

Second Edition

Antisense Drug Technology

Principles, Strategies, and Applications



Edited by
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Discovery and Development of RNAi Therapeutics

Antonin R. de Fougérolles and John M. Maraganore

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16.1 INTRODUCTION

In less than a decade since its discovery, RNA interference (RNAi) as a novel mechanism to selectively silence messenger RNA (mRNA) expression has revolutionized the biological sciences in the postgenomic era. With RNAi, the target mRNA is enzymatically cleaved, leading to decreased levels of the corresponding protein. The specificity of this mRNA silencing is controlled very precisely at the nucleotide level. Given the identification and sequencing of the entire human genome, RNAi is a fundamental cellular mechanism that can also be harnessed to rapidly develop novel drugs against any disease target. The reduction in expression of pathological proteins through RNAi is applicable to all classes of molecular targets, including those that have been traditionally difficult to target with either small molecules or proteins, including monoclonal antibodies. Numerous proof-of-concept studies in animal models of human disease have demonstrated the broad potential applicability of RNAi-based therapeutics. Further, RNAi therapeutics are now under clinical investigation for age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection, with numerous other drug candidates poised to advance into clinical development in the years to come.

In this review, we will outline and discuss the various considerations that go into developing RNAi-based therapeutics starting from *in vitro* lead design and identification, to *in vivo* preclinical drug delivery and testing, and lastly, to a review of clinical experiences to date with RNAi therapeutics. While both nonviral delivery of small interfering RNAs (siRNA) and viral delivery of short hairpin RNA (shRNA) are being advanced as potential therapeutic approaches based on RNAi, this review will focus solely on development of synthetic siRNA as drugs. Synthetic siRNAs can harness the cellular RNAi pathway in a consistent and predictable manner with regard to the extent and duration of action, thus making them particularly attractive as drugs. As a consequence, siRNAs are the class of RNAi therapeutics that is most advanced in preclinical and clinical studies.

16.2 IN VITRO SELECTION OF LEAD CANDIDATES

This section highlights the various steps required to identify potent lead siRNA candidates starting from bioinformatics design through to *in vitro* characterization. The overall scheme for turning siRNA into drugs is summarized in Figure 16.1. The three most important attributes to take into account when designing and selecting siRNA are potency, specificity, and nuclease stability.

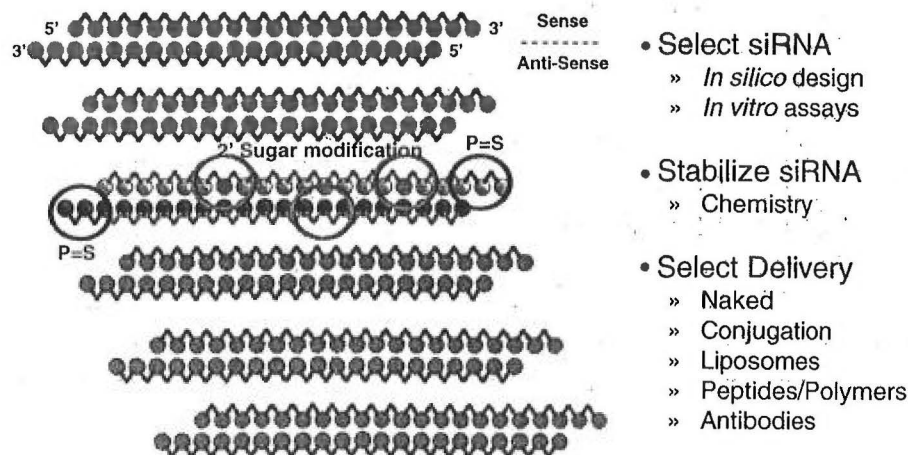


Figure 16.1 Turning siRNA into drugs. Outline of steps involved in development of an RNAi therapeutic. This three-step process begins with *in silico* design and *in vitro* screening of target siRNA, followed by incorporation of stabilizing chemical modifications on lead siRNA as required, and ending with selection and *in vivo* evaluation of delivery technologies appropriate for the target cell type/organ and the disease setting.

With regard to specificity of siRNA, the two issues of “off-targeting” due to the silencing of genes sharing partial homology to the siRNA and “immune stimulation” due to recognition of certain siRNAs by the innate immune system have been of special concern. With an increased understanding of the molecular and structural mechanism of RNAi, all issues around lead siRNA selection are better understood and also are now generally resolvable through the use of bioinformatics, chemical modifications, and empirical testing. Thus, it is now possible to very rapidly, in the span of several months, identify potent, specific, and stable *in vitro* active lead siRNA candidates to any target of interest.

16.2.1 Potency

Work by Tuschl and colleagues [1] represented the first published study to demonstrate that RNAi could be mediated in mammalian cells through the introduction of small fragments of double-stranded RNA (dsRNA), termed small interfering RNA, and that siRNAs had a specific architecture comprised of 21 nucleotides in a staggered 19-nucleotide duplex with a 2-nucleotide 3' overhang on each strand (Figures 16.1 and 16.2). Further elaboration and dissection of the RNAi pathway, including insights from X-ray crystallographic structures, have revealed that long dsRNAs are naturally processed into siRNAs via a cytoplasmic RNaseIII-like enzyme called Dicer. A multienzyme complex known as the RNA-induced silencing complex (RISC) then unwinds the siRNA duplex. The siRNA-RISC complex functions enzymatically to recognize and cleave mRNA strands complementary to the “antisense” or “guide” strand of the siRNA. The target mRNA is then cleaved between nucleotides 10 and 11 (relative to the 5' end of the siRNA guide strand). Loading of RISC with respect to the sense and antisense siRNA strands is not symmetrical. The efficiency with which the antisense or guide strand is incorporated into the RISC machinery (versus incorporation of the sense strand) is the most important determinant of siRNA potency. Through analysis of strand-specific

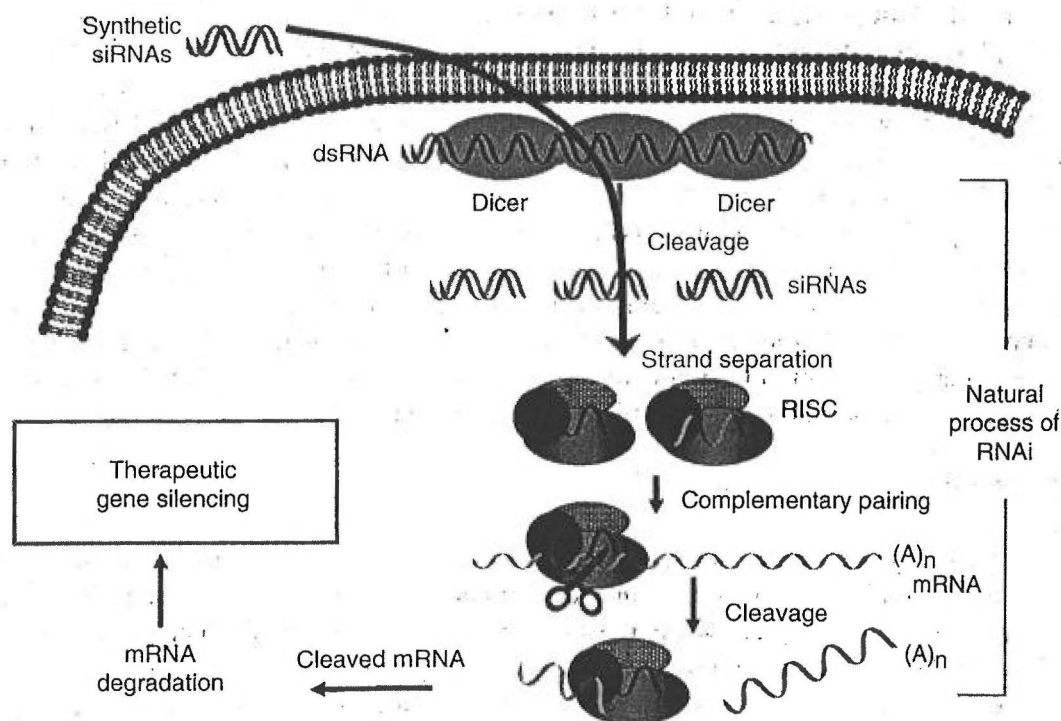


Figure 16.2 Harnessing the natural RNAi process with synthetic siRNA. Long double-stranded RNA (dsRNA) is cleaved into short stretches of dsRNA called siRNA. The siRNA interact with the RISC to selectively silence target mRNA. siRNA against any mRNA target can be chemically synthesized and introduced into cells, resulting in specific therapeutic gene silencing.

reporter constructs [2] and large sets of siRNA of varying potency [3,4], it was found that RISC preferentially associates with the siRNA duplex strand whose 5' end is bound less tightly with the other strand. A detailed description of RNAi-mediated silencing as it relates to siRNA and other small RNAs by Sigova and Zamore can be found in Chapter 3 of the book.

16.2.2 Specificity

RNAi-mediated silencing of gene expression has been shown to be exquisitely specific as evidenced by silencing fusion mRNA without affecting an unfused allele [5,6] and by studies showing ability to silence point-mutated genes over wild-type sequence [7]. Nevertheless, along with on-target mRNA silencing, siRNA might have the potential to recognize nontarget mRNA, otherwise known as "off-target" silencing. On the basis of *in vitro* transcriptional profiling studies, siRNA duplexes have been reported to silence multiple genes in addition to the intended target gene under certain conditions. Not surprisingly, many of these observed off-target genes contain regions that are complementary to one of the two strands in the siRNA duplex [8–10]. More detailed bioinformatic analysis revealed that complementarity between the 5' end of the guide strand and the mRNA was the key to off-target silencing, with the critical nucleotides being in positions 2–8 (from the 5' end of the guide strand) [11,12]. Accordingly, careful bioinformatics design of siRNA can reduce potential off-target effects. Further, published work has shown that the incorporation of 2'-O-Me ribose modifications into nucleotides can suppress most off-target effects while maintaining target mRNA silencing [13,14]. In fact, incorporation of a single 2'-O-Me modification at nucleotide 2 was sufficient to suppress most off-target silencing of partially complementary mRNA transcripts by all siRNAs tested. Thus, in summary, bioinformatics design and position-specific, sequence-independent chemical modifications can be incorporated into siRNA that reduce off-target effects while maintaining target silencing.

A second mechanism whereby siRNA can induce potentially unwanted effects is through stimulation of the innate immune system in certain specialized immune cell types. It has been demonstrated that siRNA duplexes contain distinct sequence motifs that can engage Toll-like receptors (TLRs) in plasmacytoid dendritic cells and lead to increased interferon-alpha production [15]. In much the same way that certain CpG motifs in antisense oligonucleotides are responsible for TLR-9-mediated immunostimulation, the interferon induction seen with discrete siRNA nucleotide motifs was found to occur largely via TLR-7. Much additional work remains to be done in identifying the full spectrum of immunostimulatory motifs and whether other receptors might also be involved (reviewed in [16]). Several approaches exist to circumvent the immunostimulatory properties of certain siRNA duplexes. First, *in vitro* assays exist to rank-order duplexes for their ability to induce interferon when transfected into plasmacytoid dendritic cells [15]. Second, several groups have shown that introduction of chemical modifications, such as 2'-O-Me modifications, are capable of abolishing immunostimulatory activity [15,17,18]. Third, siRNA delivery strategies can be employed that would avoid the cell types responsible for immune stimulation.

16.2.3 Stability

Not surprisingly, numerous studies have shown that the chemical modification of siRNA duplexes, including chemistries already in use with antisense oligonucleotide and aptamer therapeutics, can protect against nuclease degradation with no effect or intermediate effects on activity [19–21]. For instance, introduction of a phosphorothioate (P=S) backbone linkage at the 3' end is used to protect against exonuclease degradation and 2' sugar modification (2'-O-Me, 2'-F, others) is used for endonuclease resistance. With respect to maintenance of RNAi silencing activity, exonuclease-stabilizing modifications are all very well tolerated. Introduction of internal sugar modifications to protect against endonucleases is also generally tolerated but can be more dependent on the location of the modification within the duplex, with the sense strand being more

amenable to modification than the antisense strand. Nevertheless, using simple, well-described modifications such as P = S, 2'-O-Me, and 2'-F, it is possible in most instances to fully nuclease-stabilize an siRNA duplex and maintain mRNA silencing activity. Importantly, the degree of modifications required to fully stabilize the siRNA duplex can generally be limited in extent, thereby avoiding the toxicities associated with certain oligonucleotide chemistries.

Improved nuclease stability is especially important *in vivo* for siRNA duplexes that are exposed to nuclease-rich environments (such as blood) and are formulated using excipients that do not themselves confer additional nuclease protection on the duplex. As might be expected in these situations, nuclease-stabilized siRNA show improved pharmacokinetic properties *in vivo* (Alnylam, unpublished results). In other situations, when delivering siRNA directly to more nuclease-amenable sites such as the lung or when delivering in conjunction with delivery agents such as liposomes, the degree of nuclease stabilization that is required can be reduced significantly. While the ability of an siRNA duplex to reach its target cell type intact is vitally important, whether nuclease protection confers a measurable benefit once an siRNA is inside the cell remains to be determined. While *in vitro* comparisons of naked siRNA versus fully stabilized siRNA do not reveal significant differences in longevity of mRNA silencing [22], these studies have typically been performed using rapidly dividing cells, where dilution due to cell division, and not intracellular siRNA half-life governs the duration of gene silencing [23]. With the recent advent of fluorescence resonance energy transfer studies using siRNA [24], it should be possible in the near future to understand more completely the intracellular benefit of nuclease stabilization on the longevity of RNAi-mediated silencing.

16.2.4 Therapeutic Considerations

With the identification of active siRNA, a set of rules were initially proposed for selecting potent siRNA duplex sequences [1]. Subsequently, a number of groups have developed more sophisticated algorithms based on empiric testing to identify multiple criteria that can contribute to defining an active siRNA [25–27]. Using current algorithms, sub-nM IC₅₀ *in vitro* active siRNA can be routinely identified in a quarter to a half of the designed siRNA with a subset of siRNA often demonstrating low pM activity.

In designing siRNAs for therapeutic purposes, other considerations beyond an active target sequence exist. Where possible, it is desirable to identify target sequences that have identity across all the relevant species used in safety and efficacy studies, thus enabling development of a single drug candidate from research stage all the way through clinical trials. Other considerations in selecting a target sequence involve the presence of single-nucleotide polymorphisms and general ease of chemical synthesis.

Predicting the nucleotide sequence and chemical modifications required to yield an ideal RNAi therapeutic still remains a work in progress. While much progress has been made in understanding what attributes are required to identify an *in vitro* active and stable siRNA, much less is known about how well those attributes translate into identifying *in vivo* active siRNAs. For example, many of the issues around specificity are based on *in vitro* data and their *in vivo* relevance remains to be determined. For example, the range of off-target genes identified in tissue culture can differ dramatically depending upon the transfection method used to introduce siRNAs into cells [28]. Likewise, induction of innate immune responses by certain siRNAs has been shown to be cell-type specific [29]. At present, in order to identify robust *in vitro* active lead candidate siRNAs suitable for subsequent *in vivo* study, the practical and prudent approach is to synthesize and screen a library of siRNA duplexes for potency, specificity, nuclease stability, and immunostimulatory activity. A good example of such an empiric approach was a screen that we conducted for siRNA targeting all vascular endothelial growth factor (VEGF)-A spliced isoforms to treat AMD. Over 200 siRNA with different sequences and chemistries were evaluated from which an optimized clinical candidate, ALN-VEG01, was selected. This optimization procedure resulted in an siRNA with picomolar *in vitro* activity and sustained silencing in the relevant ocular cell type than was superior to other published VEGF siRNA compounds (Figure 16.3).

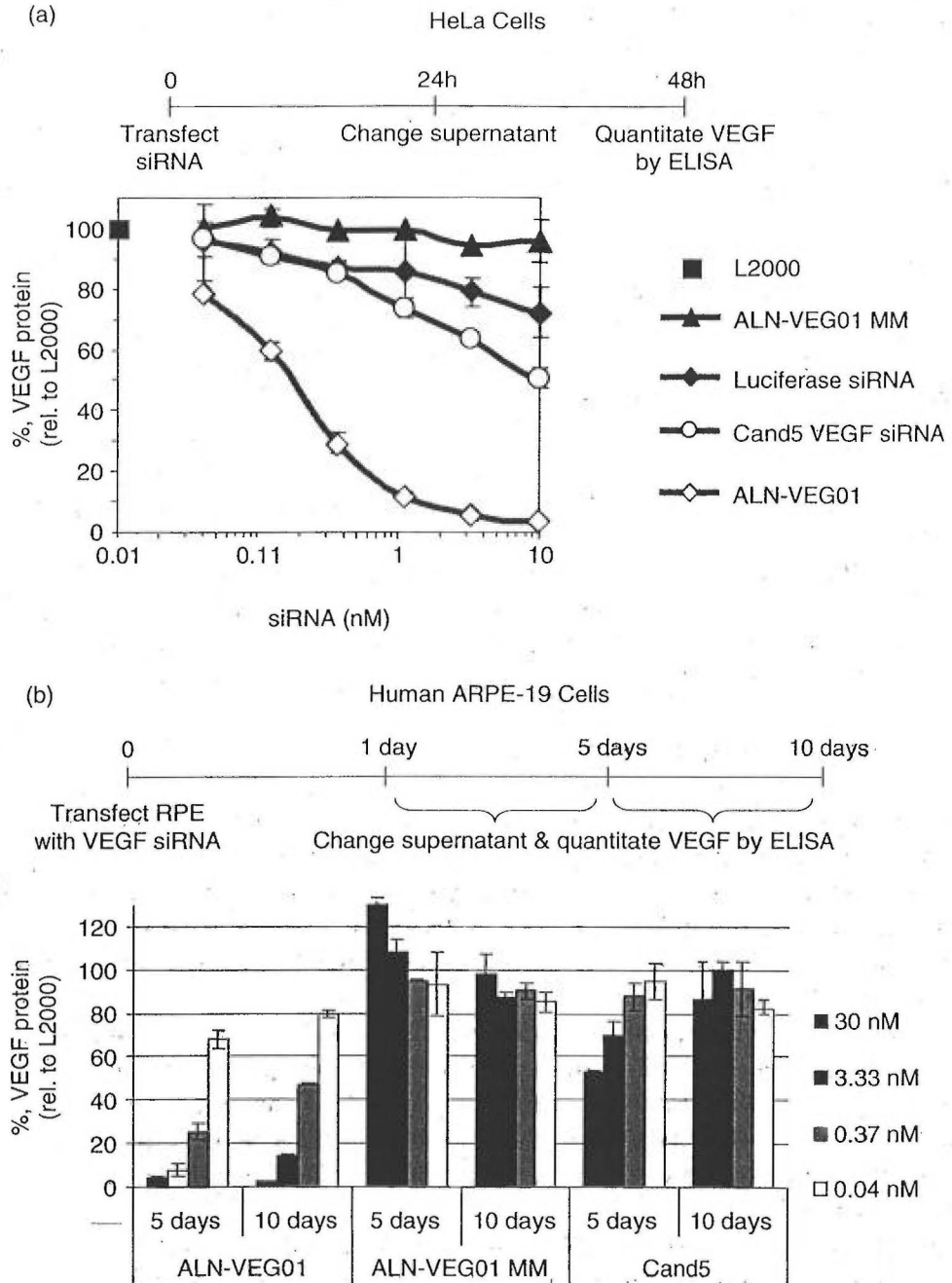


Figure 16.3 Identification of highly potent VEGF siRNA, ALN-VEG01. HeLa cells or ARPE-19 human retinal pigment epithelial cell line were plated in 96 well plates and transfected 24 h later with the indicated concentration of siRNA in Lipofectamine 2000. A Lipofectamine-alone control (L2000) is also indicated. At 24 h post-transfection, culture medium was completely removed and 100 μ l of fresh 10% FBS in DMEM added. Following this medium change, cultured supernatants from HeLa and ARPE-19 cells were collected 24 h (48 h post-transfection) and 96 h (5-day post-transfection) later, respectively. Fresh culture supernatant was added on day 5 to the confluent ARPE-19 cells and supernatants collected again 5 days later (10-day post-transfection). Thus, the effect of siRNA inhibition on VEGF protein production was measured over different time periods post-transfection (HeLa, 24–48 h; ARPE-19, days 1–5, days 6–10). Quantitation of human VEGF protein in the cultured supernatants was by ELISA. Positive control is Cand5 hVEGF siRNA [32] and negative controls include an irrelevant siRNA (luciferase) and an ALN-VEG01 siRNA containing four inverted nucleotide mismatches (ALN-VEG01 MM).

16.3 IN VIVO DELIVERY

Effective delivery is the most challenging remaining consideration in the development of RNAi as a broad therapeutic platform. To date, animal studies using siRNA either have not employed additional formulation (i.e., “naked siRNA”) or have delivered siRNA formulated as conjugates, as liposome/lipoplexes, or as complexes (peptides, polymers, or antibodies). The route of administration of siRNA has also ranged from local, direct delivery to systemic administration. Local delivery or “direct RNAi” has particular advantages for a developing technology in that as with any pharmacologic approach, doses of siRNA required for efficacy are substantially lower when siRNA are injected into or administered at or near the target tissue. Direct delivery also allows for a more focused delivery of siRNA that might circumvent any theoretical undesired side effects resulting from systemic delivery. Systemic delivery of siRNA especially with cholesterol conjugates and liposome formulations have also been widely explored with considerable success. While this section will provide a review of the different delivery approaches utilized with siRNA, it is not an exhaustive description of all *in vivo* experimentation. Several recent publications offer such a review [20,30,31].

16.3.1 Naked siRNA

Many reports describing success with RNAi *in vivo* involve direct delivery of “naked” siRNA to tissues such as eye, lung, and central nervous system. As used here, the term “naked” siRNA refers to the delivery of siRNA (unmodified or modified) in saline or other simple excipients such as 5% dextrose (D5W). The ease of formulation and administration using direct delivery of “naked” siRNA to tissues make this an attractive therapeutic approach. Not surprisingly, the initial development of RNAi therapeutics has focused on disease targets and clinical indications (AMD and RSV infection) that allow for direct administration of siRNA to the diseased organ.

16.3.1.1 Ocular

Multiple examples of efficacious local delivery of siRNA in the eye exist, where proof of concept has been attained in animal models of ocular neovascularization and scarring using both saline and lipid-based formulations [32–36]. Much evidence suggests that direct administration of “naked” siRNA is able to target cell types in the back of the eye and have profound disease-modifying effects. Using the optimized VEGF targeting siRNA ALN-VEG01 described above, we have demonstrated robust specific inhibition of pathologic retinal neovascularization in a rat oxygen-induced model of retinopathy (Figure 16.4). Following a single intravitreal injection of saline-formulated ALN-VEG01, we achieved over 75% inhibition of pathological neovascularization with no effect on the normal retinal vasculature. The inhibition seen with ALN-VEG01 was both dose-dependent and specific as a mismatched siRNA showed no inhibition. Interestingly, the degree of inhibition seen with ALN-VEG01 was dramatically more profound than that seen with either a VEGF₁₆₅-specific aptamer (pegaptanib, approved for intravitreal use in AMD patients) or a VEGF-receptor immunoglobulin fusion protein (Figure 16.4). Separate earlier studies using lipid-formulated VEGF siRNA had shown a reduction of laser-induced choroidal neovascularization (CNV) in a mouse model of AMD [32]; this initial study was followed by nonhuman primate laser-induced CNV study where it was reported that intravitreal injection of a saline-formulated VEGF siRNA was well tolerated and efficacious [33]. Lastly, intravitreal injection of saline-formulated siRNA targeting VEGF receptor-1 was effective in reducing the area of ocular neovascularization by 1/3 to 2/3 in two mouse models [34]. These encouraging proof-of-concept studies in animal models have led to clinical trials of siRNA targeting the VEGF pathway in AMD.

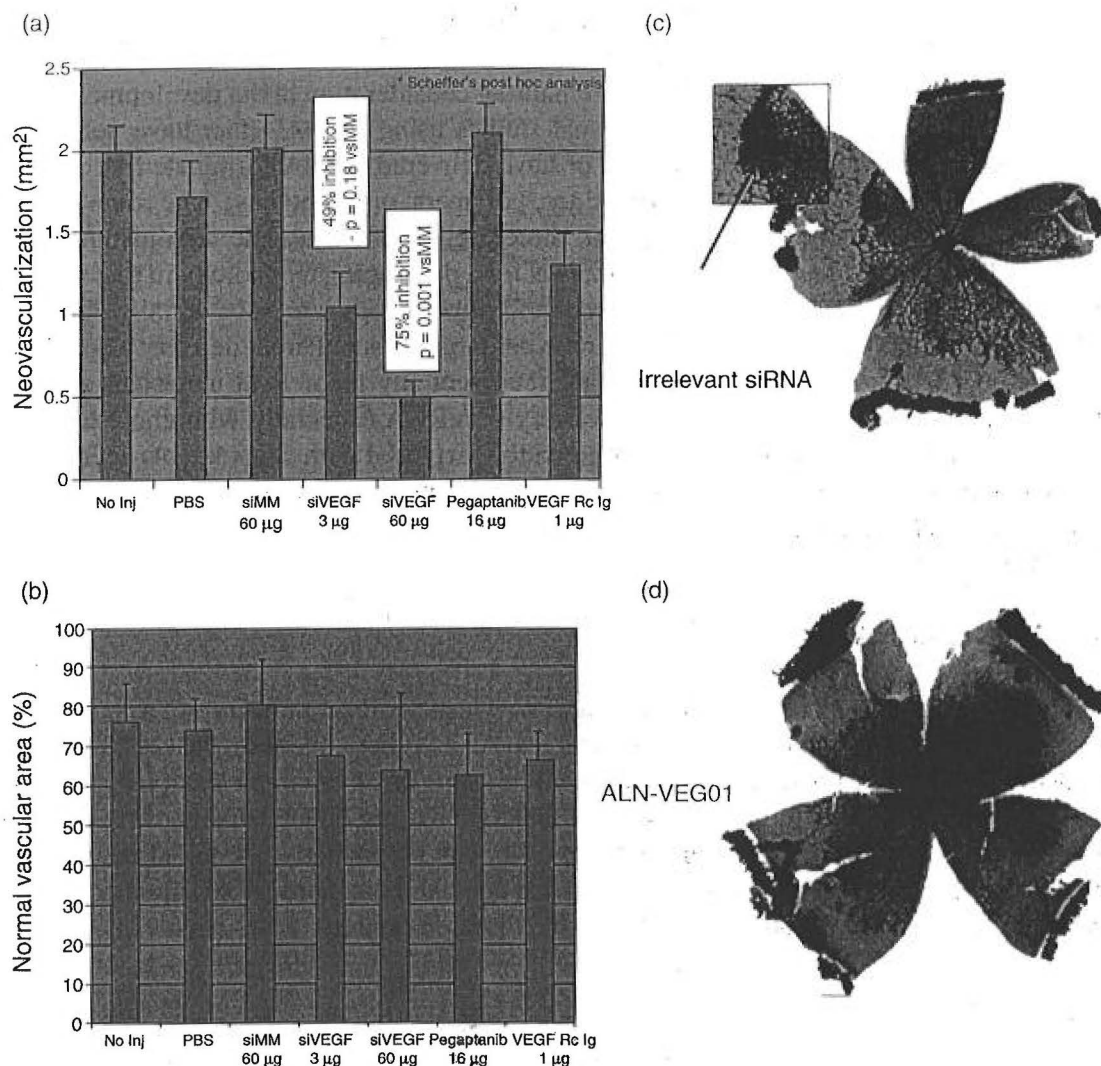


Figure 16.4 (See color insert following page 270.) ALN-VEG01 specifically inhibits retinal neovascularization in a rat oxygen-induced retinopathy model. Newborn rats were exposed to alternating high oxygen concentrations from days 0–14 as outlined previously [101]. On day 14, therapeutic agents were given once intravitreally (5 μ l volume) at the amounts indicated and rats placed in room air for the following 6 days (days 14–20). On day 20, rats were sacrificed and flat mount retinal preparations stained with ADPase was used to quantitate (a) pathologic neovascularization and (b) normal vascular development; representative ADPase flat mount preparations are shown following administration of (c) irrelevant control siRNA or (d) ALN-VEG01. Experimental groups: no injection (No Inj), saline (PBS), high- and low-dose ALN-VEG01 siRNA (siVEGF), high-dose ALN-VEG01 mismatch siRNA (siMM), clinical-grade VEGF aptamer (Pegaptanib), and research-grade VEGF receptor immunoglobulin fusion protein from R&D Systems (VEGF Rc Ig). All groups were scored blinded; N = 10 per group. Neovascularization data are expressed as mean neovascular area (mm²) \pm SE (A) and normal retinal vasculature data are expressed as percentage vascular area (\pm SE). Scheffe's post-hoc analysis was employed to identify significant differences in both neovascular area and normal vascular area. One of three representative experiments.

16.3.1.2 Respiratory

A number of studies have demonstrated that intranasal and orotracheal administration of formulated siRNA can result in a significant target gene silencing in the lung leading to distinct disease-modifying phenotypes (Table 16.1). Typically, siRNAs were administered in concentrations of \sim 100 μ g per mouse and were directed against viral or endogenous disease-related targets. Most of the examples of successful direct delivery of siRNA to lung have involved delivery of "naked" siRNA either in saline or with excipients such as D5W or lung surfactants.

Table 16.1 Successful *In Vivo* Delivery of siRNA in Pulmonary Disease

Target	Formulation	Route	Disease	Reference
RSV-P, PIV-P	Saline or TKO	Intranasal	Viral RSV and PIV	[37]
SARS	D5W or Infasurf	Intranasal	Viral SARS	[38]
HO-1	Saline	Intranasal	Hyperoxic acute lung injury	[39]
KC, MIP-2, Fas	Saline	Intranasal	Septic acute lung injury	[40,41]
Angiopoietin 2	Saline	Intranasal	Hyperoxic acute lung injury	[42]
DDR1	Saline	Intranasal	Bleomycin-induced fibrosis	[43]
FLU – NP, PA, NP + PA	PEI	Intravenous	Viral flu	[76,79]

In instances when delivery formulations such as Transit-TKO have been used, they are sometimes shown to be dispensable. For instance, a groundbreaking study reported that intranasal instillation of siRNAs directed against viral targets either unformulated or complexed with Transit-TKO reduced the viral load of RSV and parainfluenza virus (PIV), two relevant pathogens in pediatric and immune-compromised patients [37]. In these studies, viral titers in the lung were reduced by more than 3 orders of magnitude in a mouse model of viral infection using siRNA doses as low as 70 µg/animal with no observed adverse events. Intranasal instillation of the siRNAs did not induce a type I or type II interferon response measured 2 days after administration. In addition, pathological features of RSV infection such as elevated leukotriene levels, pulmonary inflammation, as well as increased respiratory rate were reduced to baseline levels after siRNA administration. The RSV-specific siRNA-mediated reduction of viral load could be achieved in both prophylaxis and treatment paradigms. In a similar approach, siRNA formulated in D5W was administered intranasally in a nonhuman primate model of SARS corona virus (SCV) infection [38]. Macaques treated with siRNA prior to, simultaneously with, or with repeated doses after viral infection showed a milder response to viral infection as judged by a reduced elevation of body temperature, a key indicator of the severity for SARS-like symptoms. In addition, siRNA treatment resulted in a significant reduction of interstitial infiltrates and pathological changes to the lung as well as in an inhibition of viral replication in the respiratory tract. Both studies demonstrate the potential of RNAi therapeutics to treat viral infection in the respiratory system.

A number of studies showed that siRNAs can achieve delivery in pulmonary tissue in certain disease settings. Lee and colleagues demonstrated that siRNA-mediated silencing of heme-oxygenase-1 (HO-1) after intranasal siRNA administration resulted in enhanced apoptosis in an ischemia-reperfusion (I-R) mouse model [39]. While I-R induced HO-1 in a number of organs, siRNA-mediated HO-1 silencing following intranasal administration was restricted to the lung and resulted in a local elevation of FAS expression and caspase3 activity. In addition, biotinylated siRNA was detected histologically in lung parenchyma up to 16 h after instillation. To further understand the pathogenesis of acute lung injury (ALI), siRNA targeting the two chemokines keratinocyte-derived chemokine (KC) and macrophage-inflammatory protein 2 (MIP-2) were administered by intratracheal instillation in a hemorrhage-induced and septic challenge model of ALI [40]. KC and MIP-2 lung mRNA levels were decreased by about 50%, resulting in a significant reduction of local IL-6 concentrations, and in the case of MIP-2 siRNA treatment also resulted in reduced neutrophil influx, interstitial edema, and disruption of lung architecture. Silencing with Fas-siRNAs in the same animal model resulted in the reduction of Fas to a level similar to control animals and ameliorated pulmonary apoptosis and inflammation [41]. Recently, in a hyperoxia-based mouse model of ALI, intranasal administration of an angiopoietin 2 (Ang2) siRNA in saline specifically and dramatically ameliorated hyperoxia-induced oxidant injury, cell death, inflammation, vascular permeability, and mortality [42]. Expression of Ang2 in this model is dramatically induced in lung epithelial cells and this was specifically inhibited by Ang2 siRNA but not control siRNA. Lastly, it was demonstrated that intranasal instillation of siRNA targeting Discoidin Domain Receptor 1 (DDR-1) mRNA

resulted in 60–70% reduction of DDR1 protein in the lung of animals treated with bleomycin to induce pulmonary fibrosis [43]. DDR1 silencing was accompanied by an attenuation of infiltration of inflammatory cells and a reduction of cytokines. As a consequence, bleomycin-induced TGF-beta up-regulation was suppressed and collagen deposition was significantly reduced. In sum, these studies exhibit the potential for direct instillation of “naked” siRNA to effectively silence endogenous lung genes and have disease-modifying effects.

16.3.1.3 Nervous System

The nervous system is a third area where direct instillation of siRNA in saline has proven successful in validating disease targets *in vivo*. Direct administration of saline-formulated siRNA by intracerebroventricular, intrathecal, or intraparenchymal infusion resulted in silencing of specific neuronal mRNA targets in multiple regions of the peripheral and central nervous system [44–47]. While the “naked” unformulated siRNA dose typically required for target silencing in these rodent studies is on the order of ~0.5 mg/day, use of polymer or lipid-based delivery systems seems to facilitate cellular uptake as effective *in vivo* doses are ~50 µg with these delivery systems [48–50].

16.3.2 Conjugation

Direct conjugation with molecular entities designed to help target or deliver drug into the appropriate target cell type is naturally appealing. Through conjugation, the therapeutic can be fixed as a one-component system, thereby significantly reducing the complexity from a chemistry, manufacturing, and controls perspective. For siRNA, conjugation is especially attractive, given the fact that only one of two strands in the duplex is active. Thus, conjugates can be attached to the sense strand without disrupting the activity of the antisense strand. Typically, conjugates have been placed on either the 5' or 3' end of the sense strand, though they can in some instances also be tolerated on the antisense strand. To date, siRNA conjugates have been made using lipophilic molecules, proteins, peptides, and aptamers.

16.3.2.1 Cholesterol

While the use of cholesterol conjugates to aid *in vivo* delivery to liver had been established using antisense oligonucleotides [51–53], the report in 2004 by Soutschek and coworkers [54] using a cholesterol-siRNA conjugate provided the first mechanistic *in vivo* proof of concept for RNAi. Effective silencing of the apolipoprotein B (ApoB) in mice was demonstrated following intravenous administration of cholesterol-conjugated siRNA duplexes. In these experiments, injections of cholesterol-conjugated ApoB siRNA at a relatively high dose of 50 mg/kg resulted in silencing of the ApoB mRNA by ~55% in the liver and ~70% in the jejunum, the 2 principal sites for ApoB expression; control cholesterol-conjugated siRNA demonstrated no silencing activity. Critically, the authors went on to demonstrate that the reduction in ApoB mRNA was a result of RNAi, through 5'RACE detection of specific mRNA cleavage product. Paralleling the mRNA decrease in ApoB mRNA, ApoB protein levels in the plasma dropped by ~70%, and in addition, consistent with the biological function of ApoB, a 35–40% decrease in serum cholesterol levels was also seen. Cholesterol conjugation imparted critical pharmacokinetic and cellular uptake properties to the duplex as evidenced by the fact that the unconjugated ApoB siRNA was rapidly cleared and unable to effect mRNA silencing. The mechanism by which cholesterol conjugates promote improved distribution and cellular uptake is an area of active research, though speculation has centered on incorporation of the cholesterol-siRNA conjugate into circulating lipoprotein particles and then delivery through receptor-mediated processes into hepatocytes. Whether other tissues or cell types can be efficiently targeted by cholesterol conjugates is also another area of interest.

16.3.2.2 Other Natural Ligands

Beyond cholesterol, other natural ligands have been successfully directly conjugated to siRNA duplexes. Among some siRNA conjugates described are membrane-permeant peptides such as penetratin and transportan [55], and shielding groups such as poly ethylene glycol (PEG) [56]. While these conjugates were successfully synthesized with RNAi-silencing activity preserved, robust *in vitro* or *in vivo* evidence indicative of an impact on cellular delivery is still lacking. Nevertheless, work done using natural ligands as targeting agents for siRNA formulated in conjunction with delivery vehicles has been positive. Molecules such as transferrin [57], folate [58], and RGD peptide [59] have been introduced into particle-based delivery vehicles to help provide specific targeting of siRNA to cells bearing the natural receptor.

16.3.2.3 Aptamers

The use of RNA aptamers as a conjugate for delivery and targeting of siRNA is a promising and elegant approach. In contrast to most of the other described delivery methods, this approach allows for specific targeting of particular cell types and yet, unlike a similar antibody-based approach, has an additional advantage that the therapeutic can be composed entirely of RNA (i.e., RNA aptamer linked to an siRNA). Recently, *in vitro* and *in vivo* proof of concept has been generated using aptamers to PSMA, a cell-surface receptor overexpressed in prostate cancer cells and tumor vascular endothelium [60,61]. PSMA aptamers, when either directly linked to siRNA [60] or conjugated through a modular streptavidin bridge [61], were capable of promoting specific cellular uptake and RNAi-mediated silencing of target mRNA *in vitro*. Using siRNA directed against survival genes (plk1 and bcl-2) directly linked to PSMA aptamer, it was found these RNA chimeras were internalized by cells and resulted in RNAi-mediated target mRNA silencing and cell death [60].

One concern about aptamer-siRNA chimeras is the potential difficulties in the synthesis of long RNA molecules. Short aptamers (25–35 bases) that bind to a wide variety of targets with high affinity have been described [62]. Thus, in theory it is possible to design siRNA-aptamer chimeras that would have a long strand of 45–55 bases, a synthesis length that is within the range of technical and commercial feasibility.

16.3.2.4 Small Molecules

Beyond natural ligands and aptamers as targeting vehicles for siRNA, there also exists the possibility of using small-molecule drugs in this fashion. Conceptually, targeting siRNA to specific cell types through conjugation to small molecules (or as discussed below through complexation to antibodies) is appealing. Developing small-molecule conjugates capable of promoting entry of siRNA into cells requires selection of an appropriate small molecule that is capable of efficient cellular internalization and endosomal escape.

16.3.3 Liposomes and Lipoplexes

Drugs have traditionally been formulated in liposomes to provide increased pharmacokinetic properties and decreased toxicity profiles. With the advent of RNAi, research in the use of liposomes to deliver drugs into cells has surged. Liposomes are vesicles that consist of an aqueous compartment enclosed in a phospholipid bilayer with drug typically entrapped in the center aqueous layer. When lipids simply complex with nucleic acids to form particles, they are known as lipoplexes and are typical of most commercial transfection agents, such as Lipofectamine 2000 and TKO. Liposomes are multicomponent lipid-based nanoparticle delivery systems typically comprising a lipid component (often containing a cationic or a fusogenic lipid), cholesterol, and a PEG-lipid. Each of these components has a critical function to play in the fusogenicity

and pharmacokinetic properties of the liposome (for reviews, see [63,64] and Chapter 9 by MacLachlan in this book).

Both liposomes and lipoplexes have been extensively utilized to deliver siRNA *in vitro* and *in vivo*. *In vitro* transfection of siRNA using lipid-based delivery agents is now a routine laboratory procedure. More recently, significant success has been demonstrated with both local and systemic administration of siRNA. One of the most important advances in the development of RNAi as a therapeutic came with the demonstration by Zimmermann et al. that systemically delivered siRNA in stable nucleic acid lipid particles (SNALPs [17]) was able to dramatically silence apoB in mice and nonhuman primates [65]. Most importantly, a single intravenous dose of 2.5 mg/kg of SNALP-formulated siRNA in cynomolgus monkeys was able to reduce ApoB mRNA levels in the liver by >90%. As expected, accompanying ApoB mRNA silencing, levels of serum cholesterol and low-density lipoproteins were reduced by greater than 65% and 85%, respectively. Remarkably, the durability of silencing following a single intravenous dose of 2.5 mg/kg SNALP-formulated siRNA was shown to last for at least 11 days. Treatment with liposomally formulated siRNA was well tolerated in these experiments, with transient increases in liver enzymes as the only reported evidence of toxicity, and these effects may be related to the targeting of ApoB itself. The general applicability of SNALP formulations for hepatic delivery of siRNA was also demonstrated in animal models of HBV and Ebola virus infection [66,67]. Other "non-SNALP" liposomes have also been demonstrated to be effective in delivering siRNA to different organs. Systemic delivery of a cationic liposome-containing TNF α siRNA specifically inhibited TNF α production in knee joints and alleviated disease in a mouse model of rheumatoid arthritis [68], while a liposomally formulated caveolin-1 siRNA reduced target expression by 90% in mouse lung endothelia with concomitant expected physiological effects [69]. In summary, use of lipid-based formulations for systemic delivery of siRNA, especially to liver, represents one of the most promising near-term opportunities for development of RNAi therapeutics.

Local administration of liposomes and lipoplexes has also been successful in delivering siRNA to target cell types in the eye, nervous system, and tumors. Within the ocular context, subretinal injection of VEGF siRNA formulated in Transit-TKO was found to reduce choroidal neovascularization in a laser-induced mouse model [32], and subconjunctival injection of TGF- β receptor II siRNA in Transit-TKO to suppress inflammation and fibrosis in a mouse subconjunctival scarring model [35]. With regard to the nervous system, intracranial delivery of lipid-complexed siRNA targeting the viral envelope genes protected mice against Japanese Encephalitis Virus and West Nile Virus-induced encephalitis [50]. Similarly, intrathecal administration of cationic lipid-formulated delta opioid receptor (DOR) siRNA facilitated delivery and specific target gene silencing in the spinal cord and dorsal root, resulting in blocked antinociception by a DOR-selective agonist [48]. Numerous publications have reported successful antitumoral effects following either direct or systemic tumoral injection of lipid-formulated siRNA (for reviews, see [20,30,31,70]). One recent example demonstrated that either intratumor injection or intraperitoneal injection of TransMessenger-formulated siRNA directed against the human papillomavirus E6 oncogene inhibited tumor growth, suppressed E6 expression, and induced tumor apoptosis in a subcutaneous cervical cancer xenograft model [71]. It is important to note that the necessity for lipid-based delivery systems (and this holds true for other delivery systems as well) in many of these direct RNAi applications must be assessed in a target cell- and disease-specific manner. For eye, lung, and nervous system, there are numerous examples of siRNA being successfully delivered in the absence of such agents, and other examples where the converse is true.

Lastly, an area of increasing interest is direct application of lipoplexed siRNA to mucosal surfaces such as the vagina and intestine. Intravaginal delivery of lipid-complexed siRNA directed against herpes simplex 2 virus was found to protect mice when delivered before or after lethal herpes virus challenge [72]. Corroborating these results is evidence for specific lamin A/C and CCR5 silencing using the relevant targeting siRNA complexed with Lipofectamine 2000, while similarly complexed irrelevant siRNA had no effect on either lamin A/C or CCR5 expression [73]. Direct

delivery of a TNF α siRNA in a Lipofectamine formulation has also recently been demonstrated to not only reduce TNF α levels but also attenuate colonic inflammation after enema administration [73]. Intravaginal and intracolonic administration of siRNA using these lipid-based delivery systems was well tolerated in mice with no reported evidence of toxicity or activation of an interferon response [72,73]. Given the relatively easy physical access, large number of potential unmet medical disorders, general tolerability of the siRNA-lipid formulations, and, importantly, demonstrated robust *in vivo* delivery success, mucosal administration of lipid-formulated siRNA will be a fertile area for development of future RNAi therapeutics.

16.3.4 Peptides and Polymers

Complexation of siRNA with positively charged peptides or polymers has been a field of increasing interest [74]. In general, cationic peptides and polymers are used to form complexes with the negatively charged phosphate backbone of the siRNA duplex. This noncovalent association is very stable and results in nanometer-sized particles. One concern with complexation approaches is the ability to prevent aggregation and thus control particle formation in a reproducible manner. This is most often done by either maintaining a net positive charge on the surface or incorporating molecules such as PEG that help stabilize the particle and prevent aggregation. PEG groups are also used with polymers to improve the pharmacokinetic profile, while agents such as folate, transferrin, antibodies, or sugars such as galactose and mannose can be incorporated for tissue targeting [75].

One of the most widely studied polymers for delivery of nucleic acids is polyethylenimine (PEI). PEI polymers are synthetic linear or branched structures with protonable amino groups and high cationic charge densities. Following complexation with siRNA, the cationic polyplexes are thought to interact with the cell surface through electrostatic interaction and are taken up by cells through endocytosis, where intracellularly they act to buffer the low endosomal pH. Endosomal escape is hypothesized to occur due to a "proton sponge" effect, whereby PEI enhances the influx of protons and water leading to endosomal destabilization and osmotic release of the polyplexes into the cytoplasm [30]. Multiple reports of use of siRNA-PEI complexes *in vivo* now exist. PEI complexed with influenza siRNA demonstrated profound antiviral effects in infected mice [76], while similar targeting of the Ebola L gene resulted in protection against lethal Ebola infection in guinea pigs [67]. Profound antitumor activity was seen upon administration of PEI-siRNA complexes targeting pleiotrophin [77], VEGF [59], and HER2 [78]. Lastly, local injection of PEI-siRNA polyplexes targeting the NMDA receptor subunit NR2B significantly attenuated formalin-induced nociception in the rat [49]. One of the main concerns with the use of PEI as a therapeutic delivery vehicle is the extreme toxicity seen at higher doses. In an attempt to expand the safety margin, several groups are optimizing the physical structure of PEI to enable improved *in vivo* delivery of siRNA [79–81]. In addition to PEI, other synthetic polycations consisting of histidine and polylysine residues have also been evaluated for delivery of siRNA and appear to have improved *in vitro* efficacy as compared to standard PEI [82].

Three other polymer approaches that have yielded *in vivo* siRNA data are chitosan nanoparticles, cyclodextrin-based nanoparticles, and atelocollagen nanoparticles. Chitosan is a well-tolerated natural biodegradable polymer that forms cationic complexes with nucleic acids. Effective *in vivo* RNAi interference was achieved both in bronchiole epithelial cells of transgenic EGFP mice after intranasal administration of chitosan/siRNA formulations [83] and in subcutaneously implanted breast cancer cells of nude mice after intravenous administration of chitosan/RhoA siRNA complexes [84]. An elegant study using transferrin-targeted cyclodextrin-containing polycation nanoparticles demonstrated targeted silencing of the EWS-FLI1 gene product in transferrin receptor expressing Ewing's sarcoma tumor cells [57]. Systemic administration of atelocollagen-siRNA complexes had marked effects on subcutaneous tumor xenografts as well as on bone metastases [85,86]. Lastly, other related polymer approaches such as PAMAM dendrimers and Poly (DL-lactic-co-glycolic acid) (PLGA) nanoparticles are

also being investigated as vehicles for siRNA delivery [87,88], though *in vivo* evidence for RNAi-mediated silencing remains to be demonstrated using these approaches.

Peptide-based approaches, including use of cell-penetrating peptides, to deliver oligonucleotides constitute an area of active research [64,89–91]. Several peptide-based gene delivery systems such as MPG [92], Penetratin [93], and cholesteryl oligo-D-arginine (Chol-R9) [94] have been shown to promote siRNA uptake *in vitro*. In the case of Chol-R9, local *in vivo* administration of complexed VEGF-targeting siRNA, but not complexed scrambled siRNA, led to tumor regression in a mouse model [94]. Lastly, peptide-based approaches are often coupled with other delivery systems, such as liposomes, to enable more targeted delivery of oligonucleotides [59,64,95].

16.3.5 Antibodies

Antibody-based approaches for specific targeted delivery of siRNA hold much promise. Recently, elegant studies have demonstrated that a protamine-antibody fusion protein was able *in vitro* to selectively deliver siRNA to HIV-envelope expressing B16 melanoma cells or HIV-infected CD4 T cells [96]. Protamine is used for its nucleic acid-binding properties while the Fab fragment was used to mediate receptor-specific binding to only those cells expressing the gp160 HIV envelope protein. Importantly, using gp160-B16 cells as a tumor model, it was shown that siRNA-antibody-protamine complexes when delivered either intratumorally or intravenously are able to specifically deliver siRNA *in vivo* and retard tumor growth. This study demonstrates the potential for antibodies as a selective cell-type-specific means to deliver siRNA *in vivo*.

16.4 CLINICAL TRIALS

RNAi has advanced from research discovery to clinical trials in a very short span of time. To date, three different RNAi therapeutics are under investigation in clinical trials, with several more poised to enter trials in the coming years. The initial trials have focused on well-validated therapeutic targets such as the VEGF pathway for the wet form of AMD and the RSV genome for the treatment of RSV infection. Furthermore, in these initial trials, direct administration of siRNA (“Direct RNAi”) to the eye and lung for AMD and RSV, respectively, will maximize the chances of delivering sufficient and therapeutically relevant concentration of drug to the tissue of interest. RNAi therapeutics in earlier phases of development are being developed to combat a series of diseases, of which the most advanced include those targeting viral genomes for pandemic influenza (<http://www.alnylam.com/therapeutic-programs/programs.asp>) and hepatitis C (Sirna-034; http://www.sirna.com/wt/page/anti_viral), and endogenous disease targets such as RTP801 and p53 for wet AMD and acute renal failure, respectively (<http://www.quarkbiotech.com/modules/main>). Also in late-stage development are siRNA directed against TNF- α for arthritis (<http://www.nastech.com/nastech/pipeline>), VEGF pathway for oncology (ICS-283; <http://www.intradigm.com/products.html>), ApoB for dyslipidemias (Pro-B; <http://www.protivabio.com/rd/pipeline/>), and a protein kinase target for pancreatic cancer (Atu027; <http://www.atugen.com/therapeuticpp>). The status of the clinical-stage programs is summarized below.

16.4.1 Ocular

Ophthalmic indications have historically been very attractive for oligonucleotide-based therapeutics with the first approved oligonucleotide-based therapeutic being Vitravene® for the treatment of CMV retinitis [97] and the most recently (and only second ever) approved oligonucleotide-based therapeutic being Macugen® for the treatment of AMD [98]. Direct injection of drug into the vitreal eye cavity is an efficient and rapid way to get drug targeted to the back of the eye. Following intravitreal injection of ³³P-radiolabeled siRNA, we have been able to demonstrate rapid uptake of radioactivity from the vitreous cavity to the back of the eye, and using autoradiography we

confirmed localization throughout the retinal layers including the retinal pigment epithelial (RPE) cells (Figure 16.5). The RPE is an important producer of VEGF and a key cell type in the pathogenesis of wet AMD [99]. Another advantage of the ocular compartment as an initial clinical target organ is the relatively favorable nuclease environment. As compared to serum, the nucleases found in eye tissue are less aggressive than those found in serum (Alnylam, unpublished results). While this does allow for unmodified siRNA to be injected directly into the vitreal cavity, it is clear from our study that exonuclease-stabilizing chemistries can dramatically improve the amount of intact siRNA found following intravitreal injection (Figure 16.5). The ease of intraocular drug delivery,

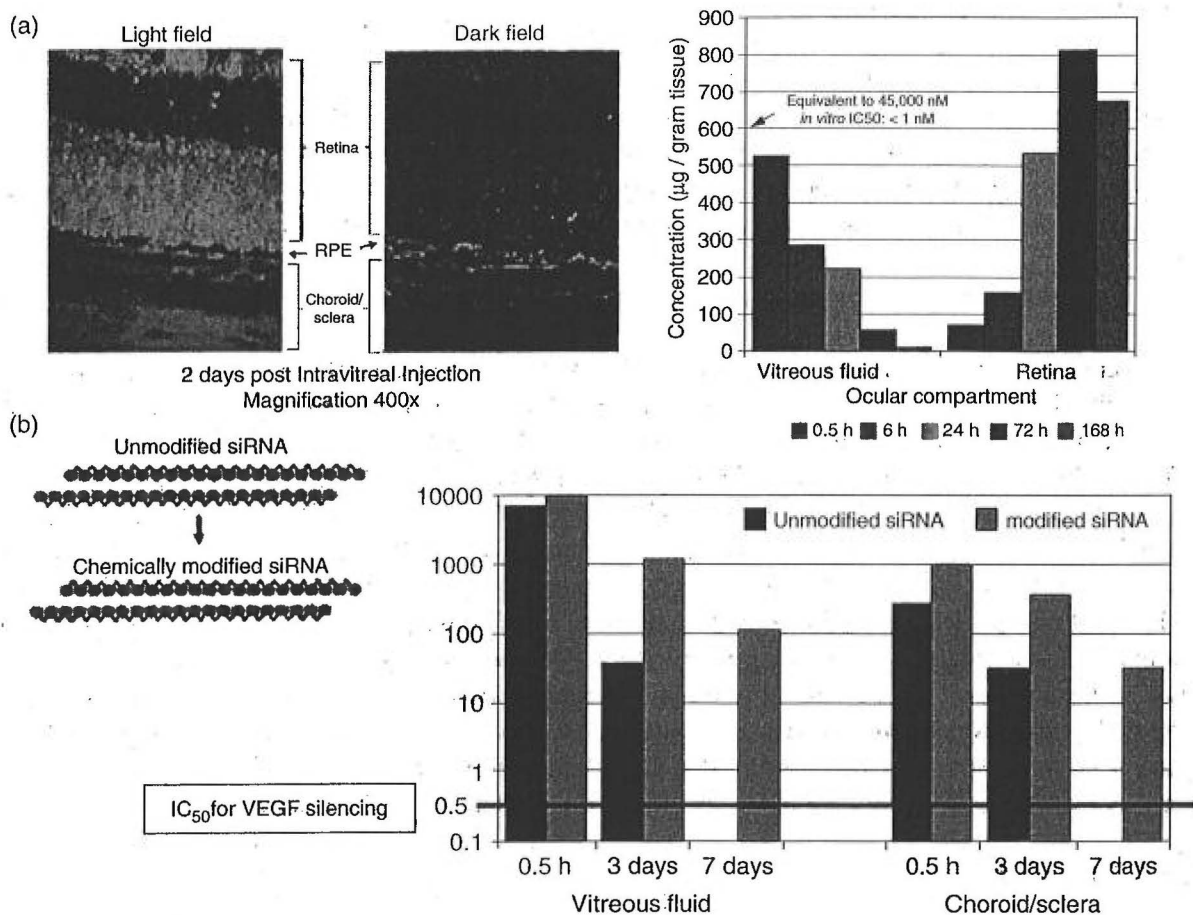


Figure 16.5 (See color insert following page 270.) Beneficial effect of nuclease stabilization on intact ocular drug levels and anatomical distribution of siRNA following intravitreal injection. A 7-day rabbit study was performed to determine the pharmacokinetics of a chemically modified siRNA ($P = S$) and its unmodified counterpart. (a) Rapid uptake and distribution of siRNA within ocular tissues. ^{33}P -radiolabeled unmodified and modified compounds (0.4 mg) were injected intravitreally and ocular tissues analyzed after 0.5, 6, 24, 72, and 168 h. Counts per minute (CPM) were measured for different eye compartments (aqueous humor, vitreous, retina, iris, sclera/choroid). Negligible counts were detected in aqueous humor and iris. Analysis of total CPM in retina for the modified siRNA is shown; unmodified siRNA exhibited a similar profile. Concentration of siRNA/gram of tissue was calculated based on CPM assuming 100% intact duplex. Microautoradiography (as visualized under light and dark field microscopy) shows distribution of radiolabeled modified siRNA throughout retina and sclera two days following intravitreal injection. Radiolabel is detected as dark spots under light field and bright spots under dark field. (b) *In vivo* benefit of exonuclease protection. Ocular tissues (vitreous and choroid/sclera) were subjected to polyacrylamide gel electrophoresis and percentage of intact ^{33}P -labeled duplex was determined by autoradioluminogram. Exonuclease protection results in greater stability within the vitreous and ocular tissue than the same duplex in its unmodified form. Estimated ocular levels of intact nucleases protected siRNA (based on $\text{CPM} \times \% \text{ intact duplex}$) are approximately 50-fold above the *in vitro* IC₅₀ 7 days after a single intravitreal injection.

combined with the clinical validation of VEGF as an attractive therapeutic target, led to the rapid development of several RNAi therapeutics in this space.

16.4.1.1 VEGF

Cand5, an siRNA targeting all VEGF-A spliced isoforms, has completed a phase-II trial in patients with serious progressive wet AMD and has been reported to provide dose-related benefits against several endpoints including near vision and lesion size (<http://www.acuitypharma.com/press/release10.pdf>). Cand5 is also being tested for efficacy against diabetic macular edema patients in a phase-II trial that began in early 2006 (<http://www.acuitypharma.com/press/release13.pdf>).

16.4.1.2 VEGF Receptor

Sirna-027, an siRNA targeting one of the VEGF receptors (VEGF-R1), has recently completed phase I trials in patients with wet AMD and was reported to be well tolerated. Additionally, it was also reported to stabilize or improve visual acuity in a subset of patients (<http://www.sirna.com/wt/page/ocular>). Whether targeting VEGFR1 alone, one of at least two signaling receptors for VEGF action, will prove sufficient to block choroidal neovascularization in AMD remains to be established.

16.4.2 Respiratory

There are many advantages in delivering siRNA therapeutics directly to the lungs for targeting lung epithelial cells. These include a noninvasive method of delivery (locally targeted delivery of drugs acting in the lungs can improve efficacy and decrease potential unwanted systemic side effects); direct access to lung epithelial cells with absence of first-pass metabolism; a local lung environment that is relatively nuclease-friendly; and, lastly, evidence that lung epithelial cells are capable of siRNA uptake and RNAi-mediated silencing [37,39]. There now exist numerous reports for *in vivo* efficacy with siRNA delivered locally to the lung (Table 16.1). While in many instances delivery of naked siRNA in saline has been shown to be effective [37,39,42], multiple approaches to optimize lung delivery are also being investigated. For instance, local delivery of naked siRNA in lung surfactants has been successful in inhibiting replication of SARS in nonhuman primates [38], and siRNA encapsulated in chitosan nanoparticles has been successful in silencing a reporter GFP gene [83]. Whether utilization of naked siRNA, additional formulations, or delivery vehicles is required will depend very much on the molecular target and disease indication being pursued.

16.4.2.1 RSV

For RSV infection, two phase-I intranasal trials with ALN-RSV01, an siRNA targeting the viral nucleocapsid (N) gene, have been completed with over 100 healthy adult volunteers, representing one of the largest human studies with an RNAi therapeutic. ALN-RSV01 was found to be safe and well tolerated (<http://phx.corporate-ir.net/phoenix.zhtml?c=148005&p=irol-newsArticle2&ID=849576&highlight=>). Clinical development of ALN-RSV01 is progressing with human experimental infection studies on-going.

16.5 SUMMARY

RNAi has advanced from research discovery to clinical trials in a very short span of time, starting with the now Nobel-prize winning discovery in 1998 by Fire and Mello that long dsRNA could mediate RNAi in *Caenorhabditis elegans* [100], to evidence in 2001 by Tuschl and colleagues that short synthetic siRNA could induce RNAi in mammalian cells [1], to the present day when three

different RNAi therapeutics are in human clinical trials and numerous others poised to enter. Since discovery of its utility in mammalian cells 5 years ago, RNAi has revolutionized biomedical research and has spawned a veritable explosion of scientific knowledge covering all aspects of *in vitro* and *in vivo* RNAi biology. The principal challenge that remains in achieving the broadest application of RNAi therapeutics is the hurdle of delivery. Tremendous progress has been made on this front through the use of different approaches, such as conjugation, complexation, and lipid-based approaches, though it is clear that delivery has yet to be solved for all cell types and tissues. Delivery solutions as they arrive will likely need to be tailored for target cell type and the disease indication. As the challenge of siRNA delivery is met, it will be possible to rapidly advance RNAi therapeutics against potentially any disease target into clinical studies. The ongoing clinical trials with siRNA for the treatment of AMD and RSV infection will be the first indicator of the potential for RNAi therapeutics to be a revolutionary new class of drug molecules.

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