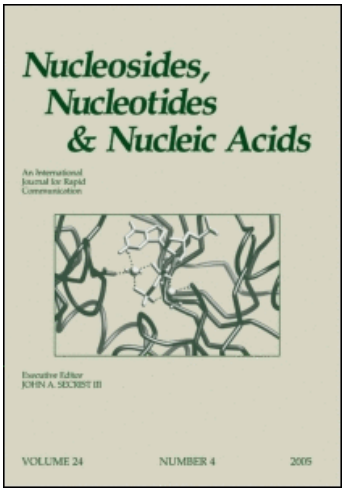


Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Antisense Properties of Morpholino Oligomers

James Summerton[a]; Dwight Weller[a]
[a] Antivirals, Inc, Corvallis, OR

To cite this Article Summerton, James and Weller, Dwight(1997) 'Antisense Properties of Morpholino Oligomers', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 889 — 898
To link to this Article: DOI: 10.1080/07328319708006105
URL: http://dx.doi.org/10.1080/07328319708006105

I. PROGRESS IN SECOND GENERATION OLIGOMER THERAPEUTICS

NUCLEOSIDES & NUCLEOTIDES, 16(7–9), 889–898 (1997)

ANTISENSE PROPERTIES OF MORPHOLINO OLIGOMERS

James Summerton and Dwight Weller*
Antivirals, Inc, 4575 SW Research Way, Corvallis OR, 97333

ABSTRACT Morpholino oligomers are third generation antisense agents designed to be cost effective, water soluble and resistant to nuclease attack. They show high potency and sequence specificity and follow predictable targeting rules when used in antisense applications in cell culture.

Antisense oligos offer the prospect of safe and effective therapeutics for a broad range of intractable diseases. However, the development of antisense therapeutics presents a number of forbidding challenges. The oligos should achieve adequate efficacy at a concentration attainable within the cells of the patient. They should inhibit their selected target sequences without concomitant attack on any other sequences in the patient's pool of approximately 200 million bases of unique-sequence RNA. They should be stable in extracellular compartments and within cells. They should be adequately soluble in aqueous solution. Lastly, they should be affordable.

First-generation antisense oligos comprised natural genetic material[1-3]. As the design challenges became more fully appreciated a number of non-natural antisense structural types such as methylphosphonates[4], phosphotriesters[5], phosphoroamidates[6] and phosphorothioates[7], were developed in an effort to improve efficacy, stability, and delivery. While each of these second-generation structural types provides one or more significant advantages over the first-generation oligos, the backbone modification substantially reduces binding affinity to target RNA[6] and/or fails to provide for adequate sequence specificity[8]. In addition, the second-generation DNA analogs may be unduly expensive due to the high cost of deoxyribonucleosides.

The use of a cheaper feedstock for oligo synthesis, such as ribonucleosides, is highly desirable, although their direct application is limited by the instability of RNA, by the multistep technology required to remove or mask the 2'-hydroxyl group, and by the difficulty of coupling to

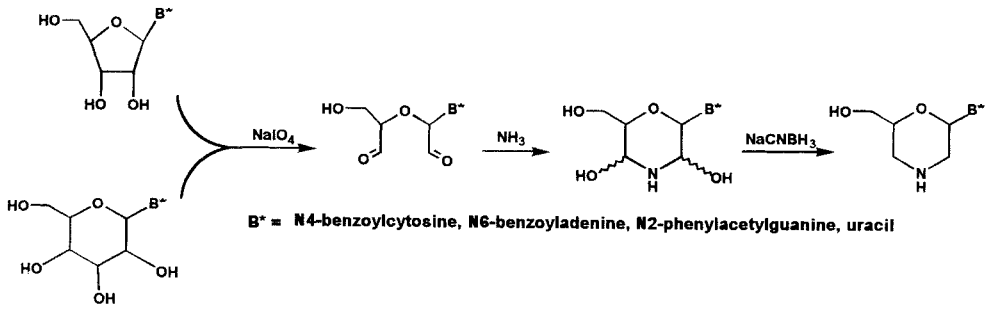


FIG. 1. Preparation of Morpholino subunits.

the 3'-hydroxyl of ribosides. We envisioned that these problems could be circumvented by converting the riboside moiety to a morpholine moiety[9,10], as illustrated in FIG. 1. The procedure is a simple one-pot process which provides the morpholino subunits in yields of 60-80%. While the ribosides are precursors for Morpholino subunits with the usual heterocyclic bases, additional subunits are available via hexopyranosides, themselves easily obtained from glucose or galactose and the appropriate heterocycle[11].

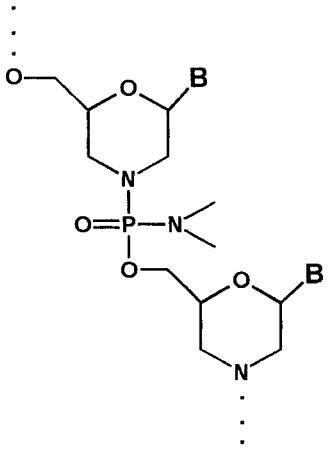
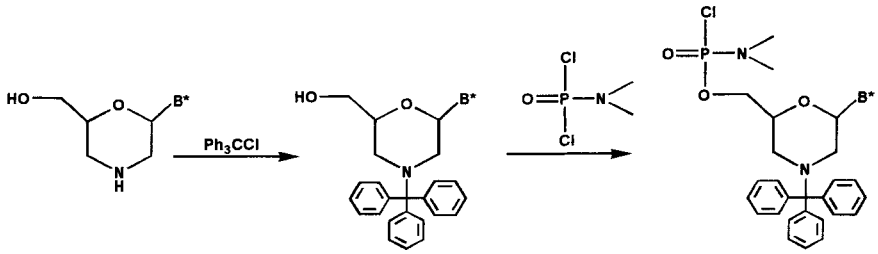


FIG. 2. Morpholino phosphorodiamidates.

A compelling advantage of the morpholino system is the combination of the secondary amine and alcohol functionalities which allows the incorporation of a large variety of intersubunit linkage types, including those based upon carbonyl, sulfonyl, and phosphoryl groups[9,12,13]. While Morpholino oligos containing a number of such linkages provide effective binding to targeted genetic sequences, consideration of cost and ease of synthesis, chemical stability, aqueous solubility, and affinity and homogeneity of binding to RNA led us to focus on the phosphorodiamidate shown in FIG. 2 as our principal linkage type for oligos targeted against single-stranded RNA sequences.

While phosphorodiamidate-linked Morpholino oligos can be assembled by a variety of methods, one relatively simple method which has proven effective[13] entails protection and activation of the morpholino subunit, as illustrated in FIG. 3. The activated subunits can be



B* = N4-benzoylcytosine, N6-benzoyladenine, N2-phenylacetylguanine, uracil

FIG. 3. Protection and activation of Morpholino subunits for conversion to Morpholino phosphorodiamidates.

stored at low temperatures for many months without significant breakdown. While they are relatively resistant to hydrolysis, they react rapidly ($t_{1/2}$ of 1 to 2 minutes) with the morpholine nitrogen of growing chains during solid phase synthesis. Oligomerization is performed on a 1% crosslinked polystyrene support loaded at 500 μMole/gram of resin, with coupling efficiencies typically about 99.7%. Because of cheaper starting materials and simpler, more efficient oligo assembly, we estimate that in large scale production the cost of these Morpholino oligos will be at least an order of magnitude lower than the cost of corresponding DNA analogs[14].

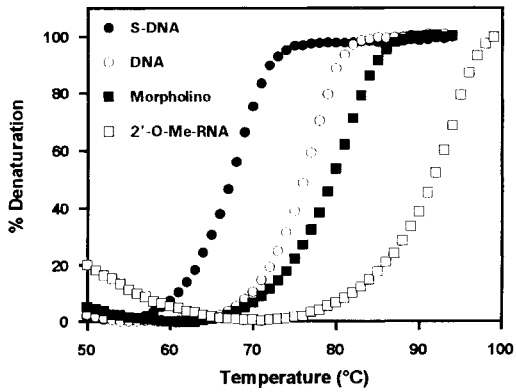


FIG. 4. Melting curves for RNA-oligo duplexes.

Although the phosphorodiamidate linkage is chiral, and both linkage stereoisomers are produced during oligomer synthesis, hybridization to nucleic acid targets is not compromised. A rigorous analysis of the effect of phosphorodiamidate stereochemistry on oligomer properties found that both stereoisomeric linkages support strong and equipotent stacking of bases on adjacent subunits[15]. FIG. 4 shows the melting curves obtained from hybrids of the Morpholino oligo and other common antisense types with a complementary RNA 20-mer. The

RNA binding affinity of the Morpholino oligo is appreciably higher than that of the phosphorothioates (S-DNA) and higher than that of DNA, but is not as great as that of 2'-O-methyl RNA.

Although conventional wisdom in the antisense field is that non-ionic antisense oligos invariably show poor water solubility, phosphorodiamidate-linked Morpholino oligos of the type shown in FIG. 2 are extremely soluble in aqueous solutions. To illustrate the exceptional aqueous solubility of morpholino oligos of this type, we have dissolved 263 mg of a heteromeric 22-mer of the sequence 5'-GCUCGCAGACUUGUUCCAUCAU in 1 ml of water (36 milliMolal) at 20°C without reaching saturation.

To achieve reasonable efficacy an antisense oligo should not be rapidly degraded either extracellularly or within cells. In this regard, it has been demonstrated that DNA and 2'-O-methyl RNA are rapidly degraded and phosphorothioate DNA is slowly degraded by nucleases in blood and within cells[16,17]. While resistance to nucleolytic degradation can be improved by adding special groups to the termini[18], or by incorporating a few nuclease-resistant intersubunit linkages near each end[19], we believe a better solution, on the basis of both function and cost, is to utilize a backbone structure which is inherently immune to a broad range of degradative enzymes present in the blood and within cells. A further advantage of using a backbone structure which is not degraded in the body is that it avoids concerns that modified nucleosides or nucleotides resulting from degradation of an antisense oligo might be toxic or might be incorporated into cellular genetic material and thereby lead to mutations and/or other undesired biological effects.

In experiments detailed elsewhere[20] it has been demonstrated that Morpholino phosphorodiamidate oligos of the type shown in FIG. 2 are immune to a wide range of nucleases, including: DNase I and II; RNases A, S1, and T1; Phosphodiesterase I and II; and P1, Mung bean, BAL-31 and Benzonase. These Morpholino oligos have also been found to be unaffected by Pronase E, Proteinase K, and Pig Liver Esterase, as well as degradative enzymes in serum and plasma.

Antisense activity in cell-free systems

In cell-free translation experiments employing a sensitive luciferase reporter, we have demonstrated that a Morpholino oligo 25 subunits in length, in either the presence or absence of RNase H, inhibits its targeted mRNA at very low concentration, and somewhat better than the corresponding S-DNA oligo in the presence of RNase H (FIG. 5). The lack of dependency on

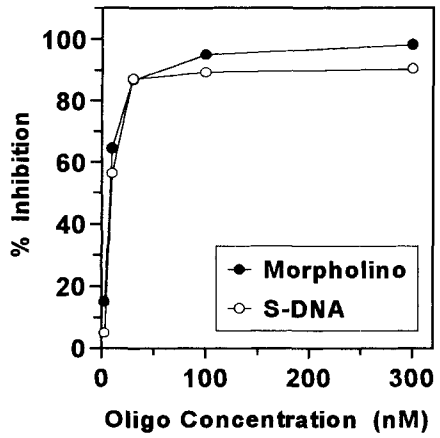


FIG. 5. Cell-free efficacy in the presence of Rnase H.

RNase H suggested that a "steric blocking" mechanism is responsible for the antisense effect of Morpholino oligos. This hypothesis is supported by a study in which an mRNA construct comprising a 5'-untranslated leader sequence from the HBV Core gene and a luciferase coding region is systematically targeted with Morpholino oligos. The resulting graph of antisense inhibition versus mRNA target position, shown in FIG. 6, clearly reveals the strong inhibition afforded by agents in or near the leader sequence. Agents directed to mRNA targets which are further downstream of the AUG are not inhibited.

Many factors are expected to limit the sequence specificity of RNase H-competent antisense structural types such as S-DNA[19,21,22]. Because of the restricted set of targets available to antisense agents which act by a steric blocking mechanism[23-25], and the lack of RNase competency, we expected that the Morpholino oligos would exhibit excellent sequence specificity. We carried out stringent specificity assays in a cell-free translation system utilizing two oligos each of S-DNA and Morpholino oligo[26]. In these experiments one oligo was perfectly complementary to its target mRNA to provide a measure of the total inhibition afforded by that oligo type. The other oligo incorporated four mispairs to that same mRNA target sequence to provide an estimate of the low-specificity component of the inhibition. The difference between these two inhibition values at each concentration then provided a measure of the high-specificity component, which we denote as "sequence-specific inhibition".

FIG. 7 shows that the S-DNA oligo, which achieves reasonable efficacy at concentrations above about 10 nM, shows a very dramatic drop in sequence specificity at concentrations above 100 nM. The corresponding Morpholino oligo, which also achieves high efficacy at concentrations above about 10 nM, maintains high sequence specificity through 10,000 nM, the highest concentration tested. Thus, in this stringent test of specificity the Morpholino oligo achieved highly effective and specific antisense activity over a concentration range more than two orders of magnitude greater than the concentration range wherein the corresponding S-DNA achieved reasonable efficacy and specificity.

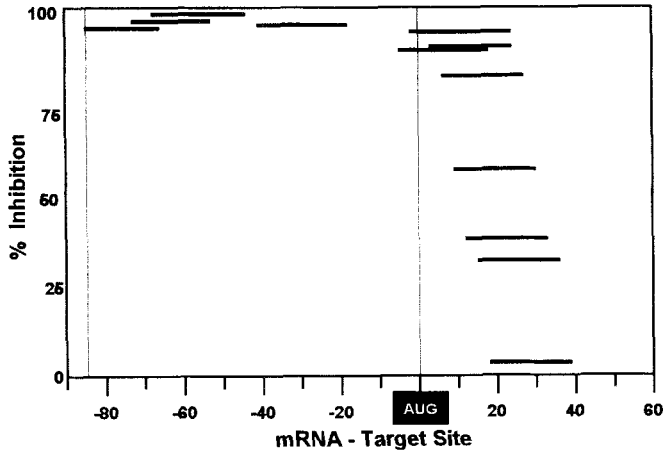


FIG. 6. Cell-free targeting study of Morpholino oligos (1μM).

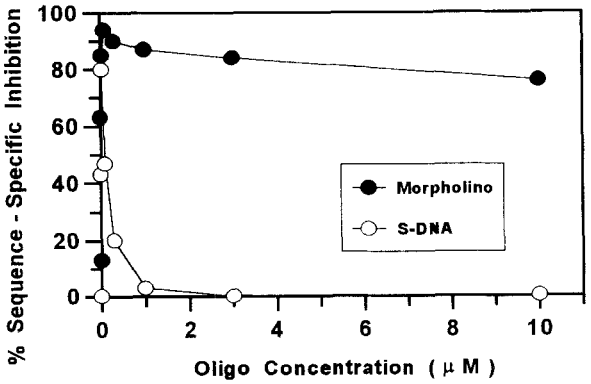


FIG. 7.
Sequence specificity of Morpholino and S-DNA oligos.

Antisense activity in cells

For effective biological activity an antisense oligo must gain entry into the cellular compartments where the target genetic sequence is synthesized, processed and/or functions - specifically, the cytosol/nuclear compartment. Our experiments with fluorescent-tagged Morpholino oligos suggested that these oligos enter mammalian cells by what appears to be endocytosis, but they do not appear to subsequently cross the endosomal or lysosomal membrane into the cytosol, based both on visualization of fluorescent-tagged oligos and a functional assay employing a transfected plasmid incorporating the luciferase reporter gene under the control of an MMTV inducible promoter[27]. This result is in agreement with limitations on uptake of antisense oligos reported by others[28-32].

Ours and others' results showing endosomal but not cytosolic uptake of antisense oligos suggest that much of the reported antisense activity with cells in culture may have been achieved because experimental manipulations inadvertently permeablized cell membranes. In this regard, we have found that simply passaging anchorage-dependent cells by the common procedure of scraping with a rubber policeman achieves significant oligo entry into the cytosolic compartment if the oligo is present during the scraping[27]. FIG. 8 shows a comparison of the efficacy and specificity of representative Morpholino and S-DNA oligos in scrape-loaded cells.

When combined with Morpholino oligos, the scrape loading technique enables reliable antisense studies to be performed in cell culture. The Morpholino oligos provide the necessary efficacy and specificity, as shown in FIG. 8, and also allow for predictable targeting. FIG. 9 shows the effect of Morpholino oligos targeted against selected sites in the HBV CORE/luciferase construct mRNA in HeLa cells. In excellent correspondence to the cell-free targeting data in FIG. 6, the Morpholino oligos appear to be effective when said agents are complementary to sequences in or near the 5'-untranslated leader region of the target mRNA.

Antisense structural types which do not support RNase H cleavage of their RNA targets may also be effective when directed against several special targets, such as splice junctions, transport signals, etc. We have recently performed a scrape loading study of NRK cells with Morpholino oligomers targeted against the *c-myc* gene. We found that an agent targeted against the splice acceptor of the first intron of the pre-mRNA to be the most effective in reducing cell proliferation, as evidenced both by direct cell quantitation and reduction in ^{3}H-thymidine incorporation[33]. In addition to expanding the scope of genetic sequences targetable with the Morpholino oligos, this result has potential therapeutic application to restenosis. This follows from a recent study which showed that antisense oligos can gain access to the cytosol of smooth muscle cells damaged during balloon angioplasty[34].

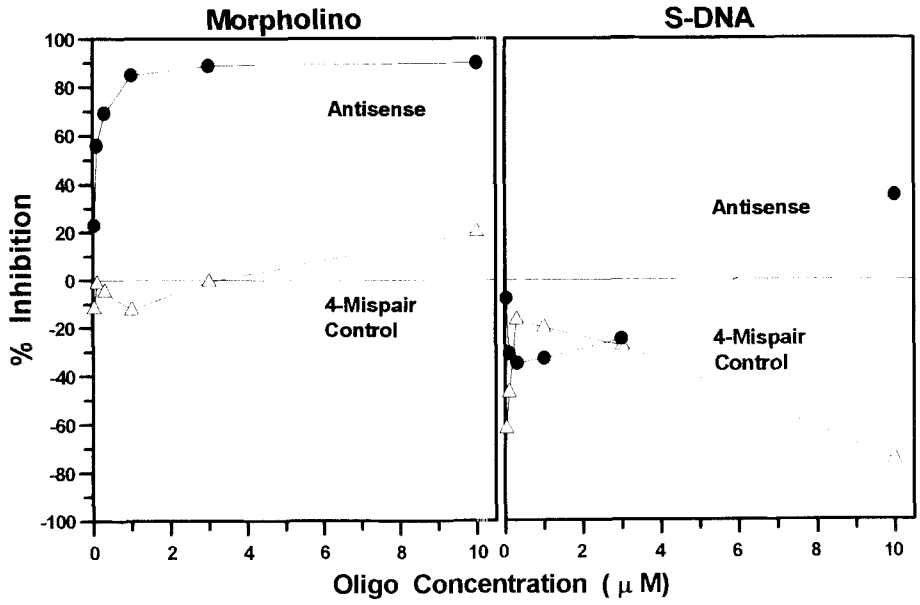


FIG. 8. In-cell activities of Morpholino and S-DNA oligos following scrape loading.

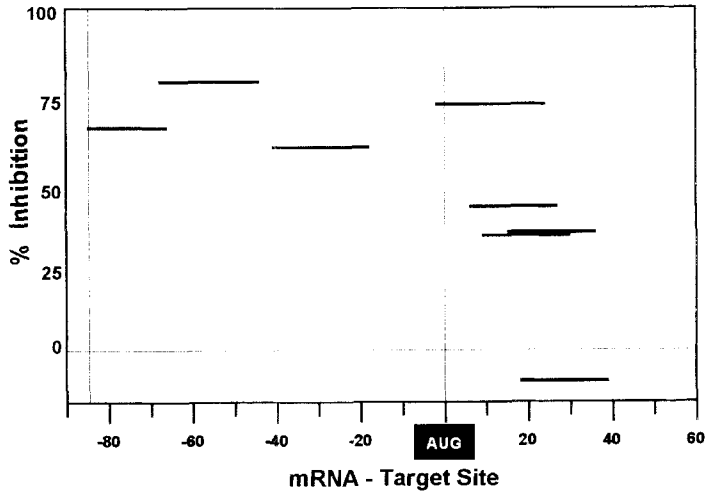


FIG. 9. In-cell targeting study of Morpholino oligos (3μM).

While there may be a few special situations where antisense oligos can easily gain access to their genetic targets within cells of a patient, it seems likely that the vast majority of therapeutic applications of antisense oligos will require a delivery technology capable of safely and efficiently delivering antisense oligos into the cytosol/nuclear compartment of cells. Accordingly, efficient delivery appears to be the next (possibly last) major challenge which must be overcome in order to achieve safe and effective antisense therapeutics.

To this end we have developed and are now optimizing a unique delivery engine designed from first principles to transport antisense oligos (and other delivery-limited drugs) from the endosome to the cytosol of cells without disruption of the endosomal membrane, with the engine being powered by the pH differential generated by proton pumps embedded in the endosomal membrane.

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