Review Article

Theme: Emerging Drug Delivery Technologies
Guest Editor: Dexi Liu

Targeted Delivery Systems for Oligonucleotide Therapeutics

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Received 7 October 2008; accepted 20 February 2009; published online 19 March 2009

Abstract. Oligonucleotides including antisense oligonucleotides and siRNA are emerging as promising therapeutic agents against a variety of diseases. Effective delivery of these molecules is critical to their successful clinical application. Targeted systems can greatly improve the efficiency and specificity of oligonucleotides delivery. Meanwhile, an effective delivery system must successfully overcome a multitude of biological barriers to enable the oligonucleotides to reach the site of action and access their biological targets. Several delivery strategies based on different platform technologies and different targeting ligands have been developed to achieve these objectives. This review aims at providing a summary and perspective on recent progress in this very active area of research.

KEY WORDS: chemical modification; liposomes; nonviral vectors; RNA interference; small interfering RNA; targeted delivery.

INTRODUCTION

Oligonucleotides (ONs) are increasingly recognized for their potential as therapeutic agents against a variety of human diseases. Early efforts have been mostly focused on antisense oligodeoxyribonucleotides (AS-ODNs), whereas development of siRNA therapeutics is rapidly gaining momentum in recent years. Other related therapeutic strategies include those that are based on microRNAs (miRNAs), immunomodulatory CpG ODNs, ribozymes, and aptamers. Oligonucleotides are molecularly targeted agents capable of modifying specific biological pathways, thus, enabling rational design of therapeutic strategies.

To facilitate efficient delivery of AS-ODNs, several strategies have been investigated. For example, AS-ODNs have been conjugated to cell-penetrating peptides, such as trans-acting activator of transcription protein (1) and antenpedia (2), or hydrophobic moieties, such as cholesterol and fatty acids, to enhance transmembrane delivery. In addition, AS-ODNs have been incorporated into electrostatic complexes with cationic agents, such as protamine and oligo-arginine, and with cationic liposomes, similar to those originally designed for plasmid DNA delivery. Furthermore,

ONs may be encapsulated into liposomes containing a moderate amount of cationic lipid to form nanoparticles that are close to neutral, e.g., stable nucleic acid lipid particles (3,4) to facilitate long systemic circulation time and improved efficiency for *in vivo* delivery.

Addition of targeting properties further improves these delivery strategies through improved pharmacokinetic properties, selective tissue distribution, and enhanced cellular uptake. Here, we briefly review recent advances on targeted delivery strategies focusing on two general approaches: (1) direct conjugation of ONs with a ligand and (2) delivery of ONs via ligand-directed nanocarriers. Various ligands, including folate, transferrin, antibodies, oligosaccharides, peptides, and polysaccharides, will be discussed.

OLIGONUCLEOTIDES: PROMISING THERAPEUTIC AGENTS HAMPERED BY INEFFICIENT DELIVERY

Oligonucleotides, including AS-ODNs and siRNAs, are promising therapeutic agents for a variety of diseases. This is especially true in the case of cancer, for which molecular mechanisms of the disease are increasingly better understood with more and more molecular targets identified. These represent a great opportunity for drug development, since this class of molecules, in theory, can act on almost any targets that otherwise will be “undruggable” by small molecule entities or by monoclonal antibodies. In fact, it has been predicted that siRNA will drive the next wave of new drug development that will soon overtake the currently popular biologics, represented by therapeutic antibodies. This, to some degree, results from the challenges facing pharmaceutical companies, such as patent expirations and low rate of success in small molecule drug development.

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However, inefficient delivery presents a major hurdle that has largely prevented the successful clinical development of ON therapeutics.

Nevertheless, several AS-ODNs have reached clinical trials. AS-ODNs are ~20 nt in lengths and act by targeting specific mRNAs through heteroduplex formation inside the cell, thereby inducing RNase H activation, translational arrest, or alternative splicing. *In vitro*, activity of AS-ODNs requires delivery via invasive methods, such as electroporation and complexation to a transfection agent. However, clinical trials on AS-ODNs invariably have utilized free ODNs. Vitravene (formiverson), a phosphorothioate AS-ODN for treatment of CMV retinitis in AIDS patients, was the first ODN to gain approval by the US FDA (5). Formiverson is somewhat unique in that it is given by direct injection into the vitreous body of the eye. For systemic administration, in order to counter rapid clearance due to renal excretion, the ODNs in clinical trials have been given via prolonged continuous intravenous infusion. Despite these measures, the clinical efficacy of AS-ODNs has been limited in most cases and target down regulation is often time not observed. For example, in a clinical trial on an AS-ODN G3139 targeting Bcl-2, a significant fraction of the patients showed up-regulation of Bcl-2, rather than the intended target down regulation (6). Several preclinical studies suggested that therapeutic efficacy of G3139 might indeed be a result of immunomodulatory effects triggered by the CpG motifs in its sequence (6,7).

Comparing to AS-ODNs, siRNA is much more efficient for gene silencing both *in vitro* and *in vivo*. RNA interference (RNAi) takes advantage of a physiological gene silencing machinery, which can efficiently mediate the cleavage of targeted mRNA molecules. siRNAs consist of duplexes of oligoribonucleotides that are 19- to 23-nt each in length, representing a sense (passenger)-strand and an antisense (guide) strand. siRNAs interact with Argonaute-2 (Ago-2) to assemble RNA-induced silencing complexes (RISCs), which degrades the sense-strand of the siRNA and then cleaves target mRNAs that are complementary to the antisense strand (8,9). Variation of RNAi-based therapeutics includes small hairpin RNAs (shRNAs), and other siRNA precursors that can be processed by the dicer endonuclease. In fact, several recent reports suggest siRNA precursors, which are several nts longer and are dicer substrates, are much more potent than the widely used siRNAs in inducing target gene silencing, suggesting direct coupling of dicer processing and Ago-2 loading mechanisms (8). miRNAs are endogenous molecules that similarly functions through RISCs via a mechanism of translational arrest that does not require perfect sequence complementarity between the target and the antisense strand. siRNAs also exhibit significant miRNA-like effects against targets that are not perfectly complementary to the guide strand. This results in off-target effects of siRNA. siRNAs are generally believed to be much more potent in inducing lasting target gene silencing on a per molar basis compared to AS-ODNs. Therefore, siRNAs are poised to replace AS-ODNs as the oligo therapeutics of choice for future clinical development. Several human clinical trials of siRNA-based drugs are currently under way. For more detailed information on AS-ODNs and siRNAs, readers are referred to other recent reviews (10–14).

CHALLENGES FOR OLIGONUCLEOTIDE DELIVERY

As polyanionic macromolecules, oligonucleotides face multiple obstacles in reaching their intracellular site of action, which presents a significant problem for development of an effective delivery system. In fact, there is no natural mechanism for these highly hydrophilic macromolecules to traverse the cellular membrane and the bioavailability of these agents on their own is minuscule. Nevertheless, the delivery of ONs is somewhat less challenging than delivery of plasmid DNA carrying therapeutic genes, which is related to gene therapy. This is because oligonucleotides, which are typically less than 30 nt or bp in length, are significantly smaller in size than plasmids (frequently >7 kb). In addition, oligonucleotides are produced by chemical synthesis, which enables large-scale production of pure materials and introduction of chemical modifications that provides greater metabolic stability or improved bioavailability. Nonetheless, ON delivery still faces many challenges. In particular, for delivery to a solid tumor, there are at least four major barriers for ONs to gain access to malignant cells and take effect on their intracellular targets, as illustrated in Fig. 1. First, the ONs must avoid rapid degradation by serum nucleases, rapid excretion by renal filtration and/or clearance by the reticulo-endothelial system (RES). Second, the ONs must gain access to the target cells by crossing the capillary endothelium and travel in the extracellular matrix. Third, the ONs must be taken up by the target cells, typically through an endocytic process. Finally, the ONs must be released from the endosomes and reach their intracellular targets, e.g., loading onto dicer/Ago-2 in the case of siRNA. An effective delivery strategy must take into account the need to overcome all of

Fig. 1. Major challenges for oligonucleotide delivery *in vivo*. After administration into blood circulation (1), the targeted ON particles are exposed to plasma components that can degrade the ON molecules. Further, they need to survive the renal excretion and RES clearance in order to reach the target tissue. To reach the target cells, they must overcome the vasculature barrier through an extravasation process (2), avoidance of the lymphatic drainage, and penetrating the interstitium either by convection or by diffusion. The targeted particles are further bound to cellular receptors, and transported into the cytoplasm through a receptor-mediated endocytosis process (3). Inside the cells, the ONs need to escape the endosome (4) and be released to the cytoplasm to reach the target mRNAs

these barriers, as well as to avoid introducing tissue toxicity or inducing undesirable immunomodulatory effects.

DELIVERY STRATEGIES FOR OLIGONUCLEOTIDES

For successful development of AS-ODNs and siRNAs as therapeutics agents, two key problems must be addressed: metabolic stability and delivery efficiency/specificity. Due to the high molecular weight and polyanionic characteristics, AS-ODNs and siRNAs are subjected to relatively rapid clearance from the blood circulation. Low cellular membrane permeability and lack of cell-type specific uptake are additional issues that need to be addressed to advance these molecules into the clinic.

To increase their metabolic stability, chemical modifications have been introduced to ONs. Examples of such modifications include the introduction of phosphorothioate linkages, and 2'-*O*-methylation of the ribose moieties (11–13,15). For siRNAs, 2'-*O*-methylation has been shown to inhibit off target effects and immunomodulatory effects due to activation of toll-like receptor 7. In addition, certain modifications to ONs have been reported to improve cellular membrane permeability (13,15,16). The chemical modifications can significantly improve the properties of ON therapeutics and may be unavoidable for their clinical exploration. Phosphorothioated AS-ODNs, such as G3139 and GTI-2040, have shown some efficacy in recent clinical trials as free oligos (17,18). Indeed, the clinical trials of AS-ODNs have been conducted without the use of any delivery systems. However, due to rapid systemic clearance, high-dosages generally have to be administered via continuous i.v. administration in order to achieve significant therapeutic efficacy. This may result in significant hemodynamic toxicity. Moreover, the problem of nonspecific tissue distribution remains to be solved (19–22). Compared to AS-ODNs, the duplexed structure of siRNAs

make them more rigid, possibly resulting in even less cellular membrane permeability. In addition, siRNAs typically have significant off-target effects, which makes nonspecific tissue distribution problematic. Therefore, a suitable delivery system becomes even more critical for siRNAs therapeutic application (23,24). Systemic delivery with selective targeting of AS-ODNs and siRNAs to specific cells or tissues provides important potential advantages and may increase tissue selectivity, lower dosage requirements, and reduce toxicity.

It is worth mentioning that ONs, e.g., shRNA, can alternatively be delivered as an mRNA transcript from a plasmid vector. In these situations, the challenges of therapeutic gene delivery would apply. Much progress has been made in gene delivery, including viral and nonviral carriers. Nevertheless, insufficient gene transfer efficiency remains a formidable obstacle for clinical applications. Here, we focus on the strategy of ON delivery rather than gene delivery because the barrier to overcome is substantially lower. Interestingly, many ON delivery systems are derived from those originally developed for plasmid DNA delivery, e.g., polyplexes, lipopolyplexes, hybrid lipopolyplexes, and more recently fusion protein/siRNA complexes. This is because ONs share many characteristics of plasmid DNA and nonviral gene delivery has been a very active field of research due to the enthusiasm for gene therapy. However, because ONs are much smaller in size, the design of an optimal carrier for ON would be very different from that of a carrier for plasmid DNA.

TARGETED DELIVERY STRATEGIES FOR ON-BASED THERAPIES

A variety of targeting strategies have been studied to facilitate efficient cell or tissue-selective delivery of ONs (Fig. 2).

Fig. 2. Delivery strategies for antisense and siRNA oligonucleotides, **a** ligand-ON conjugates; **b** antibody-protamine-siRNA complexes; **c** ligand-directed liposomal ON nanoparticles; **d** ligand-directed polymer-ON nanoparticles

These strategies are generally divided into two classes: ligand-ON conjugates and ligand-targeted nanoparticles. 3' or 5' modification of ONs is a popular approach for enhancing resistance to degradation and introducing targeting ligands such as peptides (25,26) and aptamers (27). Cationic polymers and cationic lipids are two major classes of materials commonly used for incorporating ONs into nanoparticles. Both classes of these agents have been evaluated for specific attachment of targeting moieties. The cationic polymers such as poly-L-lysine, polyethylenimine (PEI), and polyamidoamine dendrimer as well as cationic block copolymer are widely used in synthesis of polymer-ONs nanoparticles (28–30). Liposomal nanoparticles comprise electrostatic complexes of nucleic acids and cationic lipids, such as dioleoyl trimethylammonium propane and *N,N*-dimethylaminopropane carbamoylcholesterol (16,31,32). Several other cationic lipids have been synthesized, and the corresponding ODN-lipoplex formulations have been evaluated in preclinical studies in animal models (16,31). Lipopolyplex nanoparticles are ternary complexes of lipids, cationic polymer, and nucleic acid. They have also been examined for the delivery of ONs (33,34). The following are some examples of strategies for targeted delivery of ONs.

HDL/LDL-MEDIATED DELIVERY

Terminal (5' or 3') modification of ONs using cholesterol is a useful strategy for increasing their stability and cellular uptake. The improvement in cellular uptake is mediated through *in vivo* interaction and incorporation into low-density lipoproteins (LDL) and high-density lipoprotein (HDL). Cholesterol conjugated siRNAs (Chol-siRNAs) have been shown to induce silencing of apolipoprotein B (apoB) in the liver after intravenous injection in mice (35). Although a relatively high dose of Chol-siRNA (50 mg/Kg) was required, this report provided important proof-of-concept that *in vivo* RNAi can be achieved through systemic administration. The association of Chol-siRNAs with serum proteins such as LDL and HDL was thought to have played an important part in determining the pharmacokinetics, biodistribution and uptake by the liver cells (35). Besides Chol-siRNAs, siRNAs conjugated with bile acids and long-chain fatty acids (lipidic siRNAs) also have been shown to mediate the enhanced cellular uptake and gene silencing *in vivo*. Studies showed that HDL and LDL were the primary carriers for cholesterol-linked siRNA, whereas albumin served as primary carriers of ODN conjugates of medium chain fatty acids (36). This report further demonstrated that the efficient and selective uptake of these siRNA conjugates depended on the interactions with lipoprotein particles, lipoprotein receptors, and transmembrane protein Sid1. HDL directed siRNA delivery into liver, gut, kidney, and steroidogenic organs, whereas LDL targeted siRNA mainly to the liver. Sterghios *et al.* (37) examined Chol-siRNAs in the lung via intratracheal instillation. In this case, 30–45% knockdown of p38 MAP kinase mRNA was observed at 6 h by Chol-siRNAs but not by cell penetrating peptide (CPP)-conjugated siRNAs. Therapeutic effect of cholesterol conjugated siRNAs was also shown in a transgenic mouse model of Huntington's disease (37). More recently, Nishina *et al.* (38) presented a new lipid conjugated siRNA (Toc-siRNA) based on terminal modification by α -tocopherol. This siRNA derivative, Toc-siRNA, mediated

efficient reduction of apoB protein *in vivo* upon intravenous injection at 2 mg/kg.

FOLATE-MEDIATED ON DELIVERY

Folate receptor (FR), a GPI-anchored glycoprotein, is an attractive target for drug delivery. FR is overexpressed in a number of human tumors such as ovarian, colorectal, and breast cancer, while its distribution in normal tissues is minimal (39,40). Folate-mediated targeting provides advantages including small size of folic acid, lack of immunogenicity, convenient availability as well as simple chemical conjugation (40,41). Linking of folic acid to liposomes or polymers enabled their binding to FR on the surface of tumor cells, which is followed by endocytosis. Folate has been evaluated in tumor-targeting of ONs (39). For instance, Rait *et al.* (42) showed that FR-targeted cationic liposomes were more efficient in delivering anti-HER ODNs into breast cancer cells, compared to the nonselective Lipofectin transfection reagent. In addition, folate-coated cationic liposomal ODNs exhibited longer systemic circulation time and increased tumor localization compared to free ODNs (43). Kim *et al.* (44) has recently developed polyelectrolyte complex micelle (PEMC)-based nanoparticles (~70 nm in diameter) for folate-targeted delivery of As-ODNs. The PEMC was formed by complexing FOL-PEG-ODN conjugates with cationic lipids or lipofectamine. The PEMC induced a significant reduction of GFP expression in both human lung cancer A549 and human oral carcinoma KB cells. In the latest study, Zhang *et al.* (45) demonstrated a new strategy for cell-type-specific delivery of siRNAs, which involves the non-covalent attachment of siRNAs to folate-conjugated ODNs. The resultant complexes led to specific cellular uptake and gene silencing in human vein endothelial cells (HUVECs) and KB cells that highly express the FR.

TRANSFERRIN-MEDIATED DELIVERY

Transferrin receptor (TfR) is a glycoprotein that is abundantly expressed on many tumor cells. The intracellular routing of TfR-mediated endocytosis has been well characterized. The endocytosis involves the recycling of TfR to the cell surface without intracellular degradation (46,47). Compared to antibodies to the TfR, transferrin is more affordable and is commercially available in large quantities. Thus, Tf-mediated delivery has been extensively explored for a variety of targets, including tumors, endothelial cells and the brain (48). The approaches used typically involve the chemical conjugation of either transferrin itself or of anti-TfR antibodies or antibody fragments to the surface of nanoparticles.

Heidel *et al.* and Hu-Lieskovan *et al.* (49,50) condensed siRNA with cationic cyclodextrin polymers to form nanoparticles and then used transferrin for targeting these to Ewing's sarcoma tumor cells that express high levels of the TfR. Sequence-specific knockdown of EWS-FL 11 and the corresponding inhibition of tumor growth were observed in mice after tail-vein injection of the siRNA at a 2.5 mg/kg dose. Interestingly, prolonged systemic administration of the cyclodextrin-based polycation delivery system did not induce detectable immune response. The Tf-targeted nanoparticles were confirmed safe in non-human primates for M2 siRNA delivery (49).

Pirollo *et al.* (51) reported the development of a tumor-specific, nanosized immunoliposome complex (scL) for systemic delivery of anti-HER-2 siRNA, which self-assembles into ~100 nm size particles. The anti-HER-2 siRNA encapsulated scL could sensitize human tumor cells to chemotherapeutics by silencing the target gene. This targeted knockdown caused significant inhibition of tumor growth in a pancreatic cancer model. In addition, Tietze *et al.* (52) applied an oligoethyleneimine derivative, OEI-HD, to optimize siRNA polyplexes for systemic delivery of RAN siRNA into tumors. Again, Tf was incorporated in the polyplexes as a targeting ligand. Tf-conjugated OEI-HD/siRNA polyplexes specifically reduced RAN protein expression both *in vitro* and in a Neuro2A xenograft tumor model. *In vivo* downregulation of RAN protein resulted in increased apoptosis and reduced tumor growth (52).

ANTIBODY-MEDIATED DELIVERY

Antibody-based therapeutics has been increasing adopted in cancer and leukemia treatment, because of their high specificity and affinity to target antigens and demonstrated therapeutic efficacy. Therapeutic antibodies such as trastuzumab (Herceptin®), rituximab (Rituxan®), and alemtuzumab (Campath®) have been routinely used in the clinical treatment of breast cancer and leukemia (53). In addition, many therapeutic monoclonal antibodies are currently in clinical development. Antibodies can be used as targeting agents for nanoparticles. Antibody fragments, such as Fab and scFv, can also be used and may have advantages over whole antibodies (54,55). Compared to whole antibodies, they are less bulky and lack an Fc domain, which may interfere with *in vivo* delivery. There is voluminous literature (32,56–58) related to antibody-targeted delivery of drugs and nucleic acids. Nonetheless, some challenges are known for this type of strategies, such as the potential for inducing immunogenicity and the high cost of antibodies.

The use of monoclonal antibodies to direct the delivery of As-ODNs and siRNAs to specific cell types has already been successfully demonstrated *in vivo*. For example, Song *et al.* (59) designed a protamine-antibody fusion protein to deliver siRNAs to HIV-infected primary CD4 T cells and HIV-envelope-expressing B16 melanoma cells. These siRNA-antibody-protamine complexes, administered either intratumorally or intravenously, specifically delivered siRNA to HIV envelope-expressing tumor cells *in vivo* and inhibited tumor growth (59). Furthermore, they fused the single chain scFv antibody that specifically targets human integrin lymphocyte function-associated antigen-1 in their antibody-protamine system (60). This system exhibited excellent selectivity for all human white blood cells and for activated leukocytes, efficiently silencing genes in primary lymphocytes, monocytes, and dendritic cells (60).

In another recent study by Peer *et al.* (34), cyclin D1-targeted siRNAs were systemically administered shown to selectively target activated leukocytes when delivered using liposomes conjugated to an antibody against $\beta 7$ integrin. The preparation of siRNA-loaded liposomal nanoparticles involved several steps. First, small unilamellar liposomes with uncharged lipids were obtained by extrusion. This was followed by the attachment of hyaluronan. Then, $\beta 7$ integrin antibody was covalently linked to hyaluronan. Finally, a protamine-siRNA complex was incorporated via rehydration of lyophilized antibody-targeted liposomes. These nanoparticles were used to selectively deliver cyclin D1 siRNA to beta7-positive leukocyte both *in vitro* and *in vivo*. With siRNA-mediated silencing of cyclin D1 expression, leukocyte proliferation was suppressed in a mouse model of colitis, which revealed that CyD1 was a potential anti-inflammatory target.

Other examples of antibody-mediated delivery of ONs include anti-HER2 for targeting mammary carcinoma cells (61), anti-CD19 for targeting malignant B cells (62), and anti-GD2 for targeting neuroblastoma or melanoma (63) (See Table I).

Table I. Summary of Targeted Delivery Approaches for ONs

Targeting moieties	Target tissues/cells	Delivery Strategies	References
LDL/HDL	Liver	Cholesterol-linked siRNA Tocopherol-linked siRNA	(35,36) (38)
Folate	A549 and KB cells HUVECs and human nasopharyngeal carcinoma cells (KB)	Folate-conjugated AS-ODN/siRNA Folate-coated liposomal ODN	(44,45) (42,43)
Transferrin	Ewing's sarcoma tumor cells Pancreatic cancer model Neuro2A xenograft tumor model	Cyclodextrin nanoparticles ScL-encapsulated HER2 siRNA Tf-OEI-HD polyplex	(52) (49,50) (51)
Antibody	Activated leukocytes/K562 Leukocytes Human B-lymphoma cell lines Neuroblastoma or melanoma	Antibody-protamine-siRNAs complexes Beta7 integrin Ab-HA coated liposomes CD19-targeted liposomes GD2-targeted immunoliposomes	(59) (60) (62) (63)
Peptide	HUVEC and N2A cells Hela cells	Tat peptide-siRNA conjugates RGD-PEG-PEI nanoparticles	(66,67) (68)
Aptamer	Human prostate cancer cells (LNCaP)	PSMA-specific aptamer-conjugated siRNA	(69,70)
Carbohydrate	Human hepatocarcinoma cell line (HuH-7)	Lactose-PEG-siRNA	(71,72)
Sigma receptor ligand	Human lung cancer cells (NCI-H460) xenograft model	Anisamide-coated nanoparticle anti-EGF receptor siRNA	(73–76)

LDL low-density lipoproteins, HDL high-density lipoproteins, HUVEC human vein endothelial cell, AS-ODN antisense oligodeoxyribonucleotide, Tf transferrin, OEI oligoethyleneimine, RGD arginine-glycine-aspartic acid, PEG poly(ethylene glycol), PEI polyethylenimine, PSMA prostate-specific membrane antigen, LNCaP lymph node carcinoma of the prostate, EGF epidermal growth factor

PEPTIDE-MEDIATED DELIVERY SYSTEMS

Cell-penetrating peptides are able to cross the cellular membrane. Therefore, they have attracted considerable attentions in the field of drug and nucleic acid delivery. The peptides include CPP, protein transduction domains, or membrane translocation sequences (64,65). They are usually short positively charged peptide sequences, mostly containing a stretch of arginine and/or lysine residues. Peptide-directed delivery of ONs can be carried out either by direct complexation or by conjugation with ONs or in combination with other delivery systems, such as liposomal or polymeric nanoparticles. Chiu *et al.* (66) linked Tat peptide, containing the sequence, YGRKKRRQRRR, to the 3'-terminus of the antisense strand of an siRNA. The siRNA-Tat peptide conjugate was rapidly internalized by cells and efficiently silenced the target gene.

Another group of peptides such as the arginine-glycine-aspartic acid (RGD) motif has been used for the purpose of targeting integrin receptors that are expressed on the activated endothelial cells found in tumor vasculature. Moschos *et al.* (37,67) attached a RGD motif to the end of PEG-conjugated forms of branched polyethylenimine and used the resultant nanoparticles to deliver siRNA *in vivo*.

Kumar *et al.* (68) reported the successful *in vivo* delivery of siRNA using a synthetic chimeric peptide consisting of a 29-amino acid rabies virus glycoprotein (RVG-9R) peptide. siRNAs were complexed with the positively charged RVG-9R peptide and found to be capable of delivering siRNA to neuronal cells and specifically silencing gene after intravenous administration (68). In addition, it was found that robust protection from lethal infection was achieved after treatment of mice with RVG-9R/siRNA complexes that targets the Japanese encephalitis virus.

APTAMERS-MEDIATED DELIVERY

Aptamers have been used for site-specific delivery of ONs because, like antibodies, they possess high affinity and specificity for their targets. Prostate-specific membrane antigen (PSMA) is a cell-surface receptor overexpressed in prostate cancer cells and in tumor vascular endothelium. PSMA-specific aptamers were capable of promoting specific cellular uptake and RNAi-mediated silencing of target mRNA *in vitro*. The RNAi-mediated silencing can be realized either by directly linking of an aptamer to the siRNA (69,70) or by coupling through biotin-streptavidin affinity (27). Aptamers have some advantages over other classes of targeting ligand such as peptides and antibodies. For example, they have low immunogenicity and can be easily synthesized in large quantities at relatively low cost. In addition, they are amenable to a variety of chemical modifications. Furthermore, the size of aptamers (<15 kDa) is much smaller than that of antibodies (150 kDa). Aptamer mediated delivery of ONs is promising but some issues such as pharmacokinetics and biodistribution of aptamer-ON conjugates need to be further studied.

OTHER LIGANDS-MEDIATED DELIVERY

Several groups have exploited the asialoglycoprotein receptors for nucleic acid delivery to hepatocytes, using ligands

containing galactose motifs. High-affinity asialoglycoprotein and mannose receptors are selectively expressed at high densities on the surface of hepatocytes. Oishi *et al.* (71,72) constructed a lactosylated poly(ethylene glycol)-siRNA (Lac-PEG-siRNA) conjugate through an acid-labile linkage. Under the slightly acidic conditions of the intracellular endosomal compartment, the covalent linkage of Lac-PEG-siRNA was disrupted and free siRNA was released in cultured HuH-7 cells enabling efficient target silencing.

Li *et al.* (16,33,73-75) developed a sigma receptor targeted lipopolyplex nanoparticle (NP) formulation for systemically delivering siRNA into lung metastatic tumors. Anti-EGF-receptor siRNA (as well as carrier DNA) was complexed with protamine and then with liposomes. The particles were passivated with PEG and targeted using anisamide as a ligand for lung cancer cells. Anisamide is a ligand for the sigma receptor. The targeted NP effectively penetrated the lung metastasis and resulted in 70-80% gene silencing in the metastasis model after a single i.v. injection (150 µg siRNA/kg) (16,33).

In a recent study, Sato *et al.* (76) reported vitamin-A mediated *in vivo* targeting of siRNA into stellate cells. Using this strategy, specific and significant gene silencing was found in stellate cells, which reversed liver cirrhosis.

CONJUGATION CHEMISTRY FOR ATTACHING LIGAND TO ONs

Thioether, disulfide, and amide covalent linkages are frequently used for connecting targeting ligands to the termini of ONs or the surface of nanoparticles (77). To overcome steric hindrance, PEG is usually used as a linker between the ligand and ONs or nanoparticles. An acid-labile linkage based on a β -thiopropionate was developed to link the lactosylated PEG and siRNA. The β -thiopropionate linkage is confirmed to be readily cleaved at acidic pH of the endosomal compartment (71,72). The streptavidin-biotin affinity based noncovalent conjugation strategy can also be used for conjugate synthesis (27).

BIODISTRIBUTION AND PHARMACOKINETIC (PK) DIFFERENCES BETWEEN FREE ONs AND FORMULATED ONs

Due to its polyionic and hydrophilic properties, normal ONs are quickly eliminated from the blood flow by nuclease degradation, renal clearance, and by the RES. Although siRNA duplexes are more stable than single strand RNAs (ssRNAs), they still have very short half-lives *in vivo* (12,20). One study (51) indicated that ¹²⁵I-labeled siRNAs were widely distributed in the body after intravenous injection into mice, with preferential accumulation in the liver and kidney. The radiolabeled siRNA was also observed in the heart, spleen, and lung. However, the level of siRNA in the brain was not detectable. In another study using rats, naked siRNA showed a serum half-life of 6 min and a clearance of 17.6 mL/min. The poor pharmacokinetic properties of free siRNAs are due to both poor stability and rapid clearance. ONs with chemical modifications such as phosphorothioation and 2'-O-methylation have improved serum stability. However, they are still subjected to rapid clearance by the kidneys and the liver (20,22,78). In contrast, cholesterol

modified AS-ODNs and siRNAs preferentially accumulate in the liver due to interactions with lipoproteins (35,79).

Reformulation of the ONs into delivery vehicles can prolong their systemic circulation time, alter the biodistribution, and potentially enhance their accumulation in tumor sites. One important mechanism for tumor specific accumulation of the nanoparticles is based on the “enhanced permeability and retention” effect. Nanoparticles carrying ONs in blood flow can enter tumor tissues through gaps between and fenestrations on endothelial cells and are not cleared from the tumor site due to reduced lymphatic drainage. To determine PK and biodistribution properties of ON-loaded nanoparticles, several important factors including particle size, surface charge (zeta potential), and surface chemistry (e.g., degree of PEGylation, ligand conjugation) need to be considered.

Targeting tumor *in vivo* is much more challenging than targeting tumor cells *in vitro*. For example, many biodistribution studies revealed no difference in the levels of tumor accumulation between nontargeted liposomes and targeted liposomes (16). As shown by Kirpotin *et al.* (61), the PK and tissue distribution of the nontargeted and anti-Her targeted siRNA liposomes showed very similar profiles. The enhanced therapeutic effects of targeted siRNA liposome were primarily due to increased internalization of siRNA liposome with the aid of anti-HER antibody. Coating of anti-HER antibody on nanoparticles did not increase the level of accumulation of siRNAs in the target tumor but increased the rate of intracellular delivery. Similar findings have been reported on Tf coated polymeric nanoparticles (24) and anisamide directed LPD nanoparticles (16,33,74,75). Thus, optimized internalization of nanoparticle delivery system may be critical for the effective siRNA delivery.

CONCLUDING REMARKS AND PERSPECTIVES

With better understanding of molecular events associated for human diseases, antisense and siRNA oligonucleotides can be rationally designed to interfere with specific targets. A major challenge to the development of therapeutic ONs is specific and efficient *in vivo* delivery to the target cells. Chemical modifications, nanoparticle incorporation, and conjugation of ligand to ONs and/or their nanoparticle carriers are promising strategies for improving the stability, pharmacokinetic properties, and target specificity of these agents. Given the significant advantages shown in recent studies, targeted delivery technologies will likely drive the future development of oligonucleotide therapeutics.

ACKNOWLEDGEMENT

This work was supported in part by NSF grant EEC-0425626.

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