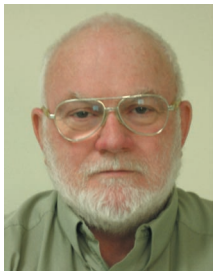
Morpholino Antisense Oligos: Applications in Biopharmaceutical Research

Morpholinos constitute a radical re-design of DNA, providing decisive advantages over the more conventional oligo types used for modulating gene expression.

By Dr James Summerton at Gene Tools LLC



James E Summerton (PhD) devised his antisense therapeutics strategy in 1969, while a graduate student at the University of Arizona – though it later became apparent that several other scientists were also developing similar ideas around this time. He began full-time experimental work on antisense in 1974 as a postdoctoral fellow at the University of California at Berkeley. In 1978, Summerton and Bartlett were issued the first patent in the antisense field (US Patent 4,123,610). Dr Summerton founded the pioneer antisense company, Antivirals Inc (now AVI BioPharma) in 1980. On 1 January 1985, he devised the Morpholino structural type, and four years later he and Weller developed an improved linkage type. In 1997, he left Antivirals Inc to form the spin-off company, Gene Tools, LLC, which has focused on commercialising Morpholinos for use as research tools, as well as developing technologies for delivering antisense oligos into cells. In 2005, Dr Summerton completed development of the Endo-Porter system for non-toxic delivery of Morpholinos (and other large polar molecules) into the cytosol of cells; his next goal is to adapt Endo-Porter for delivery of Morpholinos *in vivo*.

Oligos which bind to complementary RNA sequences are commonly called 'antisense' oligos because they are typically used to bind the 'sense' sequence of a messenger RNA. Antisense oligos are used for identifying the function and studying the control of genes, as well as for validating prospective protein targets in drug development programmes. Such oligos also promise (but have yet to deliver) therapeutics for a broad range of currently intractable diseases. Of the several hundred oligo structural types developed over the past three decades, less than half a dozen have gained acceptance for research and biopharmaceutical development. Morpholinos are in this select group, with over a thousand scientific publications wherein Morpholinos were used.

Morpholinos constitute a radical re-design of DNA. Key structural features, shown in Figure 1, are: 1) the 5-membered deoxyribose rings of DNA are replaced by 6-membered morpholine rings; and 2) the negatively-charged inter-subunit linkages of DNA are replaced by non-ionic inter-subunit linkages. These changes provide decisive advantages over the more conventional oligo types used for modulating gene expression. As a consequence of their novel backbone structure, Morpholinos are completely stable in biological systems, allowing rigorous long-term applications. Because they do not interact with proteins, they are free of the host of off-target effects, which plague both the historically popular Phosphorothioate DNA and the currently popular RNAi and siRNA structural types.

A BRIEF HISTORY: CONSERVATIVE ANTISENSE DESIGNS

In the early days of antisense research (the 1970s and 1980s), most of the focus was on improving the stability of DNA and RNA oligos, which have half-lives of only a few minutes in living systems. Most early efforts entailed making minimal changes in the structure of DNA or RNA oligos in an effort to block enzymatic degradation, while still retaining the oligo's ability to bind its complementary RNA sequence. Such modifications included modifying just one end of the oligo (Zamecnik & Stephenson), replacing one oxygen of the phosphodiester inter-subunit linkages with methyl (Miller & Ts'o) or with sulfur (Stec, Zon & Egan) or with alkylamines (Froehler & Matteucci), or entirely replacing the phosphodiester linkages with carbamates (Stirchak, Summerton & Weller (1)).

While these minimal structural modifications did increase resistance to degradation, oligos utilising these conservative modifications also suffered from serious limitations. As a case in point, the most popular of the early structural types was S-DNA (phosphorothioate DNA), wherein an oxygen on the phosphate was replaced by a sulfur (see Figure 2). This modest change

improved stability (increasing the half-life from minutes for DNA oligos to hours for S-DNA oligos), while retaining good activity against targeted RNA sequences. However, continuing research with S-DNAs brought to light a host of serious problems. Because of their limited sequence specificity, numerous off-target effects and low targeting predictability, it appears that many of the biological effects generated by any given S-DNA are typically *not* due to inhibition of the targeted mRNA.

By the mid-1980s, there was reason to suspect that the conservative structural modifications to DNA and RNA being pursued by most antisense research groups might never be adequate for achieving optimal antisense activity, particularly for therapeutic applications. This led a few adventurous souls to attempt a radical re-design of genetic material in the hope of developing a truly optimal antisense structural type. The most notable successes from such attempts over the past 20 years are Morpholinos, developed in Oregon by myself and Weller (2, 3, 4), and Peptide Nucleic Acids (PNAs), developed some years later in Denmark by Nelsen and Egholm.

MORPHOLINOS

Why the Morpholine Ring?

In the 1980s, it appeared that the very high cost of producing DNA-like antisense oligos might be a major impediment to their future therapeutic applications. In 1984, I postulated that most of these costs would be substantially reduced if one could utilise the far cheaper ribonucleosides (about 30-fold cheaper), and if one could devise an oligo structure which was assembled via couplings to relatively reactive amines, instead of far less reactive hydroxyls, as is required for assembly of DNA and RNA-type oligos. This postulate led to my devising the Morpholino structural type, which my preliminary molecular modelling suggested should be capable of binding to complementary DNA. Thus, a cheap ribonucleoside subunit could be converted in a single-pot synthesis to a Morpholino subunit, as illustrated in Figure 3. After addition of protective groups and an activated linker, such Morpholino subunits can be efficiently coupled into oligos.

As the assembly of Morpholino oligos entails couplings to the relatively reactive amine of the morpholine ring, the Morpholino subunits can be joined under mild conditions in extremely high yields without the need for catalysts, subsequent oxidations or capping after every coupling. Further, Morpholinos can be assembled on an inexpensive high-load polyacrylamide synthesis resin using a minimum of solvents and reagents. Most important, subunits can be activated in bulk and stored for up to a year before use. Because they are coupled without a catalyst, excess subunit used to drive the coupling to completion can be largely recovered, purified and re-used – leading to substantial further savings not achievable with DNA and RNA-type oligos.

Figure 1: Comparison of DNA and Morpholino structures

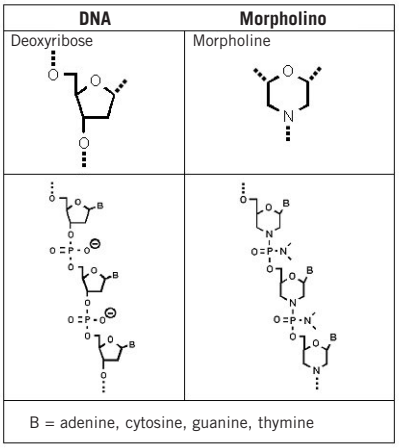
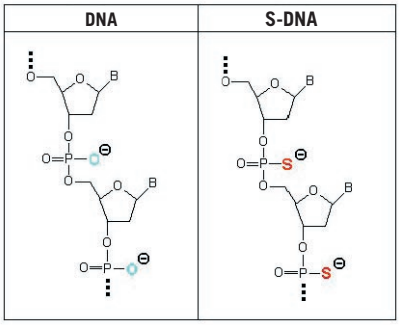


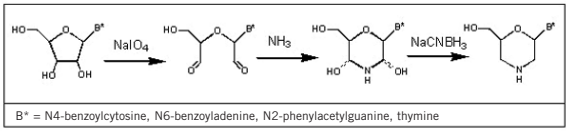
Figure 2: Comparison of DNA and S-DNA structures



Why the Non-Ionic Backbone?

By the mid-1980s, it was apparent that antisense oligos could be rendered completely resistant to enzymatic

Figure 3: Conversion of ribonucleoside to Morpholino subunit



degradation simply by using non-ionic inter-subunit linkages. However, that desirable stability was counterbalanced by the fact that, up to that point, all non-ionic antisense oligos that had been developed had suffered from very low aqueous solubilities.

When I prepared the first version of a Morpholino (having non-ionic carbamate linkages (5)) it was found, as expected, that the oligo was completely stable in biological systems and, also as expected, that it had poor aqueous solubility. Regrettably, while that oligo had a good affinity for DNA, it had a depressingly low affinity for its complementary RNA. This was a severe disappointment because RNA would be the true target in most applications. This poor binding led me to carry out molecular modelling in an effort to understand the cause of the poor binding to RNA. The results led to the postulate that the poor binding to RNA might be remedied by using a more flexible type of inter-subunit linkage. To test this postulate, a number of additional Morpholino varieties were prepared with more flexible linkage types (2).

As hoped, going to more flexible linkage types did indeed greatly improve binding to RNA (2). Surprisingly, the best of these new Morpholino varieties also exhibited a several-hundred-fold increase in aqueous solubility – resulting in excellent solubility on a par with that of DNA and RNA. Subsequent biophysical studies (6) suggested that the poor aqueous solubility of non-ionic oligos comes not from the lack of charges on their backbones, but instead from poor stacking of their nucleobases (their four genetic letters, designated 'B' in Figure 1, page 33). That same study showed that the Morpholino backbone with the current linkage type (see Figure 1) affords excellent stacking of these nucleobases.

Current Morpholinos have excellent aqueous solubility *and* complete stability in biological systems. In contrast, S-DNA and siRNA are degraded at a moderate rate in biological systems, which limits their value in longer-term applications.

COMPARISON WITH S-DNAs AND siRNAs

Oligos for modulating the expression of genes should have a number of key properties. For most research applications these include: 1) high efficacy; 2) very high sequence specificity; 3) good (preferably complete) stability in biological systems; 4) predictable targeting; and 5) general lack of off-target effects. Because most genes in higher organisms are now estimated to code for an average of three or more different proteins via an alternative splicing mechanism, it is also highly desirable that such oligos also provide: 6) a capability for precisely modifying the splicing of RNA transcripts. For therapeutic applications oligos should have the additional properties of: 7) non-toxicity; and, 8) affordability.

Efficacy

Conventional wisdom in the antisense field is that some sort of natural cofactor, such as RNase H with S-DNAs or the RISC complex with siRNAs, is required to achieve a high level of gene knockdown. However, Morpholinos function independently of natural cofactors and still generally achieve appreciably higher levels of gene knockdown than either S-DNA (3) or siRNA (7).

Sequence Specificity

To achieve very high sequence specificity, an antisense oligo should bind a minimum of about 14 contiguous genetic letters before it acts on an RNA sequence (4). Both S-DNAs in conjunction with RNase H, and siRNAs in conjunction with the RISC complex, act on appreciably shorter sequences – which virtually assures that essentially every oligo of these types will also inhibit the expression of multiple non-targeted RNAs (4, 8). In sharp contrast, when used at 37^{0}C, Morpholinos have been shown to require minimum target sequences of about 14 to 16 bases (3, 4), and so Morpholinos satisfy this basic requirement for very high sequence specificity. However, it should be noted that Morpholinos were optimised for use at about 37^{0}C; when used at much lower temperatures (such as in frog embryos at 18^{0}C), a few Morpholinos have been reported to inhibit some non-targeted genes.

Stability

As discussed earlier, Morpholinos are completely stable in biological systems, while S-DNAs and siRNAs are degraded at a moderate rate.

Targeting

Targeting success rates for S-DNAs are commonly of the order of 10% to 20%, meaning that typically 80% to 90% of S-DNAs are ineffective. Targeting success rates for siRNAs are typically reported to be in the range of about 20% to 33%, so a majority of siRNAs are also

ineffective. In contrast, targeting success rates for Morpholinos are generally in the range of 70% to 80%, which means that generally the first Morpholino you try is quite effective against its targeted RNA.

Off-Target Effects

As discussed previously, because S-DNAs interact with so many extracellular, cell surface and intracellular proteins, they are notorious for causing a host of off-target effects. As siRNAs become more widely studied, there has been an increasing number of reports describing their off-target effects. What has been found is that a typical siRNA can cause the down-regulation of dozens to hundreds of non-targeted genes, as well as the up-regulation of a substantial number of other non-targeted genes (8). In contrast, because Morpholinos do not interact appreciably with biological structures other than their targeted RNA sequences, and because they work by a simple steric block mechanism, they are generally free of such off-target effects.

Splice Modification

S-DNAs and siRNAs act in concert with their cellular co-factors to destroy their targeted RNAs, and so they cannot be used to modify splicing. Morpholinos, on the other hand, are becoming well-known as the premier tools for modification of splicing.

Non-Toxicity

Early toxicity studies with S-DNAs in mice occasionally led to convulsions and death within a matter of minutes after intravenous injection. To preclude such deaths, S-DNAs are now slowly infused over long periods of time in order to render the toxic effects sub-lethal by distributing them over time. To date, there appears to be little information on the toxicity or lack of toxicity of siRNAs *in vivo* – but I understand that such studies are underway and results should soon be available. In regard to Morpholinos, AVI BioPharma and its collaborators have run a substantial number of toxicity studies on Morpholinos in a variety of animals and in humans (Phase I and Phase II clinical trials). In all cases that I am aware of, the Morpholinos exhibited essentially no toxicity.

Affordability

The cost advantages of Morpholino oligos over DNA and RNA-type oligos have been discussed in the section above describing the selection of the Morpholino backbone structure.

DELIVERY INTO CULTURED CELLS

Until the late-1980s, most antisense experiments were carried out in cell-free test systems, where the focus was on assessing prospective structural types for directly inhibiting the function of their targeted messenger RNAs. However, as the antisense field matured and antisense experiments began to be carried out in cultured cells, careful experiments indicated that antisense oligos were ineffective in cultured cells. The problem turned out to be that most of the oligos were not getting into the cells, and those that did enter cells were only getting into endosomes/lysosomes, where they had no access to their targeted RNA sequences, which reside in the cytosol/nuclear compartment of the cell.

These findings led to wide-ranging efforts to develop effective methods for delivering oligos into the cytosol/nuclear compartment of cultured cells, and at this time, effective methods are available for delivering essentially all oligo types into cultured cells. Most such delivery systems are, however, quite complex to use, do not work well in the presence of serum and most are relatively toxic to the cells after just two to four hours of contact.

Until the late-1980s, most antisense experiments were carried out in cell-free test systems, where the focus was on assessing prospective structural types for directly inhibiting the function of their targeted messenger RNAs. However, as the antisense field matured and antisense experiments began to be carried out in cultured cells, careful experiments indicated that antisense oligos were ineffective in cultured cells.

Quite recently, Gene Tools LLC introduced a delivery reagent for cultured cells (Endo-Porter), which is very simple to use, works well in the presence of serum and causes little or no toxicity to cells, even after continuous contact for 30 to 40 hours. The key to avoiding the substantial toxicity that plagues most delivery systems is to co-deliver both the delivery reagent and the cargo (Morpholino oligo) via the natural endocytotic pathway.

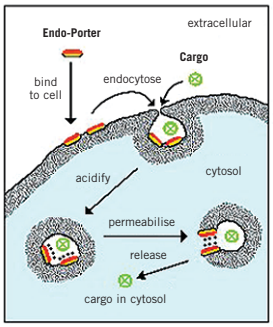


Figure 4: The 'Endo-Porter' delivery system

Once both the delivery reagent and the cargo have entered an endosome, and that endosome has undergone its natural acidification process, the lowered pH converts the delivery reagent to a form which permeates the endosomal membrane – thereby releasing the cargo into the cytosol of the cell. This non-toxic delivery process is illustrated in Figure 4, and the experimental work underlying the development of this advanced delivery reagent is described in a paper soon to be published in the Annals of the New York Academy of Sciences (9).

DELIVERY *IN VIVO*: THE FINAL CHALLENGE?

While methods for delivering antisense oligos to the cytosol/nuclear compartment of cultured cells are now fairly well developed, most of these methods appear to be ineffective in the presence of serum and/or are too toxic for use *in vivo*. In light of these limitations, it came as a surprise to many in the antisense field when claims began to circulate in the mid-1990s that by some as yet undefined mechanism, bare antisense oligos are able to readily gain entry into the cytosol/nuclear compartment of cells *in vivo* without the need for any delivery reagent or procedure. Subsequently there have been many additional claims of successful delivery *in vivo* simply by injecting bare oligos.

Given what we know about the barriers to delivery into the cytosol/nuclear compartment in cultured cells, and the possibility that off-target extracellular effects can be misinterpreted as antisense effects, I believe that all claims of successful delivery of antisense oligos *in vivo* should be viewed with great scepticism, unless the evidence is both unequivocal and can be repeated by independent investigators. In my opinion, this level of proof for effective *in vivo* delivery of antisense oligos has not yet been met.

IN CLOSING...

The bad news as I see it is that there are good reasons to doubt that safe, effective and practical delivery of antisense oligos has yet been achieved *in vivo* – and until it is achieved, the great potential of antisense therapeutics will remain only a distant promise.

The good news is that all eight of the key properties desired for antisense oligos are now present in a commercially available structural type – so that if and when safe and effective *in vivo* delivery is finally achieved, then the long-promised deluge of antisense therapeutics should rapidly come to fruition.

The author can be contacted at jsummerton@gene-tools.com

References

1. **Stirchak, Summerton and Weller (1987). Uncharged stereoregular nucleic acid analogs: 1. Synthesis of a cytosine-containing oligomer with carbamate internucleoside linkages. *J Organic Chemistry*, 52, 4,202.**

2. **Summerton and Weller (1997). Morpholino antisense oligomers: design, preparation and properties. *Antisense & Nucleic Acid Drug Development*, 7, 187.**

3. **Summerton (1999). Morpholino antisense oligomers: the case for an RNase H-independent structural type. *Biochemica et Biophysica Acta*, 1,489, 141.**

4. **Summerton (2004). Morpholinos and PNAs compared. *Letters in Peptide Science*, 10, 215.**

5. **Stirchak, Summerton and Weller (1989). Uncharged stereoregular nucleic acid analogs: 2. Morpholino nucleoside oligomers with carbamate internucleoside linkages. *Nucleic Acids Research*, 17, 6,129.**

6. **Kang, Chou, Johnson, *et al*. (1992). Stacking interactions of ApA analogues with modified backbones. *Biopolymers* 32, 1,351.**

7. **Munshi, Graeff and Lee (2002). Evidence for a causal role of CD38 expression in granulocytic differentiation of human HL-60 cells. *J Biological Chemistry*, 277, 49,453.**

8. **Jackson *et al*. (2003). Expression profiling reveals off-target gene regulations by RNAi. *Nature Biotechnology*, 21, 635.**

9. **Summerton (in press). Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. *Annals of the New York Academy of Sciences*.**