to improve splice correction.[109] Incorporation of LNA into 2′-*O*-Me-PS ONs and chemistries like the tricyclo-DNA have also been used with promising results.[33] Splice correction by pseudo-exon skipping was recently demonstrated in a mouse model for XLA, the first *in vivo* example of splice switching in hematopoietic cells.[100,101]

The approach to change the ratio of naturally occurring isoforms has been tried to develop treatments for several diseases, for example, in cancer.[110,111] This has also recently been studied as a means to reduce the PCSK9-mediated downregulation of the LDL receptor in order to reduce blood cholesterol levels.[112] This shift of a splicing pattern to a minor, naturally occurring isoform was thus also achieved by exon skipping.

Exon inclusion can be achieved by blocking of intronic splicing silencer sequences as for the treatment of SMA.[113,114] Isis Pharmaceuticals recently reported promising results for the treatment of SMA in clinical studies.[115] Bi-functional SSOs equipped with a tail containing target sites for splicing factors have also been used to enhance specific exon inclusion at weak splice sites.[116–118] Additional diseases where SSO treatments are also investigated have recently been reviewed.[56]

Worth considering is also the possibility of targeting multiple sites by the combination of several SSOs,[119] or using bispecific antisense sequences targeting different mRNAs from within the same ON.[120] An interesting design in this regard was the synthesis of disulfide-linked ONs to allow the delivery of two different SSOs in a 1:1 ratio into the same cell. The rationale is that the linked SSOs would subsequently be separated when reaching the reducing environment inside the cell.[121]

APTAMER ONs

Aptamers constitute a special group of ONs whose effect and target affinity lie in their ability to form 3D structures that enable them to recognize and bind strongly to both small and large molecules. Selected through *in vitro* or cell-based SELEX approaches, a substantial amount of DNA and RNA aptamers have been generated since the early 1990s (Fig. 1). As a result, several therapeutic aptamers are currently in clinical trials for various disorders.[122]

Aptamers typically act as ligands for proteins, often receptors, in either the intra- or extracellular environment, allowing the aptamer to affect the functionality of downstream effectors. For example, the first-in-class FDA-approved aptamer pegaptanib works by blocking vascular endothelial growth factor (VEGF), preventing it from binding to VEGF receptors on the cell surface,[49,123] which in turn inhibits intracellular signaling and blocks neovascularization. Conversely, the AS1411 aptamer is believed to bind nucleolin overexpressed in tumor cells, thereby interfering with nucleolin intracellular signaling, eventually leading to apoptosis.[17]

Apart from therapeutical applications where the aptamer is the drug, aptamers have also been used for targeted delivery of ONs. For this purpose, an aptamer capable of entering the target cell through an endosomal pathway (receptor-mediated) is connected to a therapeutic ON by (1) direct conjugation or hybridization, (2) formulation with nanoparticles containing the functionalized aptamer on the surface, or (3) by synthesis as a chimeric ON including both sequences.[122] Using these strategies, ONs with different functions, including siRNA,[124–126] SSO,[127] and antagomir,[128] have been delivered into cells.

Just like other ONs, the stability and degradation of the aptamer are major factors influencing their biological effect. ONs rich in G-base repeat stretches have been shown to form G-quadruplexes by positioning four G's in a planar conformation (combined Watson–Crick and Hoogsteen base pairing) stabilized by a monovalent cation (Na^+ or K^+) in the center. While the structure of G-quadruplexes was delineated already in 1962,[129] implications for therapeutic ONs were first noted in 1993.[130] In this report, using G-rich antisense ONs, the activity was shown not only to be dependent on the ON sequence, but also to strongly correlate with presence of the G-rich stretches. Later, it was discovered that aptamers able to form G-quadruplex

structures not only specifically interact with their protein target, but also gain dramatic increases in their serum half-life. This phenomenon was first described for the nucleolin-interacting AS1411 aptamer (formerly GRO29A).[131]

OLIGONUCLEOTIDES WITH ANTI-GENE CAPACITY

Anti-gene ONs (AGOs) act by sequence-specific binding to genomic duplex DNA. They can block the binding of transcription factors, that is, interfere with the initiation of transcription, or act by stalling RNA polymerases. AGO conjugates have also been used to induce targeted gene repair.[132] Given the notion that noncoding, antisense RNA species can have a gene regulatory function,[133] ONs can interfere with expression also by other means than direct binding to dsDNA.

TFOs bind in the major grove of dsDNA, at homopurine–homopyrimidine sequences, through either Hoogsteen or reverse-Hoogsteen interactions. Different chemical modifications to increase nuclease stability and improve binding affinity have been used, for example, in the form of PNA[134] and various LNA-based chemistries.[33] Pyrimidine containing TFOs form Hoogsteen interactions through parallel binding to the DNA target, whereas purine base TFOs instead bind in antiparallel mode via reverse Hoogsteen interactions. For such TFOs, a high G content presents a risk for aggregation through G-quartet formation. This might be overcome by inserting monomers of twisted intercalating nucleic acids, as described for ONs targeting the *KRAS* promoter.[135] So far, TFOs have not been used clinically, but it was recently reported that a TFO targeting the *MYC* promoter in combination with gemcitabine potentiated the antitumor activity in a mouse model.[136]

AGOs with capacity to invade into dsDNA and bind to one of the strands via WC base pairing have also been studied. The early reports on PNA as AGOs have been previously reviewed.[134,137] The poor dsDNA invasion (DSI) capacity of PNA-ONs at physiological salt concentrations was somewhat improved by conjugation with the intercalator 9-aminoacridine,[138] and using short LNA-containing ONs as openers enhanced invasion into supercoiled DNA.[139] For LNA-modified ONs, DSI can occur also at physiological salt concentration, and new LNA-containing AGOs were developed in the form of, for example, the Zorro-LNA. Such ONs targeting adjacent sites on both DNA-strands were described to block both RNA polymerase II and III,[34,140,141] and were also demonstrated to impair binding of the CCCTC-binding factor CTCF, involved in long-range DNA interactions.[142] Bean et al. demonstrated that LNA and ENA (2′-*O*, 4′-C-ethylene-bridged nucleic acids) oligomers were able to reduce RNA polymerase and SP1 transcription factor occupancy in the promoter of both the progesterone and androgen receptor genes,[143] and LNA-based AGOs were recently reviewed.[33]

An interesting approach is to combine the TFO and WC binding modes in a clamp-like ON. The two parts are connected with a flexible linker and target the same poly-purine stretch. This type of ON was first reported for PNA-ONs and was named bisPNA.[144] In 2013, Moreno et al. reported that an LNA-containing tail-clamp version, called bisLNA, in contrast to bisPNA, was able to strand-invade into supercoiled DNA at physiological salt concentrations.[145] Like TFOs, bisPNA and pseudo-complementary PNA-ONs together with a "donor DNA" have been used to induce homologous repair, as reported for a thalassemia-associated beta-globulin mutation.[146] Site-directed repair of the dystrophin gene has also been reported, after PNA-ssDNA ON injections into the muscle of mdx mice.[147]

However, as compared with antisense approaches for single-stranded RNA, targeting dsDNA is considerably more challenging. Thus, AGOs must reach the nuclear compartment, access their targets in a chromatin context, and overcome the hybridization between the complementary DNA strands in order to strand-invade. This means that substantial further optimization is needed before AGOs have reached the stage where they could enter into clinical trials.

CONCLUDING REMARKS

The history of therapeutic ONs demonstrates that this new class of molecular medicines has numerous potential applications in the clinic. Similar to other novel fields, there is a lag phase from the early discoveries until such therapies enter into human studies. Given the highly different modes of action, that is, antisense, as ligands for TLRs, and aptamers, it is not surprising that the lag phase may differ from one application to another, as exemplified by the rapid introduction of siRNAs into clinical trials. A common denominator for several of the ON-based therapeutics is the use of the same synthetic nucleotide chemistries, such as the phosphorothioate modification. In the main, it is the backbone that has been altered by replacing and modifying both the phosphate and the carbohydrate,

whereas the bases normally correspond to those naturally occurring, an exception being the (L)-ribonucleic acids.

In this review, we have only briefly addressed the delivery aspect and this remains an important hurdle, since ONs are rather large entities and do not enter into cells by diffusion. Different delivery approaches have recently been reviewed elsewhere.[86,148] However, while cellular uptake remains a limitation, it is also clear that several compounds already yield significant treatment effects. Collectively, this bodes well for the field, and we predict that the development is likely to continue, not only for targets within the liver, but also for correction of regulatory genes in, for example, bone marrow cells and within the CNS. This would fulfill the initial conceptual dream that ON medicines, compared to low molecular "traditional" drugs as well as to biologics, are much more straightforward to both design and develop.

ACKNOWLEDGMENTS

This work was supported by the Swedish Medical Research Council and by The Swedish Cancer Society. We are grateful to Samir El Andaloussi, Anna Berglöf, and Roger Strömberg, Karolinska Institutet; Mark A. Behlke, Integrated DNA Technologies (IDT), Inc.; Peter B. Dervan, California Institute of Technology; Scott Henry, ISIS Pharmaceuticals; Per Trolle Jørgensen, Erik B. Pedersen, and Jesper Wengel, University of Southern Denmark; and Rula Zain, Karolinska University Hospital, for valuable comments. We apologize to all those who have contributed in many ways to the development of ON therapies that we were unable to cite their work owing to space limitations.

AUTHOR DISCLOSURE

The authors have no conflicting interests to declare.

REFERENCES

1. Avery OT, Macleod CM, McCarty M. Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. J Exp Med 1944;79:137–158.
2. Watson JD, Crick FH. Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. Nature 1953;171:737–738.
3. Reist EJ, Benitez A, Goodman L. The synthesis of some 5′-thiopentofuranosylpyrimidines. J Org Chem 1964;29:554–558.
4. Codington JF, Doerr IL, Fox JJ. Nucleosides. XVIII. Synthesis of 2′-fluorothymidine, 2′-fluorodeoxyuridine, and other 2′-halogeno-2′-deoxy nucleosides. J Org Chem 1964;29:558–564.
5. Eckstein F. Nucleoside phosphorothioates. J Am Chem Soc 1966;88:4292–4294.
6. Watanabe TA, Geary RS, Levin AA. Plasma protein binding of an antisense oligonucleotide targeting human ICAM-1 (ISIS 2302). Oligonucleotides 2006;16:169–180.
7. Geary RS, Norris D, Yu R, et al. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. Adv Drug Deliv Rev 2015. [Epub ahead of print]
8. Bobst AM, Rottman F, Cerutti PA. Effect of the methylation of the 2′-hydroxyl groups in polyadenylic acid on its structure in weakly acidic and neutral solutions and on its capability to form ordered complexes with polyuridylic acid. J Mol Biol 1969;46:221–234.
9. Field AK, Tytell AA, Lampson GP, et al. Inducers of interferon and host resistance. II. Multistranded synthetic polynucleotide complexes. Proc Natl Acad Sci USA 1967;58:1004–1010.
10. Alexopoulou L, Holt AC, Medzhitov R, et al. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 2001;413:732–738.
11. Tokunaga T, Yamamoto H, Shimada S, et al. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984;72:955–962.
12. Krieg AM, Yi AK, Matson S, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–549.
13. Hemmi H, Takeuchi O, Kawai T, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–745.
14. Takeda K, Akira S. Toll-like receptors. Curr Protoc Immunol 2015;109:141211–141210.
15. Skold AE, Hasan M, Vargas L, et al. Single-stranded DNA oligonucleotides inhibit TLR3-mediated responses in human monocyte-derived dendritic cells and *in vivo* in cynomolgus macaques. Blood 2012;120:768–777.
16. Hoogsteen K. The crystal and molecular structure of a hydrogen-bonded complex between 1-methylthymine and 9-methyladenine. Acta Cryst 1963;16:907–916.
17. Bates PJ, Laber DA, Miller DM, et al. Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. Exp Mol Pathol 2009;86:151–164.
18. Moser HE, Dervan PB. Sequence-specific cleavage of double helical DNA by triple helix formation. Science 1987;238:645–650.
19. Le Doan T, Perrouault L, Praseuth D, et al. Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-[alpha]-thymidylate covalently linked to an azidoproflavine derivative. Nucleic Acids Res 1987;15:7749–7760.
20. Khorana HG, Agarwal KL, Buchi H, et al. Studies on polynucleotides. 103. Total synthesis of the structural gene for an alanine transfer ribonucleic acid from yeast. J Mol Biol 1972;72:209–217.
21. Sinha ND, Biernat J, McManus J, et al. Polymer support oligonucleotide synthesis XVIII: use of beta-cyanoethyl-N,N-dialkylamino-/N-morpholino phosphoramidite of deoxynucleosides for the synthesis of DNA fragments simplifying deprotection and isolation of the final product. Nucleic Acids Res 1984;12:4539–4557.
22. Usman N, Ogilvie KK, Jiang MY, et al. Automated chemical synthesis of long oligoribonucleotides. J Am Chem Soc 1987;109:7845–7854.
23. Zamecnik PC, Stephenson ML. Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. Proc Natl Acad Sci USA 1978;75:280–284.
24. Stephenson ML, Zamecnik PC. Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide. Proc Natl Acad Sci USA 1978;75:285–288.
25. Donis-Keller H. Site specific enzymatic cleavage of RNA. Nucleic Acids Res 1979;7:179–192.

26. Walder JAIC, Walder RY (inventor), University of Iowa Research Foundation (assignee). Nucleic acid hybridization and amplification method for detection of specific sequences in which a complementary labeled nucleic acid probe is cleaved. 1995.
27. ISIS Pharmaceuticals (assignee). Application number US 08/861,306. Filed 1991.
28. Cook PD (inventor), ISIS Pharmaceuticals, Inc. (assignee). Gapped 2′-modified oligonucleotides, Application number US 58564551999.
29. Stirchak EP, Summerton JE, Weller DD. Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages. Nucleic Acids Res 1989;17:6129–6141.
30. Nielsen PE, Egholm M, Berg RH, et al. Sequence-selective recognition of DNA by strand displacement with a thymine-substituted polyamide. Science 1991;254:1497–1500.
31. Obika S, Nanbu D, Hari Y, et al. Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3′-endo sugar puckering. Tetrahedron Lett 1997;38:8735–8738.
32. Koshkin AA, Singh SK, Nielsen P, et al. Synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. Tetrahedron 1998;54:3607–3630.
33. Lundin KE, Hojland T, Hansen BR, et al. Biological activity and biotechnological aspects of locked nucleic acids. Adv Genet 2013;82:47–107.
34. Ge R, Heinonen JE, Svahn MG, et al. Zorro locked nucleic acid induces sequence-specific gene silencing. FASEB J 2007;21:1902–1914.
35. Lennox KA, Behlke MA. Chemical modification and design of anti-miRNA oligonucleotides. Gene Ther 2011;18:1111–1120.
36. Renneberg D, Leumann CJ. Watson-Crick base-pairing properties of tricyclo-DNA. J Am Chem Soc 2002;124:5993–6002.
37. Goyenvalle A, Griffith G, Babbs A, et al. Functional correction in mouse models of muscular dystrophy using exon-skipping tricyclo-DNA oligomers. Nat Med 2015;21:270–275.
38. Tuerk C, Gold L. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 1990;249:505–510.
39. Ellington AD, Szostak JW. *In vitro* selection of RNA molecules that bind specific ligands. Nature 1990;346:818–822.
40. Bielinska A, Shivdasani RA, Zhang LQ, et al. Regulation of gene expression with double-stranded phosphorothioate oligonucleotides. Science 1990; 250:997–1000.
41. Gray AW. Modeling, synthesis, and hybridization properties of (L)-ribonucleic acid. J Am Chem Soc 1992;114:9731–9736.
42. Vater A, Klussmann S. Turning mirror-image oligonucleotides into drugs: the evolution of Spiegelmer® therapeutics. Drug Discov Today 2015;20:147–155.
43. Dominski Z, Kole R. Restoration of correct splicing in thalassemic pre-mRNA by antisense oligonucleotides. Proc Natl Acad Sci USA 1993; 90:8673–8677.
44. Bayever E, Iversen PL, Bishop MR, et al. Systemic administration of a phosphorothioate oligonucleotide with a sequence complementary to p53 for acute myelogenous leukemia and myelodysplastic syndrome: initial results of a phase I trial. Antisense Res Dev 1993;3:383–390.
45. Fomivirsen approved for CMV retinitis: first antisense drug. AIDS Treat News 1998:7.
46. Halperin SA, Van Nest G, Halperin B, et al. Immunostimulatory Oligonucleotide (ISS ODN) Co-Injection Enhances Protective Antibody Response to Hepatitis B Surface Antigen (HBsAg) and Is Well-Tolerated by Seronegative Individuals. Presented in part at the 41st Annual Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC), American Society of Microbiology, Chicago, IL, 2001.
47. Halperin SA, Van Nest G, Smith B, et al. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 2003;21:2461–2467.
48. Gragoudas ES, Adamis AP, Cunningham ET Jr., et al. Pegaptanib for neovascular age-related macular degeneration. N Engl J Med 2004;351: 2805–2816.
49. Ruckman J, Green LS, Beeson J, et al. 2′-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165). Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. J Biol Chem 1998;273:20556–20567.
50. Elbashir SM, Harborth J, Lendeckel W, et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001;411:494–498.
51. ClinicalTrials.gov. 2005. Safety and efficacy study of small interfering ribonucleic acid (RNA) molecule (Cand5) to treat wet age-related macular degeneration. https://clinicaltrials.gov/ct2/show/NCT00259753?term=bevasiranib&rank=5.
52. Singerman L. Combination therapy using the small interfering RNA bevasiranib. Retina 2009;29: S49–S50.
53. Kleinman ME, Yamada K, Takeda A, et al. Sequence- and target-independent angiogenesis suppression by siRNA via TLR3. Nature 2008;452:591–597.
54. Ozcan G, Ozpolat B, Coleman RL, et al. Preclinical and clinical development of siRNA-based therapeutics. Adv Drug Deliv Rev 2015. [Epub ahead of print]; doi: 10.1016/j.addr.2015.01.007.
55. van Deutekom JC, Janson AA, Ginjaar IB, et al. Local dystrophin restoration with antisense oligonucleotide PRO051. N Engl J Med 2007; 357:2677–2686.
56. Disterer P, Kryczka A, Liu Y, et al. Development of therapeutic splice-switching oligonucleotides. Hum Gene Ther 2014;25:587–598.
57. Hildebrandt-Eriksen ES, Bagger YZ, Knudsen TB, et al. A unique therapy for HCV inhibits microRNA-122 in humans and results in HCV RNA suppression in chronically infected chimpanzees: results from primate and first-in-human studies. Hepatology 2009;50 Suppl:228A.
58. Janssen HL, Reesink HW, Lawitz EJ, et al. Treatment of HCV infection by targeting microRNA. N Engl J Med 2013;368:1685–1694.
59. DeYoung C. Mirna Therapeutics presents interim phase 1 data on first-in-class microRNA-34 mimic, MRX34. 2014. www.mirnarx.com/pdfs/releases/2014%201119%20Mirna%20EORTC-AACR.pdf (accessed on July 28, 2015).
60. Jinek M, Chylinski K, Fonfara I, et al. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science 2012;337:816–821.
61. Farooqi AA, Rehman ZU, Muntane J. Antisense therapeutics in oncology: current status. Onco Targets Ther 2014;7:2035–2042.
62. Evers MM, Toonen LJ, van Roon-Mom WM. Antisense oligonucleotides in therapy for neurodegenerative disorders. Adv Drug Deliv Rev 2015. [Epub ahead of print]; doi:10.1016/j.addr.2015.03.008.
63. Burnett JC, Rossi JJ. RNA-based therapeutics: current progress and future prospects. Chem Biol 2012;19:60–71.
64. Martinez T, Wright N, Lopez-Fraga M, et al. Silencing human genetic diseases with oligonucleotide-based therapies. Hum Genet 2013;132:481–493.
65. Campbell JM, Bacon TA, Wickstrom E. Oligodeoxynucleoside phosphorothioate stability in subcellular extracts, culture media, sera and cerebrospinal fluid. J Biochem Biophys Methods 1990;20:259–267.
66. Fluiter K, Mook OR, Vreijling J, et al. Filling the gap in LNA antisense oligo gapmers: the effects of unlocked nucleic acid (UNA) and 4′-C-hydroxymethyl-DNA modifications on RNase H recruitment and efficacy of an LNA gapmer. Mol Biosyst 2009;5:838–843.
67. Chauhan NB, Siegel GJ. Antisense inhibition at the beta-secretase-site of beta-amyloid precursor protein reduces cerebral amyloid and acetyl cholinesterase activity in Tg2576. Neuroscience 2007;146:143–151.
68. Hu J, Matsui M, Gagnon KT, et al. Allele-specific silencing of mutant huntingtin and ataxin-3 genes by targeting expanded CAG repeats in mRNAs. Nat Biotechnol 2009;27:478–484.
69. Gagnon KT, Pendergraff HM, Deleavey GF, et al. Allele-selective inhibition of mutant huntingtin expression with antisense oligonucleotides targeting the expanded CAG repeat. Biochemistry 2010;49:10166–10178.
70. Sun X, Marque LO, Cordner Z, et al. Phosphorodiamidate morpholino oligomers suppress mutant

huntingtin expression and attenuate neurotoxicity. Hum Mol Genet 2014;23:6302–6317.

71. Miller VM, Xia H, Marrs GL, et al. Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci USA 2003;100:7195–7200.
72. Ostergaard ME, Southwell AL, Kordasiewicz H, et al. Rational design of antisense oligonucleotides targeting single nucleotide polymorphisms for potent and allele selective suppression of mutant Huntingtin in the CNS. Nucleic Acids Res 2013;41:9634–9650.
73. Bramsen JB, Kjems J. Development of therapeutic-grade small interfering RNAs by chemical engineering. Front Genet 2012;3:154.
74. Bramsen JB, Laursen MB, Damgaard CK, et al. Improved silencing properties using small internally segmented interfering RNAs. Nucleic Acids Res 2007;35:5886–5897.
75. Turunen MP, Lehtola T, Heinonen SE, et al. Efficient regulation of VEGF expression by promoter-targeted lentiviral shRNAs based on epigenetic mechanism: a novel example of epigenetherapy. Circ Res 2009;105:604–609.
76. Liu J, Pendergraff H, Narayanannair KJ, et al. RNA duplexes with abasic substitutions are potent and allele-selective inhibitors of huntingtin and ataxin-3 expression. Nucleic Acids Res 2013; 41:8788–8801.
77. Hu J, Liu J, Narayanannair KJ, et al. Allele-selective inhibition of mutant atrophin-1 expression by duplex and single-stranded RNAs. Biochemistry 2014;53:4510–4518.
78. Westerlind U, Westman J, Tornquist E, et al. Ligands of the asialoglycoprotein receptor for targeted gene delivery, part 1: Synthesis of and binding studies with biotinylated cluster glycosides containing N-acetylgalactosamine. Glycoconj J 2004;21:227–241.
79. Lonnberg H. Solid-phase synthesis of oligonucleotide conjugates useful for delivery and targeting of potential nucleic acid therapeutics. Bioconjug Chem 2009;20:1065–1094.
80. Rajeev KG, Nair JK, Jayaraman M, et al. Hepatocyte-specific delivery of siRNAs conjugated to novel non-nucleosidic trivalent N-acetylgalactosamine elicits robust gene silencing in vivo. Chembiochem 2015;16:903–908.
81. Juliano RL, Ming X, Carver K, et al. Cellular uptake and intracellular trafficking of oligonucleotides: implications for oligonucleotide pharmacology. Nucleic Acid Ther 2014;24:101–113.
82. Alvarez-Erviti L, Seow Y, Yin H, et al. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol 2011;29:341–345.
83. El Andaloussi S, Lakhal S, Mager I, et al. Exosomes for targeted siRNA delivery across biological barriers. Adv Drug Deliv Rev 2013;65:391–397.
84. Kanazawa T, Akiyama F, Kakizaki S, et al. Delivery of siRNA to the brain using a combination of nose-to-brain delivery and cell-penetrating peptide-modified nano-micelles. Biomaterials 2013;34:9220–9226.
85. Aaldering LJ, Tayeb H, Krishnan S, et al. Smart functional nucleic acid chimeras: enabling tissue specific RNA targeting therapy. RNA Biol 2015;12:412–425.
86. Colombo S, Zeng X, Ragelle H, et al. Complexity in the therapeutic delivery of RNAi medicines: an analytical challenge. Expert Opin Drug Deliv 2014;11:1481–1495.
87. Lorenzer C, Dirin M, Winkler AM, et al. Going beyond the liver: progress and challenges of targeted delivery of siRNA therapeutics. J Control Release 2015;203:1–15.
88. Piascik P. Fomiversen sodium approved to treat CMV retinitis. J Am Pharm Assoc (Wash) 1999;39: 84–85.
89. Raal FJ, Santos RD, Blom DJ, et al. Mipomersen, an apolipoprotein B synthesis inhibitor, for lowering of LDL cholesterol concentrations in patients with homozygous familial hypercholesterolaemia: a randomised, double-blind, placebo-controlled trial. Lancet 2010;375:998–1006.
90. Shan Y, Zheng J, Lambrecht RW, et al. Reciprocal effects of micro-RNA-122 on expression of heme oxygenase-1 and hepatitis C virus genes in human hepatocytes. Gastroenterology 2007;133:1166–1174.
91. Krutzfeldt J, Rajewsky N, Braich R, et al. Silencing of microRNAs *in vivo* with "antagomirs." Nature 2005;438:685–689.
92. Piva R, Spandidos DA, Gambari R. From microRNA functions to microRNA therapeutics: novel targets and novel drugs in breast cancer research and treatment (Review). Int J Oncol 2013;43:985–994.
93. Poltronieri P, D'Urso PI, Mezzolla V, et al. Potential of anti-cancer therapy based on anti-miR-155 oligonucleotides in glioma and brain tumours. Chem Biol Drug Des 2013;81:79–84.
94. Lukiw WJ. NF-kappaB-regulated, proinflammatory miRNAs in Alzheimer's disease. Alzheimers Res Ther 2012;4:47.
95. McGregor RA, Choi MS. microRNAs in the regulation of adipogenesis and obesity. Curr Mol Med 2011;11:304–316.
96. Ling H, Fabbri M, Calin GA. MicroRNAs and other non-coding RNAs as targets for anticancer drug development. Nat Rev Drug Discov 2013;12:847–865.
97. Cohn RD, Campbell KP. Molecular basis of muscular dystrophies. Muscle Nerve 2000;23:1456–1471.
98. Porensky PN, Burghes AH. Antisense oligonucleotides for the treatment of spinal muscular atrophy. Hum Gene Ther 2013;24:489–498.
99. Khoo B, Krainer AR. Splicing therapeutics in SMN2 and APOB. Curr Opin Mol Ther 2009;11:108–115.
100. Bestas B, Moreno PM, Blomberg KE, et al. Splice-correcting oligonucleotides restore BTK function in X-linked agammaglobulinemia model. J Clin Invest 2014;124:4067–4081.
101. Bestas B, Turunen JJ, Blomberg KE, et al. Splice-correction strategies for treatment of x-linked agammaglobulinemia. Curr Allergy Asthma Rep 2015;15:510.
102. Koenig M, Beggs AH, Moyer M, et al. The molecular basis for Duchenne versus Becker muscular dystrophy: correlation of severity with type of deletion. Am J Hum Genet 1989;45:498–506.
103. Koo T, Wood MJ. Clinical trials using antisense oligonucleotides in Duchenne muscular dystrophy. Hum Gene Ther 2013;24:479–488.
104. Kollberg G, Holme E. Antisense oligonucleotide therapeutics for iron-sulphur cluster deficiency myopathy. Neuromuscul Disord 2009;19:833–836.
105. Sanaker PS, Toompuu M, McClorey G, et al. Antisense oligonucleotide corrects splice abnormality in hereditary myopathy with lactic acidosis. Gene 2012;494:231–236.
106. Palhais B, Praestegaard VS, Sabaratnam R, et al. Splice-shifting oligonucleotide (SSO) mediated blocking of an exonic splicing enhancer (ESE) created by the prevalent c.903 + 469T > C MTRR mutation corrects splicing and restores enzyme activity in patient cells. Nucleic Acids Res 2015. [Epub ahead of print]; doi: 10.1093/nar/gkv275
107. El-Andaloussi S, Johansson HJ, Lundberg P, et al. Induction of splice correction by cell-penetrating peptide nucleic acids. J Gene Med 2006;8:1262–1273.
108. Moreno PM, Wenska M, Lundin KE, et al. A synthetic snRNA m3G-CAP enhances nuclear delivery of exogenous proteins and nucleic acids. Nucleic Acids Res 2009;37:1925–1935.
109. Hammond SM, McClorey G, Nordin JZ, et al. Correlating in vitro splice switching activity with systemic in vivo delivery using novel ZEN-modified oligonucleotides. Mol Ther Nucleic Acids 2014;3:e212.
110. Zammarchi F, de Stanchina E, Bournazou E, et al. Antitumorigenic potential of STAT3 alternative splicing modulation. Proc Natl Acad Sci USA 2011;108:17779–17784.
111. Nielsen TO, Sorensen S, Dagnaes-Hansen F, et al. Directing HER4 mRNA expression towards the CYT2 isoform by antisense oligonucleotide decreases growth of breast cancer cells *in vitro* and *in vivo*. Br J Cancer 2013;108:2291–2298.
112. Rocha CS, Wiklander OP, Larsson L, et al. RNA therapeutics inactivate PCSK9 by inducing a unique intracellular retention form. J Mol Cell Cardiol 2015;82:186–193.
113. Singh NK, Singh NN, Androphy EJ, et al. Splicing of a critical exon of human survival motor neuron is regulated by a unique silencer element located in the last intron. Mol Cell Biol 2006;26:1333–1346.
114. Hua Y, Vickers TA, Okunola HL, et al. Antisense masking of an hnRNP A1/A2 intronic splicing silencer corrects SMN2 splicing in transgenic mice. Am J Hum Genet 2008;82:834–848.
115. Walke DW. Isis Pharmaceuticals reports interim results from ISIS-SMN Rx multiple dose study in children with spinal muscular atrophy. 2014.

ir.isispharm.com/phoenix.zhtml?c=222170&p=irol-newsArticle&ID=1902197 (accessed on July 28, 2015).

116. Skordis LA, Dunckley MG, Yue B, et al. Bifunctional antisense oligonucleotides provide a trans-acting splicing enhancer that stimulates SMN2 gene expression in patient fibroblasts. Proc Natl Acad Sci USA 2003;100:4114–4119.

117. Cartegni L, Krainer AR. Correction of disease-associated exon skipping by synthetic exon-specific activators. Nat Struct Biol 2003;10:120–125.

118. Brosseau JP, Lucier JF, Lamarche AA, et al. Redirecting splicing with bifunctional oligonucleotides. Nucleic Acids Res 2014;42:e40.

119. Echigoya Y, Yokota T. Skipping multiple exons of dystrophin transcripts using cocktail antisense oligonucleotides. Nucleic Acid Ther 2014;24:57–68.

120. Rubenstein M, Tsui P, Guinan P. Bispecific antisense oligonucleotides having binding sites directed against an autocrine regulated growth pathway and bcl-2 for the treatment of prostate tumors. Med Oncol 2007;24:189–196.

121. Bestas B, McClorey G, Tedebark U, et al. Design and application of bispecific splice-switching oligonucleotides. Nucleic Acid Ther 2014;24:13–24.

122. Zhou J, Rossi JJ. Cell-type-specific, aptamer-functionalized agents for targeted disease therapy. Mol Ther Nucleic Acids 2014;3:e169.

123. Ng EW, Shima DT, Calias P, et al. Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. Nat Rev Drug Discov 2006;5:123–132.

124. Pastor F, Kolonias D, Giangrande PH, et al. Induction of tumour immunity by targeted inhibition of nonsense-mediated mRNA decay. Nature 2010;465:227–230.

125. Zhou J, Neff CP, Swiderski P, et al. Functional *in vivo* delivery of multiplexed anti-HIV-1 siRNAs via a chemically synthesized aptamer with a sticky bridge. Mol Ther 2013;21:192–200.

126. Ni X, Zhang Y, Ribas J, et al. Prostate-targeted radiosensitization via aptamer-shRNA chimeras in human tumor xenografts. J Clin Invest 2011;121:2383–2390.

127. Kotula JW, Pratico ED, Ming X, et al. Aptamer-mediated delivery of splice-switching oligonucleotides to the nuclei of cancer cells. Nucleic Acid Ther 2012;22:187–195.

128. Pofahl M, Wengel J, Mayer G. Multifunctional nucleic acids for tumor cell treatment. Nucleic Acid Therapeutics 2014;24:171–177.

129. Gellert M, Lipsett MN, Davies DR. Helix formation by guanylic acid. Proc Natl Acad Sci USA 1962;48:2013–2018.

130. Yaswen P, Stampfer MR, Ghosh K, et al. Effects of sequence of thioated oligonucleotides on cultured human mammary epithelial cells. Antisense Res Dev 1993;3:67–77.

131. Dapic V, Bates PJ, Trent JO, et al. Antiproliferative activity of G-quartet-forming oligonucleotides with backbone and sugar modifications. Biochemistry 2002;41:3676–3685.

132. Cannata F, Brunet E, Perrouault L, et al. Triplex-forming oligonucleotide-orthophenanthroline conjugates for efficient targeted genome modification. Proc Natl Acad Sci USA 2008;105:9576–9581.

133. Pelechano V, Steinmetz LM. Gene regulation by antisense transcription. Nat Rev Genet 2013;14:880–893.

134. Nielsen PE. Sequence-selective targeting of duplex DNA by peptide nucleic acids. Curr Opin Mol Ther 2010;12:184–191.

135. Paramasivam M, Cogoi S, Filichev VV, et al. Purine twisted-intercalating nucleic acids: a new class of anti-gene molecules resistant to potassium-induced aggregation. Nucleic Acids Res 2008;36:3494–3507.

136. Boulware SB, Christensen LA, Thames H, et al. Triplex-forming oligonucleotides targeting c-MYC potentiate the anti-tumor activity of gemcitabine in a mouse model of human cancer. Mol Carcinog 2014;53:744–752.

137. Lundin KE, Good L, Stromberg R, et al. Biological activity and biotechnological aspects of peptide nucleic acid. Adv Genet 2006;56:1–51.

138. Bentin T, Nielsen PE. Superior duplex DNA strand invasion by acridine conjugated peptide nucleic acids. J Am Chem Soc 2003;125:6378–6379.

139. Lundin KE, Hasan M, Moreno PM, et al. Increased stability and specificity through combined hybridization of peptide nucleic acid (PNA) and locked nucleic acid (LNA) to supercoiled plasmids for PNA-anchored "Bioplex" formation. Biomol Eng 2005;22:185–192.

140. Ge R, Svahn MG, Simonson OE, et al. Sequence-specific inhibition of RNA polymerase III-dependent transcription using Zorro locked nucleic acid (LNA). J Gene Med 2008;10:101–109.

141. Zaghloul EM, Madsen AS, Moreno PM, et al. Optimizing anti-gene oligonucleotide "Zorro-LNA" for improved strand invasion into duplex DNA. Nucleic Acids Res 2011;39:1142–1154.

142. Ling JQ, Hou A, Hoffman AR. Long-range DNA interactions are specifically altered by locked nucleic acid-targeting of a CTCF binding site. Biochim Biophys Acta 2011;1809:24–33.

143. Beane R, Gabillet S, Montaillier C, et al. Recognition of chromosomal DNA inside cells by locked nucleic acids. Biochemistry 2008;47:13147–13149.

144. Egholm M, Christensen L, Dueholm KL, et al. Efficient pH-independent sequence-specific DNA binding by pseudoisocytosine-containing bis-PNA. Nucleic Acids Res 1995;23:217–222.

145. Moreno PM, Geny S, Pabon YV, et al. Development of bis-locked nucleic acid (bisLNA) oligonucleotides for efficient invasion of supercoiled duplex DNA. Nucleic Acids Res 2013;41:3257–3273.

146. Lonkar P, Kim KH, Kuan JY, et al. Targeted correction of a thalassemia-associated beta-globin mutation induced by pseudo-complementary peptide nucleic acids. Nucleic Acids Res 2009;37:3635–3644.

147. Kayali R, Bury F, Ballard M, et al. Site-directed gene repair of the dystrophin gene mediated by PNA-ssODNs. Hum Mol Genet 2010;19:3266–3281.

148. Juliano RL, Carver K. Cellular uptake and intracellular trafficking of oligonucleotides. Adv Drug Deliv Rev 2015.

Received for publication May 18, 2015;
accepted after revision July 4, 2015.
Published online: July 8, 2015.