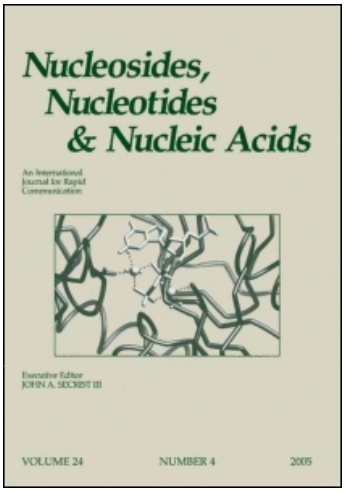

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CARBOHYDRATE MODIFICATIONS IN ANTISENSE OLIGONUCLEOTIDE THERAPY: NEW KIDS ON THE BLOCK

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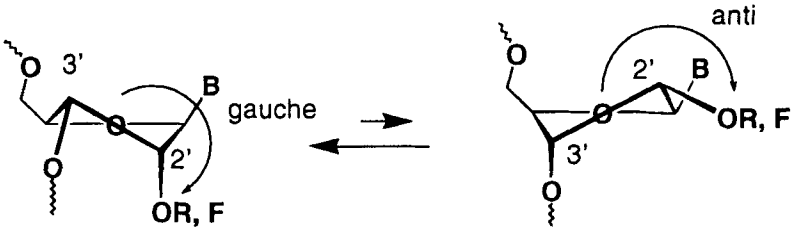
Chemical modifications to improve the efficacy of an antisense oligonucleotide are designed to increase the binding affinity to target RNA, to enhance the nuclease resistance, and to improve cellular delivery. Among the different sites available for chemical modification in a nucleoside building block, the 2'-position of the carbohydrate moiety[1] has proven to be the most valuable for various reasons: (1) 2'-modification can confer an RNA-like 3'-endo conformation to the antisense oligonucleotide. Such a preorganization for an RNA like conformation[2,3,4,5] greatly improves the binding affinity to the target RNA; (2) 2'-modification provides nuclease resistance to oligonucleotides; (3) 2'-modification provides chemical stability against potential depurination conditions



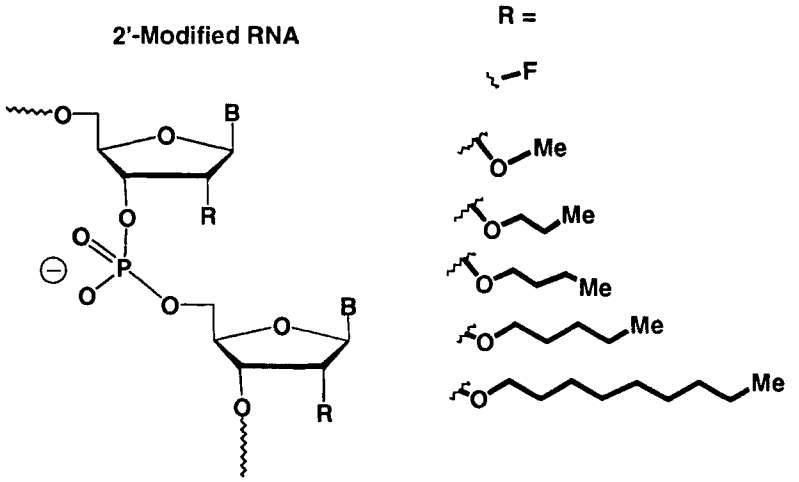
C_3'-endo **N form** **A type, RNA like**

C_2'-endo **S form** **B type, DNA like**

2'-OR, 2'-F favor *C_3'-endo* (O/O or O/F prefer gauche)

either during synthesis or after administration as the drug; (4) 2'-modification changes the lipophilicity of the oligonucleotides which relates to favorable pharmacokinetic properties.

Among the possible 2'-modifications, 2'-*O*-alkyls, 2'-*O*-alkyls with glycol ether linkages, 2'-F and 2'-*O*-aminoalkyls have been studied for their pharmacokinetic, pharmacodynamic and pharmacological properties. Within 2'-*O*-alkyls, with increase in size of the alkyl chain, the binding affinity[6] drops while the nuclease resistance increases with increase in alkyl chain[7]. On the other hand, the 2'-F modification[8], which locks the sugar conformation in a very high 3'-*endo* conformation (3'-*endo*>90%) offers the greatest increase in binding affinity. Unfortunately, this high affinity modification does not offer any resistance to nucleases as a phosphodiester (P=O). It requires the phosphorothioate backbone (P=S) to exhibit sufficient nuclease resistance.

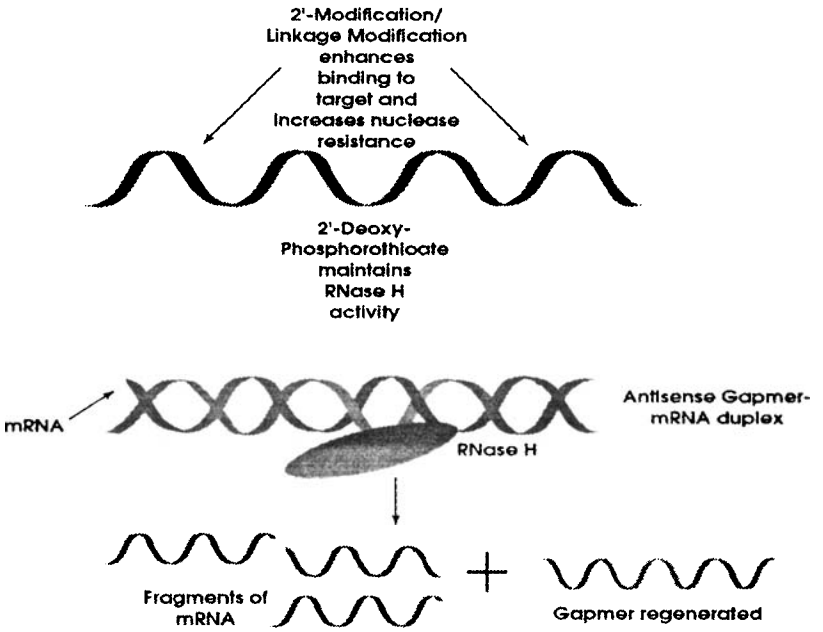


Binding Affinity: -F > -OMe > - O-Propyl > -O-Butyll > -O-Pentyl > -O-Nonyl

Nuclease Resistance: -O-Pentyl > - O-Propyl >-OMe > -F

RNaseH dependent mechanism of action of 2'-modified oligomers: Gapmer Technology.

While the 2'-modifications offer increased binding and high nuclease resistance, they fail to activate RNase H in cleaving the target RNA after hybridization. This limitation has been overcome by the development of gapmer technology[9,10,11] in which chimeric oligonucleotides with the placement of 2'-modifications only at the terminal



ends ("wings"), leaving a 2'-deoxyphosphorothioate gap-usually 6 to 10 bases long-("Gap") in the middle for RNase H activation.

2'-MOE and 2'-AP RNA

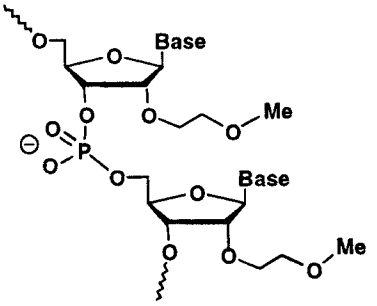
Among the 2'-modifications well-studied at Isis, two modifications stand out in terms of binding affinity to target RNA and nuclease resistance. These are 2'-*O*-(methoxyethyl) or MOE modification[12,13,14] and 2'-*O*-(aminopropyl) or AP, modification[15].

The MOE modification offers +2°C increase in binding affinity/modification compared to the first generation of 2'-deoxyphosphorothioate drug compounds.[3, 7,8] This modification as a phosphodiester (P=O) linkage, exhibits nuclease resistance at approximately the same level as a 2'-deoxyphosphorothioate (P=S) modification. The 2'-*O*-aminopropyl (AP-RNA) modification exhibits the highest nuclease resistance compared to P=S (6 to 8 times) and a modest increase in Tm (+1°C increase in binding affinity/modification)[15].

RNaseH independent mechanism of action of 2'-modified oligomers: Consequences of direct high-affinity binding to the target: Inhibition of translation

There are two reports of an RNase H-independent 2'-modified antisense oligonucleotides exhibiting antisense activity. The first one is seen in targeting 5'-cap

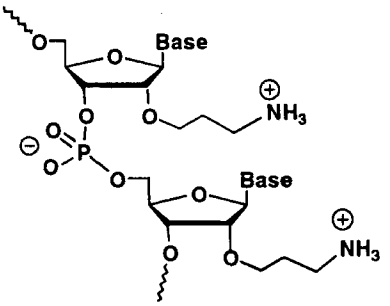
region of human ICAM-1 transcript in HUVEC cells with a series of uniformly 2'-*O* modified 20-mer oligonucleotides[16]. The 2'-MOE/P=O oligomer demonstrated the greatest activity with an IC_{50} of 2.1 nM (T_m = 87.1°C) and its P=S analog had an IC_{50} of 6.5 nM (T_m = 79.2°C). The unmodified parent oligonucleotide, (2'-deoxy P=S compound), which can activate RNase H, exhibited an IC_{50} = 41 nM. The inhibition of protein expression by the RNase H independent oligonucleotides was due to selective interference with the formation of the 80 S translation initiation complex. The other metabolic processes such as splicing and transport of the transcript RNA formed were not affected by the 2'-modification. Another example is found in the case of human HCV as the target[17] where an uniformly modified 2'-MOE phosphodiester antisense oligonucleotide complementary to the initiator AUG codon reduced HCV core protein levels without reducing HCV RNA levels.



MOE: 2'-*O*-(2-Methoxyethyl)-RNA

Gauche Effect; No Charge Effect

ΔTm = 2.0° C (rel. to P=S)



AP: 2'-*O*-(Aminopropyl)-RNA

Charge Effect; No Gauche Effect

ΔTm = 1.0° C (rel. to P=S)
$t_{1/2}$ = highest known

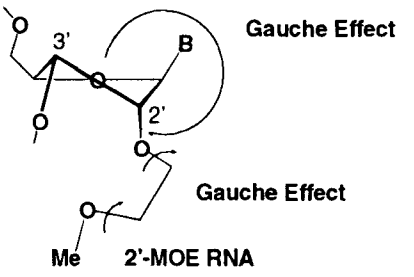
RNaseH independent mechanism of action of 2'-modified oligomers: Consequences of direct high-affinity binding to the target: Modulation of Splicing

Yet another mechanism by which uniformly modified oligonucleotides can interfere with gene expression is via modulation of splicing events[18]. Modification of splicing in the dystrophin gene in cultured *mdx* muscle cells by 2'-OMe modified phosphorothiaoates has been recently demonstrated. An antisense 2'-OMe oligonucleotide complementary to the 3'splice site of intron 22 induces dystrophin expression at the sarcolemma of transfected *mdx* myotubes. The mutant exon 23 of mdx dystrophin is skipped following transfection with an antisense 2'-OMe modified

phosphorothiaoate complementary to the 3' splice site of intron 22. Other research groups have also reported similar observations in modulating splicing as a potential mechanism of controlling gene expression[19,20,21]

MOE-RNA: What factors contribute to the extraordinary stability and binding affinity of MOE-RNA?

Egli *et al.* have rationalized[22] the extraordinary nuclease stability and binding affinity of MOE-RNA by studying crystal structures of MOE-RNA molecules. The stabilizing 2'-MOE substituent as well as the modified furanose ring (C_3-endo pucker) are conformationally preorganized for an A-form duplex. The conformation of the torsion angles around the ethyl C-C bonds in the side chain fall into the syn-clinal conformation. The resulting conformation is compatible with the minor groove topology in an A-form RNA duplex. The orientation of the side chain is further constrained by the coordination of water molecules involving O2', O3' and the ether oxygen. The water molecule lies within 3 angstroms of these three atoms. Such a hydrogen bonding would significantly contribute to the preorganization of the modified nucleoside into an A form. This complexation will also inhibit nucleases from cleaving the phosphate below the O3' atom.



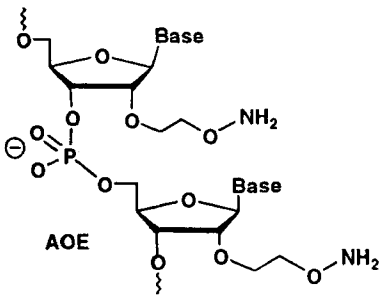
What factors contribute to the extraordinary nuclease stability of 2'-AP RNA? "The Charge Effect":

As mentioned above, the 2'-*O*-aminopropyl (AP-RNA) modification[15], even as a diester (P=O) exhibits the highest nuclease resistance compared to P=S oligonucleotides, due to the existence of positive charge on this group ("the charge effect"). The increase in

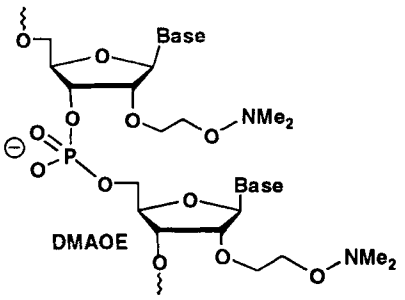
resistance is not a steric effect, since the propyl or longer pentyl modifications do not offer such an increase in nuclease resistance. Similarly the sugar conformation (C3'-endo population) does not seem to contribute to this improved stability because other C3'-endo substituents such as –F, -O-alkyls do not exhibit such a robust stability. The side chain of 2'-AP-RNA has an amino group (pKa=9.4) and protonated at physiological pH. This positive charge can interact with metal ions in an unproductive fashion at the active site[23] of the nucleases, contributing to the high nuclease resistance. Other cationic oligonucleotide analogs are also expected to contribute to increase in nuclease resistance[24,25,26]. However, the 2'-*O*-aminopropyl (AP-RNA) modification lacks the gauche effect of the -O-CH_2-CH_2-O- (MOE-RNA) linkage and this compromises the binding affinity of this modification. The lack of gauche effect minimizes the entropic advantage of the preorganization to form RNA-like duplex.

Going beyond 2'-MOE and 2'-AP modifications

To improve the pharmacokinetic and pharmacodynamic properties of 2'-modified oligomers even further by incorporating the virtues of MOE (improved binding affinity due to the gauche effect) and AP modifications (nuclease resistance due to the charge effect), we are synthesizing many modifications, some of which are shown below:



2'-*O*-(2-Aminooxyethyl)-RNA



2'-*O*-(2-Dimethylaminooxyethyl)-RNA

(1) 2'-*O*-AOE (Aminooxyethyl)[27]

This modification exhibits same binding affinity and same nuclease resistance as MOE RNA. This is again because of the maintenance of gauche effect and favored preorganization. The oxy-amino group is not expected to be protonated at physiological conditions.

(2) 2'-*O*-DMAOE (Dimethylaminooxyethyl)

In designing the DMAOE modification we desired to maintain the following features:

i. An electronegative atom at 2'-connecting site, which is absolutely necessary for C_3-endo conformation via O_4-O_2· gauche effect (Increase in binding affinity).
ii. Gauche effect of the 2'-substituent -O-CH_2-CH_2-O- (increase in binding affinity/nuclease resistance)
iii. Restricted motion around N-O bond as in the natural product calicheamicin, which will lead, to conformational constraints in side chain.
iv. Lipophilicity of the modification which relates to protein binding/absorption properties of oligonucleotides.

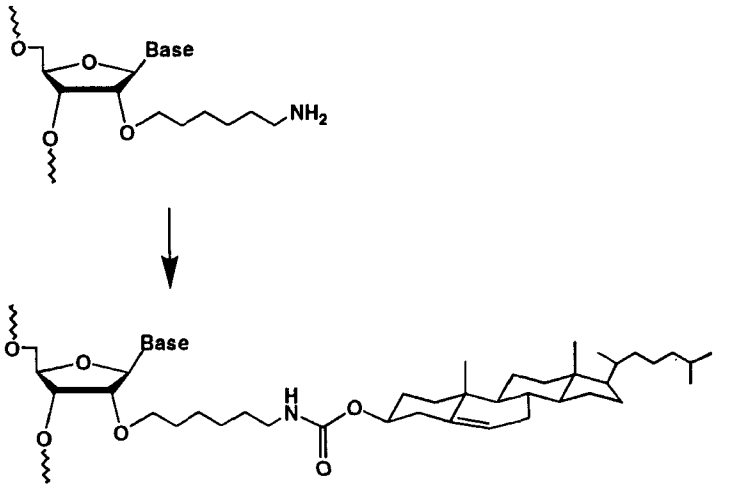
DMAOE modification shows higher binding affinity and higher nuclease resistance than MOE. The maintenance of gauche effect and favored preorganization causes the binding affinity advantage. Also, the improved steric effect (compared to MOE and AOE modifications) may be contributing to the enhanced nuclease resistance. This modification supports both an RNase H dependent (as a Gapmer) and an RNase H independent mode of action in biological assays. Message walks have been started with this modification in several new targets to choose the appropriate target site which is optimal for this modification. Thus the 2'-*O*-(dimethylaminooxyethyl) modification (DMAOE-RNA) exhibits the attractive features of both MOE-RNA and AP-RNA although it also lacks the charge effect like the 2'-AOE modification.

Oligonucleotide Conjugates as Carbohydrate Modifications[28]

Finally, in addition to the above chemical modifications, the carbohydrate 2'-position has been used as a site for conjugating other ligands via an amino group to improve the antisense properties of oligonucleotides. For example, the cholesterol conjugate, changes the biodistribution and pharmacokinetic properties of antisense oligonucleotides dramatically.

Conclusions and Perspectives

The 2'-position of the carbohydrate residue continues to be a valuable site for chemical modifications for antisense technology and other genome-based drug discovery efforts. Many chemical processes, which are not directly presented here, have evolved for simpler and efficient methods of these key modifications[29]. *In vitro* and *in vivo*



pharmacology evaluations and correlation with pharmacokinetic changes are emerging from these novel chemical modifications. Analytical chemistry of modified oligonucleotides before and after biological administration of antisense oligonucleotides with techniques such as capillary gel electrophoresis (CGE) and mass spectrometry help to determine the purity as well as the *in vivo* fate of these complex molecules. Large-scale synthesis is becoming a tangible reality for antisense oligonucleotides. Nucleic acid chemists and biologists alike are beginning to understand the structure-biological activity in terms of basic physical-organic parameters such as the gauche effect, the charge effect and conformational constraints. Synthesis of chimeric *designer* oligonucleotides bringing the attractive features of different modifications to a given antisense oligonucleotide sequence to generate synergistic interactions is forthcoming[30]. These advances along with the potential availability of complete human genome sequence information promise a bright future for the widespread use of nucleic acid based therapeutics.

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