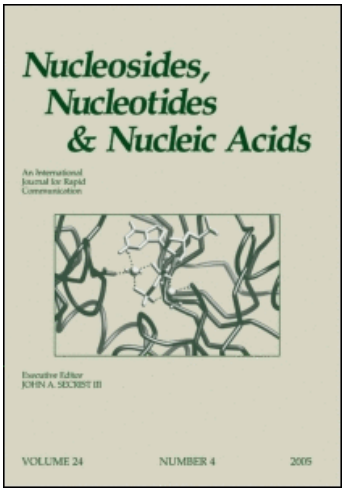

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

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To cite this Article Cook, P. Dan(1999) 'Making Drugs Out of Oligonucleotides: A Brief Review and Perspective', Nucleosides, Nucleotides and Nucleic Acids, 18: 6, 1141 — 1162
To link to this Article: DOI: 10.1080/07328319908044652
URL: http://dx.doi.org/10.1080/07328319908044652

NUCLEOSIDES & NUCLEOTIDES, 18(6&7), 1141–1162 (1999)

MAKING DRUGS OUT OF OLIGONUCLEOTIDES: A BRIEF REVIEW AND PERSPECTIVE

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ABSTRACT: I provide a brief review and perspective thoughts concerning the antisense oligonucleotide, drug discovery paradigm.

INTRODUCTION

The notion that oligonucleotides could be made into drugs and along with the discovery and development of two essential, enabling technologies, automated DNA sequencing[1] and automated oligonucleotide synthesis and purification, set the stage for oligonucleotide drug discovery (reviewed by Zon).[2] Having these enabling technologies, intensive efforts to modify short strands of nucleic acids to enhance their properties as drug candidates began in earnest about 10 years ago. Thus the field of modified oligonucleotides for drug applications has a very short history. Subsequently, oligonucleotide medicinal chemistry and pharmacology, which form the basis of structure-activity-relationship studies, have been highly developed in the process of converting oligonucleotides into drugs. Before 1989, little value was given to synthetically changing the structure of short strands of DNA or RNA except for some early modifications, primarily directed to enhance structural studies and diagnostic applications. Now, great interest in making drugs out of oligonucleotides has spurred intense structure-activity/property-relationship studies (S-A/P-R) to optimize drug properties.[3, 4]

Given that oligonucleotides are gene-based materials (or informational materials, as described by **Cohen, 1991**)[5] and considering the emergence of genomic target

selection, which is also gene-based, it is surprising that it has taken so long for the drug discovery community to recognize the potential value of modified oligonucleotides as drugs. However, when traditional medicinal chemists first consider modifying oligonucleotides, they are confronted with a number of daunting chemical, biophysical and biochemical properties of oligonucleotides, which surely has slowed progress in this area. In addition to the application of novel ligands to drug discovery, the nucleic acids targets which serve as receptors, particularly RNA molecules, are novel. In spite of the early difficulties in exploring the use of oligonucleotides as drugs, much progress has been made in recent years. The first antisense oligonucleotide drug, **Vitravene™**, recently won approval from the FDA for treatment of cytomegalovirus retinitis in AIDS patients. Several additional oligonucleotides for anti-cancer and anti-inflammatory indications are in late phase II and III clinical studies.[4]

INFORMATIONAL DRUG DISCOVERY APPROACHES

Several discovery approaches, which employ the encoded-information of oligonucleotides as the drug agent, are being pursued. In the process of transcription, complementary RNA (message RNA) is derived from the antisense DNA strand by Watson-Crick base-pair recognition[6] and binding. The antisense approach targets the initial sense complementary RNA strand (primary transcript), as well as many down-stream sites that are available, as RNA is metabolized in the process of protein production. Oligonucleotides may also inhibit gene expression at the DNA level by several antigene approaches. In this case, the most often examined approach is triple strand formation by oligonucleotides binding in the major groove of dsDNA via several Hoogsteen type base-pairing rules.[7] A less often examined approach is the use of oligonucleotides to bind via Watson-Crick base pairing rules to a single-strand of DNA available from the formation of transcription bubbles or locally open-chain sites of dsDNA.

Utilizing nucleic acids as decoys to compete with natural *cis* acting sites on dsDNA for essential regulatory proteins is referred to as the sense approach. In this case, synthetic oligonucleotides (typically dsDNA) are designed to bind sequence-specifically to DNA-binding proteins, which will prevent gene expression.[8]

In another protein binding approach, aptamers, derived from nucleic acid selection processes, can specifically target regulatory proteins. Common steps to all selection methods for nucleic acid binding species are: generation of large pools of sequence diversity from chemically synthesized DNA pools, transformation of the pools by enzymatic manipulations such as PCR or *in vitro* transcription, selection of functional shapes and amplification. As each RNA sequence folds into a distinct three-dimensional shape and because of the great numbers of structures (millions) generated, highly selective binding to a protein target may occur, and provide useful biological activities (**reviewed by Bacher and Ellington**).[9]

Antisense Approaches

A strict definition of the antisense approach describes the inhibition of gene expression by targeting a predetermined sequence in a RNA. Several types of antisense approaches, which may be differentiated by their modes of actions, are of interest. Antisense oligonucleotides primarily operate by sequence specifically binding to the targeted RNA and the resulting modified oligomer-RNA heteroduplex recruits a ubiquitous endonuclease, ribonuclease H (RNase H), which binds and cleaves the targeted RNA strand. Other potential antisense modes of action are by direct, sequence specific binding (or occupancy only) of the oligonucleotides to the targeted RNA, such that the function of essential RNA binding proteins is prevented or that secondary structure required for gene expression of the RNA is disrupted. A variety of distinct sites in the intermediary metabolism of RNA can potentially be targeted by antisense oligonucleotides with RNase H-dependent or -independent modes of actions.

Ribonuclease L (RNase L), like RNase H, is another cellular nuclease that cleaves RNA. It's endonucleolytic cleavage is triggered by binding to 2′,5′-linked, polyadenylic sequences. Torrence and coworkers have conjugated the RNase L activating moiety to oligonucleotides to provide sequence-specific cleavage of targeted RNA.[10]

Ribozymes are RNA molecules with catalytic RNA cleaving activities[11] and thus represent another type of antisense oligonucleotide. These molecules are typically much larger RNA molecules which contain, both a catalytic region that can cleave a bound RNA segment, and an adjacent sequence, or two sequence arms that allow Watson-Crick base-

pair binding to the target RNA. A variety of ribozymes have been described that differ primarily in their three-dimensional structures and length (**reviewed in ref 12).** The sequence-specific cleavage of a predetermined RNA target is thought to proceed without assistance from proteins.

Of the drug discovery approaches based on oligonucleotides, the antisense efforts, which utilize RNase H as a mode of action, are the most advanced. Sixteen first generation phosphorothioate oligonucleotides have entered human clinical trials and one antisense oligonucleotide has achieved FDA approval.[4] Much less success has been accomplished targeting DNA with triple strand-forming oligonucleotides, antisense ribozymes and RNase L-modified oligonucleotides. From a chemical point of view, these and other less successful approaches suffer from not being amenable to the readily available, first generation backbone-modified oligonucleotides such as methyl phosphonates, amidates, phosphorothioates, and alpha oligonucleotides. Success in these drug discovery approaches, assuming that the biological rationale is valid, will require much more intensive chemical efforts.

SCOPE OF OLIGONUCLEOTIDE MODIFICATIONS AND DRUG PROPERTIES THAT MAY BE ALTERED

A diverse range of modifications, at all possible modification sites of an oligonucleotide have been reported (**reviewed in reference 3,4**). A dimer of an oligonucleotide depicting subunits that may be modified to enhance oligonucleotide drug properties is depicted in **Figure 1**. These subunits are composed of heterocycles, carbohydrates, phosphodiesters, and sugar-phosphates (a 4-atom linkage or backbone). Modifications can be performed on these subunits, as well as modifications that relate to how these units are connected (connection sites). Complete removal of the sugar-phosphate backbone with appropriate replacements and attaching, or conjugating, drug enhancing moieties at various positions in the subunits, are additional important modification areas. Finally, prodrug modifications may be employed to enhance drug properties. Most of the positions available in a G-C or A-T dimer (approximately 26 positions for each dimer), that do not directly interfere with Watson-Crick base pair-hydrogen bonding, have been modified.

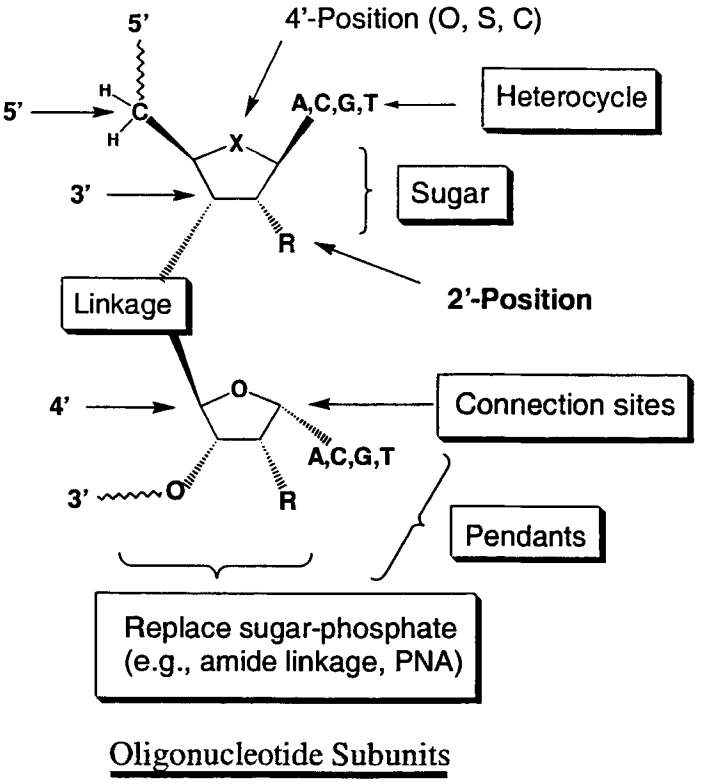

Figure 1

The nucleobases or heterocycles of nucleic acids provide the recognition points for the Watson-Crick base pairing-rules and any oligonucleotide modification must maintain these specific hydrogen-bonding interactions. Thus, the scope of heterocyclic modifications is quite limited; this is verified by the fact that only four or five types of modified heterocycles have demonstrated useful oligonucleotide binding properties. These heterocyclic modifications can be grouped into three structural classes; enhanced base stacking, additional hydrogen bonding, and the combination of these. Modifications that enhance base stacking by expanding the π-electron cloud are represented by conjugated, lipophilic modifications in the 5-position of pyrimidines[12-16] and the 7-position of 7-deaza-purines.[17-20] 5-Substituted pyrimidine modifications include propynes, hexynes, azoles and simply a methyl group **(see Figure 2a)** and 7-substituents in the 7-deaza position include iodo, propynyl, and cyano groups **(see Figure 2b)** The Gilead

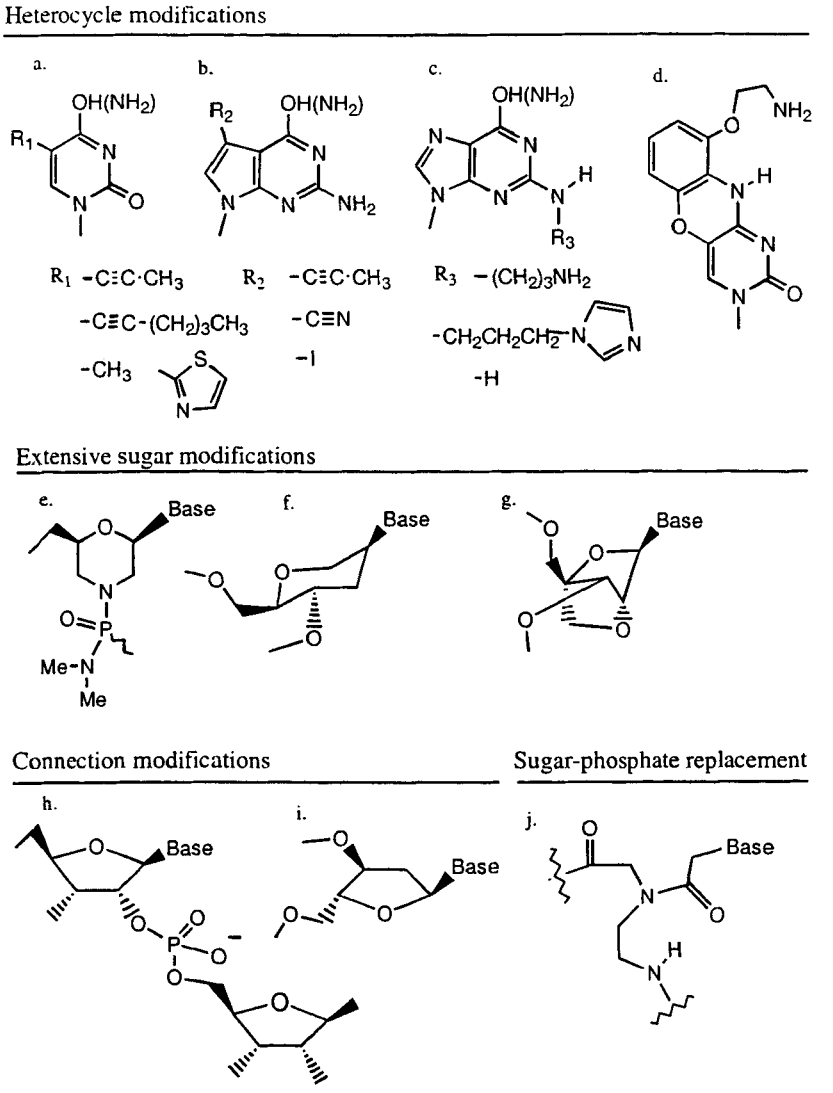

Figure 2. Examples of oligonucleotide modifications. (a) 5-substituted pyrimidines; (b) 7-substitute-7-deaza deazapurines; (c) N2-modified purines; (d) tricyclic cytosine clamps; (e) morpholino-bis-amidates; (f) hexopyranosyl-DNA; (g) locked nucleic acids, LNA; (h) alpha-DNA; (i) 2',5'-linked-DNA; (j) peptide nucleic acids, PNA

research group, focusing on heterocyclic modifications, have continued to build out of the 5-position of cytosine by going from the propynes to five-membered heterocycles to the most recently reported, tricyclic fused systems (**Figure 2d**) emanating from the 4,5-positions of (cytosine clamps).[21-24] A second type of heterocyle modification is represented by the 2-amino-adenine (**Figure 2c**) where the additional amino group provides another hydrogen bond in the A-T base pair, analogous to the three hydrogen bonds in a G-C base pair. Heterocycle modifications providing a combination of effects are represented by 2-amino-7-deaza-7-modified A[20] (**Figure 2b**) and the tricyclic cytosine analog having hydrogen bonding capabilities in the major groove of heteroduplexes[24] (**Figure 2d**). Furthermore, N2-modified 2-amino adenine (**Figure 2c**) modified oligonucleotides have exhibited interesting binding properties.[25, 26] All of these modification are positioned to lie in the major or minor groove of the heteroduplex, do not affect sugar conformation of the heteroduplex, provide little nuclease resistances, but will generally support an RNase H cleavage mechanism.

Modifications in the ribofuranosyl moiety have provided the most value in the quest to enhance oligonucleotide drug properties (**Figure 3**). In particular, certain 2′-*O*-modifications have greatly increased binding affinity, nuclease resistance, altered pharmacokinetics, and are potentially less toxic.[3] Pre-organization of the sugar into a 3′-*endo* pucker conformation is responsible for the increased binding affinity. Unfortunately, no sugar modification has been reported that is useful in supporting RNase H cleavage. Thus, these modifications currently are employed in a gap motif (see below). More recently, the 2′-*O*-(dimethylaminooxyethyl) (**Figure 3cc**) has shown a combination of binding affinity and nuclease resistance, superior at this stage, to all other 2′-modifications.[27]

More extensive sugar modifications, other than the 2′-position, are hexopyranosyl-like DNA (**Figure 2f**)[28] and the more recently reported, locked nucleic acids (LNA), which bridges the 4′C to the 2′-*O* with a methylene group, of the ribofuranosyl sugar (**Figure 2g**).[29-32]

Phosphorus atom modifications involve changes to the non-bridging oxygen atoms, such as P=S (phosphorothioate), Me-P (methylphosphonate), N-P

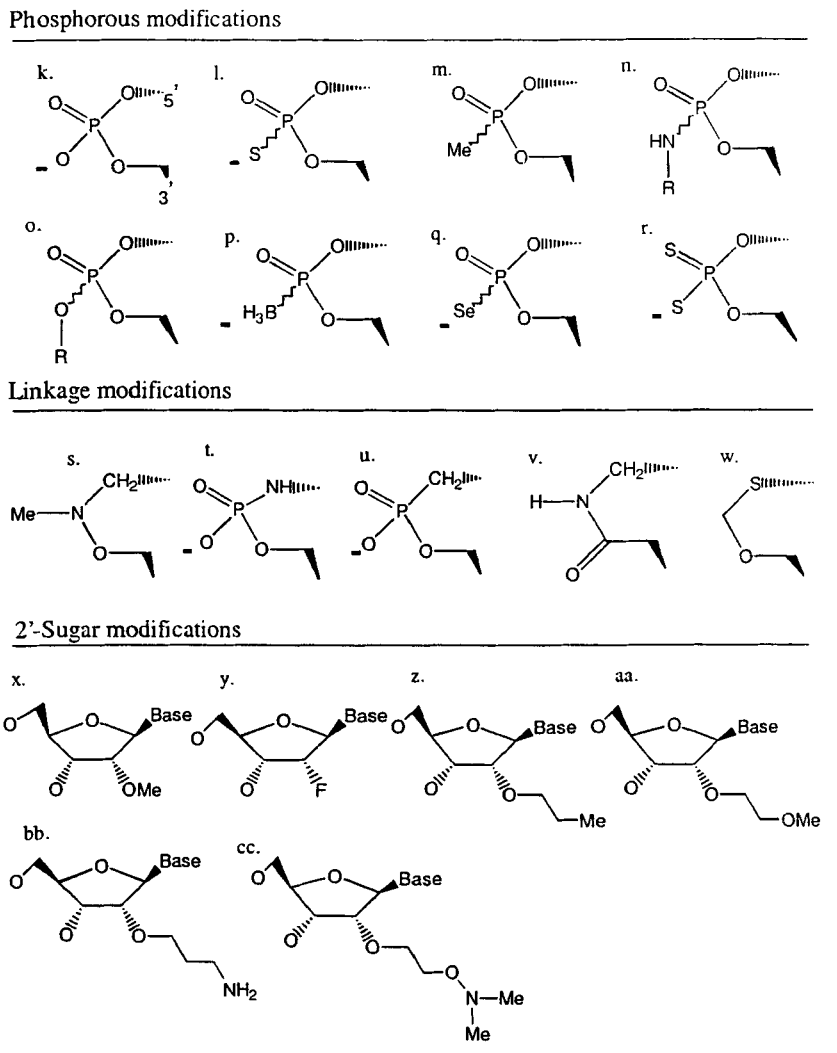

Figure 3. Examples of oligonucleotide modifications. (k) natural phosphodiester; (l) chiral phosphorothioate; (m) chiral methyl phosphonate; (n) chiral amidate; (o) chiral triester; (p) chiral borano; (q) chiral seleno; (r) phosphorodithioate; (s) methylene-imino-methyl (MMI); (t) 3'-amidate; (u) 3'-methylene; (v) amide-3; (w) thioformacetal; (x) 2'-*O*-methyl; (y) 2'-fluoro; (z) 2'-*O*-propyl; (aa) 2'-*O*-methoxyethyl (MOE); (bb) 2'-*O*-aminopropyl (AP); (cc) 2'-O-dimethylaminooxyethyl (DMAOE)

(phosphoramidate) and others (**depicted in Figure 3**). More extensive changes have been considered, in which the entire four-atom linkage between sugar moieties is replaced. Of the phosphorous atom changes, only P=S phosphorothioates are useful in the support RNase H cleavage. The most useful linkage-replacement modification, MMI-*bis*-O-methyl[33, 34] (**Figure 3s**), provides greatly enhanced binding affinities, removes the nuclease-cleavable phosphodiester linkage and the chirality of the phosphorothioate linkage, and allows adjustments of oligonucleotide lipophilicity by controlling the negative charge.

Examples of connection modifications in oligonucleotides are the α-nucleosides (reverse connection of the heterocycle at the C-1′ position),[35] **Figure 2h**, and 2′,5′-phosphodiester connection rather than the normal 3′,5′-phosphodiester connection[9] (**Figure 2i**).

Peptide nucleic acids (PNA) is an example of the complete removal of the sugar-phosphate backbone and replacement with a peptide linkage (**Figure 2j**).[36] In addition to the above classes, pendants or conjugation groups can be attached at many positions in the various subunits to enhance drug properties.

Oligonucleotides, particularly phosphorothioates, have considerable avidity to a variety of serum and tissue proteins. This is due to the highly negatively charged nature of the oligonucleotides as well as the lipophilicity of the sulfur atom of the thiophosphate linkages. Prodrug approaches, which are designed to mask the negative charge to allow altered pharmacokinetics, are of considerable interest.[37]

The antisense concept has well-defined structural requirements for the oligonucleotide ligand that binds to a reasonably characterized RNA receptor. Although this knowledge of rather precise binding is of great value, and certainly sets this drug discovery approach apart from current approaches, additional modifications are limited to those, that do not interfere with Watson-Crick base-pairing rules.

Modifications of oligonucleotides may be expected to address essentially every facet of antisense drug properties. Biophysical, biochemical and chemical properties that may be affected by modifications include binding affinity, specificity, nuclease resistance, support of RNase H, chemical stability, solubility, lipophilicity and others. Drug properties, that will be affected by altering these parameters, would include the general

areas of pharmacokinetics, pharmacodynamics, and toxicology. In addition, certain modifications may represent cost and proprietary patent advantages.

RATIONALLY MODIFYING OLIGONUCLEOTIDES

Modifications of short strands of DNA and RNA for drug purposes should rationally be directed by drug property-deficiencies of the parent or first generation oligonucleotides.[4] Biological deficiencies are discovered from pharmacologic, pharmacokinetic, and toxicologic studies of the parent oligonucleotides at the biophysical, biochemical, *in vitro, in vivo* and clinical levels. In addition, as modified oligonucleotides move from discovery into the development pipeline, chemical development deficiencies such as larger-scale synthesis, purification and analytical processes emerge. Finally, in contemplating research in oligonucleotides, one should, in addition to examining the traditional scientific literature, search the patent literature, as considerable proprietary positions of various aspects of the technology have been achieved. Hopefully, the days of incorporating nucleosides, novel or known, into oligonucleotides – just because of availability – are over. This non-rational approach was of value in the beginning of oligonucleotide structure-activity modifications, simply to develop important databases. But as knowledge has been gained about what is required to achieve enhanced properties, current and future modification or structure-activity relationship studies of oligonucleotides, should be soundly and rationally based on one or more of the known deficiencies or emerging drug problems.

CONTINUED OPTIMIZATION OF OLIGONUCLEOTIDE DRUG PROPERTIES ARE IMPORTANT MEDICINAL CHEMISTRY OBJECTIVES.

Oligonucleotide Binding Affinities -

In considering ligand-receptor theory for pharmacological activity, increasing the affinity of an oligonucleotide for its RNA target should increase potency. A relatively simple physical-chemistry experiment is employed to determine the level of binding and specificity of a modified oligonucleotide. A complementary oligonucleotide is the simplest target to which an antisense oligonucleotide can hybridize (form a duplex according to Watson-Crick base-pairing rules). Oligonucleotide affinity, as measured by

melting curves,[38] is increased as the length of the oligonucleotide-RNA heteroduplex increases. This has been experimentally verified in that 15- to 25-mers are typically used in antisense experiments rather than shorter oligonucleotides which may have melting transitions (T_ms) close to or below physiological temperature and therefore may only form low levels of the required heteroduplex. However, examples of short oligomers (12-mers or less) have exhibited interesting biological activity. These required modifications leading to high affinity per nucleotide unit. One interesting example was the report of potent and selective inhibition of gene expression by 7- and 8-mers phosphorothioate containing only the heterocyclic modifications, 5-propynyl substituted uracil and cytosine bases[39, 40] (**Figure 2a**). Furthermore, several recent studies correlate binding affinities of a series of 2′-*O*-modified oligonucleotides with increased *in vitro* and *in vivo* activity.[41-48] An up-to-date, extensive, comparative listing of binding affinities of 2′-*O*-modifications was recently published.[49]

Oligonucleotide Nuclease Resistance -

A number of *in vivo* pharmacokinetic studies in several animal species indicate that P=S oligonucleotides (phosphorothioates) are not as stable as initially thought.[50, 51] Although the stability of P=S oligonucleotides may be sufficient for many drug applications, greater stability of P=S oligonucleotides will be helpful in expanding dosage regiments (longer duration of action relates to less frequent dosing), in efforts to develop oral bioavailability, and less degradation of modified oligomers will minimize metabolite toxicity.

In summary, the preponderance of published antisense biological data suggests oligonucleotides with higher binding affinities and greater stabilities towards nucleases are important medicinal chemistry objectives to pursue.

STANDARDS FOR BINDING AFFINITY, NUCLEASE RESISTANCE, AND OTHER OLIGONUCLEOTIDE PROPERTIES HAVE BEEN ESTABLISHED

The intense oligonucleotide research performed in the past ten years has provided a remarkable enhancement of several of the desired antisense drug properties. The results achieved with modified oligonucleotides concerning binding affinity, base-pair specificity,

nuclease resistance, and support of RNase H cleavage of the targeted RNA is impressive. The level of binding affinity (as represented by T_m), nuclease resistance (as represented by $t_{1/2}$), and support of a RNase H mechanism, that a new modification should possess, to be of interest, as a potential drug should be considered. A number of «winners» have been identified (**Figure 2,3**) and discussed in several reviews.[3,4]

Certain modifications of oligonucleotides provide an increase in T_m of greater than 1.5 °C/mod relative to a P=S oligonucleotide (~1.0 °C/mod relative to a phosphodiester, P=O, linkage) and nuclease resistance ($t_{1/2}$) of greater than 24 hours with SVPD (snake venom phosphorodiesterase, about the level of P=S). In view of these values, I would suggest that a novel modification should exhibit a T_m > 1.5 °C/mod compared to its P=S oligonucleotide parent. In evaluation of the binding properties, the modified oligonucleotides should be hybridized with a RNA complement, as this is the receptor required for the antisense approach. A clear correlation between T_ms derived from hybridization to a DNA or to a RNA complement, has not been established. Also, correlations of T_ms of oligonucleotides having just one modification (point or pendant modification) or several modifications distributed throughout the sequence or a contiguous placement of the modification in the sequence have not been established.[17, 22, 52-54] Since the application of a modification will likely require its placement uniformly in the sequence, or at least several contiguous bases in a row for a gapmer strategy [54] (described below), measurements should be taken with these types of modified oligonucleotides. Furthermore, the modification must not compromise base pair specificity. In this regard, information from a number of papers suggests, that base-pair specificity actually increases as T_m values are increased. A specificity level comparable to base mismatches of a P=S phosphorothioate would appear to be a useful standard.

Nuclease resistance of a novel modification in a P=O oligonucleotide backbone should at least be at the level of uniformly modified P=S phosphorothioates. As $t_{1/2}$ values, unlike T_m measurements, are performed by several procedures and differing conditions, use of P=S oligonucleotide controls (standards) are necessary. In addition, several concentrations of the enzyme should be employed to minimize complications of inhibiting the nuclease.[55] Values of $t_{1/2}$ of ~ 24 hours are often reported for P=S

phosphorothioates in snake venom phosphodiesterase (SVPD) assays. When the 3′-end of an oligonucleotide is modified to have sufficient resistance to 3′-exonucleases, then endonucleolytic cleavage becomes evident. Thus, modifications should also protect against endonucleases. In a gapmer strategy, this is accomplished by phosphorothioate linkages which will also support an RNase H cleavage mechanism. Nuclease resistance of a modified oligomer, if not also provided by the modification, may in many cases, be provided by employing a P=S oligonucleotide backbone.

In considering the relative importance of nuclease resistance of an antisense oligomer and its level of affinity to its RNA target, recent biological results suggest that stability of an oligonucleotide may be a more important property to enhance than binding affinity.[56] Modifications that provide high binding oligonucleotides but with low nuclease resistance have not provided significant biological activity. On the other hand, oligonucleotides such as phosphorothioates, have provided significant biological activity. Although some modifications provide high binding affinities and high nuclease resistance, they may not exhibit useful antisense activities because they do not support an RNase H mechanism. A modification, which will support a RNase H mode of action and possess high T_ms and $t_{1/2}$s values, has not been reported. Thus, an ideal oligonucleotide modification would provide an oligomer that hybridizes to target RNA with high binding affinity and specificity, be stable to nucleolytic degradation, and would allow a RNase H cleavage of the RNA target. This has lead to the concept, that to optimize the antisense activity of an oligomer, a combination of oligonucleotide modifications will be required.[52, 54]

Additional standards that one should be aware of are the impact modified oligonucleotide modifications may have on the cost of future antisense drugs. Again, as a standard, the cost of P=S oligonucleotides should be considered. The cost of phosphorothioates has been dramatically reduced in the past ten years due to improvements in the process, cost reduction of key reagents and the economics of larger scale syntheses. Currently, the cost of goods for a 20-mer P=S oligonucleotide is less than $300/gram and is projected to be less than $50/gram as larger quantities are required. The 2′-*O*-(methoxyethyl) modified oligonucleotides are derived from the ribonucleosides in RNA and should eventually be substantially less expensive than modified

oligonucleotides derived from deoxyribofuranosyl nucleosides from DNA. P=S oligonucleotide antisense drugs are expected to be cost competitive when considering parental treatment, three times/week, with a 1 mg/kg dose. Modified oligonucleotides must also be cost effective and will need to be less expensive to synthesize (e.g., shorter), or have a dosing advantage (less often), or greater therapeutic index, or have other important advantages to offset a possible increase in cost of synthesis.

When considering research to modify oligonucleotides, I believe it is important to know the extent that proprietary protection of a modification can be obtained. Important patent positions for many types of modifications have been established during the past ten years as making drugs out of oligonucleotides has become of interest.[57-59]

OLIGONUCLEOTIDE MOTIFS

As noted above, having developed high binding, nuclease resistance, 2′-*O*-modified oligonucleotides, it was rather disappointing, that oligomers uniformly modified were inactive or less active than their first generation parent phosphorothioates. It is now well known that uniformly 2′-*O*-modified-oligonucleotides do not support an RNase H mechanism. The 2′-*O*-modified oligonucleotide-RNA heteroduplex has been shown to present a structural conformation that is recognized by the enzyme but cleavage is not supported. [60, 61] The lack of activity of 2′-*O*-modified oligonucleotides has led to the development of a chimeric oligonucleotide strategy (gapmer technology).[47, 54, 62] This approach focuses on the design of high binding, nuclease resistant antisense oligonucleotides, that are «gapped» with a contiguous sequence of 2′-deoxy phosphorothioates (**Figure 4**). On hybridization to target RNA, a heteroduplex is presented, that supports an RNase H-mediated cleavage of the RNA strand. The stretch of the modified oligonucleotide-RNA heteroduplex, which is recognized by RNase H may be placed anywhere within the modified oligonucleotide. The modifications in the flanking regions of the gap should not only provide nuclease resistance to exo- and endonucleases, but also not compromise binding affinity and base-pair specificity.[63]

Modifications of the phosphorus atom of the natural phosphodiester linkage to provide methyl phosphonates, phosphorothioates, and phosphoramidates destabilize heteroduplexes –0.7 to –1.5°C for each modification[64-66] **(Figure 3).** The decreased

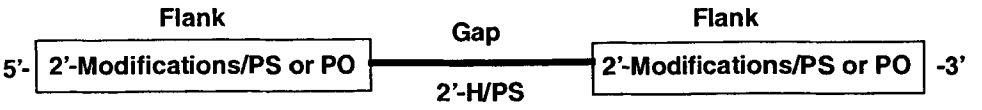

Figure 4. Gapmer technology

binding affinity of these modified oligonucleotides could be expected to reduce antisense effectiveness. In the case of chimeric 2′-*O*-methyl or 2′-fluoro modified oligonucleotides, an enhancement in the binding affinity of about 1.5 to 2.3°C (compared to P=S phosphorothioates) for each modification is obtained.[41, 66-68] However, it is now clear that 2′-*O*-methyl and 2′-F modified DNA are not sufficiently nuclease resistant to have antisense value as P=O backbones.[42, 68-71] This problem can be circumvented by the use of 2′-O-methyl or 2′-F modified phosphorothioates in the flanking regions to provide doubly modified oligonucleotides.[41, 67]

More recent research has focused on 2′-*O*-modifications, such as methoxyethyl[43, 46] and aminopropyl,[72] which not only provide relatively high binding affinities but also a level of nuclease resistance that allows the replacement of the thiophosphate with the natural phosphodiester linkage. 2′-*O*-Modifications, with a favorable combination of T_m and $t_{1/2}$, can be employed in the chimera strategy (**Gap Technology, Figure 4**), such that a significant portion of phosphorothioate linkages can be replaced with P=O linkages. Just how many sulfurs can be replaced depends on the length of the oligomer and the gap size or RNase H cleavage site. In a typically 21-mer with a 7-nucleoside gap, 65% of the P=S linkages replaced with P=O linkages. As noted in the limitations of P=S oligonucleotides, reduction of the sulfur content in a P=S oligonucleotide could have important implications in the pharmacokinetic and pharmacodynamic properties as well as the toxicity profile of oligonucleotides.[43, 73]

A very important aspect to the gapmer technology is that the gap or RNase H cleavage site must be protected from endonucleolytic cleavage. Phosphodiester linkages and even an alternating P=S/P=O motif did not provide a useful level of nuclease resistance for biological activity.[63, 64] A recent report of the lack of activity of «gapped»

3'-amidate phosphodiesters is likely due to endonuclease degradation.[74] Uniform phosphorothioates are the only useful modification to allow a reasonable combination of binding affinity, nuclease resistance and also support an RNase H mechanism. Hence, as noted above, most antisense activities require an RNase H mechanism, which in turn require sulfur in the form of thiophosphate, somewhere in the chimeric molecule for nuclease resistance.

GENERAL PATHWAY FOR SRUCTURE-ACTIVITY/PROPERTY RELATIONSHIPS STUDIES TO ENHANCE OLIGONUCLEOTIDE DRUG PROPERTIES

The excitement of oligonucleotides as drugs stems from the fact that, unlike other drug discovery approaches, they are informational materials, i.e., chemicals having a specific set of rules that clearly govern their binding to a specific receptor. These oligonucleotides bind to their nucleic acid targets via Watson-Crick base pairing rules. In addition, the fact that DNA and RNA molecules are relatively new molecular targets, as opposed to proteins, is of great interest. Thus, the first test to determine if a modified oligonucleotide will be of interest is whether it maintains its sequence specificity according to Watson-Crick rules. A newly modified oligonucleotide not possessing this fundamental specificity property is an immediate failure in the SAR study. Having ascertained that a particular modified oligonucleotide has an acceptable level of specificity (level of P=S oligonucleotide), the next step in the SAR study is to determine how tightly the modified oligonucleotide binds to its target nucleic acid. As noted, the binding affinity of an oligomer is a physical-chemical property, determined by measuring a modified oligomer sequence specific interaction with its length-matched RNA complement. The hybridization or melting process is performed under a rather standard set of conditions designed to mimic an intracellular environment.[38] As this is a measurement taken under artificial conditions, it may not accurately represent the binding of an oligomer to a native RNA inside a cell.

The next essential property that an oligonucleotide must possess is sufficient resistance to degradation by exo- and endo-nucleolytic plasma and tissue nucleases. Nuclease resistance of an oligonucleotide is determined in various assays, such as, heat-

inactivated fetal calf serum, cellular extracts, or with purified exonucleases or endonucleases. These results are also likely to differ from the stability of an oligomer in an *in vivo* situation. However, these T_m and $t_{1/2}$ assays do allow structure-property/activity-relationship studies to proceed, and thus provide reasonable methods to compare various oligonucleotide modifications at an early stage of the drug discovery process. This is quite different from traditional drug discovery approaches.

Conjugated Oligonucleotides -

The sugar, heterocycles, and backbone (linkages) subunit modifications as depicted in **Figure 1** are core modifications of oligonucleotides, which have been modified to greatly enhance binding affinities and nuclease resistance of antisense oligonucleotides. To enhance additional antisense drug properties of an optimized, core-modified oligonucleotide, a variety of molecules (pendants) have been attached (conjugated) in a point modification motif, (i.e. only one pendant in an antisense oligonucleotide). Pendant modifications have primarily been directed to enhancing oligonucleotide uptake. Other potential applications of pendants include increased solubility, lipophilicity and means to attach synthetic cleavers, intercalaters (for improvements in binding affinity), and cross-linking and alkylating groups. Several reviews have discussed oligonucleotide pendants.[75-77]

Prodrugs of Oligonucleotides –

Also, once the core subunits have been optimized, including conjugations, prodrug modifications, which may serve to further enhance various drug properties, particularly pharmacokinetics, can be employed. The most advanced approach is the pro-oligonucleotide described by the Imbach laboratory.[37]

PERSPECTIVE

A P=S oligonucleotide (phosphorothioate) is now an available drug (**Vitravene**^{TM}) and others will follow in the next several years. However, to continually improve this novel and exciting drug class and to overcome certain limitations, structural changes are required. In the past ten years a diverse range of modifications, at all possible

modification sites of an oligonucleotide (**Figure 1**), have been reported. This application of traditional medicinal chemistry (structure-activity-relationship studies) to drug discovery in antisense oligonucleotides and oligonucleotides in general, has answered many important questions. For example, as a result of this rather intense effort, we are now aware of modifications that stabilize oligonucleotides towards nucleolytic degradation (2′-*O*-(methoxyethyl), MOE; 2′-*O*-(aminopropyl) AP; 2′-*O*-propyl; and 2′-*O*-(dimethylaminooxyethyl), DMAOE, are examples), modifications that greatly enhance binding affinities while maintaining base-pair specificity (MOE, DMAOE and LNA are examples), and modifications that support endonucleolytic cleavage by RNase H (5-propynyl pyrimidines are examples). Although these are biochemical and biophysical properties, a large volume of cellular and animal studies support the notion that enhancing these properties, correlate with enhanced antisense biological activity *in vivo*. Unfortunately, a single modification that would provide high binding, nuclease-resistant antisense oligonucleotides and also would support an RNase H mechanism is not available. A modification of this nature is of current interest in the antisense approach. We are also aware that, changing the structure of phosphorothioate oligonucleotides provides an opportunity to alter their pharmacokinetic profile. In structural changes, which remove sulfur (as thiophosphate) and/or change lipophilicity (e.g., by 2′-*O*-modifications), more favorable toxicity profiles have also resulted.[42] Although we are aware of these important antisense properties, (and there may be many more to learn about), and we have learned how to control them, we are unaware of the optimum values at which to aim our modifications. In addition, antisense oligonucleotides that are orally available and/or penetrate the blood brain barrier, present the most current, important deficiency of antisense oligonucleotides. Recent reports of antisense oligonucleotides doubly modified at the 3′ and 5′ ends with 2′-*O*-methyl or 2′-*O*-methoxyethyl and P=S phosphorothioates, to provide a high level of nuclease resistance, have provided encouraging results that these pharmacokinetic deficiencies will soon be solved by appropriate chemical modifications.[78] Furthermore, considerable formulation research is underway in several laboratories.

One should be aware of the level of accomplishments achieved in oligonucleotide medicinal chemistry research in the past ten years. I have discussed these (binding

affinities, nuclease resistance, support of RNase H, and cost of synthesis) and suggest that they be considered (as standards) before initiating or continuing certain oligonucleotide modification research. In addition, understanding the proprietary patent positions that have been established is an important research consideration. I believe that at this stage of oligonucleotide medicinal chemistry, it is highly unlikely that a single modification will be discovered that will significantly impact all of the important drug properties described above. The types of modified oligonucleotides currently being pursued (going beyond P=S oligonucleotides) possess a combination of modifications and this trend will certainly continue as pendants will be conjugated to oligonucleotides with optimized core subunits to obtain a «completely» optimized oligonucleotide drug, which still may benefit from prodrug modifications.

I view the current «winners» or the first modifications most likely to be incorporated into antisense oligonucleotides, that will undergo clinical trials, as the RNA mimics, MOE, DMAOE, and AP, and the backbone modification, MMI *bis*-methoxys. These will likely be utilized in a gap strategy (Gap Technology). However, efforts to prepare uniform modifications, such as RNA mimics (2′-*O*-modifications), MMI and PNA are of considerable interest, in that reliance on RNase H for a mode of action would not be required. In addition to these modifications which act, either by direct binding (RNase H-independent) or RNase H, I believe modifications of the pendent subunit, such as cholesterol-conjugates, folic acid-conjugates, etc., and prodrug modifications, will become increasingly more important for optimizing multiple-modified oligonucleotides. It remains to be seen how valuable the newest, high binding modifications such as LNA, tricyclic cytosine clamps, and DMAOE will be, in view of the current need for a gapmer strategy for useful biological activities.

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