

Minireview

Antisense Oligonucleotides: Basic Concepts and Mechanisms

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Conceptual simplicity, the possibility of rational design, relatively inexpensive cost, and developments in the sequencing of human genome have led to the use of short fragments of nucleic acid, commonly called oligonucleotides, either as therapeutic agents or as tools to study gene function. Furthermore, in the past decade, the development of antisense oligonucleotide technologies as therapeutic agents has led to Food and Drug Administration approval for the commercialization of the first antisense oligonucleotide, Vitravene (for cytomegalovirus retinitis; Refs. 1 and 2), and to numerous clinical trials of therapeutic oligonucleotides (3).

The concept underlying antisense technology is relatively straightforward: the use of a sequence, complementary by virtue of Watson-Crick bp hybridization, to a specific mRNA can inhibit its expression and then induce a blockade in the transfer of genetic information from DNA to protein. However, although antisense oligonucleotides are commonly in use now both in the laboratory and clinic, this theoretical simplicity belies the many questions concerning the molecular mechanisms of action of these compounds. It is our contention that a highly critical approach must still be taken in interpreting data derived from experiments using antisense oligonucleotides. It remains relatively easy to claim that experimentally observed biological effects occur by an antisense mechanism; nevertheless, it is also easy to ignore those nonspecific effects that can provide a virtually identical explanation of the observed phenotype, especially when oligonucleotides with phosphorothioate backbones are used. These themes will be expounded in some detail in this review.

Oligonucleotide Chemistries

Oligonucleotides are unmodified or chemically modified single-stranded DNA molecules. In general, they are relatively short (13–25 nucleotides) and hybridize (at least in theory) to a unique sequence in the total pool of targets present in cells. Although it is not a complicated matter to synthesize phosphodiester oligonucleotides, their use is limited as they are rapidly degraded by the intracellular endonucleases and exonucleases, usually via 3'→5' activity (4–6). In addition, the degradation products of phosphodiester oligonucleotides, dNMP² mononucleotides, may be cytotoxic and also exert antiproliferative effects (7). Koziolkiewicz *et al.* (8) have

demonstrated that the toxic effects of dNMPs can be correlated with mononucleotide dephosphorylation by the cell-surface enzyme ecto-5'-nucleotidase. In human umbilical vein endothelial cells, and in HeLa cells, this enzyme dephosphorylates dNMP to the corresponding nucleoside, which then inhibits the function of other critical proteins, such as thymidine kinase (9), an event that can result in inhibition of cell growth. Deoxyribonucleotide phosphodiester oligonucleotides should therefore not be used in antisense experiments. However, many successful (to some extent) chemical modifications (Fig. 1) have been developed to attempt to overcome these problems.

The first chemically synthesized modified oligonucleotides were the methylphosphonates. Methylphosphonate oligonucleotides are noncharged oligomers, in which a nonbridging oxygen atom is replaced by a methyl group at each phosphorus in the oligonucleotide chain. Although these oligonucleotides have excellent stability in biological systems (10), the absence of charge reduces their solubility. Absence of charge also reduces their cellular uptake (11, 12), which appears to occur predominately via the process of adsorptive endocytosis (13) and not by diffusion through membranes (14). Whereas the absence of charge eliminates charge-charge repulsions that would ordinarily occur during the formation of an RNA-DNA duplex, methylphosphonate linkages are also inherently helix-destabilizing and, most importantly, cannot activate RNase H activity (see below). These features severely restrict their use as antisense effector molecules.

The phosphorothioates are the most widely studied oligonucleotides, because of their nuclease stability (although they are by no means nuclease proof) and relative ease of synthesis. However, the replacement of one of the nonbridging oxygens by sulfur at each phosphorus in the oligonucleotide chain introduces chirality at phosphorus. In fact, it is only the Sp phosphorothioate diastereomer that is nuclease resistant. The Rp diastereomer is as nuclease sensitive as a phosphodiester linkage. However, the Sp linkage is, like the methylphosphonate linkage, sterically helix destabilizing, a property that tends to decrease the melting temperature (T_m) of the oligonucleotide/mRNA complexes relative to the natural phosphodiester oligomer (15). However, phosphorothioate oligonucleotides are highly soluble and have excellent antisense activity. Critically, they are also capable of activating RNase H activity. During the last 2 decades, many reports have been published using this backbone to generate antisense effects both in tissue culture and *in vivo*. These data have led to the introduction of phosphorothioate oligonucleotides into clinical therapeutic trials. At the present time, the most promising of these are G3139, an 18-mer targeted to the initiation codons of the *bcl-2* mRNA (now being evaluated clinically in melanoma, chronic lymphocytic leukemia, and other tumors), and Isis 3521, a 20-mer targeted to the 3'

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² The abbreviations used are: dNMP, deoxyribonucleoside-5'-phosphate; ICAM, intercellular adhesion molecule; PNA, peptide nucleic acid; PN, phosphoramidate.

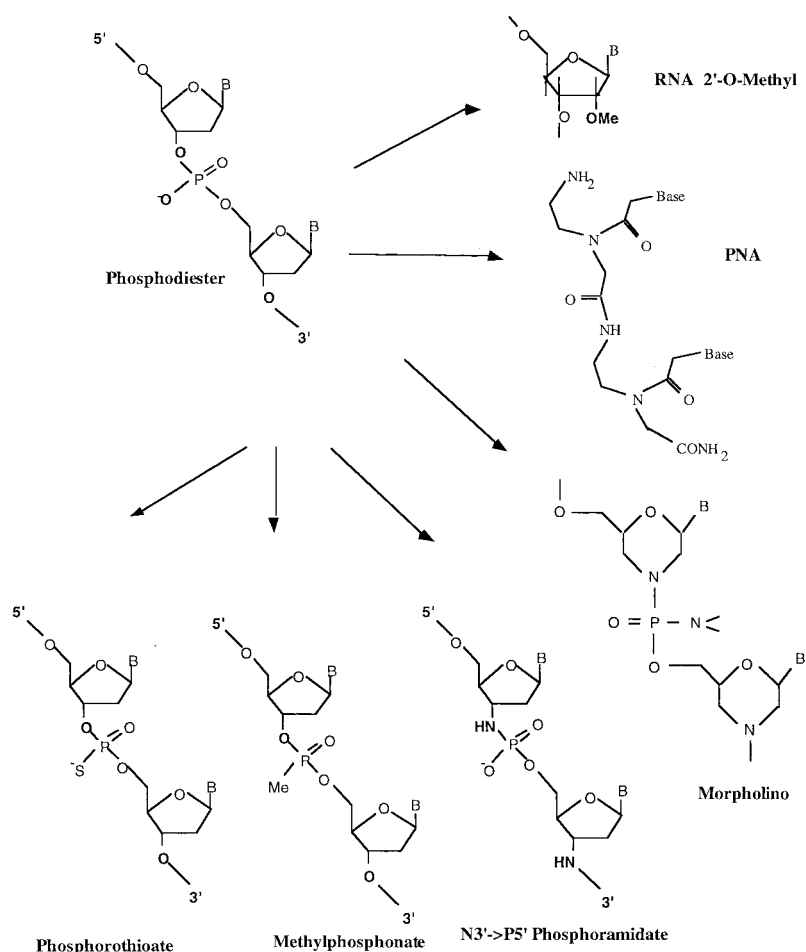


Fig. 1. Chemical structures of the oligonucleotides.

untranslated region of the protein kinase C- α isoform, which is being evaluated currently in non-small cell lung cancer. In preliminary studies with G3139 in combination with dimethyl triazeno imidazole carboxamide in advanced melanoma, several striking objective responses have been observed (16). In advanced lung cancer, early results with Isis 3521 when added to combination chemotherapy have raised speculation that life expectancy may be prolonged as much as 50% (17).

Despite the fact the phosphorothioates are the most widely used oligonucleotides, they have many properties which render them suboptimal antisense effector molecules. The phosphorothioate backbone is known to induce sequence-independent effects attributable to its length-dependent high affinity for various cellular proteins, especially heparin-binding growth factors, such as acidic fibroblast growth factor, basic fibroblast growth factor, platelet-derived growth factor, and vascular endothelial growth factor, and a host of other heparin-binding molecules, such as laminin, fibronectin, and Mac-1 (18–21). However, it also may well be true that these “problems” may actually contribute to observed clinical responses by synergizing with the down-regulation of the specific target. Indeed, it is difficult to

credit the notion that the extremely specific down-regulation of a single target (and virtually no other targets) will lead to much in the way of clinical responses. Viewed in this light, the attractiveness of the phosphorothioate backbone markedly increases, and we contend that despite the interesting properties of chemically modified backbones that we will describe, the phosphorothioates will continue to be extensively, perhaps even exclusively, used in the clinical trial setting.

Nevertheless, in an attempt to overcome the various non-specific problems, new chemical modifications have been developed. These “second-generation” oligonucleotides are resistant to degradation by cellular nucleases and hybridize specifically to their target mRNA with higher affinity than the isosequential phosphodiester or phosphorothioate. However, such antisense effects result from RNase H-independent mechanisms.

Extremely important, increasingly common oligonucleotide modification involves replacement of the hydrogen at the 2'-position of ribose by an O-alkyl group, most frequently methyl. These oligonucleotides form high melting heteroduplexes with targeted mRNA (22) and induce an antisense effect by a non-RNase H-dependent mechanism. Baker *et al.* (23) demonstrated that the 2'-O-(2-methoxy)ethyl oligonu-

cleotides, ISIS11158 and ISIS 11159, targeted to the 5' cap region of the human ICAM-1, modulate one or more of the earlier steps in mRNA translation, probably as a result of interference with translation initiation, more specifically with ribosomal assembly. This was demonstrated by the use of a polysome profile, in which a major portion of the targeted transcript in ISIS 11158- and 11159-treated cells was localized to the 40S and 60S subpolysome fractions. In contrast, the polysome profile with scrambled control oligonucleotide demonstrated that most of the full transcript could be found in the monosome or polysome fractions. Nevertheless, despite their inability to induce RNase H activity, these oligonucleotides were still potent inhibitors of ICAM-1 expression in human umbilical vein endothelial cells.

Stable oligonucleotides have also been produced that do not possess the natural phosphate-ribose backbone. PNAs are nucleic acid analogues that contain an uncharged, flexible, polyamide backbone comprised of repeating *N*-(2-aminoethyl)glycine units to which the nucleobases are attached via methylene carbonyl linkers (24, 25). These oligomers can form very stable duplexes or triplexes with nucleic acids: single or double-strand DNA or RNA (25, 26). The property of high-affinity nucleic acid binding can be explained by the lack of electrostatic repulsion because of the absence of negative charges on the PNA oligomers. Because PNAs are not substrates for the RNase H or other RNases, the antisense mechanism of PNAs depends on steric hindrance. PNAs can also bind to DNA and inhibit RNA polymerase initiation and elongation (27–29), as well as the binding and action of transcription factors, such as nuclear factor κ B (30). PNAs can also bind mRNA and inhibit splicing (31) or translation initiation and elongation (32–36).

Phosphorodiamidate morpholino oligomers also have several properties considered desirable for antisense purposes. Here, the deoxyribose moiety is replaced by a morpholine ring, and the charged phosphodiester intersubunit linkage is replaced by an uncharged phosphorodiamidate linkage (37). These oligonucleotides are very stable in biological systems (38) and exhibit efficient antisense activity in cell-free translation systems and in a few cultured animal cell lines (39, 40). However, as the morpholino oligomers are not charged, they should have far fewer nonspecific properties that phosphorothioate. Nevertheless, they are not substrates for RNase H and do not form complexes with cationic lipids or other commonly used cationic delivery reagents. To overcome this problem, Partridge *et al.* (41) have used a scrape loading technique to facilitate oligonucleotide penetration, thus maximizing their antisense effects. The permeation of cells with streptolysin O is another method that has been used for the cellular delivery of morpholino oligonucleotides (42).

Another example of a "second-generation" oligonucleotide is the N3' \rightarrow P5' PN, which result from the replacement of the oxygen at the 3' position on ribose by an amine group. These oligonucleotides can, relative to their isosequential phosphodiester counterparts, form very stable complexes with RNA and single- or double-stranded DNA (43, 44). Under some conditions, PN can exhibit highly selective and specific antisense activity *in vitro* and *in vivo*. An 11-mer PN, complementary to junction region of the *bcr-abl* mRNA, a

protein thought to be a determinant of the chronic myelogenous leukemia phenotype, efficiently inhibited the growth of treated BV173 cells. This inhibition has been associated with a decrease in the levels of the *bcl-abl* mRNA. Because PNAs are not inducers of RNase H activity, the authors explained these results by suggesting the existence of a hitherto unknown enzyme, which is able to cleave the heteroduplex formed by the PN and the mRNA (45). Another PN, a 15-mer oligonucleotide targeted to the start codon of the *c-myc* proto-oncogene selectively inhibited the *c-myc* protein expression and the proliferation of HL-60 cells. The control, a mismatched PN oligonucleotide, had no effect on protein expression and cellular proliferation. These *in vitro* experiments were extended to an *in vivo* mouse model. Leukemic severe combined immunodeficiency mice bearing HL-60 cells were treated with different doses of the anti-*c-myc* and control PN oligonucleotides. The antisense PN demonstrated high sequence specific activity, as the mice treated with 900 μ g/day the antisense oligonucleotide survived 30 weeks, compared with the control mice, treated with the mismatched oligonucleotide, which survived only 7 weeks (46). However, subsequent studies with the PN backbone, have demonstrated that these oligomers exert far more non-sequence-specific effects than were thought initially.

The plethora of nonspecific effects observed with the RNase H-dependent phosphorothioate oligonucleotides have led to the mixing and matching oligonucleotide chemistries to create increased specificity. Specificity, as well as efficacy, can be increased by using a chimeric oligonucleotide, in which the RNase H-competent segment, usually a phosphorothioate moiety, is bounded on one or both termini by a higher-affinity region of modified RNA (47, 48), frequently 2'-O-alkyloligoribonucleotides (22, 49). This substitution not only increases the affinity of the oligonucleotide for its target but reduces the cleavage of nontargeted mRNAs by RNase H (49, 50). Two chimeric 2'-O-Methyl/phosphorothioate antisense oligonucleotides are now in clinical trials: one targets the R1 α subunit of protein kinase A, and the other targets cytomegalovirus-induced retinitis (3).

Mechanism of Action

Oligonucleotides are in theory designed to specifically modulate the transfer of the genetic information to protein, but the mechanisms by which an oligonucleotide can induce a biological effect are subtle and complex. Although some of these mechanisms of inhibition have characterized, rigorous proof for others is still frequently lacking. On the basis of mechanism of action, two classes of antisense oligonucleotide can be discerned: (a) the RNase H-dependent oligonucleotides, which induce the degradation of mRNA; and (b) the steric-blocker oligonucleotides, which physically prevent or inhibit the progression of splicing or the translational machinery.

The majority of the antisense drugs investigated in the clinic function via an RNase H-dependent mechanism. RNase H is a ubiquitous enzyme that hydrolyzes the RNA strand of an RNA/DNA duplex. Oligonucleotide-assisted RNase H-dependent reduction of targeted RNA expression can be quite efficient, reaching 80–95% down-regulation of

protein and mRNA expression. Furthermore, in contrast to the steric-blocker oligonucleotides, RNase H-dependent oligonucleotides can inhibit protein expression when targeted to virtually any region of the mRNA. Thus, whereas most steric-blocker oligonucleotides are efficient only when targeted to the 5'- or AUG initiation codon region, phosphorothioate oligonucleotides, *e.g.*, can inhibit protein expression when targeted to widely separated areas in the coding region (51, 52).

The importance of RNase H-induced cleavage of mRNA has been demonstrated in at least four systems, including wheat germ extract (53), rabbit reticulocyte lysate (54), *Xenopus* oocytes (55), and human leukemia cells (56). RNase H competent backbones include oligodeoxynucleotide phosphodiester and phosphorothioates. 2'-fluorooligodeoxynucleotides are also RNase H competent (57, 58). Other modifications, including methylphosphonates, 2'-O-methyloligoribonucleotides, PNAs, and morpholino oligonucleotides, are not RNase H competent. The precise mechanism by which RNase H recognizes duplexes, however, is not well understood. Using chimeric oligonucleotides in which 2'-O-methyloligoribonucleotide phosphorothioates are placed at the 3' and 5' termini of the oligonucleotide, while the central region remains phosphorothioate oligodeoxyribonucleotide, Monia *et al.* (22) demonstrated that a 5-bp region of homology is sufficient to induce RNase H activity. However, it is unclear if such a remarkable lack of "stringency" also occurs in living human cells and/or cell lines. Despite this caveat, it has been shown that Isis 3521, a 20-mer phosphorothioate targeted to the protein kinase C (PKC)- α mRNA, can also down-regulate PKC- ζ , with which it shares 11 bases of contiguous homology (59). This phenomenon of the cleavage of nontargeted mRNAs because of partial hybridization may be of major concern if oligonucleotides are used to validate gene function. Furthermore, although the use of chimeric oligonucleotides can suppress this problem, it does not appear to eliminate it altogether (22, 48, 60, 61).

Other oligonucleotide modifications (2'-O-alkyl, PNA, and morpholinos) may use different mechanisms to inhibit protein expression, *e.g.*, they can inhibit intron excision, a key step in the processing of mRNA. Splicing occurs during the maturation step and can be inhibited by the hybridization of an oligonucleotide to the 5' and 3' regions involved in this process (62). Such inhibition can lead to the lack of expression of a mature protein (42, 63) or, as numerous reports have shown, to the correction of aberrant splicing and the restoration of a functional protein (64, 65). This approach has been also developed in mice. Most of the oligonucleotides capable of inhibiting splicing are non RNase H dependent (31, 42, 66).

Numerous reports in the literature also demonstrate that "second-generation" oligonucleotides can efficiently inhibit mRNA translation. This inhibition may be attributable to the disruption of the ribosomes or by physically blocking the initiation (23) or elongation steps of protein translation. Steric blockade of translation can be demonstrated by the arrest of the polypeptide chain elongation, as shown by Dias *et al.* (34) in 1999. In an *in vitro* system assay, the authors identified a truncated protein after incubating a PNA oligonucleotide with

the Ha-*ras* mRNA. This truncated product had the same size as a truncated peptide produced by the RNase H-mediated cleavage obtained when using an isosequential phosphodiester oligonucleotide.

However, despite the use of oligonucleotides that target the translation initiation codon (70% of all oligonucleotide sequences reported in the literature according to Tu *et al.*; Refs. 23, 67, and 68), it may not always be an optimal site for targeting. For some mRNAs, sites in the 3'- or 5'-untranslated region appear to be just as reasonable a target than the translation initiation codon region (46, 68). The coding region itself appears to be somewhat less targetable. In fact, compared with the 5'-untranslated or initiation codon regions, there exists just a few examples of modified oligonucleotides able to induce an antisense effect when targeted to coding regions (22, 45, 69, 70). This may be attributable to the ability of the ribosomal machinery to unwind the oligonucleotide from its targeted mRNA.

A novel, and potentially remarkable, development in oligonucleotide technology is the relatively recent finding that 21–23-mer double-stranded RNA molecules, known as siRNA, can effectively silence gene expression (71, 72). This effect appears to be quite profound, occurring at very low concentrations of oligoribonucleotide (frequently <50 nM), and extremely specific, both with regard to mismatches and backbone. Interestingly, the low concentration required and formation of the duplex seems to help to evade complete digestion by RNases. Mechanistically, it is not yet clear, especially in mammalian cells, just how siRNA can be so apparently effective. In *Drosophila*, *e.g.*, it has been proposed that one of the strands of the siRNA acts as a primer for an RNA-dependent RNA polymerase. The resulting RNA-mRNA duplex is then believed to be cleaved to a series of 21–23-mers by the RNA III-related enzyme Dicer (73–75). These cleaved 21–23-mers then repeat and effectively amplify the gene-silencing process. Whether or not siRNA technology will supplant classic oligonucleotide approaches is an open question at the moment. There will probably be advantages and disadvantages for each technology, and considerations of cost (which disfavor the use of siRNAs) must always be considered.

Delivery of Oligonucleotides to Cells

In order for an antisense oligonucleotide to down-regulate gene expression, it must penetrate into the targeted cells. To date, the precise mechanisms involved in oligonucleotide penetration are not clear. Uptake occurs through active transport, which in turn depends on temperature (76, 77), the structure and the concentration of the oligonucleotide (78), and the cell line. At the present time, it is believed that adsorptive endocytosis and fluid phase pinocytosis are the major mechanisms of oligonucleotide internalization, with the relative proportions of internalized material depending on oligonucleotide concentration. At relatively low oligonucleotide concentration, it is likely that internalization occurs via interaction with a membrane-bound receptor (76, 77). De Diesbach *et al.* (79) have recently purified and partially characterized one of these receptors. At relatively high oligonu-

cleotide concentration, these receptors are saturated, and the pinocytotic process assumes larger importance.

Numerous reports have demonstrated that naked oligonucleotides are internalized poorly by cells whether or not they are negatively charged (80–82). More specifically, naked oligonucleotides tend to localize in endosomes/lysosomes, where they are unavailable for antisense purposes. Numerous experiments have demonstrated that the *sine qua non* of antisense activity appears to be nuclear localization. To improve cellular uptake and oligonucleotide spatial and temporal activity, a range of techniques and transporters have been developed. Simultaneously, the use of these vectors increases the stability of oligonucleotides against nuclease digestion and permits the use of far lesser (~10-fold) concentrations of oligonucleotides.

The first generation of vectors developed were liposomes, which are vesicular colloid vesicles generally composed of bilayers of phospholipids and cholesterol. Liposomes can be neutral or cationic, depending on the nature of the phospholipids. The nucleic acid can be easily encapsulated in the liposome interior, which contains an aqueous compartment, or be bound to the liposome surface by electrostatic interactions. These vectors, because of their positive charge, have high affinity for cell membranes, which are negatively charged under physiological conditions. As these vectors use the endosomal pathway to deliver oligonucleotides into cells, certain “helper” molecules have been added into the liposomes to allow the oligonucleotides to escape from the endosomes; these include species such as chloroquine and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine. These “helper” molecules ultimately induce endosomal membrane destabilization, allowing leakage of the oligonucleotide, which then appears to be actively transported in high concentration to the nucleus (82–86). Many commercial vectors, such as Lipofectin and compounds known collectively as Eufectins, Cytofectin, Lipofectamine, etc., are commonly used in laboratory research studies. With some of these delivery vehicles, and under defined conditions, oligonucleotide concentrations of ≤ 50 nM may be successfully used.

The use of other cationic polymers, including poly-L-lysine (87, 88), PAMAM dendrimers (89), polyalkylcyanoacrylate nanoparticles (90, 91), and polyethyleneimine (92), have been also developed for drug delivery. Nucleic acids interact with these vectors via electrostatic interactions. Activity has been demonstrated in various cell lines (91, 93, 94) and in a nude mice (95) model, but unfortunately, these polyamines, which appear to cause endosomal rupture via a “molecular sponge” mechanism, tend to be somewhat toxic and are less commonly in use than are the cationic liposomes.

All of these cationic delivery systems internalize oligonucleotides via an endocytotic mechanism. To avoid the resulting compartmentalization problems, consideration has been given to modulating plasma membrane permeability. By using basic peptides, one can increase oligonucleotide passage through the plasma membrane by a receptor- and transporter-independent mechanism. As these peptides have membrane translocation properties, covalent coupling with an oligonucleotide can increase the latter’s penetration into the cell, delivering them directly into the cytoplasm and

hence ultimately the nucleus. Several of these peptides, such as the *Drosophila melanogaster* homeotic transcription factor, the Antennapedia peptide (96), and the Tat protein of HIV-1 (97), have been identified and studied. In another example, using fluoresceinylated oligonucleotides coupled to the E5CA peptide, which corresponds to the NH₂-terminal segment of the HA2 subunit of the influenza virus agglutinin protein, Pichon *et al.* (98) demonstrated that oligonucleotides were rapidly taken up by cells and diffused into the nucleus.

An additional approach to oligonucleotide internalization is to generate transient permeabilization of the plasma membrane and allow naked oligonucleotides to penetrate into the cells by diffusion. This approach involves the formation of transitory pores in the membrane, induced either chemically by streptolysin O permeabilization (99, 100), mechanically by microinjection (101) or scrape loading (41), or produced by electroporation (102, 103). All of these methods, under defined circumstances, can permit charged or uncharged oligonucleotides to enter cells rapidly and localize in the nucleus, where they produce antisense inhibition of gene function. They are obviously not useful *in vivo*, and their relevance for purposes of validation of gene function must also be questioned.

The use of vectors in antisense drug delivery *in vivo* remains, at this point, a somewhat open question. In contrast to *in vitro* studies, all of the clinical trials with antisense oligonucleotides are carried out with naked oligonucleotides (51, 104). A delivery vehicle does not appear to be needed as endosomal/lysosomal sequestration, and lack of nuclear localization does not appear to be a problem. It has been proposed that endogenous molecules can promote physiological delivery, but the evidence for this conjecture at present is scant.

Efficiency of Antisense Oligonucleotides

In practice, only a few complementary oligonucleotides can successfully hybridize to a targeted mRNA (67). It is assumed that this is largely because of problems of target accessibility, which in turn may be because of the secondary or tertiary mRNA structure and/or to the proteins bound to the RNA.

To define the best mRNA hybridization sites, several predictive methods have recently been developed. Ding and Lawrence (105) proposed a method based on the determination of the RNA structures using algorithms and thermodynamic and structural properties of the RNA.³ Sczakiel *et al.* used a similar approach to select effective antisense oligonucleotides. They used a systemic alignment of computer-predicted secondary structures of local sequences of the targeted RNA to identify favorable local target sequences and then designed more effective antisense oligonucleotides. Using this method, they found that 17 of the 34 antisense oligonucleotides tested showed significant inhibition (>50%) of ICAM-1 expression in mammalian cells (106–108). Another selection method is based on the determination of the melting temperatures (109) or the free energies of

³ Internet address: <http://bioinfo.math.rpi.edu/~zuckerm/rna/>.

formation of the oligonucleotide/RNA duplexes (110, 111). Additional recent methods have used combinatorial oligonucleotides, which are used to identify the hybridization sites directly within the RNA. These sites are revealed by RNase H cleavage (112), microarrays (113, 114), or MALDI-TOF mass spectrometry (115). Despite the fact these methodologies are somewhat cumbersome, they may indeed ultimately identify excellent target sites.

Questions of Specificity and Controls in Antisense Oligonucleotide Experiments

To determine whether observed biological effect results from an antisense mechanism and not from nonspecific effects, experiments must be performed with adequate controls. The nature of these controls has recently been summarized by Stein (116) and will not be reviewed again here. However, it is important to remember that the "gold standard" of antisense efficacy is down-regulation of protein expression combined, if phosphorothioate oligonucleotides are used, with RNase H-dependent down-regulation of mRNA expression as demonstrated by Northern blot. Biological end points cannot be used as proof of antisense efficacy because of the intrinsic activity of the phosphorothioate backbone.

When sterically blocking oligonucleotides are used, Northern blotting becomes irrelevant as these oligonucleotides do not induce mRNA cleavage by the RNase H. To demonstrate antisense efficacy, one can, *e.g.*, demonstrate the synthesis of a truncated peptide (34). However, if the targeted site is at the 5' region or the AUG initiation start codon, then only Western blots will be useful.

Toward the Future

Over the past 2 decades, the antisense oligonucleotide technology has emerged as a valid approach to selectively modulate gene expression. By adhering to a strict set of specific rules, ongoing *in vitro* studies using antisense oligonucleotides permit the characterization of new targets and new potential therapeutic compounds. The number of *in vitro* experiments has increased continuously, and this has led to numerous therapeutic trials, a few of which now appear preliminarily to be positive. However, the optimal use of antisense oligonucleotides in the treatment of disease requires the resolution of problems relating to effective design, enhanced biological activity, and efficient target delivery. These issues are currently being actively addressed and will hopefully continue to shed light on ways to increase therapeutic efficacy and specificity.

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