

## SURVEY AND SUMMARY

# Mechanisms and strategies for effective delivery of antisense and siRNA oligonucleotides

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### ABSTRACT

**The potential use of antisense and siRNA oligonucleotides as therapeutic agents has elicited a great deal of interest. However, a major issue for oligonucleotide-based therapeutics involves effective intracellular delivery of the active molecules. In this Survey and Summary, we review recent reports on delivery strategies, including conjugates of oligonucleotides with various ligands, as well as use of nanocarrier approaches. These are discussed in the context of intracellular trafficking pathways and issues regarding *in vivo* biodistribution of molecules and nanoparticles. Molecular-sized chemical conjugates and supramolecular nanocarriers each display advantages and disadvantages in terms of effective and nontoxic delivery. Thus, choice of an optimal delivery modality will likely depend on the therapeutic context.**

### OVERVIEW

Antisense and siRNA oligonucleotides hold great promise as therapeutic agents. Several first generation (phosphorothioate) antisense oligonucleotides are in late phase clinical testing (1–4), while newer oligonucleotide chemistries are providing antisense molecules with higher binding affinities, greater stability and lower toxicity as clinical candidates (5–7). The rapid development of mammalian RNA interference (RNAi) opens the path to a powerful new strategy for therapeutic regulation of gene expression (8–12). Promising results have been attained with small interfering RNAs (siRNAs) in animal models (13–15) and several clinical trials are underway (13). However, despite abundant promise, a number of problems and hurdles remain for oligonucleotide-based therapeutics. Perhaps the most important issue concerns the effective delivery of antisense or siRNA oligonucleotides to their respective sites of action in the nucleus or cytoplasm. In studies of cells in culture, delivery agents such as cationic lipids or polymers are required in order to attain significant

antisense or siRNA effects. However, the large size and/or considerable toxicity (16,17) of cationic lipid particles and cationic polymers may render them problematic candidates for *in vivo* utilization. In contrast, many animal studies and virtually all of the clinical studies thus far have used ‘free’ antisense or siRNA compounds (without a delivery agent), thereby demonstrating that oligonucleotides can function in that form. However, many investigators believe that appropriate delivery platforms could be very helpful for oligonucleotide-based therapeutics (18–20). In this Survey and Summary, we review and analyze chemically based approaches to oligonucleotide delivery, including use of nanocarriers and molecular conjugates. These approaches will be considered both in terms of intracellular delivery to cultured cells and in terms of *in vivo* biodistribution. Obviously, another important therapeutic strategy will be to use viral vectors for siRNA expression (10,12,21–24), but we will not further consider the viral approach in this review.

### BACKGROUND

#### Antisense and siRNA mechanisms

Here, we briefly summarize aspects of the chemistry and biology of antisense and siRNA oligonucleotides that are salient to their potential as therapeutic agents.

*Antisense.* RNaseH-mediated degradation of complementary mRNA is the major mode of action of antisense oligonucleotides. However, oligonucleotides that do not support RNaseH can affect gene expression by translation arrest or by altering splicing (25). Target site selection in the mRNA is an important issue and remains rather empirical. Simple phosphodiester oligonucleotides are unstable in the biological milieu; thus, a number of chemically modified oligonucleotides have been developed to enhance stability and to confer other desirable properties (3,5,6). Substitution of sulfur for oxygen forms phosphorothioate oligonucleotides, the most common modification. However, several other highly improved oligonucleotide chemistries have emerged including 2'-OH modifications, locked nucleic acids (LNAs), peptide nucleic acids (PNAs),

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morpholino compounds and hexitol nucleic acids (HNAs). All of these entities have high affinities for RNA and are more stable than phosphorothioates; however, they do not support RNaseH activity (5–7). Thus oligomers based entirely on these chemistries cannot be used as ‘classic’ antisense agents (although they may be very effective for modification of splicing or translation arrest). Introduction of several central phosphodiester residues into these agents, thereby creating ‘gapmers’, results in antisense oligonucleotides that activate RNaseH but that also retain many of the desirable properties of the parent compounds (7).

*siRNA*. Suppression by double-stranded RNA (dsRNA) is an important endogenous mechanism of gene regulation, acting through pathways involving mRNA degradation and/or sequestration, translation arrest and effects on chromatin and transcription (26). The mRNA cleaving action of interfering dsRNA in mammals involves two enzymatic steps. First, the ‘Dicer’ enzyme and its co-factors cleaves dsRNA to 21- to 23-mer segments (siRNA) and assists its loading on to the Argonaute 2 (Ago 2)-containing ‘RISC’ complex. RISC removes the sense strand, pairs the antisense (guide) strand with a complementary region in the cognate mRNA and initiates cleavage (‘slicing’) at a site between bases 10 and 11, relative to the 5’ end of the antisense strand (21,27–29). The resulting 5’ and 3’ mRNA fragments are subsequently fully degraded by several cellular nucleases (26,30).

In addition to ‘slicer’ activity, which requires essentially complete complementarity between the siRNA guide strand and its target, short dsRNAs can also display miRNA activity against partially complementary sequences in the 3’-regulatory regions of mRNAs. While bound to Ago protein complexes, the ‘seed region’ of the antisense strand (positions 2–7, 8 from the 5’ end) pairs precisely with the target, while some mismatches are tolerated in other regions. This process can lead to arrest of translation, sequestration of the target mRNA in cytosolic P-bodies (which are key sites of RNA processing), and possibly to de-capping and degradation (26,31). Thus, miRNA-mediated actions can potentially lead to off-target effects of siRNAs. In addition to the ‘slicer’ and miRNA pathways, dsRNA can also regulate transcription at the chromatin level via processes that are not yet fully elucidated (32). There is also an interesting conjunction between siRNA effects and antisense mechanisms. Thus, antisense oligonucleotides can be designed to interrupt the function of endogenous miRNAs. Since miRNAs often reduce gene expression, these antisense agents (sometimes termed ‘antagomirs’) can cause upregulation of expression of some of the genes that are regulated by a particular miRNA (11,33–35).

In mammals, long dsRNAs elicit highly toxic responses related to the effects of viral infection and interferon production (8,28). To avoid this, Tuschl and colleagues initiated the use of short interfering RNAs (siRNAs), comprised of 19-mer duplexes with 2 base 3’ overhangs on each strand, that associate with Ago2 and selectively degrade targeted mRNAs (36). Short, chemically synthesized, siRNAs do not require the Dicer step, although Dicer-enhanced RISC loading may contribute to efficiency.

To enhance siRNA effectiveness a variety of chemical modifications have been pursued including alterations in the backbone chemistry, 2’-sugar modifications, altered ring structure, nucleobase modifications and others (37–40), and the importance of these modifications to potential clinical applications has been emphasized (41). In terms of overall design, understanding of the biochemical mechanism of RNA interference has provided important guidelines; first, the siRNA must maintain an A-form (RNA-like) duplex, second the 5-position on the antisense strand must be able to be phosphorylated, third to be effective siRNAs should have low thermodynamic stability in the 5’ antisense region (26,40,42). These general design principles can then be further refined through the use of appropriate chemical modifications (39).

It is important to note, however, that siRNA biology is complex, and that it is essential to validate the mechanisms underlying observed biological effects before attributing them to RNA interference. A dramatic example of this concerns a recent study of siRNAs designed to ameliorate macular degeneration by targeting VEGF or its receptor and thus suppressing angiogenesis in the eye. A comprehensive analysis of the situation revealed that the observed reduction of angiogenesis was not due to sequence-specific RNA interference at all, but rather was caused by sequence-independent stimulation of Toll-like Receptor 3 and its downstream signaling pathway by dsRNA-like molecules, leading to an interferon- $\gamma$  and IL-12 mediated suppression of neovascularization (43). Thus, the possible interplay of oligonucleotides with various members of the Toll-like receptor family of cell surface proteins must be considered in the design of experiments.

### Mechanisms of endocytosis and intracellular trafficking

In almost all instances, oligonucleotides (and their various delivery agents) are taken up by some form of endocytosis. Ultimately, the oligonucleotide must exit from the endosome to reach its site of action in the cytosol or nucleus. Thus, in order to understand key issues in the intracellular delivery of oligonucleotides it is important to consider the multiple routes of endocytosis and the complex trafficking pathways that exist in cells.

Endocytosis is a blanket term that covers multiple distinct uptake pathways including: (i) the ‘classic’ clathrin-coated pit pathway; (ii) the caveolar pathway; (iii) one or more noncaveolar, clathrin-independent pathways (CLIC pathways); (iv) macropinocytosis and (v) phagocytosis (that mainly takes place in ‘professional phagocytes’ such as macrophages and granulocytes) (44,45). When the molecule being internalized is simply dissolved in the ambient medium, the uptake process is usually termed pinocytosis. When the molecule is bound to a cell surface receptor the process is termed receptor-mediated endocytosis. Each of the endocytotic pathways is actin-dependent (with the possible exception of one CLIC pathway), and the clathrin and caveolar pathways are dependent on the GTPase dynamin to pinch off budding vesicles, while macropinocytosis is not dynamin dependent. Caveosomes and CLIC vesicles are enriched in ‘lipid raft’ components including glycosphingolipids and

cholesterol (46,47). Initial uptake of receptor and ligand is followed by sequential intracellular trafficking into a variety of low pH endomembrane compartments, including early/sorting endosomes, late endosomes/multivesicular bodies, and lysosomes; in some cases, receptors/ligands can traffic to the Golgi complex. In many instances, receptor and ligand are dissociated in the low pH endosome environment, and in some cases the receptor can recycle back to the cell surface via the sorting endosomes (Figure 1).

The complex flow of endomembrane traffic (48) is regulated by the Rab family of small GTPases and by tethering complexes, while vesicular fusion events are controlled by SNARE proteins (49–51). The SNX (sorting nexin) proteins also are important in sorting and cargo retrieval from endosomes (52). The trafficking of internalized receptor and ligand can often be very complex and dependent on the nature of the receptor, the physiological status of the cell and the cell type. Some receptors can enter cells via multiple pathways, for example, via both clathrin-coated vesicles and caveolae (53). The route of entry can affect the ultimate fate and function of the ligand–receptor complex. For example, at low ligand levels the EGF-receptor is internalized via coated pits and can continue to signal, while at high ligand concentrations the receptor is ubiquitinated, internalized via a CLIC or caveolar pathway, and degraded (54).

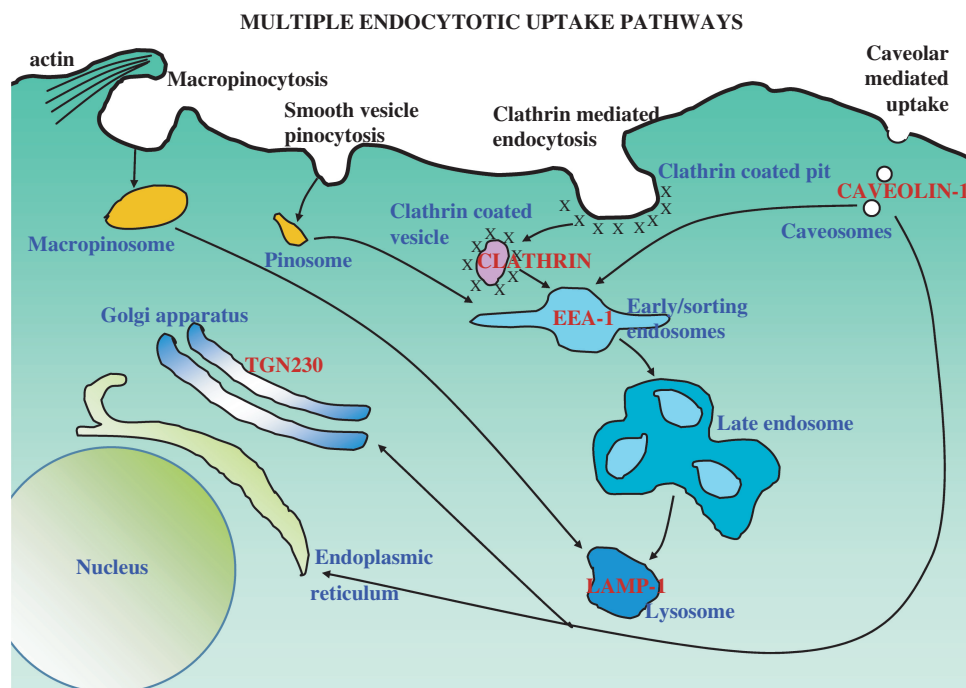
There are a number of tools commonly used to probe pathways of internalization. For example, many studies of oligonucleotide uptake have used pharmacological inhibitors that putatively block specific endocytotic pathways. In reality, however, such inhibitors often affect multiple

uptake pathways, as well as having many other effects on the cell. For example, although  $\beta$ -cyclodextrin is often used to block uptake involving lipid rafts, it also affects clathrin-mediated endocytosis (55). Likewise, agents that block macropinocytosis by affecting actinomyosin function will also block other endocytotic pathways, as well as having generalized effects on the cytoskeleton (56,57). Thus, results obtained with pharmacological inhibitors need to be interpreted conservatively. Potentially, more powerful approaches include using antibodies to key marker proteins to co-visualize fluorophore-tagged oligonucleotides in specific endomembrane compartments (58), and use of dominant-negative Rab proteins to interfere selectively with trafficking patterns (59).

Although general aspects of the cellular uptake of oligonucleotides have been studied for a long time and extensively reviewed (7,60,61), it is only recently that investigators have probed oligonucleotide internalization in light of current understanding of endosomal trafficking pathways. While not enough work has been done to achieve a broad consensus, some interesting examples of more detailed trafficking studies have recently emerged (62–64).

#### Ligands for enhancing and targeting delivery

In this section, we will examine ligands that have been used to attain cell-type selective targeting or to enhance the uptake of oligonucleotides. For the sake of simplicity, we have divided the discussion into agents that target specific receptors (CTLs, cell targeting ligands) and agents that enhance transmembrane permeation (primarily cell penetrating peptides, CPPs).



**Figure 1.** Endocytotic pathways. The figure depicts the multiple endocytotic pathways that may be involved in uptake of oligonucleotides. The black arrows represent well-documented trafficking routes within the cell. The names in red indicate well-known protein markers for various endomembrane compartments; in most cases, antibodies to these marker proteins are commercially available.

**CPPs.** The chemistry and actions of CPPs have been extensively reviewed (65–69) and here we touch on only a few key aspects. The prototypical CPPs derived from the Tat and antennepedia transcriptional regulators have been joined by a large number of new moieties. Most of these are relatively short (9–30 amino acids) polycationic peptides rich in arginine and lysine, although some include membrane-interactive hydrophobic sequences (Figure 2). CPPs have been linked to proteins by recombinant DNA techniques or chemically coupled to peptides, oligonucleotides or nanocarriers, which then comprise the ‘cargo’ for the CPP. Initially, CPPs were thought to convey their cargo directly across the plasma membrane. However, it is now clear that polycationic CPPs initially bind to cell surface glycosaminoglycans; this is followed by endocytotic uptake (possibly macropinocytosis) (70), and eventual release of cargo from the endosome to the cytosol. Although initial reports emphasized the great promise of CPPs for delivery of macromolecules, recently there has been some controversy as to just how effective they are (71). Certainly, the nature of the cargo (in terms of size, charge and other molecular characteristics) has an important impact on the effectiveness and the toxicity of CPP-mediated delivery (72–75). In a section below, we will discuss how CPPs have been applied to oligonucleotide delivery.

**CTLs.** A promising strategy is to deliver antisense and siRNA oligonucleotides by use of a CTL that binds with high affinity to a cell surface receptor that is capable of undergoing efficient internalization. A wide variety of potential ligands are available including antibodies (76), polypeptides derived from phage display libraries (77) and small organic molecules. Since various receptors are often preferentially expressed on particular cell types, this approach offers the possibility of improved selectivity for the oligonucleotide reagents. While a rich variety of cell surface receptors are expressed in the human body, work thus far involving delivery of oligonucleotides has primarily focused on lipoprotein receptors (particularly those in the liver) (78), integrins (79,80) and receptor tyrosine kinases (81). Another potentially rich source of

targets is the G-protein coupled receptor (GPCR) superfamily, by far the largest family of receptors in the human genome with approximately 850 members (82). GPCRs have long been a major interest of the pharmaceutical industry and thus a vast number of high affinity small organic molecule ligands for GPCRs are available or are emerging via high-throughput screening procedures (83).

## CELLULAR DELIVERY OF OLIGONUCLEOTIDE CONJUGATES AND COMPLEXES

In this section, we discuss the use of CPPs and CTLs in the delivery of antisense and siRNA oligonucleotides. Emphasis here is on cellular studies, while *in vivo* work is discussed in a following section. As a prelude, it is important to recall that oligonucleotides do not permeate intact cell membranes to any significant degree via simple diffusion, primarily because of the hydrophobic nature of the membrane lipid bilayer. This is true for both negatively charged siRNA or antisense moieties as well as for molecules with uncharged backbones such as methylphosphonates, PNAs and morpholinos (84,85).

### CPP—oligonucleotide conjugates or complexes

A considerable effort has gone into the preparation and evaluation of conjugates of CPPs and oligonucleotides; however, on the whole this has been only moderately successful (86,87). Our laboratory has reported biological effects of conjugates of CPPs with anionic antisense oligonucleotides (88,89), and others have reported on CPP–siRNA conjugates (90,91). However, the bulk of the literature suggests that CPPs are primarily able to deliver oligonucleotides with uncharged backbones, such as peptide nucleic acids and morpholino compounds (92–96).

CPPs have been studied in connection with both antisense and siRNA, and as chemical conjugates or noncovalent complexes with the oligonucleotide. In addition to the usual assays based on ‘knockdown’ of mRNA by antisense or siRNA, another popular approach has been the use of an assay based on the splice-correcting properties of certain types of antisense oligonucleotides (25). Briefly, an aberrant intron is placed into a reporter gene (luciferase, GFP) cassette and stably expressed in cells. The aberrant intron results in incorrect splicing and failure to produce functional mRNA and protein. However, appropriate splice switching oligonucleotides (SSOs) can correct splicing leading to expression of the reporter gene. Since splicing only takes place in the nucleus, this splice correction assay provides a convenient positive read-out for delivery of the SSO to the nuclear compartment.

Early work from our laboratory explored conjugates of the CPPs Tat and Antennepedia (also known as penetratin) with either standard phosphorothioate oligonucleotides targeting the MDR1 drug resistance gene (89,97) or with SSOs comprised of 2'-O-Me phosphorothioates (88); in both cases, the peptide and oligonucleotide were joined by bio-reversible S–S bridges. In both types of assay, the presence of the CPP enhanced biological effects over those

### Cell Penetrating Peptides (CPPs)

- Small peptides (usually less than 30 aa)
- Net positive charge (in most cases)
- Translocate across plasma or endo-membranes
- Transport cargos into cytoplasm and nucleus

Name	Sequence	Length	Charge	MW
Penetratin	RQIKIWFQNRRMKWKK	16	7 (+)	2247
TAT	GRKKRRQRRRPPQ	13	8 (+)	1719
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	27	5 (+)	2842
R9 peptide	RRRRRRRRR	9	9 (+)	1428
MPG peptide	GALFLGWLGAAGSTMGAPKKRKV	24	5 (+)	2445
KALA peptide	WEAKLAKALAKALAKHLAKALAKALAKACEA	30	7 (+)	3132

**Figure 2.** Cell penetrating peptides. This describes some of the more commonly used cell penetrating peptides in terms of molecular weight and net charge.

attained with unconjugated 'free' antisense oligonucleotide; additionally, a limited amount of microscopy was done to evaluate intracellular distribution and evidence was found for nuclear accumulation of the oligonucleotide. In contrast, a later study from another group examined a number of disulfide bridged conjugates between various CPPs and 2'-O-Me or LNA oligonucleotides complementary to the HIV-1 TAR element (98). In this case, there was little biological effect unless endosome disrupting agents were used; further, the oligonucleotides were observed by fluorescence microscopy to be restricted to cytoplasmic vesicles, with no sign of nuclear localization. The reason for the discrepancies between these two sets of early studies is unclear. One possibility is that, in our early studies, the peptide-oligonucleotide conjugates became aggregated during use, and this actually enhanced their effectiveness. Interestingly, there have been several reports of noncovalent complexes or aggregates between anionic siRNA oligonucleotides and cationic CPPs that seem to have provided moderately effective delivery to cells in culture. In one case, a modified version of the CPP penetratin having endosomolytic properties was superior to other CPPs that bound the siRNA equally well but lacked endosome lytic ability (99). In another case, a designed peptide comprised of both positively charged residues and a fusion peptide sequence was found to complex with siRNA and deliver it to the cytosol (100); this approach has also been followed by other groups (101). Whether conjugates or complexes are likely to provide more effective delivery of anionic oligonucleotides *in vivo* is an important issue and will be further explored below.

A number of investigators have evaluated conjugates of CPP with oligonucleotides having uncharged backbones. In one study, both stable and bioreversible disulfide linkages were used to produce conjugates between various CPPs and a PNA targeting the HIV-1 Tar motif (92). Certain conjugates, particularly an R6-penetratin version, demonstrated clear-cut biological effects, although little nuclear localization was seen by fluorescence microscopy. Several other CPP-oligonucleotide conjugates, with either stable or disulfide linkages, were able to attain biological effects when chloroquine was used to enhance their release from endosomes. An additional study used an R6-penetratin conjugate of a PNA SSO to activate a luciferase reporter gene; good effects were attained at micromolar concentrations (96). Another study also utilized PNA SSOs coupled via disulfide bridges to various CPPs to activate luciferase (93); here the transportan CPP was found to be particularly effective. Confocal microscopy and use of endosomal markers indicated that the CPP-PNA conjugates were most likely taken up by macropinocytosis, but there was little evidence of nuclear localization despite the observed effects on splicing. Studies from another group examined additional conjugates between PNA SSOs and various CPPs; they also found that a transportan-PNA conjugate linked via a bioreversible disulfide bridge was most effective (94). Recent studies have described a novel CPP termed 'M918' (63). When conjugated to PNA SSOs, M918 did not require binding to cell surface glycosaminoglycans for

uptake (in contrast to Tat or penetratin). Nonetheless, it entered cells by endocytosis and attained good biological effects. An interesting variation used CPP-PNA conjugates to target chromosomal DNA and cause effects at the transcriptional level (102).

Similar studies have also been done with CPP conjugates of morpholino oligonucleotides. In one very comprehensive investigation, a variety of linkages were formed between a morpholino SSO and several CPPs (103). A peptide containing nine arginines was particularly effective and resulted in splice correction activity at micromolar concentrations; fluorescence microscopy indicated some delivery of the oligonucleotide to the nucleus as well as to intracellular vesicles. More recently, this group has investigated the properties of conjugates comprised of morpholino SSOs linked to CPPs containing 6-aminohexanoic acid residues (104,105), finding that these have superior properties in terms of stability and effects on splicing.

The strategy of using CPP conjugates of SSOs having uncharged backbones has recently been reviewed (106). The overall picture seems to be that conjugates of various CPPs with uncharged backbone oligonucleotides can enter cells and effectively alter RNA splicing processes. Various CPPs differ somewhat in their potency in this regard; however, in most cases biological effects are only attained when the conjugates are used at micromolar concentrations. This may indicate that most of the material taken up by cells remains in endosomal compartments, with only a tiny fraction reaching the nucleus where RNA splicing occurs. One technical issue with many of these studies is their reliance on a single model system involving correction of splicing in a modified HeLa cell line. As discussed earlier, this system allows facile evaluation of whether a splice switching oligonucleotide can reach the nucleus and correct splicing of the aberrant reporter gene present there. However, it seems unwise to rely so heavily on a single cell type.

### CTL-oligonucleotide conjugates or complexes

A number of studies have appeared recently using CTLs for the delivery of antisense or siRNA. Some of these studies had *in vivo* components that will be more fully discussed in a section below. Here, we will focus on the cell targeting and uptake aspects.

An aptamer-siRNA chimera targeting prostate-specific membrane antigen (PSMA) was able to effectively deliver the associated siRNA to LNCaP prostate cancer cells; use of plk-1 siRNA triggered apoptosis and resulted in cell death both in culture and in a prostate tumor model (107). In this case, concentrations in the 2-400 nM range were effective in attaining gene silencing in cultured LNCaP cells, but not in PC-3 cells that lack PSMA. Other interesting approaches to aptamer-mediated siRNA delivery have also been described (108). The conjunction of nucleic acid aptamer technology and siRNA could potentially be a very powerful avenue for developing reagents for cell type selective regulation of gene expression.

Another approach involved a chimeric protein comprising the highly positive peptide protamine and an antibody Fab fragment directed against the HIV-envelop glycoprotein; this proved to be an effective carrier for siRNA that is complexed noncovalently with the protamine moiety. The chimeric protein was able to deliver an HIV *gag* siRNA to HIV infected CD4+ T cells causing inhibition of HIV replication (109). A later version of this approach used a conformation-sensitive single chain antibody directed against the LFA-1 integrin to specifically target siRNA to activated leukocytes (110); in this case, the complexed siRNA was directed against the CCR5 chemokine receptor. Effective gene silencing was obtained with amounts of siRNA in the sub-nanomol range, although the exact concentrations used are unclear.

In another example, a small cyclic peptide that binds the IGF1-receptor was able to deliver a PNA antisense moiety to the cytoplasm of cells expressing this receptor (111). A similar approach was also used for delivery of siRNA directed to the signaling protein IRS1 (112). Here, the peptide was conjugated via an NHS linker to a 5'-aminolinked sense strand. Significant silencing of the target gene in MCF7 breast cancer cells was observed using concentrations of the conjugate in the 100 nM range.

In a similar vein, work from our laboratory has recently shown that a bivalent RGD peptide having high affinity for the  $\alpha_5\beta_3$  integrin can effectively deliver conjugated SSOs to melanoma cells that express this integrin (62). Significant effects on splicing were attained with concentrations of conjugated SSO in the 10 nM range. The uptake process of the conjugates was traced via confocal fluorescence microscopy; this indicated that the RGD-conjugates entered via caveolae and other lipid raft-rich structures and then eventually trafficked to the *trans*-golgi. While substantial nuclear localization was not seen, the biological effects observed make it clear that some of the SSO reached the nucleus. An important point is that conjugates of this type display very little cytotoxicity, even when used at concentrations far higher than those needed to obtain a biological effect.

Another study used a polymer as a carrier for both siRNA and a targeting ligand. Thus, the polymer was covalently 'decorated' with siRNA, polyethylene glycol (PEG) and *N*-acetylgalactosamine as a ligand to target the hepatic asialo-glycoprotein receptor (113). This approach permitted effective silencing of two endogenous genes in cultured hepatocytes.

A particularly impressive study involved delivery of siRNA to neurons in culture, and to the brain, by complexation with a peptide that comprises a positively charged (Arg9) sequence to bind the oligonucleotide and a sequence that binds with high affinity to the nicotinic acetylcholine receptor in neurons (114). The chimeric peptide selectively delivered siRNA to neural cells expressing the acetylcholine receptor, but not to other cells, and could silence a GFP reporter gene in the neuronal cells when used at 10 pmol levels.

There is a striking functional contrast between the studies utilizing CTLs for oligonucleotide delivery and those using CPPs. In many cases, biological effects were

attained using nanomolar concentrations of oligonucleotides when delivery took place via receptor targeting, whereas delivery using various CPPs attained strong biological effects only at micromolar concentrations. The reason for this differential is unclear and may have little to do with total uptake (although it is not possible to reliably compare this parameter for the different studies). Possibly, a key issue is the intracellular trafficking pathway(s) accompanying the various initial uptake processes.

### Nanocarriers for oligonucleotide delivery

A variety of supramolecular nanocarriers including liposomes (115), cationic polymer complexes (116) and various polymeric nanoparticles (117) have been used to deliver antisense and siRNA oligonucleotides, as more fully described in several recent reviews (3,9,38,118–122). There is an enormous literature on use of various nanocarriers to deliver nucleic acids; thus here we can only touch on selected recent examples. Complexation of oligonucleotides with various polycations is a popular approach for intracellular delivery; this includes use of PEGylated polycations (123), polyethyleneamine (PEI) complexes (124,125), cationic block co-polymers (126) and dendrimers (127–130). Several cationic nanocarriers including PEI and polyamidoamine dendrimers exert a so called 'proton sponge effect' that helps to release contents from endosomes (131). Thus, as the nucleic acid-polymer conjugate enters the low pH endosome compartment, secondary amino groups in the polymer are titrated; the necessary proton influx also brings chloride and water into the endosome, causing swelling and increased leakiness. Other widespread approaches include use of polymeric nanoparticles (132), polymer micelles (133), quantum dots (134,135) and lipoplexes (136,137). Lipoplexes comprised of cationic lipids also exert endosome destabilizing effects (138); in this case, the cationic lipids interact with anionic lipids of the endosome membrane, leading to the formation of nonbilayer structures and consequent endosome instability. In some cases, nanoparticle approaches have been coupled with targeting strategies. As one example, a lactosyl-PEG-siRNA conjugate was complexed with polylysine to form nanoparticles; these were effectively delivered to liver tumor cells via interaction with the asialo-glycoprotein receptor (139). In considering the various types of nanocarriers, it is important to keep in mind that the carrier systems themselves can have significant effects on gene expression, and may potentially cause toxicity. This has been emphasized in two excellent recent reviews that comprehensively describe effects of polymers and nanocarriers on gene expression (17,140).

### IN VIVO DELIVERY OF OLIGONUCLEOTIDES

In this section, we will consider recent investigations regarding the *in vivo* behavior of various oligonucleotide conjugates and nanocarrier formulations. We will place particular emphasis on several studies that have

demonstrated clear-cut enhanced pharmacological effects of systemically administered siRNA as a result of use of delivery modalities. Some of these reports have been discussed above in terms of results at the cell culture level. Prior to initiating this discussion it is important to realize that there is a dichotomy in the behavior of oligonucleotides when comparing the *in vivo* situation to cell culture. Thus, almost without exception, effective use of antisense or siRNA in cell culture requires a delivery agent such as a cationic lipid; in contrast, many of the successful *in vivo* studies with oligonucleotides have used 'free' compounds (7,60). There seem to be two possible interpretations of this dichotomy. One version suggests that cells undergo radical changes in organization and gene expression as they go from a 3D tissue environment to a 2D culture environment and that in this process key oligonucleotide transporters are lost. While it is clear that cells do undergo dramatic changes in the transition from 3D to 2D (141), it seems unlikely to us that the same oligonucleotide transport systems would be lost in every single type of cell. Another interpretation is a pharmacodynamic one. Because of experimental constraints, in culture cells are only briefly exposed to the oligonucleotides; in contrast, most *in vivo* therapeutic experiments involve multiple doses and protracted exposures, thus perhaps allowing gradual intercellular accumulation of oligonucleotides. Surprisingly, this important dichotomy has not been carefully addressed via experimentation.

### Biological barriers to *in vivo* delivery of oligonucleotides

In planning for the effective delivery of oligonucleotides, it is essential to understand key aspects of endocytosis and intracellular trafficking at the cellular level. However, *in vivo* there are a number of other important parameters to consider as well (Figure 3). Essentially, a series of

#### In Vivo Barriers to the Effective Delivery of Antisense and siRNA Oligonucleotides

##### 1. Rapid excretion via the kidney

##### 2. Degradation by serum and tissue nucleases

##### 3. Uptake by the phagocytes of the reticuloendothelial system leading to sequestration in liver and spleen

##### 4. Failure to cross the capillary endothelium

##### 5. Slow diffusion/binding in extracellular matrix

##### 6. Inefficient endocytosis by tissue cells

##### 7. Inefficient release from endosomes

**Figure 3.** *In vivo* barriers. The figure lists key barriers to effective *in vivo* delivery of oligonucleotides. Rapid excretion is an issue for low molecular weight compounds. Clearance by phagocytes, capillary permeability and slow diffusion in the extracellular matrix apply to larger molecules and nanoparticles. Both small and large delivery agents can be affected by poor cellular uptake and inefficient release from endosomes.

biological barriers stand between the newly administered oligonucleotide and its ultimate site of action in the cytosol or nucleus of tissue cells (142). For 'free' oligonucleotides or small conjugates, an important limitation is rapid excretion via the kidney. Molecules less than 5000 molecular weight are rapidly ultrafiltered in the glomerulus and, in the absence of re-uptake, are accumulated in the urine (143). This picture is somewhat altered with oligonucleotides that bind strongly to plasma proteins thus retarding ultrafiltration (144). The vascular endothelial wall comprises another major barrier, especially for larger carriers. In general, molecules with a diameter of  $>45 \text{ \AA}$  (equivalent to about the size of an immunoglobulin) do not readily pass across the capillary endothelium and thus cannot efficiently enter the extracellular fluid that bathes tissue cells (145). In addition, it is not only the size but also the shape of the macromolecule or nanocarrier that affects its ability to traverse the endothelium (146,147). In a few tissues, including liver and spleen, the vascular endothelium is 'fenestrated' with gaps that allow the egress of macromolecules and nanoparticles up to about 200 nm diameter (148). In addition, work in xenograft tumors has given rise to the concept that the tumor vasculature is far 'leakier' than normal vasculature, thus also allowing egress of relatively large macromolecules and nanoparticles, the so-called 'EPR effect' (enhanced permeation and retention) (149). However, it is not clear that spontaneously occurring tumors in animals or humans uniformly display such increased leakiness. Further, tumors often exhibit other properties, such as increased interstitial pressure, that would tend to oppose delivery of nanocarriers to the tumor (150). Although one must be concerned about the issue of vascular permeability, it is also important to realize that the vascular endothelial cells themselves can be portals for therapy. This is especially true in sites of inflammation, where the endothelium upregulates key cell surface proteins including VCAM, ICAM and P-Selectin (151) or in angiogenic endothelium where the avb3 integrin is upregulated (152); thus, in both situations there is enhanced expression of receptors that can be addressed via CTLs linked directly to oligonucleotides or to nanocarriers. There are already many examples in the literature where endothelial receptors, especially avb3, have been targeted by various macromolecular or nanoparticle carriers bearing drugs or imaging agents (153–155).

Even if a nanocarrier exits the vasculature, it still needs to diffuse through the extracellular matrix to reach tissue cells; the ECM is comprised of a dense meshwork of proteins and proteoglycans that can hinder nanocarrier diffusion and in some cases may even tightly bind the carrier (142). Another key barrier is presented by the phagocytes of the reticuloendothelial system (RES). These cells monitor the blood and remove foreign materials such as bacteria and viruses; unfortunately, they also tend to treat administered macromolecules and nanoparticles as foreign, thus accumulating these materials in hepatic Kupffer cells, splenic macrophages and other sites rich in phagocytes (156,157). This process can be attenuated to some degree by 'passivating' or 'stabilizing' the surfaces

of nanoparticles with hydrophilic polymers such as PEG (158); PEGylation serves to reduce the adsorption of 'opsonins', plasma proteins that enhance phagocytosis, but this tactic is never completely effective. PEG-conjugated nanocarriers can remain in the circulation much longer than unmodified versions, but ultimately significant clearance by the RES takes place. In summary, a variety of considerations at the both cellular and whole organism levels are involved in the design of effective *in vivo* delivery strategies for therapeutic oligonucleotides.

### ***In vivo* delivery of oligonucleotide conjugates**

At this point, relatively little is known about the *in vivo* behavior of ligand-oligonucleotide conjugates. A recent study examined the biodistribution of a conjugate between an arginine-rich CPP and a morpholino oligonucleotide and suggested increased uptake in many tissues as compared to free oligonucleotide (159). Studies of cholesterol-linked siRNAs indicated that their association with serum proteins plays an important part in their pharmacokinetics, biodistribution and ultimate effects on gene expression in liver cells (14). Thus, the elimination half-life and tissue accumulation of an apolipoprotein B-targeted siRNA was substantially increased via cholesterol conjugation, leading to enhanced reduction of target mRNA and protein and consequent effects on blood cholesterol levels. A more detailed analysis of the behavior of lipidic conjugates of siRNA indicated that HDL and LDL were the primary carriers for cholesterol-linked siRNA, while conjugates of medium chain fatty acids primarily bound albumin (160). This report further demonstrated the key role of the LDL- and HDL-receptors in tissue uptake of cholesterol siRNA, with uptake via the LDL-receptor predominating in liver. Interestingly, this report also suggests a very novel mechanism for cell entry of siRNA via lipoprotein receptors. Thus, instead of simple receptor-mediated endocytosis of the siRNA-loaded lipoprotein, the authors suggest that the siRNA is passed from the lipoprotein receptor to Sid 1, a multispreading plasma membrane protein whose homolog in *C. elegans* can potentiate RNAi uptake and effects. Interestingly, other groups, working with mammalian cell cultures, have also linked Sid1 to intracellular uptake of siRNA (161,162). If these observations are generalizable, it would have a profound effect on our understanding of the transport of siRNA and on approaches to delivery and therapeutics.

Cholesterol-conjugated siRNA has also been administered directly into the lung via intratracheal instillation. In this case, the target was p38 MAP kinase mRNA. Significant target reduction was attained with the cholesterol siRNA but not with CPP-conjugated siRNA; indeed, in this case, potentially toxic results were observed (163). An interesting variation on delivery via lipid conjugation is embodied in a recent report on  $\alpha$ -tocopherol modified siRNA; this lipidic material also promotes siRNA delivery via binding to serum proteins and lipoproteins, but may involve a different set of binding partners (164).

### ***In vivo* delivery of oligonucleotides using nanocarriers**

A variety of nanocarriers have been developed to promote the effective *in vivo* delivery of oligonucleotides, with the emphasis on siRNA. An impressive early study involved complexation of siRNA with cationic cyclodextrin polymers to form nanoparticles, and used transferrin to target the nanocomplex to Ewing's sarcoma tumor cells that express high levels of the transferrin receptor (165). Delivery of siRNA targeting the EWS-FLI1 oncogene product resulted in reduced tumor growth. This same technology has more recently been tested for safety in primates, but with the active material being siRNA targeting the M2 subunit of ribonucleotide reductase (166).

In another study involving *in vivo* targeted delivery, 'self assembled nanoparticles' (a form of lipoplex) were used to deliver siRNA to tumors (167). Anti-EGF-receptor siRNA (as well as carrier DNA) was complexed with protamine and then with lipid. The particles were passivated with PEG and targeted using anisamide as a ligand. The nanoparticles were used to treat mice bearing xenografts of the NCI-H460 tumor, which expresses high levels of a cell surface receptor that binds anisamide; this treatment resulted in partial reduction of tumor growth. In addition, this study examined the pharmacokinetics and biodistribution of the administered siRNA, observing extensive tumor uptake for the targeted nanoparticles but not untargeted controls.

A very recent study also used a liposome-type carrier for targeted *in vivo* delivery of siRNA (168). Here, uncharged lipids were used to form small unilamellar liposomes. These were covalently linked to hyaluronan to stabilize and passivate the liposomes, which were then further conjugated to an antibody that binds the beta7 integrin subunit. The antibody-targeted liposomes were then 'loaded' with a protamine-siRNA complex. This technology was used to selectively deliver cyclin D1 siRNA to beta7-positive leukocyte subsets involved in intestinal inflammation, resulting in amelioration of an experimentally induced colitis.

Stable nucleic acid lipid particles (SNALPs), a type of liposome, have proven very effective for delivery of siRNA to the liver (169). In this case, apoB siRNA was used to treat cynomolgus monkeys resulting in substantial silencing of apoB mRNA expression followed by reduced protein levels and reductions in blood cholesterol. Extensive pharmacokinetic studies were also performed. The SNALPs are not a targeted nanocarrier, but rather rely on stability and long circulation time to attain effective delivery. Another promising approach using liposomes involved the suppression of liver fibrosis via delivery of siRNA targeting a heat-shock protein (HSP47) in hepatic stellate cells. These cells express a receptor for vitamin A and the liposomes were thus complexed with this substance in order to attain targeting both in hepatic cell cultures and *in vivo* (170). This study was notable for the very extensive validation of the *in vivo* therapeutic response.

Another interesting approach, discussed previously, involves creating protein chimeras of Fab or scFv antibody components with protamine, followed by



complexation with siRNA (109). This 'nanocomplex' approach was used to deliver growth inhibitory siRNAs *in vivo* to B16 melanoma cells engineered to express the HIV envelop glycoprotein, or to breast tumor cells over-expressing ErbB2, using the appropriate antibody component in each case. A modification of this approach (110) used a protamine chimera with a conformation-sensitive single chain antibody directed against the LFA-1 integrin to specifically target siRNA *in vivo* to lymphoid tumor cells that express the activated form of LFA-1 (110).

Polymer systems have also been used for targeted *in vivo* delivery. Thus, as mentioned earlier, a polymer nanocarrier linked to *N*-acetylgalactosamine was used to promote selective uptake by hepatocytes (113). *In vivo*, this system was able to effectively deliver apoB siRNA to mouse liver, resulting in gene silencing and reduced systemic cholesterol levels. Somewhat similarly, a system involving an RGD-conjugate of PEI was used to deliver siRNA targeting VEGF-receptor to tumor vasculature (125).

Finally, as mentioned earlier, delivery of siRNA to the brain was attained by formation of a nanocomplex with a peptide comprising positively charged sequence to bind the oligonucleotide and a sequence that binds with high affinity to the nicotinic acetylcholine receptor in neurons (114). Use of an antiviral siRNA provided protection against a potentially fatal viral encephalitis in mice. It is very surprising that the siRNA nanocomplex was able to cross the blood-brain barrier since even most small molecule drugs fail to do so. However, in this case it is possible that the presence of an active viral infection altered the permeability of the barrier.

In summary, several types of nanoparticle technologies have effectively delivered siRNAs to the liver, to tumors, or to inflamed tissues. It should be noted, however, that the vasculature in these tissues differs substantially from that more generally present in the body, as has been discussed earlier. Thus, it is unclear whether these approaches can be generalized to other tissues. Several of the studies mentioned above have also been reviewed recently in another venue (171).

## CONCLUSIONS

As reflected in this article, a great deal of effort is currently focused on the delivery of therapeutic oligonucleotides. Significant strides have been made, but the issue has not been fully resolved. Thus, it seems valuable to compare and contrast in broad terms the various strategies that are being pursued. Perhaps the most significant parameter to consider is the size of the moiety being delivered, contrasting nanoparticles that have diameters in the 50–200 nm range and molecular weights in the millions to monomolecular oligonucleotide conjugates with molecular weights in the thousands. Another key issue is charge since both polyanionic and polycationic molecules or nanocarriers can interact with blood proteins to incite toxicity or affect biodistribution (172,173).

Nanoparticles of various types offer many advantages as delivery agents. They can carry a large 'payload' comprising hundred or thousands of copies of the siRNA

or antisense oligonucleotide. They can be decorated with multiple copies of targeting ligands, thus providing high-avidity interaction with the target cells. Nanoparticles can be designed to release their contents at prescribed rates and can also be engineered to assist in the release of their contents from endosomes. Novel approaches for producing extremely uniform nanoparticles with controllable drug release characteristics are becoming available (174). Technologies for producing nanoparticles are reasonably mature thus permitting relatively facile scale-up for clinical studies (although reliable large-scale formulation of nanoparticles under GMP conditions is not without problems). Additionally, regulatory agencies are familiar with nanoparticles as delivery agents, based in part on the several liposomal drugs now on the market (175). Many of these useful aspects of nanoparticle-mediated oligonucleotide delivery are implicit in the studies reviewed earlier. However, the many positive features of nanoparticles are counterbalanced by some important negative ones. First, despite advances in using PEG or other hydrophilic polymers for stabilization, a large fraction of the injected dose of nanoparticles will accumulate in the liver, and much of that will be taken up by hepatic phagocytes. Thus, a significant portion of the dose of oligonucleotide will wind up at the wrong site, where toxic effects could potentially occur. Second, because of the vascular endothelial barrier, nanoparticles can only reach certain tissues, such as liver and spleen, where gaps in endothelium occur. This is one of the reasons that the various siRNA and antisense companies have focused so strongly on diseases that involve the liver. Nanoparticles that have long circulation times can also accumulate in some types of tumors due to the EPR effect; inclusion of a ligand that binds a receptor on the tumor cells can then promote uptake and intracellular delivery (176). However, what nanoparticles cannot do is to access parenchymal cells in most normal tissues; they are simply excluded by the endothelial barrier. Thus, many potential disease targets will not be addressable by oligonucleotide-based therapeutic platforms that involve nanoparticles.

In contrast, oligonucleotide conjugates are usually far smaller than the pores in normal vascular endothelium; thus, in principle, they should be able to access virtually all tissues, just as conventional drugs do (with the exception of the central nervous system). It should be noted that in some cases the oligonucleotide conjugate may be rapidly excreted via the kidney. Clearly, the detailed physical and chemical properties of individual oligonucleotide conjugates will influence their interactions with plasma proteins and their overall biodistribution; however, to a first approximation, the relatively small size of oligonucleotide conjugates implies a fundamental difference in their *in vivo* behavior as compared to nanoparticles. Nonetheless, there are some liabilities associated with this approach. First, each conjugate requires a separate synthesis, whereas a particular type of nanoparticle can potentially accommodate a variety of different oligonucleotides. Second, since only a single ligand is conjugated to the oligonucleotide this implies a lower-affinity interaction with target receptors than is the case for multivalent nanoparticles. Another issue concerns release from endosomes subsequent to

cell uptake. It is hard to visualize building both a targeting moiety and an endosome escape moiety into a monomolecular oligonucleotide conjugate; at minimum, the chemistry will be quite difficult.

Thus, both nanocarriers and molecular conjugates exhibit pluses and minuses as delivery agents. Ultimately, the most attractive delivery system may turn out to be neither a relatively small monomolecular oligonucleotide conjugate nor a very large nanoparticle or nanocomplex. Rather it may be an intermediate-sized moiety, perhaps comprised of oligonucleotides and targeting agents covalently linked to a small polymer (113) or protein that is large enough to avoid rapid excretion but yet small enough to be able to pass the vascular endothelial barrier. This approach may offer some of the high payload and high-affinity targeting aspects of nanoparticles without the constraints due to relatively large particle size. This is certainly an appealing approach and one that our group is actively pursuing.

An issue that applies to both conjugates and nanocarriers is the choice of accessory ligands. As mentioned earlier, there have been a number of attempts to improve both cellular uptake and endosomal release of oligonucleotides using CPPs. Surprisingly, however, this has not proven very effective, at least for monomolecular oligonucleotide conjugates. On the other hand, studies of polymolecular complexes of CPPs with oligonucleotides have seemed more promising; perhaps it requires multiple copies of CPPs to attain strong endosome destabilizing effects. As discussed earlier, use of cell targeting ligands that bind to specific receptors seems a more productive strategy. However, in most of the examples cited, while the targeting ligand can certainly enhance uptake, it is not clear that endosomal release is also enhanced. Perhaps one aspect of the receptor targeting strategy may entail differential opportunities for release from endosomes as the internalized oligonucleotide trafficks through different endomembrane compartments. Thus, it would be interesting to see if the same oligonucleotide, taken up via two different receptors, had the same or different ultimate biological effect. Certainly, it will be important to use current cell and molecular biological approaches to learn more about the details of intracellular trafficking of oligonucleotides and conjugates.

Another issue concerns the use of conjugates versus complexes. Some of the most exciting *in vivo* observations to date have involved noncovalent complexes between an oligonucleotide and a delivery agent (109,114). In most cases, however, the stoichiometry and physical characteristics of these complexes are essentially unknown. This raises issues concerning scale-up, reproducibility and the pharmaceutical acceptability of these approaches. Conjugates have the advantage of being well-defined molecular entities that may be easier to move along the path toward large-scale production, commercialization and clinical utilization.

In summary, while much progress has been made in the area of delivery of antisense and siRNA, much remains to be done. It will be important to pursue basic studies concerning both subcellular trafficking of oligonucleotides and their detailed biodistribution in animals. A measured

approach may, in the long run, serve the field better than hasty attempts to 'hit a home run' by bringing poorly characterized delivery strategies prematurely into the clinic.

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