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reviews

Nonviral Methods for siRNA Delivery

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Abstract: RNA interference (RNAi) as a mechanism to selectively degrade mRNA (mRNA) expression has emerged as a potential novel approach for drug target validation and the study of functional genomics. Small interfering RNAs (siRNA) therapeutics has developed rapidly and already there are clinical trials ongoing or planned. Although other challenges remain, delivery strategies for siRNA become the main hurdle that must be resolved prior to the full-scale clinical development of siRNA therapeutics. This review provides an overview of the current delivery strategies for synthetic siRNA, focusing on the targeted, self-assembled nanoparticles which show potential to become a useful and efficient tool in cancer therapy.

Keywords: RNA interference; small interfering RNA; delivery; nanoparticles

Introduction

Ever since RNA interference (RNAi) was discovered by Fire et al. in 1998,[1] this technology has rapidly become a powerful tool in basic research and potentially a new strategy for clinical trials. Small interfering RNA (siRNA), produced from cleavage of longer dsRNA precursors by the RNaseIII endonuclease dicer, can enter the RNA-induced silencing complex (RISC), which is activated upon guide (antisense) strand selection.[2] The selection is based on the relative thermodynamic stabilities of the two duplex ends, and it is the least stable 5′ end of the duplex that is recognized and asymmetrically unwound by the Piwi-Argonaute-Zwille (PAZ) domain of argonaute 2, a multifunctional protein within the RISC. The incorporated strand acts as a guide for the activated RISC complex to selectively degrade the complementary mRNA.[3] By targeting an oncogene, siRNA could be applied as a therapeutic agent in cancer therapy.[4] However, due to its relatively large molecular weight and polyanionic nature, naked siRNA does not freely diffuse across the cell membrane, and thus a delivery system is required to facilitate siRNA access to its intracellular sites of action.

The success of gene therapy is highly dependent on the delivery vector, which can be generally categorized into viral and nonviral vector.[5] Viral vectors are highly efficient; they are currently still the most powerful tool for gene transfection. However, some viral vectors show a limited loading capacity, are difficult to produce in large scale and, most importantly, pose severe safety risks due to their oncogenic potential and their inflammatory and immunogenic effects, which prevent them from repeated administration. To overcome these limitations, nonviral vectors have emerged as a promising alternative for gene delivery. A number of

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Table 1. Delivery Systems for siRNA in Vivo

delivery system	mechanism	target tissue or model	characteristics
hydrodynamic iv or direct injection	High pressure contributes to penetration across the cell membrane.	rat brain,[86] mouse liver,[16] mouse lung[66]	relative simplicity of local administration
cholesterol conjugation with siRNA	Promote distribution and cellular uptake via lipoprotein as a carrier.	dyslipidemia in mice[18]and nonhuman primate[20]	Significantly decrease the complexity by conjugated with the sense strand.
liposomes and lipoplexes	Improve pharmacokinetic properties and/or reduce toxicity profiles.	dyslipidemia in monkeys,[40] pancreatic tumor xenografts in mice,[32]breast cancer xenografts in mice,[64]prostate cancer xenograft in mice.[77]	similarity to commercial transfection agents
polymers and peptide delivery systems for siRNA	Endosomal escape takes place because of "proton sponge" effect. Improve selectively and specifically deliver siRNA in vivo.	Ewing sarcoma in mice,[60]mouse brain,[56]melanoma xenografts in mice[50]	Condensed nanoparticles with siRNA. Can be modified with a targeting element for receptor mediated uptake.
surface modified LPD nanopartcles	siRNA condensed with protamine to form a core, which is wrapped with cationic lipid membrane. Final PEGylation provides surface protection and targeting specificity.	Oncogenes in solid and metastatic tumors can be effectively silenced in mouse models.[21,80,93,95]	very high tumor uptake and low immunotoxicity

nonviral siRNA delivery approaches have now been reported in vivo, including in nonhuman primates and humans.[6−14]

Nonviral Vectors for siRNA Delivery

Of primary consideration in deciding on a drug delivery system for siRNA is whether the intended disease target lends itself to systemic or local administration. In the case of delivery of DNA encoding for the short hairpin RNA (shRNA) by nonviral delivery systems, nuclear translocation of the DNA is often inadequate. Most attention will be given to nonviral delivery of siRNA. Various strategies for delivering siRNA to specific tissue and organ systems in vivo following systemic administration are summarized here (Table 1). The delivery strategy can be classified into the categories presented in the following paragraphs.

A. Hydrodynamic Injection

Quick injection of siRNA in a large volume of physiological buffer effectively localizes duplex siRNA in the liver. In rats, administration of a VEGF-specific siRNA resulted in more than 75% inhibition of pathological neovascularization. Due to the invasiveness of the injection technique, hydrodynamics-based transfection is not appropriate for clinical applications at this point.[15,16] However, recent advances in using a computer-controlled, catheter-guided injection device have greatly improved the precision and

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reproducibility of this approach.[17] To date, the device has only been used for the delivery of DNA, but siRNA should be equally applicable in this approach.

B. Cholesterol Conjugation with siRNA

A siRNA with chemically modified backbone conjugated to a lipophilic cholesterol moiety at the 3′ end of the sense strand led to suppression of apoB mRNA by approximately 60% in the liver after iv injection. Soutschek et al. reported that no immune stimulation or off-target effect occurred in mice at a high dose of 50 mg/kg.[18,19] However, safety evaluation of this potential approach needs further study. Recent studies indicated that the cholesterol-conjugated siRNA is delivered into the hepatocytes as a complex of lipoproteins.[20]

C. Cationic Delivery Systems

Cationic lipids and liposomes, cationic polymers, cationic dendrimers, and cationic cell-penetrating peptides have been used for the delivery of siRNA. A common characteristic among these vectors is their net positive charge, which contributes to both complex formation with the polyanionic nucleic acid, such as siRNA, and interaction with the negatively charged cell membrane.[6,10–13,21–26] Nanoparticles and complexes should be <100 nm to reduce renal excretion and be taken up by cells,[21] because they are taken into the cell via endocytosis and/or macropinocytosis.

1. Liposomes and Lipoplexes. Liposomes consist of an aqueous compartment enclosed in a phospholipid bilayer with hydrophilic drug typically entrapped in the center aqueous layer. The bilayer often contains a lipid component (regularly is a cationic and/or a fusogenic lipid), cholesterol and polyethylene glycol-lipid. The liposomes are particles with stable physicochemical characteristics. In contrast, lipoplexes are spontaneously formed via interaction of positively charged lipids and negatively charged nucleic acids. Lipoplexes should be prepared immediately before use because lipoplexes are unstable.[27] Lipofectin, RNAifect, Oligofectamine, Lipofectamine and TransIT TKO are commercially available as potential enhancers of siRNA delivery in vitro.[24,26,28–42] DOTAP (*N*-[1-(2,3-dioleoyloxy)]-*N*,*N*,*N*-trimethyl ammonium propane) and Oligofectamine were some of the first lipid formulations to be used for the in vivo delivery of siRNA and effective gene silencing of TNF-α and β-catenin in mice.[29,30] Cationic liposomes termed "solid

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nucleic acid lipid particles" (SNALPs) that have been stabilized by PEGylation for improved pharmacokinetics have also been successfully used to deliver siRNA in mice and nonhuman primates. In monkeys, ApoB were markedly suppressed at a does of 2.5 mg/kg of SNALP-formulated siRNA. Furthermore, it should be noted that PEGylated liposomes are a clinically approved delivery system for doxorubicin and therefore represent a viable option for delivering siRNA in humans.[28]

2. Polymers and Peptide Delivery Systems. Of the many cationic polymers, polyethyleneimine (PEI) has been widely examined for DNA, oligonucleotide, and siRNA delivery.[13,23,24,26,43−64] Now, a novel delivery strategy using PEGylated PEI with an RGD (Arg-Gly-Asp) peptide to deliver siRNA targeting VEGF has been demonstrated inhibit tumor growth and reduce angiogenesis after iv administration. The use of polyamidoamine polymers in vivo might be hindered owing to their nonspecific toxicity. Atelocollagen (300 kDa) has been used to administer siRNA systemically and locally in tumor models.[48,49,53,65] In vivo, this polymer was also able to effectively deliver siRNA targeting VEGF to tumor vasculature in an

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orthotopic model of human testicular cancer, to bone metastases, and to a xenograft model of prostate cancer.[49,53,65] A cyclodextrin polycation delivery system is well tolerated, and even repeat doses failed to elicit a significant delivery system-specific antibody response. More recently, there was a report that repeated administration of RVG (rabies virus glycoprotein)-9R-bound antiviral siRNA did not induce inflammatory cytokines or antipeptide antibodies. The study also reported for the first time that systemically delivered siRNA-peptide conjugates can cross the blood−brain barrier. (BBB).[56]

D. Other Delivery Systems

1. Intraocular Delivery. Local delivery to the eye of naked and lipid-complexed siRNA is possible, and such studies have paved the way for human clinical trials.[52,56,66–88] siRNA targeting VEGF was delivered to prevent laser-induced choroidal neovascularization in an experimental model of age-related macular degeneration (AMD).[67] There is also an ongoing clinical trial in a similar direction. (ALN-RSV01; Alnylam Pharmaceuticals [Cambridge, MA] press release.AKI-i5; Quark Pharmaceuticals press release.) FDA recently approved a clinical study of a cyclodextrin-containing nanoparticle formulation for siRNA (RONDEL). Calando's RONDEL is two-part siRNA delivery system. The first component is a linear, cyclodextrin-containing polycation that binds to the anionic backbone of the siRNA. The polymer and siRNA self-assemble into nanoparticles with size smaller than 100 nm in diameter that fully protect the siRNA from nuclease degradation in serum. The nanoparticles are additionally modified by adding adamantane and conjugated PEG with or without the targeting ligand transferrin. The siRNA delivery system has been designed to allow for intravenous injection. Upon delivery to the target cell, the targeting ligand binds to membrane receptors on the cell

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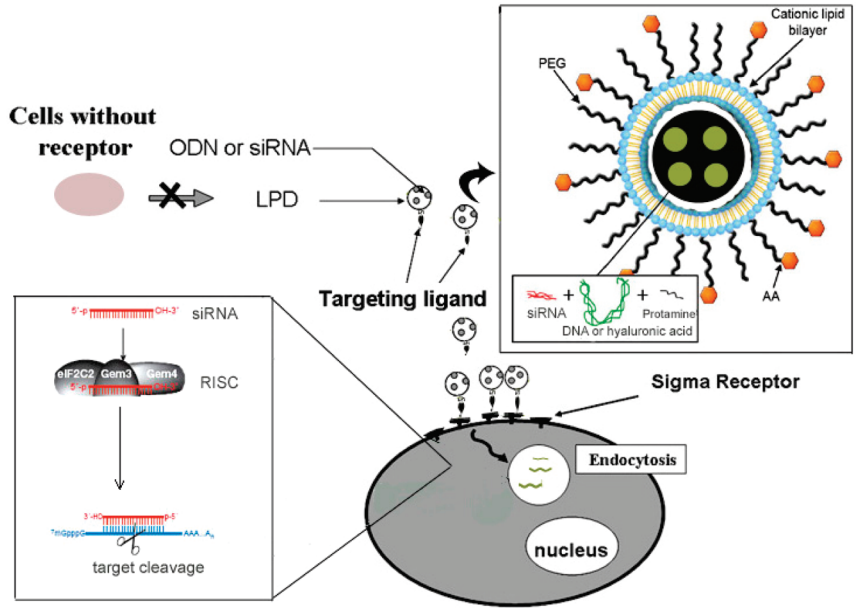

Figure 1. A schematic depiction of the structural components required of a targeted cationic complex or nanoparticle for siRNA delivery in vivo. Optimally designed and formulated siRNA can cross the endosome membrane and provide a sequence-specific gene-silencing effect.

surface and the RNA-containing nanoparticle is taken into the cell by endocytosis.

2. Intratumoral Delivery. Direct local injection of siRNA, with or without a delivery system, into the tumor has been an effective anticancer approach in vivo. Delivery of siRNA targeting RasA and RasC in a xenograft breast cancer model in mice has been shown to successfully reduce tumor volume.[74] However, clinically, only a few tumors are suitable for local delivery; systemic approaches described above are necessary for most solid tumors in vivo.

3. Local in Vivo Electroporation to Muscle. Electroporation has been used to deliver siRNA to mouse muscle tissue. Golzio et al. showed silencing effects lasted 23 days after administration of siRNA cotransfected with a plasmid encoding GFP in mice.[81]

4. Local Delivery to the CNS. Local delivery of siRNA to the CNS is a feasible option for targeting CNS-based diseases, though systemic delivery strategies may be clinically more attractive. The delivery of siRNA targeting the NR2B subtype of the NMDA receptor using intrathecal injection led to a 83% decrease in the protein's expression level in the rat brain.[70]

5. Intranasal Delivery to the Airway. Delivering siRNA to the airway could be a treatment for a number of diseases including asthma, cystic fibrosis, ischemic reperfusion injury, and infection with respiratory viruses.[66,84,87] Thomas reported marked gene silencing in an acute model of lung injury, and Li et al. showed the treatment of experimental infection with SARS virus in a rhesus macaque model.[68,81,89]

E. Self-Assembled LPD Nanoparticles

We have used surface-modified LPD nanoparticles to deliver siRNA to solid and metastatic tumors. The major components of the delivery systems are cationic lipids and protamine (a cationic polypeptide), which can interact with negatively charged siRNA. Surface steric stabilization is introduced by PEGylation to prevent the aggregation of the resulting complex with serum components. Ligands are attached to the distal end of the PEG chain to increase cellular bioavailability. Cationic lipid is necessary for endosome lysis and intracellular release of siRNA. The mechanism of the endosome membrane destabilization is most likely due to the formation of ion pair complex between the cationic lipid in the nanoparticles and the negatively charged anionic lipids in the endosome membrane, as hypothesized by Xu and Szoka.[90] We have used anisamide as a targeting ligand for tumor cells expressing the sigma receptor.[91,92] Our ligand targeted, PEGylated LPD formulation showed significant increase in cellular uptake via specific receptor-mediated pathway. It is important to note that the function of anisamide is not the tumor uptake, since nontargeted LPD nanoparticles accumulated in the tumor as efficiently as the targeted particles.[91] The surfaced modified LPD nanoparticles showed a strong enhanced permeability and retention (EPR) effect in that approximately 60−80% of the injected dose per gram

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of tissue was found in the tumor.[93] This targeted formulation also demonstrated its strong gene-silencing effect mediated by RNAi (Figure 1 and see below). Preliminary data showed that the surface-modified LPD delivered siRNA predominantly to the tumor, which was the major uptake organ, after intravenous administration. Our formulation provides an advantage of high tumor targeting and low RES uptake, which implied its potential for RNAi-based tumor therapy.[93]

Our subsequent study confirmed that the celluar entry mechanism for the targeted nanoparticles was via a sigma receptor dependent pathway.[80] The gene silencing activity was significantly improved, and nanoparticle formulations were not immunotoxic. In B16F10 melanoma cells targeted nanoparticles showed a 4-fold increase in delivery efficiency compared to nontargeted nanoparticles. Addition of free haloperidol, a known agonist for the sigma receptor, significantly reduced the delivery efficiency of targeted nanoparticles but not other formulations. Since the B16F10 cells were stably marked by a luciferase gene by using a retrovirus vector, the in vivo activity of the nanoparticle formulations was assessed by the luciferase gene silencing in the whole B16F10 tumor loaded lungs. Targeted nanoparticles silenced about 75% luciferase activity, whereas none of the other control formulations showed any significant gene silencing activity. None of the formulations, including the targeted nanoparticles, induced significant proinflammatory cytokines (IL6, IL12, TNF and IFN-α) in the serum.

We combined three different siRNA sequences (MDM2/c-myc/VEGF = 1:1:1, weight ratio) into different formulations to evaluate the therapeutic outcomes.[80] In the B16F10 tumor-loaded lung, siRNA in the targeted nanoparticles significantly reduced the tumor load to 20−30% as compared to the untreated control ($P < 0.01$). The therapeutic outcome was also analyzed in terms of animal survival. On day 23, the survival rate for the therapeutic siRNA and the control siRNA in the targeted nanoparticles was 90 and 40%, respectively, and the mean survival time was 28 and 22.5 days, respectively ($P < 0.01$). Furthermore, we demonstrated that, at the therapeutic dose (0.45 mg/kg), the targeted nanoparticles did not induce significant production of all analyzed cytokines, including IL-6, IL-12, TNF-α, and interferon-α. Even after two consecutive injections, the IL-6 and IL-12 levels were not significantly elevated. At the therapeutic dose, none of the formulations caused elevation in cytokine production in the lung. Additionally, the body weight did not significantly decrease during treatment at the therapeutic dose. The targeted nanoparticle formulation did not damage the major organs (heart, liver, spleen, lung, and kidney).

Since the self-assembly of the nanoparticles requires the presence of a high molecular weight polyanion for stability, calf thymus DNA was initially used as a carrier for the siRNA which is a low molecular weight nucleic acid. Calf thymus DNA as a foreign DNA to human may cause unwanted toxicity and immune stimulation. Plasmid DNA contains a high amount of unmethylated CpG motifs that is hostile to the cell.[94] Recently, we have successfully replaced calf thymus DNA with a high molecular weight anionic polysaccharide, i.e., hyaluronic acid.[93,94] The resulting formulation contains cationic liposome, protamine and hyaluronic acid and is called LPH. LPH showed the same gene silencing activity as the corresponding LPD formulation. The ED_{50} for the luciferase silencing in the B16F10 melanoma model was 75 μg/kg in siRNA, the same as the LPD formulation. However, the induction of both IL-6 and IL-12 cytokines by LPH was significantly lower than that of the corresponding LPD containing the calf thymus DNA.

Conclusions

Since the discovery of RNA interference, research groups worldwide have sought to invent efficient delivery systems to enhance the ability of siRNA to traverse the cell membrane and elicit a biological response. The most widely used siRNA delivery methodologies consist of cationic lipids and/or cationic polymers that package siRNA into stable nanoparticles capable of translocating across the cellular membrane. Encapsulation of siRNA into the naked nanoparticles dramatically increased the intracellular delivery and the gene silencing activity through the charge−charge interaction of the formulation with the endosome membrane. PEGylation of the naked nanoparticles abolished the nonspecific interaction with negatively charged cells or proteins and also enhanced the uptake of siRNA by the tumor due to the EPR effect. Introduction of a targeting ligand at the distal end of the PEG chain restored the intracellular delivery to the receptor positive cells, while the tissue selectivity was maintained. This targeted nanoparticle formulation with improved tissue specificity and delivery efficiency silenced the target gene in the lung metastasis effectively without any significant immunotoxicity. The immunotoxicity of the nanoparticles was further reduced by replacing the high molecular weight DNA with hyaluronic acid. We therefore conclude that siRNA formulated in the targeted nanoparticles has the potential to become a useful tool in clinical cancer therapy.

The almost ideal specificity of RNAi is not entirely true in reality. Silencing of off-targets is clearly unwanted. Importantly, off-target effect remains a critical issue for therapeutic applications of RNAi. Hopefully, novel protein array technology will provide a better picture of siRNA effects on cellular protein expression profiles and provide a better way of screening siRNA. Developing and identifying potent or hyperfunctional siRNA will help resolve unwanted off-targeting since these siRNAs works at subnanomolar

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concentration. Likewise intelligent and effective design of siRNA that improves strand selectivity may be possible to significantly avoid off-target effects. While knowledge of immune stimulatory properties calls for research to proceed to animal models, in vitro use of human primary cells with a full repertoire for immune stimulation is also needed. Long dsRNA would induce interferon responses by binding to double-stranded-RNA-activated protein kinase (PKR), 2′,5′-oligoadenylate synthetase RNase L system or several Toll-like receptors (TLRs). A particular sequence motif (5′-GUCCUUCAA-3′), GU-rich regions and CpG motifs can stimulate innate immune responses. Recently, Dharmacon published that even short 23 nt long siRNA may invoke interferon responses in cell culture assays. Great care and thorough testing are clearly needed before proceeding to clinical use. Importantly, the length threshold seems to vary among cell types, which makes it hard to predict the outcome of dicer substrate in vivo.

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