Strategies for *In Vivo* siRNA Delivery in Cancer

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Abstract: A better understanding of the mechanisms involved in small interference RNA (siRNA) gene silencing opens new horizons for the development of the targeted therapy of malignant and benign diseases. As a research tool, siRNA has proven to be highly effective in silencing specific genes and modulating intracellular signaling pathways. However, *systemic* delivery of siRNA has been more problematic due to degradation and poor cellular uptake. In order to overcome these limitations, a variety of strategies are being developed including new delivery vehicles and chemical modifications. Here, we review potential approaches for the systemic delivery of siRNA for cancer treatment.

I. INTRODUCTION

RNA interference (RNAi) was first discovered in the nematode *Caenorhabditis elegans* by Fire, Mello and colleagues in 1998 as a natural mechanism leading to post-transcriptional gene silencing [1]. In plants and invertebrates, small interference RNA (siRNA) is a natural defense mechanism that protects cells against viruses and transposable elements [2]. The natural siRNA machinery is also present in mammalian cells and can be activated with the introduction of 21 - 22 base nucleotide siRNAs to induce sequence specific messenger RNA degradation [3].

The mechanism by which siRNA inhibits the conversion of messenger RNA (mRNA) into protein is well characterized and has been recently reviewed [4]. Double stranded RNA is recognized by the endoribonuclease, Dicer and cleaved into small fragments of around 21 base pairs in length called siRNA [5]. These RNA fragments bind a protein complex called RISC (RNA-induced silencing complex) by which one strand of the duplex is cleaved and discarded [6]. The remaining antisense strand guides the RISC complex to the complementary messenger RNA (mRNA). Next, a RNA endonuclease within the complex, Argonaute 2 (Ago2) [7], cleaves the target mRNA, which loses its protection against specific RNases that cleave mRNA and gets degraded [8, 9].

The explosion in knowledge generated by a growing understanding of the human genome has led to a concomitant interest in the potential therapeutic applications of siRNA. While traditional therapeutic approaches such as small molecule inhibitors [10, 11] and monoclonal antibodies [12, 13] have been successfully used for cancer therapy, there are limitations to these approaches. For example, many tyrosine kinases can have kinase-independent oncogenic functions such that a small molecule that inhibits receptor phosphorylation will not restrict its entire function. Moreover, most small molecule inhibitors are not specific with regard to target modulation, which can contribute to toxicity. Additionally, many targets are simply not "druggable" because the structure is not known or the protein is quite large. Therefore, siRNA is a powerful modality that provides another option for silencing difficult, yet important targets.

The development of this technology is rapidly evolving and is supported by many biotechnology and pharmaceutical companies. However, despite the enormous interest, siRNA technology has been difficult to implement due to several limitations including rapid enzymatic degradation of naked siRNA and poor cellular uptake. The use of a delivery system to improve siRNA pharmacokinetics, avoid degradation by endonucleases and to enhance tissue target specificity is needed to move this technology into the clinic. In this paper, we review the different approaches that have been made using synthetic carriers for *in vivo* siRNA delivery.

II. *IN VIVO* DELIVERY OF SIRNA

Some of the key elements for successful use of siRNA as a therapeutic tool are careful selection of targets and good sequence design. Appropriate targets are proteins that are believed to play a role in growth and progression of cancer. To avoid toxicity, an ideal target should be selectively expressed or overexpressed in tumors.

The proper design of siRNAs can be accomplished using established algorithms that take into account the sequence length, C and G content and localization of the target sequence within the mRNA. Elbashir and collaborators were the first to describe certain guidelines for siRNA design. SiRNA sequences must be 21 to 22 nucleotides long [3]. These guidelines include: 1) selection of the DNA target sequence starting in the region that begins with AA or TT; 2) siRNAs with 30-50% GC content as these are more effective than those with a higher or lower GC content; 3) use of 3' terminal dinucleotide overhang as these can contribute to more effective activation of the RISC complex [14, 15]. Several commercial and academic sources now provide free web based designer tools that follow these algorithms. Examples of excellent databases for siRNA design include siDirect (http://design.RNAi.jp/) [16] and DEQOR (cbg.de/Deqor/deqor.html) [17].

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For *in vivo* administration, siRNA must be chemically modified or coupled to a carrier or vehicle. Unmodified and uncomplexed siRNA has limited value as a drug because: 1) most mammalian cells do not take up naked siRNA efficiently enough to achieve gene silencing, and 2) naked siRNA has a very short half-life because of rapid renal clearance and degradation by endogenous serum RNases [18]. Stabilizing siRNA by chemical modification is one approach to overcome these problems. The other is to deliver siRNA incorporated in a carrier such as liposomes or nanoparticles that guarantee protection, improvement of cellular uptake and in some cases, tissue specificity.

Chemical modifications of siRNAs have been performed to increase stability, resistance to nucleases and reduce off-target effects. A full review of these modifications is outside the scope of this paper, but has been recently summarized [19]. Some examples of chemical modifications that have been performed to increase resistance to endonucleases are phosphorothioate linkages and 2'-O-methylation [20]. Modifications to reduce off-target effects include the addition of a methyl group to the 2' position of the ribosyl ring of the second base of the siRNA [21, 22]. Most modified siRNAs have been successfully tested *in vitr*o, but their *in vivo* effects are sometimes unpredictable. For example, Layzer and associates demonstrated that siRNA modified with 2'-flouro (2'-F) pyrimidines showed higher resistance to endonucleases when cultured in plasma. However, when it was injected intravenously in mice, the potency and inhibitory capacity of these siRNAs was similar to unmodified siRNAs [23]. However, some modifications have been successful for *in vivo* models. Morrisey and colleagues [24, 25] modified siRNAs to improve elimination half-life and reduced undesirable immunostimulatory properties by substituting 2'OH residues for 2'F, 2'O-Me or 2'H residues. The stabilized siRNA was more effective than unmodified siRNA when tested for inhibition of hepatitis B virus (HBV) replication in a mouse model.

As with any other therapy, toxicity can develop with siRNA administration. Two potential problems can arise: induction of interferon (IFN) and off-target effects [26]. Induction of IFN is believed to be caused by siRNA-mediated activation of the double-stranded RNA-activated protein kinase (PKR), an important modulator of the INF pathway [27, 28]. However, not every siRNA sequence induces interferon activation, suggesting that this process may be sequence dependent [29-32]. Off-target effects are another concern with siRNA administration and are also sequence dependent. Studies with array-based systems suggest that siRNA can modulate the expression of genes that are not directly related with the primary target [21, 33-35]. Off target effects may cause undesired effects and toxicity that would be hard to avoid and predict. In an attempt to address this problem, computer algorithms have been developed that predict off-target effects for a siRNA sequence. These tools are accessible as webtools (for example, dsCheck (http:// dsCheck.RNAi.jp/), a web-based online software for estimating off-target effects) [36].

III. SYNTHETIC CARRIERS TO DELIVER siRNA

Viral vectors and particulate carriers have been used for the systemic delivery of siRNA. In both instances, improved siRNA tissue delivery was observed in experimental models. Successful siRNA delivery into malignant cells has been achieved using amplicon vectors and adenovirus [37, 38]. However, the use of viral vectors for systemic siRNA delivery may be of limited value due to the risk of toxicity, insertion mutagenesis, immunogenicity, and limitations in large-scale manufacturing [39]. Therefore, in search of safe and effective alternatives, many investigators have focused on using synthetic particles such as liposomes or polymer nanoparticles. These particles are not only safer, but also easier to manufacture. Hence, they are the preferred choice for *in vivo* siRNA delivery.

1. Delivery of siRNA Using Liposomes

Liposomes are lipid structures characterized in most instances by assembly of phospholipid bilayer membranes with a varying aqueous compartment. These carriers have already been used for delivery of chemotherapeutic agents. Liposomal incorporation of chemotherapeutic agents and antimicrobials modifies the pharmacokinetics of the drug that is incorporated and as a result, these carriers can allow modification of tissue distribution and pharmacokinetics. Moreover, some of the side effects that are typically associated with the use of these anticancer agents can also be reduced with the use of these carriers [49]. Some chemotherapy drugs administered in liposomal formulations have been successfully used in the clinic to treat cancer patients, like the cisplatin analog L-NDDP [50] and doxorubicin [51].

Liposomes constitute an effective carrier for *in vivo* siRNA delivery. Incorporating siRNA into liposomes provides protection from degradation, resulting in a reduction of the elimination half-life and increased potency [52]. The effectiveness of this approach has been established using a variety of cancer animal models and administration routes (summarized in Table **1**). With the use of fluorescent tags, studies have demonstrated that siRNA can be effectively delivered into a variety of tumor types when administered in liposomal formulations using either cationic lipids or neutral lipids. Gene silencing properties were conserved and all target proteins were down regulated after liposomal siRNA administration (Table **1**). Moreover, decreased tumor growth was reported as well as increased sensitivity to certain chemotherapy drugs.

Despite the encouraging pre-clinical data regarding the use of liposomes for siRNA delivery, some concerns should be considered. For example, cationic lipids have been widely used, but several have been associated with toxicity including pulmonary inflammation by oxygen radical release, complement activation *via* the alternative pathway and acute systemic inflammatory reactions [53, 54]. Systemic delivery of siRNAs using cationic lipids also seems to potentiate the induction of interferon response [55].

To achieve highly efficient *in vivo* delivery while minimizing toxicity, our group has focused on the use of a neutral lipid, 1,2-Dioleoyl-*sn*-Glycero-3-Phosphocholine (DOPC), for liposomal siRNA delivery. We had previously reported excellent transfection using this lipid for DNA antisense delivery [56]. More recently, we have demonstrated that siRNA targeted against EphA2 incorporated into DOPC (L-EphA2-

Table 1. Published Studies Using Liposomes for *In Vivo* siRNA Delivery in Cancer

Reference	Liposome	Dose	Target	Route	Animal Model	Outcome	Toxicity
[40]	Neutral lipid (DOPC)	150 ug/kg	EphA2	IP	Orthotopic model of ovarian cancer	Decreased tumor size	None
[41]	Neutral lipid (DOPC)	150 ug/kg	ADRB1 and ADRB2	IP	Chronic stress animal model for ovarian cancer	Reduced tumor weight and number of nodules	None
[42]	Cationic liposomes	2.17 mg/kg	CD31	IV	Orthotopic model of human prostate cancer	Reduction of tumor growth and metastasis	Slight increase AST and ALT
[43]	Cationic liposome LIC-101	10 mg/kg	Bcl-2	IV	Model of liver metastasis	Decreased liver weight	None
[43]	Cationic liposome LIC-101 (as above)	0.1 mg per mouse	Bcl-2	SC	Subcutaneous model of prostate cancer	Decreased tumor size	None
[44]	Cationic liposomes containing a cationic lipid analogue	600 uM	PLK-1	Intravesical	Orthotopic bladder cancer model		None
[45]	Cationic cardiolipin analog (CCLA)	7.5 mg/kg	c-ras	IV	Subcutaneous breast cancer model	Decreased tumor size	None
[46]	Neutral lipid (DOPC)	150 ug/kg	FAK	IP	Orthotopic model of ovarian cancer	Reduced tumor weight and improved response to docetaxel and cisplatin	None
[47]	Cationic cardiolipin liposomes (NeoPhectin-AT)	7.5 mg/kg,	Raf-1	IV	Orthotopic model of human prostate cancer	Inhibition of tumor growth	None
[48]	Cationic liposomes	1 μg per injection	Integrin αV	Intratumoral	Prostate cancer xenographs (flank and tibia)	Reduced tumor growth both subcutaneous and bony sites	None

siRNA) was effective in an orthotopic murine model of ovarian cancer [57]. The gene modulation capacity of siRNA was conserved when administered *in vivo* using neutral liposomes. For our initial studies, we targeted the EphA2 receptor tyrosine kinase because it is commonly overexpressed in ovarian and other cancers [58]. Using an orthotopic mouse model of ovarian carcinoma, we demonstrated reduction in tumor growth by 30-50% following intravenous delivery of EphA2 siRNA-DOPC. The anti-tumor effect was at least additive when the EphA2 siRNA-DOPC was administered concomitantly with paclitaxel chemotherapy. Using the same animal model, we were also able to demonstrate that the administration of DOPC-siRNA given into the peritoneal cavity was equally effective as the intravenous route [40]. Since the initial studies, we have demonstrated the utility of this approach for many other targets in ovarian [41, 59] and other cancers (unpublished data). Apart from improved delivery, DOPC has other advantages including minimal toxicity. Unlike cationic lipids, DOPC does not induce renal or hepatic toxicity or changes in hematological parameters [60]. In our studies to date, no obvious hematological or organ toxicities have been observed.

The stability of liposomes may be further improved by the incorporation of polyethylene glycol (PEG) units to the surface, a process called pegylation [61]. Following administration, liposomes rapidly enter the liver, spleen, kidney and reticuloendothelial systems. Pegylation is a method that has been developed to overcome these problems, PEG is non-toxic, biodegradable, non-immunogenic and FDA approved. Some pegylated drugs such as Egaspargase for leukemia and pegademase for severe combined immunodeficiency disorder have been already FDA approved [62]. Pegylated liposomes have increased half-life, are less taken up by the liver and spleen, allowing higher accumulation of the drug in the tumor tissue [63]. Pegylation changes particle surface charge and size because PEG structure absorbs water. In consequence, pegylated particles show less aggregation [64], reduced renal clearance and decreased reticuloendothelial system uptake and complement activation [65]. We and others are examining the utility of pegylation for siRNA delivery.

2. Cationic Polymers

Positively charged polymers are potentially viable carriers for the negatively charged DNA and RNA. Typically, biode-

gradable and non-toxic carriers are more desirable. Among them, polyethyleneimine (PEI) and chitosan have been used [69]. A summary of the published studies using cationic polymers for systemic siRNA delivery for cancer therapy can be found in Table **2**.

Conjugation of nucleic acids with PEI is achieved by electrostatic interactions between the positively charged amino groups of PEI and the negatively charged phosphate groups of DNA or RNA [70]. The advantage of using PEI among other polymers is the high transfection efficiency observed. PEIs have the unique characteristic of buffering the low endosomal pH, a property called "proton sponge effect" that can lead to the destruction of the endosomes and release of the nucleic acid into the cytoplasm [71]. *In vivo* delivery of low molecular weight PEI/siRNA complex was evaluated in a subcutaneous xenograft model of ovarian cancer [67]. Following systemic administration of Her-2 siRNA/PEI complex using IV and IP routes, successful intra-tumoral siRNA delivery was achieved with no signs of acute toxicity [67]. In another study, siRNA specific for the secreted growth factor pleiotrophin (PTN) conjugated with PEI reduced tumor growth and cell proliferation after intracerebral delivery in an orthotopic glioblastoma mouse model. No toxicity or abnormal animal behavior was reported in this model [66].

Chitosan, a polysaccharide extracted from the exoskeleton of crustaceans and insects, displays many advantages as a siRNA carrier including a positive charge, biodegradability and low toxicity [72, 73]. Recent studies demonstrate that *in vitro* chitosan nanoparticles were able to deliver siRNA with almost 100 % transfection efficiency [74]. To date few *in vivo* studies have been performed using chitosan for siRNA delivery. In one study, chitosan/siRNA aministered *via* nasal administration was effective in delivering siRNA into bronchiolar epithelial cells [80]. Although not related to cancer, this work demonstrated that chitosan could potentially be used for local siRNA delivery in lung disease. Another study using a breast cancer model, Pille and colleagues showed that anti-RhoA siRNA in chitosan-coated polyisohexylcyanoacrylate (PIHCA) nanoparticles injected intravenously (IV), substantially reduced the size of the tumor without any reported toxicity [68].

VI. Targeted Delivery of siRNA in Cancer

Target specificity may have different meanings according to their application. So the elusive "magic bullet" is still far from being developed. At least three characteristics should be looked at in a targeted system: First, the drug delivery vehicle should only reach and deliver therapeutic agents to target cells. Second, the targeted siRNA should be preferentially (or only) expressed in tumor cells. Third, it is desirable that both the carrier and the siRNA are eventually degraded. Are we close to this paradigm? Hardly, but there are a number of avenues of research that could be exploited. It is possible that cancer cells can be specifically targeted because many express unique antigens or receptors that are absent in normal cells. Targeting the tumor microenvironment by selectively delivering the molecule of interest to the tumor vasculature is another attractive possibility. Angiogenic vessels from tumor vasculature expressed certain proteins that are present at low or undetectable levels in normal blood vessels (for example αv integrins) [81]. A number of the available delivery systems and siRNA are both biodegradable and some examples of these approaches are discussed below and had been summarized in Table **3**.

IV.1. Folate Modified Particles

An approach that may allow for selective delivery of therapeutic agents to tumor cells is targeting the folate receptor (FR) [82]. Folate is needed for rapid cell growth and most cancer cells overexpress the folate receptor [83]. It is hypothesized that inhibition of this receptor will lead to suppression of tumor growth. The FR can be targeted using folic acid or monoclonal antibodies against the receptor. Folic acid, which is FR high affinity ligand, can be coupled to the surface of a liposome or polymer either directly or indirectly using a PEG spacer [82]. Antibodies against the FR can in-

Table 2. Published Studies Using Cationic Polymers for *In Vivo* siRNA Delivery in Cancer

Reference	Type of Nanoparticle	Dose	Target	Route	Animal Model	Outcome	Toxicity
[66]	PEI	8 ug per injection for SC and IP administration and 0.2 ug for intracerebral injection	Secreted growth factor pleiotropin (PTN)	SC, IP and intracerebral	U87 glioblastoma cells implanted subcutaneous and intracranial	Inhibition of tumor growth	None
[67]	PEI	8 ug per injection	HER-2 receptor	IP	Subcutaneous model of ovarian cancer cell SKOV3	Inhibition of tumor growth	None
[68]	Chitosan-coated polyisohexyl-cyanoacrylate (PIHCA)	150 and 1500 ug/kg	Rho	IV	Subcutaneous model of breast cancer cell MDA-MB-231	Inhibition of tumor growth of about 90% for the lower dose and complete inhibition of tumor growth for the higher dose	No toxicity found.

Table 3. Published Studies Using Targeted Delivery Vehicles for *In Vivo* siRNA in Cancer Therapeutics

Reference	Vehicle	siRNA Dose	Gene Target	Route	Animal Model	Outcome	Toxicity
[75]	Folate conjugated pRNA nanoparticle		Survivin	Tumor cells incubated with siRNA before being introduced into the mice	Nasopharyngeal epidermal carcinoma (KB) animal model	Suppression of tumor growth with folate/pRNAsurvivin siRNA complex	None
[76]	DNA/RNA hybrid – cationic liposome with anti-transferrin antibody	3 mg/kg	Her-2	IV	Breast cancer model	Inhibition Her-2 expression	None
[77]	DNA/RNA modified hybrid with same immunoliposome (as above)	3 mg/kg – 1.5 mg/kg	Her2	IV	Pancreatic cancer model	Tumor growth inhibition	None
[78]	Protamine-antibody fusion protein. An antibody targeting HIV envelope protein was used.	80 ug per injection	c-myc, VEGF and MDM2	Intra-tumor and IV	Subcutaneous model of HIV env-expressing melanoma B16 cells	Reduced tumor size most dramatically when combining siRNAs for all 3 genes.	None
[79]	Cyclodextrin-containing polycations (CDP) self-assemble with siRNA. As a targeted ligand tranferrin was used.	2.5 mg/kg	*EWS-FLI1* fusion gene	IV	Murine model of metastatic Ewing's sarcoma	Targeted prevented tumor cell engraftment and slowed the growth of any tumors that did develop	None

clude whole antibodies, fragments or derivatives. Between both approaches to target the FR, folate is the preferred one because it is less immunogenic, cost-effective and easy to store and handle [84]. Folate targeted therapy has been evaluated for a wide number of therapeutic agents, including DNA treatment [85]. Recent studies demonstrated that it is also useful for targeted siRNA delivery. Using a Poly (ethylenimine) (PEI) nanoparticle conjugated with PEG and folate (PEI-PEG-FOL), Kim and colleagues [86] demonstrated better *in vitro* siRNA transfection and gene silencing than using the same complex without folate. This finding indicates that cell internalization of the complex was achieved *via* endocytosis mediated by the FR. Guo and colleagues [75] created a folate-conjugated packing RNA (pRNA) of bacteriophage phi29 DNA packaging motor to deliver chimeric siRNA. Phi29 DNA packaging motor refers to the motor proteins that pack DNA from the bacteriophage 29. One important part of the motor complex, made of RNA and referred to as pRNA, was used as a drug delivery vehicle because allowed the coupling of folate and siRNA. Once internalized by the cell, it was cleaved by dicer allowing the release of siRNA. These investigators treated luciferase transfected nasopharyngeal carcinoma cells with a siRNA against luciferase. Only the complex with the folate conjugate was able to silence the luciferase gene, demonstrating that folate mediated the entry of the complex into tumor cells. The *in vivo* activity of the complex was first tested *ex-vivo* by transfecting tumor cells with a siRNA specific for survivin and later implanted in mice. They found inhibition of tumor growth only when the complex included folate. However, the utility of this approach for selective systemic delivery and suppression of tumor growth is not known.

IV.2. Antibodies

Many antibodies have therapeutic properties, but can also be used as targeting approaches for drug delivery systems. To achieve tumor-specificity delivery, careful selection of the antigen has to be made. Ideal antigens should be selectively expressed in tumor tissues or overexpressed in malignant cells compared to normal cells. Examples of antigens that have been used for drug delivery include Her2 [87] and the epidermal growth factor receptor [88]. For drug delivery, either whole monoclonal antibodies or engineered fragments can be used. Fragments are considered the best approach because they can be produced in bacteria, making them less expensive to manufacture and scale up [20].

Recent studies suggest that antibodies can be used for specifically targeting tumor tissue for siRNA delivery. Pirollo and colleagues developed a cationic liposome complexed on the surface with an antibody fragment against the transferrin receptor (TfR) [77]. This is a cell membrane associated glycoprotein require for rapid cellular growth that is up-regulated in various tumor types [89, 90]. Fluorescent siRNA complexes in immunoliposomes were administered IV into an orthotopic pancreatic cancer animal model and a lung metastasis model. Efficient and specific siRNA delivery into malignant cells was achieved with minimal uptake into normal tissues. This group used the same carrier to systemically deliver a 19-mer short hybrid (DNA sense/RNA antisense) specific for Her-2 [76]. After systemic administration in a breast and pancreatic cancer animal model, this immunocomplex showed good silencing of the target gene and inhibition of tumor growth.

Another group, Song and coworkers, used an antibody fragment against the HIV envelope glycoprotein gp160 (env) for *in vivo* siRNA targeted delivery [78]. They delivered siRNA bound to a protamine-antibody fusion protein called F105-P. They were able to inhibit HIV replication in HIV infected primary T-cells with a heavy chain Fab fragment of an HIV-1 envelope antibody. *In vivo*, specific cell delivery was confirmed after injection of fluorescent siRNA into env-expressing B16 tumor model. Tumor cells took up siRNA efficiently without any significant delivery in adjacent tissue. In contrast, env negative tumors did not take up any siRNA. Tumor growth inhibition was achieved by delivering siRNAs against c-myc, MDM2 and VEGF using the same model. Furthermore, using a single chain antibody fragment that recognized the ErbB2 receptor siRNA was selectively delivered to ErbB2 positive cells. Although the latest experiment was only performed *in vitro*, it is nevertheless a promising approach for cancer treatment [78].

IV.3. Peptides and Proteins

Attachment of peptides on the surface of liposomes or nanoparticles is another approach for delivering genetic material into cancer cells. Peptides can be used as a cell penetrating aid to facilitate delivery of genetic material into any cell [91] or as a target moiety to improve siRNA specificity for tumor tissue. Cell penetrating peptides (CPP) have been used to deliver therapeutic agents into a variety of cells. CPP are peptides of about 30 or less amino acids that have the ability to translocate across the cell membrane [92]. Zhang and colleagues described a novel approach for siRNA delivery using a liposome bearing a synthetic arginine-rich CPP called R8 [91]. The particles were stable and the system showed high transfection efficiency *in vitro* into lung tumor cells. However, the *in vivo* efficiency of this approach needs to be demonstrated [91]. To date, no other reports are available for siRNA delivery using targeting peptides.

Proteins can also be used for targeted delivery into tumor cells. Proteins that are used include natural ligands for some cells surface receptors. Hu-Lieskovan and associates developed a delivery system using a particle made of cyclodextrin-containing polycation complexed with transferrin [79]. The transferin receptor is overexpressed on many cancer cells and can also be targeted using monoclonal antibodies. In a mouse model of metastatic Ewing's sarcoma, successful delivery of siRNA specific for *EWS-FLI1* was achieved with anti-tumor effects. Removal of the targeted ligand did not have any anti-tumor effects indicating that siRNA transfection was dependent on the TfR-induced endocytosis. The same group performed pharmacology and safety studies using the same targeted nanoparticle in non-human primates [93]. For siRNA doses commonly used for *in vivo* studies (3 mg/kg and 9 mg/kg), the particles were well tolerated. For higher doses of 27 mg/kg, however, the animals developed renal and liver toxicities and had increased levels of IL-6 and IFN-λ. This study is interesting because it evaluated targeted delivery of siRNA in non-human primates and suggested that therapeutic doses may be well tolerated.

FUTURE DIRECTIONS

Use of siRNA as a method of gene silencing has rapidly become a powerful tool for protein function delineation, gene discovery and drug development. Currently, remarkable efforts are being made to take this technology into the clinic. Some phase I clinical trials have been already performed using local siRNA delivery to treat macular degeneration and respiratory syncytial virus (RSV) [94], with no major toxicity reported. Although some groups are planning future phase I clinical trials for systemic siRNA delivery to treat cancer, none have been started to-date. To succeed in this task, it has become evident that siRNA requires utilization of delivery vehicles to prevent degradation and improve pharmacokinetics. SiRNA offers opportunities for targeting key oncogenic pathways that would not be "druggable" with other methods. With caution and a methodical approach, siRNA offers hope for developing new therapeutic strategies for cancer patients.

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REFERENCES

[1] Fire, A.; Xu, S.; Montgomery, M.K.; Kostas, S.A.; Driver, S.E.; Mello, C.C. *Nature,* **1998**, *391*, 806.
[2] Hamilton, A.J.; Baulcombe, D.C. *Science,* **1999**, *286*, 950.
[3] Elbashir, S.M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature,* **2001**, *411*, 494.
[4] Rana, T.M. *Nat. Rev. Mol. Cell Biol.,* **2007**, *8*, 23.
[5] Bernstein, E; Caudy, A.A.; Hammond, S.M.; Hannon, G.J. *Nature,* **2001**, *409*, 363.
[6] Martinez, J.; Patkaniowska, A.; Urlaub, H.; Luhrmann, R.; Tuschl, T. *Cell,* **2002**, *110*, 563.
[7] Liu, J.; Carmell, M.A.C.; Rivas, F.V.; Marsden, C.G.;Thomson, J.M.; Song, J.J.; Hammond, S.M.; Joshua-Tor, L.; Hannon, G.J. *Science,* **2004**, *305*, 1437.
[8] Matranga, C.; Tomari, Y.; Shin, C.; Bartel, D.P.; Zamore, P.D. *Cell,* **2005**, *123*, 607.
[9] Rand, T.A.; Petersen, S.P.; Du, F.; Wang, X. *Cell,* **2005**, *123*, 621.
[10] Druker, B.J.; Guilhot, F.; O'Brien, S.G.; Gathmann, I.; Kantarjian, H.; Gattermann, N.; Deininger, M.W.; Silver, R.T.; Goldman, J.M.; Stone, R.M.; Cervantes, F.; Hochhaus, A.; Powell, B.L.; Gabrilove, J.L.; Rousselot,P.; Reiffers, J.; Cornelissen, J.J.; Hughes, T.; Agis, H.; Fischer, T.; Verhoef, G.; Shepherd, J.; Saglio, G.; Gratwohl, A.; Nielsen, J.L.; Radich, J.P.; Simonsson, B.; Taylor, K.; Baccarani, M.; So, C.; Letvak, L.; Larson, R.A. *N. Engl. J. Med.,* **2006**, *355*, 2408.
[11] Jabbour, E.; Cortes, J.C.; Kantarjian, H. *Oncology,* **2007**, *21*, 653.
[12] Smith, I.; Procter, M.; Gelber, R.D.; Guillaume, S.; Feyereislova, A.; Dowsett, M.; Goldhirsch, A.; Untch, M.; Mariani, G.; Baselga, J.; Kaufmann, M.; Cameron, D.; Bell, R.; Bergh, J.; Coleman, R.; Wardley, A.; Harbeck, N.; Lopez, R.I.; Mallmann, P.; Gelmon, K.; Wilcken, N.; Wist, E.; Sanchez Rovira, P.; Piccart-Gebhart, M.J. *Lancet,* **2007**, *369*, 29.
[13] Fietz, T.; Thiel, E. *Recent Results Cancer Res.,* **2007**, *176*, 153.
[14] Elbashir, S.M.; Harborth, J.; Weber, K.; Tuschl, T. *Methods,* **2002**, *26*, 199.
[15] Harborth, J.; Elbashir, S.M.; Vandenburgh, K.; Manninga, H.; Scaringe, S.A.; Weber, K.; Tuschl, T. *Antisense Nucleic Acid Drug Dev.,* **2003**, *13*, 83.
[16] Yuki, N.; Tomoyuki, Y.; Kumiko, U.T.; Shinichi, M.; Kaoru, S. *Nucleic Acids Res.,* **2004**, *32*, W124.
[17] Henschel, A.; Buchlolz, F.; Habermann, B. *Nucleic Acids Res.,* **2004**, *32*, W113.
[18] Soutschek, J.; Akinc, A.A.; Bramlage, B.; Charisse, K.; Constien, R.; Donoghue, M.; Elbashir, S.; Geick, A.; Hadwiger, P.; Harborth, J.; John, M.; Kesavan, V.; Lavine, G.; Pandey, R.K.; Racie, T.; Rajeev, K.G.; Röhl, I.; Toudjarska, I.; Wang, G.; Wuschko, S.; Bumcrot, D.; Koteliansky,V.; Limmer, S.; Manoharan, M.; Vornlocher, H.P. *Nature,* **2004**, *432*, 173.

[19] Snove, O.Jr.; Rossi, J.J. *ACS Chem. Biol.,* **2006**, *1*, 274.
[20] Ikeda, Y.; Taira, K. *Pharm.Res.,* **2006**, *23*, 1631.
[21] Jackson, A.L.; Bartz, S.R.; Schelter, J.; Kobayashi, S.V.; Burchard, J.; Mao, M.; Li, B.; Cavet, G.; Linsley, P.S. *Nat.Biotechnol.,* **2003**, *21*, 635.
[22] Birmingham, A.; Anderson, E.M.; Reynolds, A.; Ilsley-Tyree, D.; Leake, D.; Fedorov, Y.; Baskerville, S.; Maksimova, E.; Robinson, K.; Karpilow, J.; Marshall, W.S.; Khvorova, A. *Nat.Methods,* **2006**, *3*, 199.
[23] Layzer, J.M.; McCaffrey, A.P.; Tanner, A.K.; Huang, Z.; Kay, M.A.; Sullenger, B.A. *RNA,* **2004**, *10*, 766.
[24] Morrissey, D.V.; Blanchard, K.; Shaw, L.; Jensen, K.; Lockridge, J.A.; Dickinson, B.; McSwiggen, J.A.; Vargeese, C.; Bowman, K.; Shaffer, C.S.; Polisky, B.A.; Zinnen, S. *Hepatology,* **2005**, *41*, 1349.
[25] Morrissey, D.V.; Lockridge, J.A.; Shaw, L.; Blanchard, K.; Jensen, K.; Breen, W.; Hartsough, K.; Machemer, L.; Radka, S.; Jadhav, V.; Vaish, N.; Zinnen, S.; Vargeese, C.; Bowman, K.; Shaffer, C.S.; Jeffs, L.B.; Judge, A.; MacLachlan, I.; Polisky, B. *Nat. Biotechnol.,* **2005**, *23*, 1002.
[26] Liu, G.; Wong-Staal, F.; Li, Q.X. *Histol. Histopathol.,* **2007**, *22*, 211.
[27] Sledz, C.A.; Holko, M.; de Veer, M.J.; Silverman, R.H.; Williams, B.R. *Nat. Cell Biol.,* **2003**, *5*, 834.
[28] Sioud, M.; Sorensen, D.R. *Biochem. Biophys. Res. Commun.,* **2003**, *312*, 1220.
[29] Judge, A.D.; Sood, V.; Shaw, J.R.; Fang, D.; McClintock, K.; MacLachlan, I. *Nat. Biotechnol.,* **2005**, *23*, 457.
[30] Sioud, M. *J. Mol. Biol.,* **2005**, *348*, 1079.
[31] Hornung, V.; Guenthner-Biller, M.; Bourquin, C.; Ablasser, A.; Schlee, M.; Uematsu, S.; Noronha, A.; Manoharan, M.; Akira, S.; de Fougerolles, A.; Endres, S.; Hartmann, G. *Nat. Med.,* **2005**, *11*, 263.
[32] Schlee, M.; Hornung, V.; Hartmann, G. *Mol. Ther.,* **2006**, *14*, 463.
[33] Xu, P.; Zhang, Y.; Kang, L.; Roossinck, M.J.; Mysore, K.S. *Plant Physiol.,* **2006**, *142*, 429.
[34] Qiu, S.; Adema, C.M.; Lane, T. *Nucleic Acids Res.,* **2005**, *33*, 1834.
[35] Du, Q.; Thonberg, H.; Wang, J.; Wahlestedt, C.; Liang, Z. *Nucleic Acids Res.,* **2005**, *33*, 1671.
[36] Naito, Y.; Yamada, T.; Matsumiya, T.; Ui-Tei, K.; Saigo, K.; Morishita, S. *Nucleic Acids Res.,* **2005**, *33*, W589.
[37] Sabbioni, S.; Callegari, E.; Manservigi, M.; Argnani, R.; Corallini, A.; Negrini, M.; Manservigi, R. *Gene Ther.,* **2007**, *14*, 459.
[38] Cho-Rok, J.; Yoo, J.; Jang, Y.J.; Kim, S.; Chu, I.S.; Yeom, Y.I.; Choi, J.Y.; Im, D.S. *Hepatology,* **2006**, *43*, 1042.
[39] Devroe, E.; Silver, P.A. *Expert Opin. Biol. Ther.,* **2004**, *4*, 319.
[40] Landen, C.N.; Merritt, W.M.; Mangala, L.S.; Sanguino, A.M.; Bucana, C.; Lu, C.; Lin, Y.G.; Han, L.Y.; Kamat, A.A.; Schmandt, R.; Coleman, R.L.; Gershenson, D.M.; Lopez- Berestein, G.; Sood, A.K. *Cancer Biol. Ther.,* **2006**, *5*, 1708.
[41] Thaker, P.H.; Han, L.Y.; Kamat, A.A.; Arevalo, J.M.; Takahashi, R.; Lu, C.; Jennings, N.B.; Armaiz-Pena, G.; Bankson, J.A.; Ravoori, M.; Merritt, W.M.; Lin, Y.G.; Mangala, L.S.; Kim, T.J.; Coleman, R.L.; Landen, C.N.; Li, Y.; Felix, E.; Sanguino, A.M.; Newman, R.A.; Lloyd, M.; Gershenson, D.M.; Kundra, V.; Lopez-Berestein, G.; Lutgendorf, S.K.; Cole, S.W.; Sood, A.K. *Nat. Med.,* **2006**, *12*, 939.
[42] Santel, A.; Aleku, M.; Keil, O.; Endruschat, J.; Esche, V.; Durieux, B.; Loffler, K.; Fechtner, M.; Rohl, T.; Fisch, G.; Dames, S.; Arnold, W.; Giese, K.; Klippel, A.; Kaufmann, J. *Gene Ther.,* **2006**, *13*, 1360.
[43] Yano, J.; Hirabayashi, K.; Nakagawa, S.; Yamaguchi, T.; Nogawa, M.; Kashimori, I.; Naito, H.; Kitagawa, H.; Ishiyama, K.; Ohgi, T.; Irimura, T. *Clin. Cancer Res.,* **2004**, *10*, 7721.
[44] Nogawa, M.; Yuasa, T.; Kimura, S.; Tanaka, M.; Kuroda, J.; Sato, K.; Yokota, A.; Segawa, H.; Toda, Y.; Kageyama, S.; Yoshiki, T.; Okada, Y.; Maekawa, T. *J. Clin. Invest.,* **2005**, *115*, 978.
[45] Chien, P.Y.; Wang, J.; Carbonaro, D.; Lei, S.; Miller, B.; Sheikh, S.; Ali, S.M.; Ahmad, M.U.; Ahmad, I. *Cancer Gene Ther.,* **2005**, *12*, 321.
[46] Halder, J.; Kamat, A.A.; Landen, C.N., Jr.; Han, L.Y.; Lutgendorf, S.K.; Lin, Y.G.; Merritt, W.M.; Jennings, N.B.; Chavez-Reyes, A.; Coleman, R.L.; Gershenson, D.M.; Schmandt, R.; Cole, S.W.; Lopez-Berestein, G.; Sood, A.K. *Clin. Cancer Res.,* **2006**, *12*, 4916.
[47] Pal, A.; Ahmad, A.; Khan, S.; Sakabe, I.; Zhang, C.; Kasid, U.N.; Ahmad, I. *Int. J. Oncol.,* **2005**, *26*, 1087.
[48] Bisanz, K.; Yu, J.; Edlund, M.; Spohn, B.; Hung, M.C.; Chung, L.W.; Hsieh, C.L. *Mol. Ther.,* **2005**, *12*, 634.
[49] Batist, G.; Ramakrishnan, G.; Rao, C.S.; Chandrasekharan, A.; Gutheil, J.; Guthrie, T.; Shah, P.; Khojasteh, A.; Nair, M.K.; Hoelzer, K.; Tkaczuk, K.; Park, Y.C.; Lee, L.W. *J. Clin. Oncol.,* **2001**, *19*, 1444.
[50] Lu, C.; Perez-Soler, R.; Piperdi, B.; Walsh, G.L.; Swisher, S.G.; Smythe, W.R.; Shin, H.J.; Ro, J.Y.; Feng, L.; Truong, M.; Yalamanchili, A.; Lopez-Berestein, G.; Hong, W.K.; Khokhar, A.R.; Shin, D.M. *J. Clin. Oncol.* **2005**, *23*, 3495.
[51] Gabizon, A.A. *Cancer Invest.,* **2001**, *19*, 424.
[52] Santel, A.; Aleku, M.; Keil, O.; Endruschat, J.; Esche, V.; Fisch, G.; Dames, S.; Loffler, K.; Fechtner, M.; Arnold, W.; Giese, K.; Klippel, A.; Kaufmann, J. *Gene Ther.* **2006**, *13*, 1222.
[53] Lv, H.; Zhang, S.; Wang, B.; Cui, S.; Yan, J. *J. Control Rel.,* **2006**, *114*, 100.
[54] Dass, C.R. *J. Pharm. Pharmacol.,* **2002**, *54*, 593.
[55] Ma, Z.; Li, J.; He, F.; Wilson, A.; Pitt, B.; Li, S. *Biochem. Biophys. Res. Commun.,* **2005**, *330*, 755.
[56] Gutierrez-Puente, Y.; Tari, A.M.; Ford, R.J.; Tamez-Guerra, R.; Mercado-Hernandez, R.; Santoyo-Stephano, M.; Lopez-Berestein, G. *Leuk. Lymphoma,* **2003**, *44*, 1979.
[57] Landen, C.N., Jr.; Chavez-Reyes, A.; Bucana, C.; Schmandt, R.; Deavers, M.T.; Lopez- Berestein, G.; Sood, A.K. *Cancer Res.,* **2005**, *65*, 6910.
[58] Thaker, P.H.; Deavers, M.; Celestino, J.; Thornton, A.; Fletcher, M.S.; Landen, C.N.; Kinch, M.S.; Kiener, P.A.; Sood, A.K. *Clin. Cancer Res.,* **2004**, *10*, 5145.
[59] Halder, J.; Landen, C.N., Jr.; Lutgendorf, S.K.; Li, Y.; Jennings, N.B.; Fan, D.; Nelkin, G.M.; Schmandt, R.; Schaller, M.D.; Sood, A.K. *Clin. Cancer Res.,* **2005**, *11*, 8829.
[60] Gutierrez-Puente, Y.; Tari, A.M.; Stephens, C.; Rosenblum, M.; Guerra, R.T.; Lopez- Berestein, G. *J. Pharmacol. Exp. Ther.,* **1999**, *291*, 865.
[61] Hamidi, M.; Azadi, A.; Rafiei, P. *Drug Deliv.,* **2006**, *13*, 399.
[62] Harris, J.M.; Chess, R.B. *Nat. Rev. Drug Discov.,* **2003**, *2*, 214.
[63] Papahadjopoulos, D; Allen, T.M.; Gabizon, A.; Mayhew, E.; Matthay, K.; Huang, S.K.; Lee, K.D.; Woodle, M.C.; Lasic, D.D.; Redemann, C. *Proc. Natl. Acad. Sci.USA,* **1991**, *88*, 11460.
[64] Heyes, J.; Hall, K.; Tailor, V.; Lenz, R.; MacLachlan, I. *J. Control Rel.,* **2006**, *112*, 280.
[65] Gbadamosi, J.K.; Hunter, A.C.; Moghimi, S.M. *FEBS Lett.,* **2002**, *532*, 338.
[66] Grzelinski, M.; Urban-Klein, B.; Martens, T.; Lamszus, K.; Bakowsky, U.; Hobel, S.; Czubayko, F.; Aigner, A. *Hum. Gene Ther.,* **2006**, *17*, 751.
[67] Urban-Klein, B.; Werth, S.; Abuharbeid, S.; Czubayko, F.; Aigner, A. *Gene Ther.,* **2005**, *12*, 461.
[68] Pille, J.Y.; Li, H.; Blot, E.; Bertrand, J.R.; Pritchard, L.L.; Opolon, P.; Maksimenko, A.; Lu, H.; Vannier, J.P.; Soria, J.; Malvy, C.; Soria, C. *Hum. Gene Ther.,* **2006**, *17*, 1019.
[69] Dang, J.M.; Leong, K.W. *Adv. Drug Deliv. Rev.,* **2006**, *58*, 487.
[70] Grayson, A.C.; Doody, A.M.; Putnam, D. *Pharm. Res.,* **2006**, *23*, 1868.
[71] Aigner, A. *J. Biomed. Biotechnol.,* **2006**, *2006*, 71659.
[72] Mansouri, S.; Lavigne, P.; Corsi, K.; Benderdour, M.; Beaumont, E.; Fernandes, J.C. *Eur. J. Pharm. Biopharm.,* **2004**, *57*, 1.
[73] Zheng, F.; Shi, X.W.; Yang, G.F.; Gong, L.L.; Yuan, H.Y.; Cui, Y.J.; Wang, Y.; Du, Y.M.; Li, Y. *Life Sci.,* **2007**, *80*, 388.
[74] Katas, H.; Alpar, H.O. *J. Control Rel.,* **2006**, *115*, 216.
[75] Guo, S.; Huang, F.; Guo, P. *Gene Ther.,* **2006**, *13*, 1553.
[76] Hogrefe, R.I.; Lebedev, A.V.; Zon, G.; Pirollo, K.F.; Rait, A.; Zhou, Q.; Yu, W.; Chang, E.H. *Nucleosides Nucleotides Nucleic Acids,* **2006**, *25*, 889.
[77] Pirollo, K.F.; Zon, G.; Rait, A.; Zhou, Q.; Yu, W.; Hogrefe, R.; Chang, E.H. *Hum. Gene Ther.,* **2006**, *17*, 117.
[78] Song, E.; Zhu, P.; Lee, S.K.; Chowdhury, D.; Kussman, S.; Dykxhoorn, D.M.; Feng, Y.; Palliser, D.; Weiner, D.B.; Shankar, P.; Marasco, W.A.; Lieberman, J. *Nat. Biotechnol.,* **2005**, *23*, 709.
[79] Hu-Lieskovan, S.; Heidel, J.D.; Bartlett, D.W.; Davis, M.E.; Triche, T.J. *Cancer Res.,* **2005**, *65*, 8984.
[80] Howard, K.A.; Rahbek, U.L.; Liu, X.; Damgaard, C.K.; Glud, S.Z.; Andersen, M.O.; Hovgaard, M.B.; Schmitz, A.; Nyengaard, J.R.; Besenbacher, F.; Kjems, J. *Mol. Ther.,* **2006**, *14*, 476.

[81] Arap, W.; Pasqualini, R.; Ruoslahti, E.*Science,* **1998**, *279*, 377.
[82] Gosselin, M.A.; Lee, R.J. *Biotechnol. Annu. Rev.,* **2002**, *8*, 103.
[83] Weitman, S.D.; Weinberg, A.G.; Coney, L.R.; Zurawski, V.R.; Jennings, D.S.; Kamen, B.A. *Cancer Res.,* **1992**, *52*, 6708.
[84] Sapra, P.; Allen, T.M. *Prog. Lipid Res.,* **2003**, *42*, 439.
[85] Zhao, X.B.; Lee, R.J. *Adv. Drug Deliv. Rev.,* **2004**, *56*, 1193.
[86] Kim, S.H.; Mok, H.; Jeong, J.H.; Kim, S.W.; Park, T.G. *Bioconjug. Chem.,* **2006**, *17*, 241.
[87] Park, J.W.; Hong, K.; Kirpotin, D.B.; Colbern, G.; Shalaby, R.; Baselga, J.; Shao, Y.; Nielsen, U.B.; Marks,J.D.; Moore, D.; Papahadjopoulos, D.; Benz, C.C. *Clin. Cancer Res.,* **2002**, *8*, 1172- 81.
[88] Mamot, C.; Drummond, D.C.; Noble, C.O.; Kallab, V.; Guo, Z.; Hong, K.; Kirpotin, D.B.; Park, J.W. *Cancer Res.,* **2005**, *65*, 11631.
[89] Daniels, T.R.; Delgado, T.; Rodriguez, J.A.; Helguera, G.; Penichet, M.L. *Clin. Immunol.,* **2006**, *121*, 144.
[90] Daniels, T.R.; Delgado, T.; Helguera, G.; Penichet, M.L. *Clin. Immunol.,* **2006**, *121*, 159.
[91] Zhang, C.; Tang, N.; Liu, X.; Liang, W.; Xu, W.; Torchilin, V.P. *J. Cont. Rel.,* **2006**, *112*, 229.
[92] Patel, L.N.; Z.J., Shen, W.C. *Pharm. Res.,* **2007**, [Epub ahead of print].
[93] Heidel, J.D.; Yu, Z.; Liu, J.Y.; Rele, S.M.; Liang, Y.; Zeidan, R.K.; Kornbrust, D.J.; Davis, M.E. *Proc. Natl. Acad. Sci. USA,* **2007**, *104*, 5715.
[94] Dykxhoorn, D.M.; Lieberman, J. *Cell,* **2006**, *28*, 231.

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