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Feature Articles

History of Research on Antisense Oligonucleotide Analogs

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Abstract. In the search for novel therapeutics, *antisense oligonucleotide* (ASO) analogs have been a major focus of research for over 40 years. They use the *antisense* strategy, namely they have a nucleic acid base sequence that is complementary to a portion of a specific mRNA that is produced in the cell, or to a viral RNA, in order to selectively inhibit gene expression. Oligonucleotides need to be chemically modified to stabilize them against hydrolysis by endogenous nucleases. Until now several phosphorothioate (PS) oligonucleotide analogs have been approved by the FDA for human use. This article seeks to provide a history of this subject to date.

Keywords: Antisense, Oligonucleotide, Analogs, Therapeutic, RNA.

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INTRODUCTION

The basic premise of the antisense approach to therapy is that since most human diseases are genetic in origin, it is necessary to use a genetic means to cure them. This includes cancer and infectious diseases, being the insertion of bacterial or viral genes into cells. Antisense depends on the intervention of information-containing drugs in the form of synthetic oligonucleotides to disrupt the flow of information transfer from DNA to RNA to protein.[1] Since DNA is highly protected, the process of transcription from DNA to mRNA is more difficult to target,[2] so the process of protein biosynthesis is the easier to target, hence antisense is a form of *translation arrest* in which the mRNA is targeted (Figure 1). Whereas usual drugs bind strongly to a protein active site and inhibit its action, the antisense approach is intended to prevent expression of the same protein by blocking its synthesis at the basic molecular level using Watson-Crick base pairing.

Antisense is a term that was introduced following the description of the double helical structure of DNA by Watson and Crick,[3] the *sense* strand being the one that is expressed into protein and the *antisense* strand being its unexpressed complement. The *first* published use of an oligonucleotide as an antisense inhibitor was by Paul Zamecnik and Mary L. Stephenson against Rous sarcoma virus in 1978.[4,5] This was before the advent of DNA synthesizers, so they had a natural phosphodiester (PO) 13-mer oligo[6] synthesized

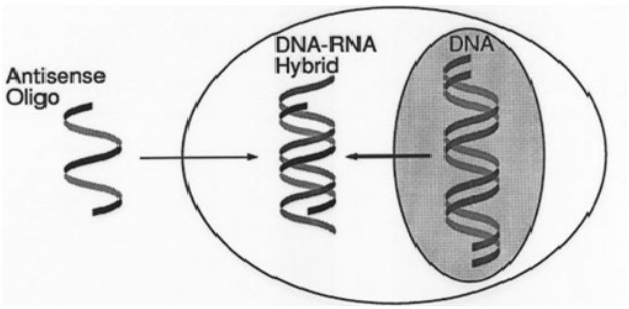
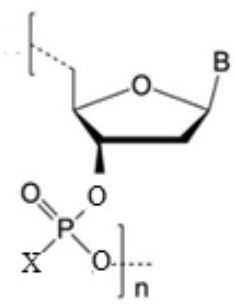


Figure 1. Schematic showing translation arrest by an antisense oligo binding to a cellular mRNA.



Name of Analogue	X
DNA	O
Phosphorothioate	S
Methylphosphonate	CH_3
Phosphoramidate	NH_2

Figure 2. DNA analogs based on minor substituents of the canonical structure.

manually for them that was complementary to a 3' and 5' reiterated terminal region, and they found it inhibited virus production. The use of a natural PO oligo no doubt resulted in hydrolysis to some extent by nucleases in the system.[7] Other work was reported on this antisense approach using specific sequence PO oligos.[8,9] Zamecnik was awarded the National Medal of Science in 1991 for his work on antisense among other things.

This story began for me in 1986,[10] when a paper appeared in *Biochemistry* reporting the use of a chemically modified antisense oligonucleotide (ASO) analog inhibiting stomatitis virus infection from Paul Ts'o and Paul Miller at Johns Hopkins University.[11] They used a methylphosphonate (PM) analog (Figure 2) to stabilize the oligo against endogenous nucleases in the cell. I was aware of this work from a symposium we had both attended in Jerusalem the previous year[12] and from their previous published work.[13] Note however that their use of 9-mers meant that they were unlikely to have bound very effectively as a duplex with the target RNA.

The potential of this approach against HIV and cancer was clear, and so because of my background and experience in DNA chemistry,[14] I was assigned to develop this approach in the NCI by my Lab and Division Chiefs.[15] I began a collaboration with my colleague Gerald Zon, Bureau of Biologics, FDA, since he was b-testing a prototype Applied Biosystems automated DNA synthesizer in his lab.[16,17]

Serendipitously, Zon and his colleagues were synthesizing phosphorothioate (PS) analogs (Figure 2) of oligos to identify signals in the ^{31}P NMR spectrum.[18] Phosphorothioate poly-ribonucleotides had been described by Eckstein and co-workers,[19] and their resistance to hydrolysis by nucleases had been noted,[20,21] but no-one before had synthesized specific sequence PS oligomers for a therapeutic application. We tested several PS oligos targeted against the *rev* gene mRNA of HIV in collaboration with Sam Broder, Head of the Oncology Program at NCI, using the PM and PS analogs with the natural phosphate (PO) oligos as controls. The *rev* gene (also known as *art/trs*) was chosen because it produces the *gag* protein that can easily be quantified. We used 28-mers since according to the calculations of Hélène and Toulmé this should reduce the probability to a single target sequence in the human genome and would be long enough to duplex.[22] This had consequences since at that length the PM oligos, which are uncharged and hydrophobic, were virtually insoluble and gave very low yields.

By contrast, the PO and PS analogs are charged and are water soluble. However, at first the yield of PS analog was low due to the inefficiency of the sulfurization reaction[23] compared to the usual oxidation reaction.[24,25] We were able to increase the yield using a different solvent.[26] The oligomers were tested in an HIV assay and the PS oligomers showed excellent inhibition against the *rev* gene, but *none* with the PM oligos or the PO oligo controls.[27] However, disturbingly we also found inhibition by the control sequence PS compounds, as well as PS homo-oligomers. This led to further research using a more appropriate assay system using chronically infected cells that gave a pure sequence-dependent antisense inhibition, with the same controls as before (Figure 3).[28]

Zon and his collaborators carried out a comparative study of inhibition of the chloramphenicol acetyltransferase (CAT) gene in a plasmid by various oligo analogs (phosphodiester, methylphosphonate, alkyltriester and phosphorothioate) and found that the PS analog was the most effective.[29] Zamecnik and his colleagues also applied PS and phosphoramidate oligo analogs to HIV.[30] We also compared the inhibition by PS oligos against the *gag, pol* and *rev* genes of HIV[31] and we also extended the use of PS oligos against other viruses.[32] We also expanded our work with PS oligos to the selective inhibition of oncogenes, c-*myc* in hematopoietic cells in culture by liposome fusion[33] and *bcl*-2 in leukemic cells.[34]

After these initial results were reported there was an immediate reaction.[35] Several companies were established[36] (**Table 1**), with billions of dollars of investment,

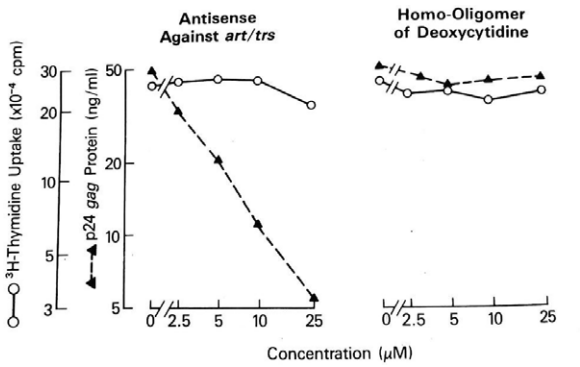


Figure 3. Sequence-specific inhibition of HIV p24 *gag* protein expression after 5 days in culture supernatant by the antisense phosphorothioate 28 mer against *rev* (5'-dTCG TCG CGT TCT CCG CTT CCT GCC A) determined using a radioimmunoassay (left). Neither the normal oligo, the sense phosphorothioate nor the homo-oligomer S-dC_{28} (right) had any inhibitory effect.[28]

to exploit the finding of stable PS oligos as therapeutic agents, which was patented by NIH.[37] Several of the original companies were subsequently taken over by larger companies and also diversified their products to other than antisense compounds.

I organized one of first conferences on this subject entitled "Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression: Therapeutic Implications," that was sponsored by NCI and NIAID and held in Rockville, MD, on June 18-21, 1989.[38] About 300 people attended, including most of those then active in the field. Many companies supported the conference and the dinner speaker was Michael Riordan of Gilead Sciences, whose subject was "Oligos: a commercial proposition." Other early conferences on this topic were held in the UK (Cambridge, organized by Dan Brown, 1987), France (Les Arc, organized by Jean-Jacques Toulmé, 1988), Russia (Akademgorodok, organized by Valentin Vlassov, 1988).[39]

If one searches the scientific literature (using CAS SciFinder) for "antisense AND oligonucleotide" there are some 43,000 hits, with a gradual increase from 1987 (15 hits) to 2001 (3,249) and then a gradual decline to 2019 (1,086) (Figure 4). Explaining this histogram is one aspect of the function of this article. Clearly it would be impossible to do justice to all these many articles, reviews (4,837) and other publications on this topic. However, it is the responsibility of the author to try to discern trends in this morass of data.

In 1989 we published the first volume on this subject, entitled "Oligodeoxynucleotides: Antisense Inhibitors of Gene Expression,"[41] which contained chapters from all the leading researchers in the field. This is an

Table 1. Companies engaged in antisense and DNA therapeutic R&D.*

Company	Location	Comment
Akcea Therapeutics	Boston MA	Owned by Ionis
Biogen	Cambridge MA	
Codex	San Diego CA	
Dynacure	Illkirch, France	
Dynavax Technologies	Emeryville CA	
Genta Inc.	San Diego CA	
Gilead Sciences	Foster City CA	Founded 1987
Helix Naonotechnologies	Cambridge MA	
Hybridon	Worcester MA	Acquired by Idera in 2004
Ionis Pharmaceuticals	Carlsbad CA	Originally ISIS Pharma
NapaJen Pharma	Burlingame CA	Japanese company
Pharmagenics	Allendale NJ	Acquired by Genzyme in 1997
Procarta Biosystems	Norwich UK	
Ranger Biotechnologies	Funen Denmark	
RogCon Biosciences	San Diego CA	
Secarna Pharmaceuticals	Martinsreid Germany	
Sterna Biologicals	Marburg Germany	
Stoke Therapeutics	Bedford MA	
Triplex Pharmaceuticals	Woodlands TX	Acquired by Argus in 1995
Zata Pharmaceuticals	Worcester MA	

* This is not a complete list.

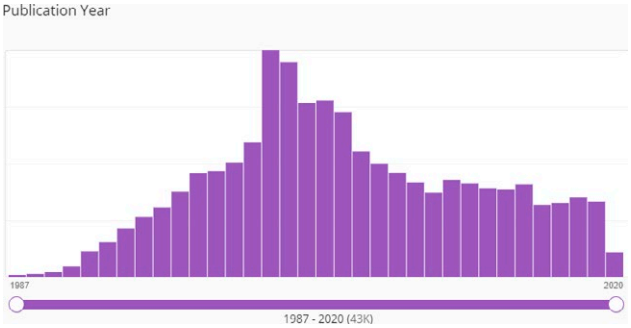


Figure 4. Number of publications per year for the literature search "antisense AND oligonucleotides." The discovery of the PS analog of oligos as a stable (slowly hydrolysable) and water-soluble (hydrophilic) alternative to the natural PO compounds, made the antisense oligo approach feasible for human therapy,[40] and led to the initial *increase* in published work on this subject.

Table 2. First Generation Oligonucleotide Analogs.

Analog	Designation	Structure	Reference
Phosphate	PO	(RO)(R'O)(HO)-P=O	Zamecnik et al., 1978[4]
Methylphosphonate	PM	(RO)(R'O)(CH3)-P=O	Agris et al., 1986[11] Tidd et al. 1988[47]
Phosphorothioate	PS	(RO)(R'O)(HO)-P=S	Matsukura et al,, 1987[27]
a-anomer	a-PO		Morvan et al., 1987[48] Rayner et al., 1990 [49]
Phosphoroselenoate	PSe	(RO)(R'O)(HO)-P=Se	Mori et al., 1989[50]
Phosphotriesters	---	(RO)(R'O)(R"O)-P=O	Miller et al., 1974[51]
Phosphorodithioate	PS2	(RO)(R'O)(HS)-P=S	Jaroszewski et al., 1996[52]
Phosphoramidate	PNH2	(RO)(R'O)-P(O)-NH2	Agrawal et al., 1988[30] Peyrotte et al. 1996[53]

excellent summary of the state of affairs in this subject at that time. A series of alternative oligo analogs were described that have come to be called "first generation analogs." (Table 2) and note that they were mostly developed prior to 1990.

Mixed alternating co-polymers, such as (PO-PS) n[42] and (PM-PS)n,[43] and end-protected analogs on natural PO oligos to protect against exonucleases[44] were also described. Since the PS analog appeared at that time to be the best analog available for truly therapeutic purposes, many applications were made with them, including clinical trials and applications to the FDA for human use.[45,46]

PROBLEMS WITH OLIGONUCLEOTIDE ANALOGS

Several factors can mainly be blamed for the subsequent gradual *decrease* in published work on antisense oligo analogs after 2001 (**Figure 4**): first, problems that arose in the application of the PS and other ASO analogs as therapeutic agents, including: 1. cellular uptake; 2. non-sequence dependent effects; 3. RNase H function; 4. cost of production; 5. primary, secondary and tertiary structure effects of oligos. The second cause of the reduction in interest in synthetic oligo analogs was the finding of endogenous antisense mechanisms within living cells, mainly so-called silencing RNA (siRNA). We will consider each of these factors as a part of the history of the development and application of ASO analogs.

1. Cellular Uptake:

Since the antisense mechanism occurs within the cell cytoplasm, it is essential that the putative inhibitory oligo must be able to enter the cell. This subject was considered early on in the history of antisense oligos.[54] There are three means for cellular uptake of oligos: a. passive diffusion through the cell membrane; b. active transport via a specific membrane mechanism; and c. specific means of delivery, for example either encapsulating the oligo within liposomes, or attaching a membrane–active agent.

a. *Passive diffusion*: This usually only occurs for small hydrophobic compounds that dissolve in the membrane lipid bilayer, and it is unlikely for any oligomer, least of all a negatively charged one to be able to enter the cell by passive diffusion.

b. *Active transport*: A fluorescent label acridine was attached to the 5' end of oligos, in this case PS oligos in order to avoid cellular degradation, and the uptake into the cell was monitored over time. After 24 hours a punctate distribution (i.e discrete points of fluorescence) was observed, and this was taken to mean that uptake not only occurred, but that it was by the formation of vesicles within the cells (non-vesicular up-take would give a homogeneous distribution) (Figure 5).[54]

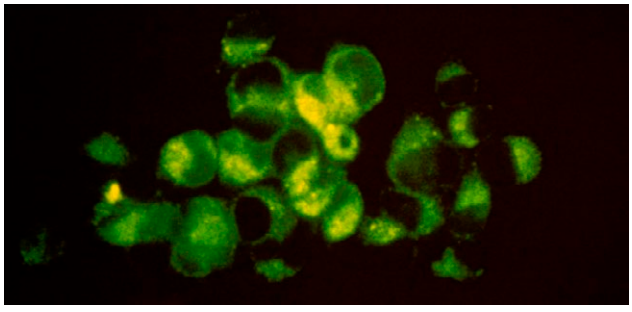


Figure 5. Photomicrograph showing punctate distribution of fluorescently labelled PS oligo within cells after 24 hrs exposure.[54]

c. *Delivery mechanisms*: The delivery of ASO into cells using liposomes was studied in detail by Yehezkel Barenholtz and co-workers, who investigated issues of sequence dependence, charge, size and composition of the liposomes, and studied comparative inhibition of the bcl-2 oncogene using different compositions of liposomes.[55-57] The authors concluded that liposomes are an effective means of improved delivery of ASOs into cells. Other delivery mechanisms include linking the ASO with membrane-active peptides to enhance cellular uptake of ASOs,[58] and binding to gold nanoparticles.[59] This general subject has been reviewed.[60,61]

2. Non-Sequence Dependent Effects

Non-sequence dependent inhibition was observed with phosphorothioate oligomers in the first study carried out with HIV.[27] In fact homo-oligomers of cytosine such as S-dC_{18} were very efficient inhibitors of HIV replication. The mechanism of this inhibition was investigated in detail, which showed that the intact poly-anion was the active agent.[62] These homo-polymers were also applied as inhibitors of other viruses.[63,64] Although these non-sequence dependent effects in viruses were at first considered a potential problem for the application of PS-oligos to cellular systems for the inhibition of oncogenes, this effect was never reported as a problem.

Another known problem is the antigenic effect of the sequence CpG in oligos, but this has been studied and can be avoided with substituents on the C base.[65-67]

3. RNase H Function

RNase H is an enzyme that specifically cleaves the RNA molecule in a DNA-RNA duplex.[68,69] Therefore, it should enhance the effectiveness of an ASO relative to purely passive binding and inhibition. However, not all DNA analogs are substrates for RNase H.[70] The PS oligo analog is a substrate,[71] but it depends on the number of PS groups present, if there are too many then the oligo can become inhibitory for RNase H action.[72] On the other hand, Summerton has argued that having an RNase H-independent antisense function of morpholino ASOs is an advantage.[73]

4. Cost of production

It was understood from the start that ASOs are large molecules compared to usual drugs, and to produce them would cost much more. At first the cost per nucleotide for automated synthesis was prohibitive, but over time, savings in chemicals and methodology greatly reduced the cost,[74] so that now it is certainly affordable.

5. Primary, secondary and tertiary effects of oligos

Oligonucleotides are not always simply in a denatured conformation, but can form secondary and tertiary structures, such as hairpin loops and tetra-G complexes. How these can affect the inhibitory function of the intended ASO is not always easy to determine, but generally hairpin-loops have been proposed to be an aid to the protection of vulnerable oligos (mainly PO) against nucleases.[75,76]

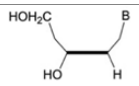
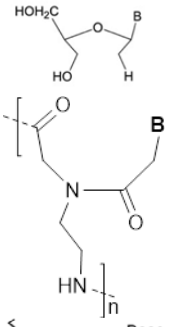
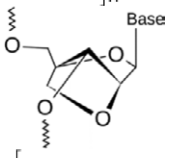
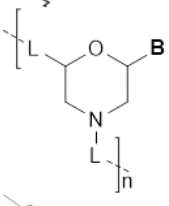
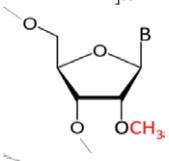
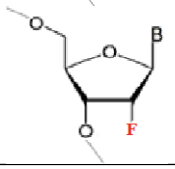
6. siRNA and its implications for ASO applications

In 2006, the Nobel Prize in Physiology or Medicine was awarded jointly to Andrew Fire and Craig Mello for their discovery of RNA interference – gene silencing by RNA.[77] It was realized that the large number of small RNA molecules, micro-RNAs, in the cell perform in effect an endogenous antisense function.[78] This finding resulted in a competitive approach to the use of antisense oligonucleotides, namely the use of the so-called silencing or small interfering RNA, known as siRNA.[79] It was soon realized that siRNA could be exploited either biologically or chemically,[80] in the form of nuclease-protected ribo-oligonucleotide analogs, much like ASOs. Currently there are drugs based on RNAi being developed.[81] It was also reported that there are synergistic effects between ASO analogs and siRNA inhibitors.[82] There are problems with control of the siRNA approach,[83] and the ASO methodology seems more "druggable." However, further consideration of this topic is beyond the scope of this article.

SECOND GENERATION OLIGONUCLEOTIDE ANALOGS

Because of the problems experienced with the PS analog, that was the first effective ASO developed, and because of the general desire to find more effective drug candidates, "second- generation analogs" were also subsequently developed, mostly after 1990 (Table 3). Here we will describe the most important of these and assess their potential as therapeutic agents relative to the first-generation ones, particularly the PS analog.

Table 3. Second Generation Oligonucleotide Analogs.

Name	Designation	Structure	References
Acyclic derivatives	___		Vandendriessche et al., 1993[84]
Peptide NA	PNA		Egholm et al., 1992[85]
Locked NA	LNA		Wengel et al., 1999[86]
Morpholino NA	___		Summerton & Weller, 1997[87]
2'O-Me RNA	___		Wagner et al., 1991[88]
2'-Fluoro RNA	___		Eckstein et al., 1991[89]

1. Acyclic nucleic acids

There have been many attempts to prepare oligomers containing ring-opened furanoside, as shown in Table 3 (opened between C1' and C4' or C2' and C3').[90] Piet Herdewijn and co-workers have examined a series of these compounds[91] and have concluded that they are too flexible to allow the formation of stable duplexes with a target DNA or RNA.[84] GNA is the oligomer in which glycol has been used to replace the ribo-sugar,[92] however this was found not to duplex with RNA, although it did form a strong homo-duplex[93] (Figure 6).

2. Covalently linked oligos

By attaching a specific chemical group to (usually) the 5'-terminus of an oligonucleotide intended for antisense inhibition, researchers have hoped to either increase its efficacy, improve its ability to enter cells, or actually carry out a catalytic reaction. The first known use of a "complementary addressed" oligo to chemically modify a tRNA was from the lab of Dr. D.G. Knorre and V.V. Vlassov.[94]

An acridine ring attached to either end of an oligo can be a reporter group of the environment, both in the isolated oligo (that might form secondary structure) or in the formation of a duplex, where is might interca-

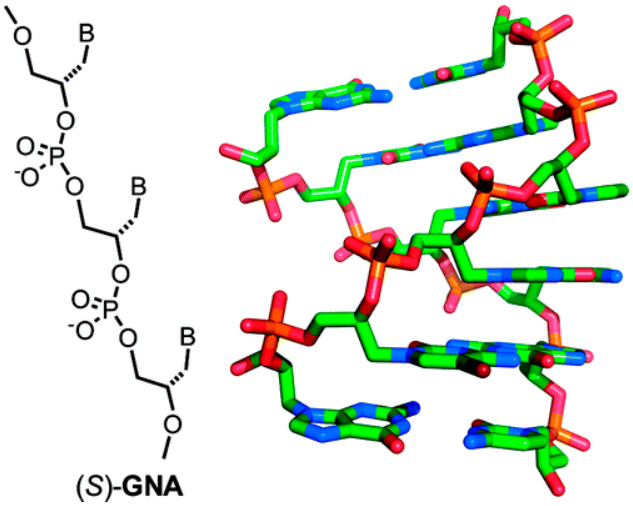


Figure 6. Homo-duplex formed by glycol nucleic acid (GNA).[93]

late between the base pairs.[95] Fluorescent acridine covalently linked to an oligo was also used to follow cellular uptake.[96] As well as several passive aromatic groups that can be attached, it is possible to attach reactive groups meant to cleave the RNA in a duplex, groups such as EDTA-Fe(II)[97-99] or porphyrin with iron.[100] Knorre et al. provide an extensive list of such reactive groups.[101] We attached imidazole to the terminus of an oligonucleotide[102] in order to try to reproduce the catalytic effect of RNase, since the catalytic effect depends on imidazoles in the active site.[103] Since then much progress has been made in synthesizing oligos containing one,[104-106] two[107] or multiple imidazole groups[108] to mimic the catalytic activity of ribonuclease using tRNA as a target. This approach has great potential for site-specific RNA cleavage.

3. Peptide nucleic acids

Oligopeptides with nucleic acid bases in place of the usual amino acid side chains have been developed for antisense applications[85] (Table 3). Their advantage is that they are of course resistant to nucleases and are water soluble, and they can easily be attached to cell-penetrating peptides in order to improve cell uptake.[109,110] A variety of biological systems, including bacteria have been targeted successfully with antisense PNAs.[111]

4. Locked nucleic acids

Locked Nucleic Acids (LNA) were developed in order to provide an analog completely resistant to nucleases, and by virtue of the locked structure of the sugar moiety (Table 3), reduce the flexibility of the ribo-furanoside ring, thus improving duplex stability.[86] Kurreck et al compared the LNAs with the PS oligos and found them to be superior, although this does not take into account the cost and difficulty of production.[112] Another report declared the LNAs were superior to other ASOs.[113]

5. Morpholino nucleic acids

Summerton and his co-workers developed the morpholino analog of oligos as a mimic of the natural structure (Table 3), and claim that they are superior to other analogs.[87] In studies of antisense activity and RNase H function they claim that the morpholino analog exceeds the function of the PS analog.[73,114] In a comparison of the PNA, LNA, and morpholino, it was found that each had advantages.[115] The morpholino analogs have been applied therapeutically in many different biological systems.[116]

6. Modified RNAs – 2'O substituted analogs

Researchers realized early on that a potential source of ASOs was the ribo-oligomer with the 2'-hydroxy position substituted with a methyl group or another alkyl group, and this has become a major area of research and development. Sproat and co-workers described the synthesis of 2'O-R analogs, where R=Me, allyl or dimethylallyl, for antisense applications, primarily because these compounds were relatively easy to synthesize and are resistant to nucleases.[117] Ohtsuka and co-workers used 2'-O-Me analogs to inhibit the expression of portions of the human β-globin gene.[118]

Lamond and Sprout expanded on the use of the 2'O-alky-lRNA analogs for applications in RNA biochemistry.[119]

Cook and co-workers described the use of combined 2'O-alkyl ribo- and deoxy- co-polymers and showed that they increased RNase H activity.[120] And others combined the PS oligos with 2'O-alkyl co-polymers as improved chimeric antisense analogs.[121,122] In a comparative study for muscular dystrophy drugs, the 2'OMe-PS combination was found to be more effective in inhibition of exon skipping than other ASOs including LNA and PNA.[123] This approach of "chimeric" combinations of analogs for improved RNase H degradation of the mRNA complex was also described by Giles and Tidd.[124] Eckstein and co-workers described the presence of a 2'-fluoro adenosine in a ribozyme[89] and Cook and co-workers synthesized uniformly modified 2'-deoxy-2'-fluoro PS oligos as nucle-

ase-resistant antisense compounds with high affinity and specificity for RNA targets.[125]

This approach was expanded upon by Stan Crooke and those at Ionis (formerly ISIS), who favor 2'O-methoxyethyl substituents (MOE) and 2'4'-constrained ethyl (cET) like the LNA and GalNAC conjugates of ASOs.[126] Their collaborators at Ciba-Geigy formulated the concept of gapmers as second generation OAS analogs, with the "wings" consisting of 2'O-alkyl PS analogs and the center "gap" consisting of PS oligo only, in order to take advantage of the increased RNase H activity.[127] Of course there are a myriad variations on these themes, but their general conclusion is that the future is with RNA oligo analogs. It should be pointed out, that many of the applications of Crooke and co-workers are targeted not at mRNA *per se*, but at pre-mRNA before splicing, and at introns or exon-intron junctions.[128] This provides unique target sequences that prove to be extremely efficient for correcting abnormal gene expression.

OTHER RELATED APPROACHES

1. Triple Helix

The formation of a triple stranded helix with a strand of an oligonucleotide binding into the major groove of the DNA duplex by Hoogsteen base pairing was first shown in 1968 [129] (Figure 7).

Dervan and coworkers showed in 1987 that an oligonucleotide with an EDTA-(Fe(II) attached could bind in the major groove of DNA and cause a double strand break.[130,131] Other workers also added to this approach as a so-called *anti-gene strategy*.[132] Hogan and co-workers sought to make triple helix formation an effective gene targeting technique.[133] Although triple helix formation has continued as an active area of research its therapeutic promise has not been realized.

2. Aptamers

Aptamers are oligonucleotides (or peptides) that bind to a specific target molecule and are selected by repetition from a large random sequence pool that was first described in 1990.[134-136] Many applications of this technique were made to evolve oligonucleotides that bind to various biological molecules.[137-140] Of more recent interesting applications are improvements in the methodology and approaches to therapeutic applications.[141-143]

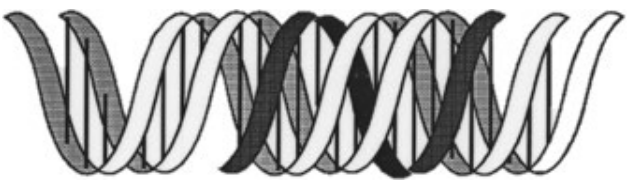


Figure 7. Triple helix showing an oligo (dark) bound in the major groove of DNA.

3. Ribozymes

Ribozymes are catalytic RNA molecules that were first described in the early 1980's as the active enzymatic component of RNase P.[144-146] There are various types of ribozymes that have been characterized from their general shape as hammerhead, pistol, twister and hairpin ribozymes.[147] Therapeutic applications of ribozymes have been described.[148-150]

4. Effects of mRNA structure

In considering the antisense mechanism, one must always bear in mind not only the antisense oligonucleotide analog, but also the mRNA being targeted. It is not enough to know the sequence of the mRNA/DNA, but also its conformation to ensure that the oligo will be able to access a target sense sequence. Studies have been made on the conformation of mRNAs and the access to target sequences.[151,152] Vlassov and co-workers showed that a binding oligonucleotide can modify and invade the structure of a tRNA target, thus making their use potentially wider.[153] It has been proposed that a statistical analysis can improve the ability to choose target sequences in mRNA.[154]

5. Molecular Dynamics Simulations

Previously we used molecular dynamics (MD) and energy minimization protocols to assess the duplexation of a PS oligo with target complementary RNA *in silico*.[155] MD calculations have also been applied to the PNA analog.[156] Recently our earlier methodology has been refined and updated [157] to assess the ability of previously unknown oligo analogs to dimerize with a target RNA. It is not generally realized that there are 10 atoms between one base and the next in DNA (Figure 8), all of which are not required for dimerization to occur in principle.

Of more radically modified analogs that can be conceived with methylene groups in place of the sugar moiety, only one has previously been characterized, namely

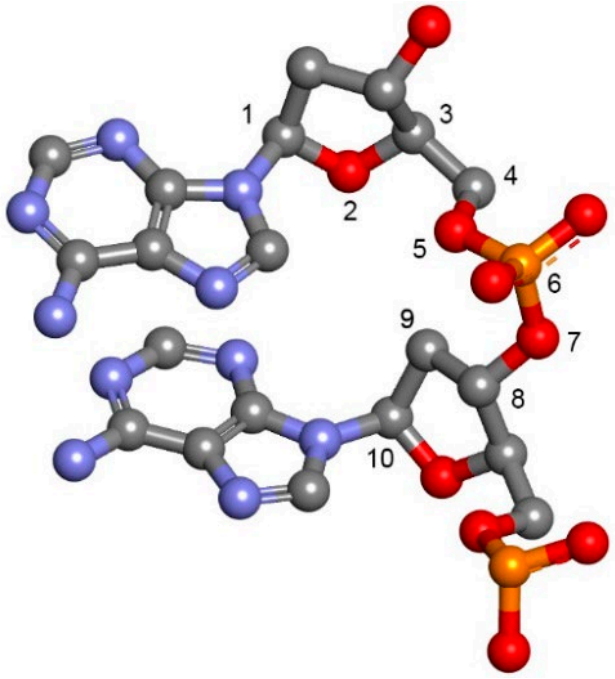


Figure 8. Showing the number of atoms/bonds between adjacent bases in DNA.

GNA.[93] Recently we have used this MD methodology to assess the capability of other analogs to duplex with complementary DNA and RNA, and we have found that only one of the eight possible structures does in fact form a viable duplex, namely that with the formulation [–CH(B)-O-P(O)(OH)-O-CH(B)-]$_n$ with RNA (Figure 9).[158] This use of computer aided molecular design (CAMD) to guide synthetic strategies is a viable predictive tool in future comparative ASO analog development and rational drug design.

CLINICAL ASPECTS

Agrawal and coworkers carried out a series of studies on *in vivo* distribution of various ASOs in animal models.[44,159-161] In 1998, the first antisense oligonucleotide, Fomivirsen, (Vitravene), a 21-mer PS oligo analog produced by Isis Pharma (now Ionis), was approved by the FDA for human ocular use against cytomegalovirus retinitis in immunocompromised AIDS patients. A detailed review of the recent FDA files[162] shows that there have been 6 ASOs approved for human use, three of them PS analogs, one a chimeric 2'OMe-PS combination and the two others were of other compositions. Among these drugs approved by the FDA are Nusinersen and Eteplirsen that are exon-skipping antisense oligonucleotides for the treatment of Spinal Muscular Atrophy and Duchenne Muscular Dystrophy.[163,164]

MicroRNA (abbreviated miRNA) are small noncoding RNA molecules (containing about 22 nucleotides) found in animals, plants, and some viruses, that

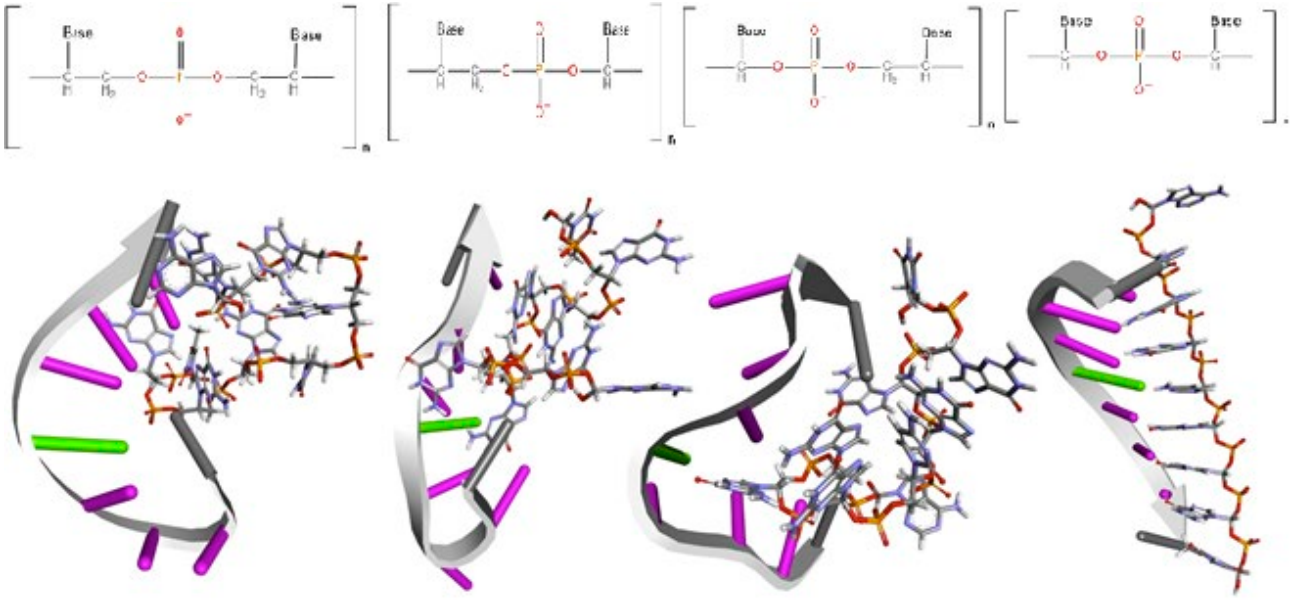


Figure 9. Representative structures from Molecular Dynamics analysis of the most populated cluster for each analog represented above, with complementary RNA. Analysis performed using the entire sampled data. Only one of the four structures with methylene groups in place of the ribo-furanoside forms a stable duplex (on the right).[158]

function in RNA silencing and post-transcriptional regulation of gene expression. miRNAs function via base-pairing with complementary sequences within mRNA molecules. miRNAs are similar to siRNAs but have different origins and specific functions. miRNAs are associated with many human diseases. miRNAs have been inhibited using locked, morpholino or 2'OMe oligos.[165,166] This is only the beginning of such inhibitions of miRNAs related to human disease.

In 1998, there were 8 PS-ASOs undergoing clinical trials.[46] A search of the NIH web-site ClinicalTrials.gov reveals the following interesting results: (a) there are 52 active or completed studies of siRNA; (b) there are 77 active or completed studies of "antisense oligonucleotides" (type not specified; also listed as AS ODNs), the majority in the US, Canada and Europe. It is to be hoped that many more will follow. It is considered possible that mixtures of antisense oligos could be used to target genetically heterogenous diseases such as cancer.

CONCLUSION

The general conclusion of this article is that the history of this subject really began dramatically with the first publication using the phosphorothioate (PS) analog of oligonucleotides against HIV in 1987.[27] For the first time this made their development as therapeutic agents feasible. This area of research then expanded rapidly, reached a peak around 2001, during which time many other analogs were developed and applied. Research then declined for two main reasons, first problems that were encountered in the application of the ASO's, such as cell uptake and cost, and secondly the development of endogenous RNAi methods that were considered to be superior. However, like other forms of endogenous biological therapy, such as gene therapy, the promise of siRNA and other techniques have also lost their initial promise. Therapeutic applications of ASO analogs is still a subject with a compelling future.

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15. My Lab Chief Charles "Snuffy" Myers and my Division Chief Samuel Broder
16. I had met Gerry Zon thru my post-doc Bill Egan, and when I was on sabbatical at the Weizmann Inst., Rehovot, Israel, in 1976-7, I agreed for him to use my laboratory at NIH for their joint research. Later in 1986 we shared a post-doc Kazuo Shinozuka.
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36. I was invited to join several start-up companies, but I became initial scientific adviser to Gilead Sciences and Pharmagenics.
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