Editorial

Making Sense Out of Anti-Sense: Challenges of Imaging Gene Translation with Radiolabeled Oligonucleotides

Watson-Crick base pair formation confer an extremely high degree of specificity to deoxynucleic acid (DNA) structure. Calculations estimate that an oligonucleotide (oligo) of 15 to 17 nucleotides in length would have a unique sequence relative to the entire human genome. In principle, this specificity has been the driving force behind current attempts to develop oligodeoxynucleotides for therapy and diagnosis of human disease. For therapeutic applications, oligos complementary to nucleotide sequences of messenger ribonucleic acid (mRNA), termed antisense oligos, are thought to interfere in a sequence-specific manner with translation of host cell or viral mRNA into protein (*1,2*). Other targets for antisense oligos include donor-acceptor sites for splicing pre-mRNA (*3*), for example, to inhibit replication of the human immunodeficiency virus-type 1 (HIV-1) (*4*) and genomic DNA, where Hoogsteen base pairing in the major groove can form triple-helical structures (*5*). Rather than interfering with physiological processes, diagnostic applications view these intracellular sites as high affinity "receptors," amenable to binding a radiolabeled antisense oligo with high specificity for use in imaging the mRNA content of the target tissue. Two recent papers in the *Journal* have begun to explore radiolabeled antisense oligos, one demonstrating how the radiotracer method can be used to document the fate of a novel class of receptor-targeted oligo conjugates designed with the intent of targeting DNA delivery to selected tissues (*6*), the other describing radiolabeled oligos for imaging (*7*). There are significant challenges ahead, many common to both therapeutic and diagnostic applications of antisense technologies. I refer the interested reader to several excellent recently published reviews pertaining to antisense oligos, largely from the perspective of chemical synthesis and therapeutic applications (*1,8,9*). This brief overview will summarize lessons derived from therapeutic oligo development with the intent of critically assessing the benchmarks that must be met for diagnostic applications.

For successful antisense imaging, at least six criteria must be fulfilled (*9*).

1. Oligos Must Be Synthesized Easily and In Bulk

Phosphoramidite chemistry (*10*) and its development into an automated technology now allow routine synthesis and purification of gram quantities of antisense oligos. This has lead to much commercial activity in the development of therapeutic uses for oligos. However, radiolabeling the oligos typically requires construction of a conjugate molecule, consisting in general of the oligo, a linker, and a metal chelating or targeting moiety for tagging with a radionuclide. These place additional constraints on synthesis and purification harsher even than those faced in therapeutic applications. While both the 5′ alkyl primary amine linker strategy (*11*) employed by Dewanjee et al. (*7*) and the poly-(L)lysine linker (*12*) employed by the Yarmush group (*6*) are well established, they nonetheless introduce potential complications in synthesis and quality control. Strategies to formulate kit preparations are important first steps toward developing these agents for routine clinical imaging (*7*).

2. The Oligos Must Be Stable In Vivo

This problem is formidable and was addressed by both investigative groups with results that point to the significant challenges (and opportunities) that remain. The problem is multifaceted. First, the human body is rich in serum and intracellular endo- and exonucleases which degrade the phosphodiester backbone of naturally occurring oligos (*13*). Much medicinal chemical and synthetic effort has been directed toward the development of nuclease-resistant oligos. Introduction of phosphate, sugar and pyrimidine modifications into the oligos has lead to significant reductions in nuclease sensitivity, however, changes in membrane permeability, binding affinity of the oligos to the target mRNA and ability to activate ribonuclease (RNase) H are simultaneously altered in complex ways (*14*). Phosphorothioate, methylphosphonate and phosphotriester substitutions for the phosphodiester backbone have been developed, the former two also readily prepared by automated synthesizers (*15,16*). Indeed, Dewanjee et al. (*7*), compared phosphodiester and phosphorothioate radiolabeled oligos under a variety of conditions and generally confirmed the enhanced stability of the phosphorothioate derivatives. Some approaches even eliminate the phosphodiester backbone completely by replacement with a polyamide analog (*17*). Second, the oligo-linker conjugate must remain stable in vivo. Lu et al. (*6*) report 30% dissociation of their glycoprotein-poly-(L)lysine-DNA complex after 7 min under chromatographic conditions and up to 85% dissociation by preincubation in phosphate buffer or media plus serum for 1 hr before chromatographic analysis. This bodes poorly for sequence-specific targeting in vivo. Dewanjee et al. (*7*) showed 15%–35% degradation of

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their oligo complexes after a 2-hr incubation in human plasma or in a P388 cell suspension. Third is a concern unique to imaging applications with oligos: the radiolabel must remain bound to the antisense oligo-chelate by designing and synthesizing complexes that minimize transmetallation or demetallation reactions. These caveats demonstrate the challenges faced in overcoming natural detoxification mechanisms in biological systems that appear poised to metabolize extracellular and intracellular DNA fragments and their derivatives.

3. The Oligos Must Be Able to Enter the Target Cells

Naturally occurring phosphodiester and synthetic phosphorothioate oligos are polyanionic and thus cannot passively diffuse across cell membranes. It has been shown that lymphoid cells bind DNA on their membranes (*18*). An ~80 kDa protein has been isolated from the cell membranes of CHO fibroblasts, HL60 cells and others (*19,20*) that binds polyanionic oligos in a calcium-dependent manner (*21,22*). However, cell surface binding cannot explain the many examples of sequence-specific inhibition of mammalian mRNA translation by antisense oligos. Thus, internalization of polyanionic oligos must occur, and has been generally found in most cells to depend on time of exposure and on oligo concentration. These observations have led to a model of cellular internalization of oligos by processes known as receptor-mediated fluid-phase pinocytosis and adsorptive endocytosis (*19*). This is the physiological process whereby cells pinch off surface membrane and engulf bulk extracellular medium into plasma membrane-derived vesicular structures found in the cytosol. Thus, early intracellular compartments for internalized oligos likely are vesicular structures such as endosomes (*19*). Using fluorescence-tagged oligos, punctate structures within cells are often observed (*23*), however, there is little evidence that oligos enter acidic compartments such as late endosomes and lysosomes (*9*). Although oligos must exit these vesicular compartments to interact with their intracellular targets, the mechanisms of vesicular transport are not known. Interestingly, it has been reported that 5′ terminus modifications of polyanionic oligos with poly(L)lysine, for example, may mask the negative charge of the oligo, thus enhancing the transit of the oligo from these vesicular compartments into the cytoplasm (*24*). Indeed, the Massachusetts General Hospital group (*6*) used glycoprotein-poly(L)lysine-FITC conjugates as reporters of their antisense conjugates and found nuclear localization of the FITC-labeled complex by confocal microscopy. However, in the absence of a sequence-specific oligo within the complex to confer nuclear localizing properties, the interpretation of this result is unclear. Dewanjee et al. (*7*) used 5′ amino-linked DTPA conjugates of oligos. Their evidence for intracellular accumulation was indirect, coming from TCA precipitation of cell lysates which showed 60%–70% of the probe associated with an insoluble fraction, taken as evidence, although not definitive, that the oligo probe was bound to c-myc mRNA.

4. The Radiolabeled Oligos Must Be Retained by the Target Cell

There is evidence that oligos undergo exocytosis in vitro (*19,25*). In cells such as HL60, greater than 90% of the exocytosis occurs with half-times of 30 min or less and consists of full-length or truncated forms of the oligos (*9*). In addition, oligo chain extension has been found on exocytosed oligos (*4*). For radiolabeled oligos, these exocytotic processes present twofold complications. If the rates of exocytosis are equal to or greater than the rates of hybridization to target mRNA, then antisense cell targeting efficiency will be compromised (*9*). In addition, if cytosolic metabolism of the oligo-radiolabel complex were to occur and the free oligo transported out of the cell while the radioactive metal-chelate complex (often charged) is trapped in cytosolic compartments, then confounding background radioactivity unrelated to the mRNA target would be imaged.

5. The Oligos Must Be Able to Interact with Their Cell Targets

Intracellular antisense targets such as mRNA, pre-mRNA and genomic DNA are often protein bound, and it has been pointed out that many sites may not be accessible for Watson-Crick base pairing (*9*). Two of the most common targets for antisense approaches have been the 5′ cap and initiation codon (AUG) regions. Data indicate that many 3′ targets have binding constants several orders of magnitude lower than these 5′ regions (*26,27*), however, the 3′ untranslated region of the mRNA of the intercellular adhesion molecule ICAM-1, was reported to be a better target than the initiation region (*28*). These data point to the difficulty in establishing generalized principles for antisense applications. In this regard, although Dewanjee et al. (*7*) report that HPLC analysis of mRNA extracts of cell lysates in vitro showed 70%–80% of radiolabeled antisense probe bound to mRNA, tumor homogenates in vivo indicated a disappointing 25%–30% of total radiolabeled antisense bound to mRNA.

6. The Oligos Should Not Interact in a Nonsequential-Specific Manner with Other Macromolecules

There exists controversy whether the specificity of oligos is truly sequence-specific. It has been shown that longer oligos, rather than increasing specificity, may actually decrease specificity through non-sequential-specific and length-dependent increases in the number of potential hybridization sites (*27*). Thus, the 67 mer oligo that Lu et al. (*6*) chose for their study may represent a worst-case analysis. In addition, during experimental validation, many "control" therapeutic oligos (typically, the sense sequence or, alternatively, the scrambled sequence, that is, an oligo containing the same base composition, but scrambled order) have been found to produce biological effects that are indistinguishable from the antisense oligo (*14*). However, Dewanjee et al. (*7*) used sense oligos as control re-

agents for their studies and obtained encouraging results. The most compelling data suggesting that the oligocomplex was binding to target mRNA was the 2.5-fold enhancement of cellular accumulation in vitro and the 10-fold greater localizing properties during biodistributions in tumor-bearing Balb/c mice in vivo of radiolabeled antisense oligos compared to sense oligos. Perhaps the 5′ modifications of these oligos for radiolabeling also conferred favorable biological targeting properties, but much work remains to prove this hypothesis. Further complications arise because charged oligos (phosphodiesters and phosphorothioates) are polyanionic and may interact with naturally occurring protein targets for endogenous polyanions such as the glycosaminoglycans heparan, dermatan and chondroitin sulfate. For example, CD4, HIV-1 RT, gp120 and PKC β1 bind both charged oligos and sulfated polyanions, although the binding constants of phosphodiester oligos to these proteins are generally lower than those of their natural ligands (*9*).

Hence, the task of directly imaging the gene has begun. This brief overview highlighting some of the general principles and difficulties that have been learned from exploration of therapeutic oligos, illustrates concerns relevant to diagnostics that must be addressed if this exciting new technology of radiolabeled antisense oligos for the imaging of gene translation and transcription is to become clinical reality.

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