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Review

Gene Knockdowns in Adult Animals: PPMOs and Vivo-Morpholinos

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Abstract: Antisense molecules do not readily cross cell membranes. This has limited the use of antisense to systems where techniques have been worked out to introduce the molecules into cells, such as embryos and cell cultures. Uncharged antisense bearing a group of guanidinium moieties on either a linear peptide or dendrimer scaffold can enter cells by endocytosis and subsequently escape from endosomes into the cytosol/nuclear compartment of cells. These technologies allow systemic administration of antisense, making gene knockdowns and splice modification feasible in adult animals; this review presents examples of such animal studies. Techniques developed with PPMOs, which are an arginine-rich cell-penetrating peptide linked to a Morpholino oligo, can also be performed using commercially available Vivo-Morpholinos, which are eight guanidinium groups on a dendrimeric scaffold linked to a Morpholino oligo. Antisense-based techniques such as blocking translation, modifying pre-mRNA splicing, inhibiting miRNA maturation and inhibiting viral replication can be conveniently applied in adult animals by injecting PPMOs or Vivo-Morpholinos.

Keywords: Antisense; Morpholino; PPMO; Vivo-Morpholino.

1. Introduction

Morpholino oligos are steric-blocking antisense molecules which bind to RNA and get in the way of cellular processes. These oligos have no electrical charge, do not interact strongly with proteins, and do not require the activity of RNase-H, Argonaute, or other catalytic proteins for their activity

Antisense molecules however do not readily cross cell membranes without delivery techniques, which has prevented their effective use in adult animals [1]. With delivery of Morpholino oligos achieved *in vivo* by Vivo-Morpholinos and Morpholino oligos linked to arginine-rich cell penetrating peptides (PPMOs), a longstanding barrier to applying Morpholino antisense techniques in adult animals has been overcome. Vivo-Morpholinos and PPMOs enter cells from the extracellular space and gain access to the cytosol and nuclear compartments. Antisense effects can be observed after systemic delivery of Vivo-Morpholinos or PPMOs.

1.1. Uses of unmodified Morpholinos

Effective techniques for delivering the oligos to the cytosol and nuclear compartments in tissue cultures have been developed, such as mechanical scraping [2], electroporation [3] or use of endosomal escape reagents [4]. Unmodified Morpholinos have been used routinely to block translation, modify splicing, inhibit miRNA activity and inhibit viral replication as well as more exotic RNA-blocking applications [5]. The Morpholino antisense structural type has been a revolutionary tool in developmental biology [6]. Success in embryos came by microinjecting Morpholino oligos into eggs or single or few celled zygotes, so that during cell division the Morpholinos were apportioned into daughter cells. This neatly avoided the problem of delivering the antisense separately into each cell of a many-celled organism [7]. In addition to their use to determine gene functions and interactions in embryos, Morpholino antisense oligos have been used for research into a broad range of diseases as well as several clinical trials (AVI BioPharma, Inc.). In most cases, carrying work from research to therapeutic applications of unmodified Morpholino oligos has been limited by difficulties with *in vivo* delivery [8]. However, delivery of relatively high doses of unmodified Morpholinos into dystrophic muscle in animal models of DMD has induced expression of functional Dystrophin in skeletal muscle [9,10] and unmodified Morpholinos are currently in clinical trial for treatment of DMD (http://clinicaltrials.gov/ct2/show/NCT00844597).

1.2. Morpholino chemistry and nomenclature

Morpholino oligos are manufactured from ribosides. The ribose ring is opened by oxidation, re-closed on ammonia and the product subsequently reduced to substituted morpholine. The base and the morpholine nitrogen are protected and the subunit is activated with a dimethylamino phosphoro-dichloridate. The activated subunits are added to a synthesis resin with washing, deprotection and activation steps for each activated base added. Oligos are cleaved from the resin and deprotected with ammonium hydroxide, then purified and quantitated, often followed by lyophilization and sterilization [11]. Substitutions such as peptides or the dendrimer scaffold for guanidinium may be added to the 3' morpholine nitrogen while the oligo is still attached to the synthesis resin. Alternatively, peptides may be added in the solution phase after resin cleavage and purification steps.

The ends of a Morpholino oligo are described as 3' and 5', but these labels do not refer to properly numbered atoms of the Morpholino backbone. Instead the atom designations of natural nucleic acids are used by analogy to label Morpholino ends; nucleic acids have a 5'-methylene hydroxyl (often phosphorylated) at one end and a 3'-ring hydroxyl at the other end. The methylene attached to the morpholine ring is designated as the 5' end of a Morpholino subunit and the morpholine nitrogen is

the Morpholino oligo familiar to molecular biologists, who are accustomed to referring to the 5' and 3' ends of RNA and DNA.

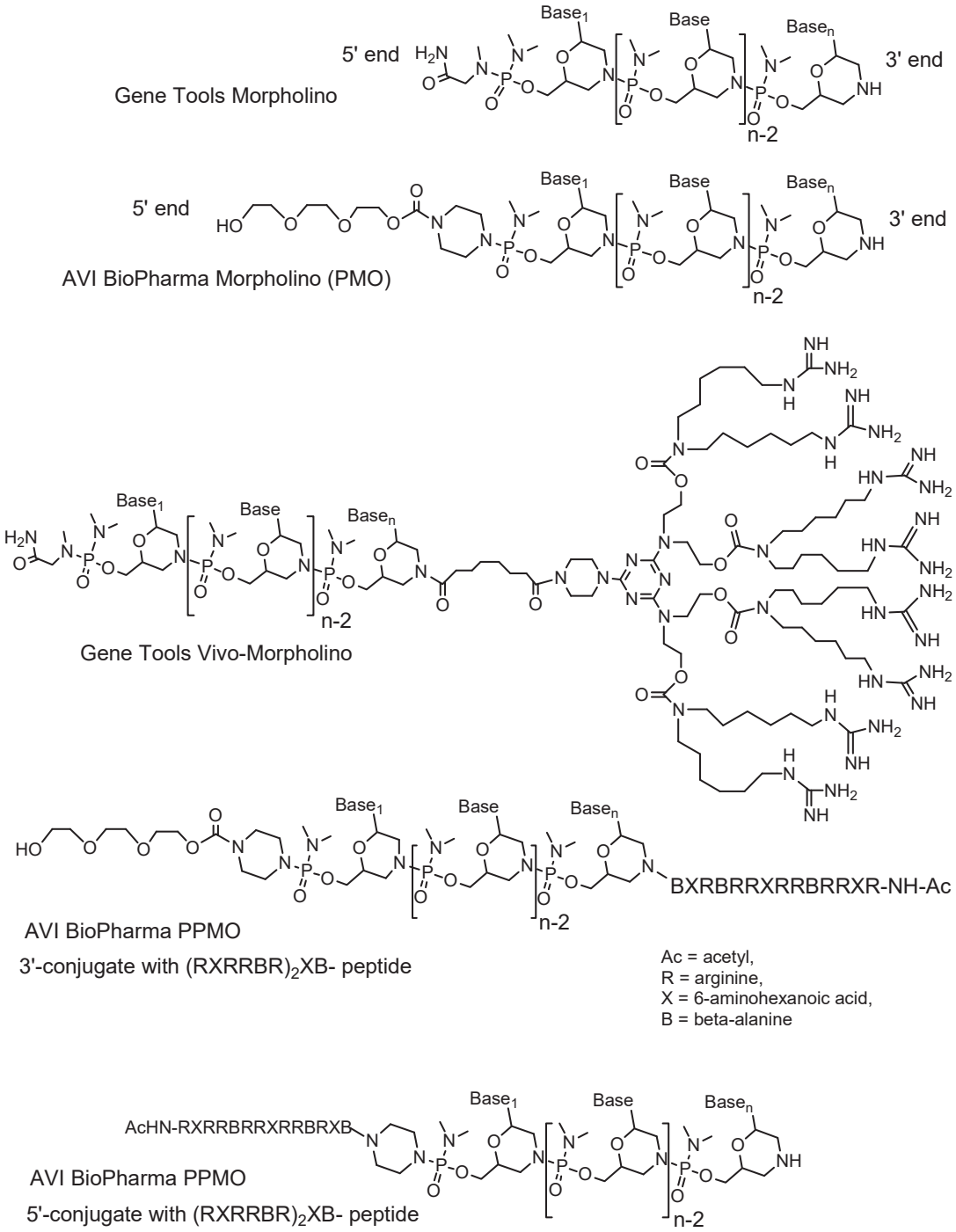
1.3. Endocytosis and the barrier to entry into the cytosol/nuclear compartment

Unmodified Morpholino oligos are endocytosed but have little to no activity against their RNA targets. Uptake by endocytosis does not mean the antisense oligos are reaching the cytoplasm or nucleus of the cell. Fluorescently-labeled Morpholinos can be detected as dim punctuate fluorescence within endosomes, but in most cells too little antisense escapes from the endosomes to the cytosol/nuclear compartment to be biologically active [2]. Morpholinos are not degraded in endosomes [12] but remain trapped. Many biological problems are best studied in adults and so a method that allows entry of antisense into most or all cells in an adult organism is very desirable. Ideally this systemic delivery could be accomplished by routine means such as intravenous or intraperitoneal injections. A technology that allows enough of the endocytosed oligos to escape from endosomes to have biological activity would enable *in vivo* delivery of oligos administered by systemic injection. Such technologies have now been developed: PPMOs and Vivo-Morpholinos. A PPMO may have an arginine-rich cell-penetrating peptide linked to either the 3' end or the 5' end of the Morpholino oligo [13]. The arginines have guanidinium moieties as part of their side chains, and the presence of these guanidiniums have been shown to increase cellular uptake of conjugated materials [14]. Studies of the mechanism of PPMO entry have shown that the cell-penetrating peptides offer two advantages over unmodified Morpholinos: enhanced uptake into endosomes and, critically, enhanced ensodomal escape [15]. A Vivo-Morpholino has an octaguanidinium dendrimer constructed on the 3' end of a Morpholino oligo [16] (Figure 1).

1.4. PPMOs

The first effective chemically-mediated method for systemic delivery of Morpholino antisense was based on covalently linking the oligos to cell-penetrating peptides, using arginine-rich cell-penetrating peptides to deliver uncharged antisense molecules [17]. These molecules are called peptide-linked phosphorodiamidate Morpholino oligomers, or PPMO for short. Research to develop more safe and effective peptide sequences for PPMOs has been the focus of Hong Moulton's group at AVI BioPharma Inc. [18,19], where preclinical work on PPMOs for Duchenne muscular dystrophy is currently ongoing. Studies on PPMOs have shown that uptake of the oligos is an energy-dependant and temperature-dependant process that can be prevented using endocytosis inhibitors; these characteristics indicate that uptake of the PPMOs is by endocytosis [20]. A fraction of endocytosed PPMOs escape from the endosome, entering the cytosol and nuclear compartment where they can block mRNA translation and modify pre-mRNA splicing [21]. Intravenous injection [22], intraperitoneal injection or intranasal administration [23] of PPMOs to mice have inhibited viral replication. Injections of PPMOs into transgenic mice carrying engineered splice-reporter genes have triggered expression of green fluorescent protein throughout the tissues [24].

Figure 1. Structures of unmodified and delivery-enabled Morpholino oligos.



Positively-charged arginine amino acid residues are not electrostatically attracted to uncharged antisense such as Morpholino oligos or peptide nucleic acid (PNA) oligos, so the positively charged arginines are free to interact with membranes [25]. At the end of an arginine amino acid's side chain is a guanidinium group, which carries the amino acid's positive charge. This charged guanidinium group is not only attracted to a phosphate because of the phosphate's negative charge, it can also form two hydrogen bonds with the phosphate's oxygens. This makes the attraction of a guanidinium and a

phosphate an unusually strong non-covalent interaction [26]. It may be this strong interaction that distorts the endosomal membrane and renders it permeable.

1.5. Vivo-Morpholinos

Vivo-Morpholinos are Morpholino antisense oligos covalently linked to a molecular scaffold that carries a guanidinium group at each of its eight tips. These custom-sequence antisense molecules enable Morpholino applications in adult animals. Vivo-Morpholinos have been shown effective in mice [27,28] and are being tested in rats, adult zebrafish and various organ explants.

To make a Vivo-Morpholino, the scaffold that will hold the guanidinium groups is added to the morpholine nitrogen at the 3'-end of a Morpholino oligo while the oligo is still bound at its 5' end to the synthesis resin. Subsequent treatment with ammonia cleaves the Vivo-Morpholino from the synthesis resin, de-protects the Morpholino's bases and de-protects the eight tips of the 3'-terminal scaffold. Next, treatment with *O*-methyl isourea converts the eight terminal amino groups to guanidinium groups [16].

2. Targets and outcomes of Morpholino experiments

Morpholino oligos bind to complementary RNA. If a molecular process normally occurs at the site where the Morpholino is bound to RNA, the Morpholino might be suitably positioned to get in the way of that process and prevent it from occurring. This mechanism is called steric blocking [29]. The effect of a Morpholino on a cell depends on where the Morpholino binds to the RNA, which determines whether it can block a process and which process it blocks. There are three common applications for Morpholino oligos: blocking translation of mRNA, modifying splicing of pre-mRNA or inhibiting miRNA activity [5]. In addition, work has been done on viral targets such as internal ribosomal entry sites [3] and cyclization sequences [30]; much of the reported work with PPMOs has involved viral targets [31]. Exotic targets have also been explored, such as binding to slippery sites to trigger ribosomal frameshifting [32] or binding to ribozymes to prevent catalytic cleavage of RNA [33].

2.1. Translation blocking

Translation blocking is the simplest method for knocking down gene expression with a Morpholino. When making a protein by cap-dependant translation, the small subunit of a ribosome binds to the 5' cap of an mRNA and, along with some other proteins, forms an initiation complex. The initiation complex moves along the 5' untranslated region (UTR) of the mRNA until a start codon is reached. At the start codon, the large subunit of the ribosome docks to small ribosomal subunit and then the process begins of linking together amino acids to form the new protein. Morpholinos that block the journey of the ribosomal initiation complex from the 5'-cap to the start codon, halting translation of the mRNA before the linking of amino acids can start. These Morpholinos can bind to RNA targets anywhere in the 5'-UTR through the start of the coding region, so long as the oligo binds onto the start codon or upstream (to the 5' direction) of the start codon [34].

The activity of a translation blocking oligo is assayed by an immunochemical method, typically Western blotting. Halting translation of a protein will not immediately cause a detectable change in the protein's signal on a Western blot. Some preexisting protein must be degraded over time before the

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