Appl Microbiol Biotechnol (2007) 76:9–21
DOI 10.1007/s00253-007-0984-y

MINI-REVIEW

Applications of RNA interference: current state and prospects for siRNA-based strategies in vivo

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Received: 2 March 2007 / Revised: 2 April 2007 / Accepted: 3 April 2007 / Published online: 25 April 2007

Abstract Within the recent years, RNA interference (RNAi) has become an almost-standard method for in vitro knockdown of any target gene of interest. Now, one major focus is to further explore its potential in vivo, including the development of novel therapeutic strategies. From the mechanism, it becomes clear that small interfering RNAs (siRNAs) play a pivotal role in triggering RNAi. Thus, the efficient delivery of target gene-specific siRNAs is one major challenge in the establishment of therapeutic RNAi. Numerous studies, based on different modes of administration and various siRNA formulations and/or modifications, have already accumulated promising results. This applies to various animal models covering viral infections, cancer and multiple other diseases. Continuing efforts will lead to the development of efficient and "double-specific" drugs, comprising of siRNAs with high target gene specificity and of nanoparticles enhancing siRNA delivery and target organ specificity.

Keywords RNA interference · RNAi · siRNA · Gene-targeting · Gene knockdown · Nonviral siRNA delivery · Nanoplexes

Introduction

After antisense technologies and ribozymes, in the late 1990s a novel mechanism for gene-targeting was discovered: RNA interference (RNAi). It soon became clear that RNAi represents a particularly efficient and—at least in vitro—easy-to-use method for the knockdown of the expression of a selected target gene. Consequently, RNAi is now a well-established method for high-throughput analyses as well as for functional studies in vitro, including mammalian cells.

Many pathological conditions rely on the aberrant expression of endogenous normal or mutant genes causing, e.g., alterations in signal transduction pathways, cellular proliferation, apoptosis, or resistance toward external factors. Additionally, the infection of an organism can lead to the introduction and expression of foreign genes. While the inhibition of the activity of (aberrant) gene products, e.g., through small molecule inhibitors or inhibitory antibodies is one major focus in therapy, much attention has now shifted to an earlier step, i.e., the initial knockdown of the specific gene of interest through RNAi. However, for the in vivo application of RNAi in mammals as a therapeutic approach for reversing a pathological condition as well as for the study of particular gene functions, sophisticated strategies for the induction of RNAi are needed.

Mechanism and induction of RNAi

RNAi is a naturally occurring, sequence-specific mechanism for gene silencing. Its discovery in the nematode *C. elegans* (Fire et al. 1998) was awarded the 2006 Nobel prize for physiology or medicine. However, soon it became obvious that RNAi, although somewhat more complicated, also exists in higher organisms including mammals. RNAi relies on an intracellular multistep process, which can roughly be divided into the initiation phase (see below) and the subsequent effector phase. In the effector phase (Fig. 1, left), which represents the actual RNAi mechanism, small, 21–23 bp double stranded RNA molecules (small interfering RNAs, siRNAs), are incorporated into the RNA-

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induced silencing complex (RISC; Hammond et al. 2000). Upon adenosine triphosphate (ATP)-dependent unwinding of the double-stranded siRNA molecule through an RNA helicase activity into a single-stranded, so-called guidance RNA (Nykanen et al. 2001), the now activated RISC (RISC*) binds to its target mRNA molecule (Martinez et al. 2002; Nykanen et al. 2001). This process is mediated through the sequence-specific hybridization of the guidance RNA to the mRNA target site and brings RISC into close proximity to its target mRNA molecule, which is then cleaved by the RISC nuclease Argonaute 2 (Ago 2) and rapidly degraded due to its now unprotected ends (Liu et al. 2004; Rand et al. 2004; Rivas et al. 2005). Since RISC is recovered for subsequent rounds, this represents a catalytical process leading to the selective reduction in specific mRNA molecules and thus resulting in decreased expression of the targeted gene. The mechanism also demonstrates the pivotal role of siRNA molecules in initiating RNAi and established the delivery of siRNA molecules as sufficient for RNAi induction (Elbashir et al. 2001a, b).

Induction of RNAi: the initiation phase

In a natural, experimental or therapeutical setting, siRNAs can be directly or as precursor molecules introduced into a target cell through different strategies (Fig. 1, upper part). This includes viral or nonviral delivery of DNAs, which are transcribed into long, double-stranded RNA molecules. In the so-called initiation phase, these dsRNAs are cleaved into siRNAs by the multiprotein complex "Dicer", which contains an N-terminal helicase domain, an RNA-binding so-called Piwi/Argonaute/Zwille (PAZ) domain, two RNAse III domains and a double-stranded RNA binding domain (Bernstein et al. 2001; Collins and Cheng 2005). Commercially available systems explore this mechanism by providing DNA vector constructs coding for short hairpin RNAs (shRNAs): The double-stranded region of the shRNA is formed through hairpin formation and intramolecular hybridization and is recognized by Dicer, leading to the formation of siRNAs homologous to the target gene of interest. Alternatively, shRNA molecules can be directly introduced into the cell. However, one major disadvantage of long double-stranded RNA molecules, either directly introduced or intracellularly transcribed, is the induction of a cellular immune response through activation of the interferon system. The direct delivery of siRNA molecules into the target cell strategy largely avoids this problem, although some interferon-stimulating sequences are known as well. Furthermore, it does not require the action of Dicer (Bridge et al. 2003; Hornung et al. 2005; Sledz et al. 2003).

Systematic studies on targeting efficacies have shown that optimal siRNAs can be deduced according to certain selection rules. This includes an optimal length of 19–25 bp and a guanine–cytosine content between 30 and 52%, symmetric two nucleotide 3′ overhangs as well as specific nucleotides at certain positions (see, e.g., Dykxhoorn and Lieberman 2006 for review). Based on these already established criteria, several computer-based algorithms allow the identification of optimal siRNA sequences for any given gene of interest. One example is the siRNA

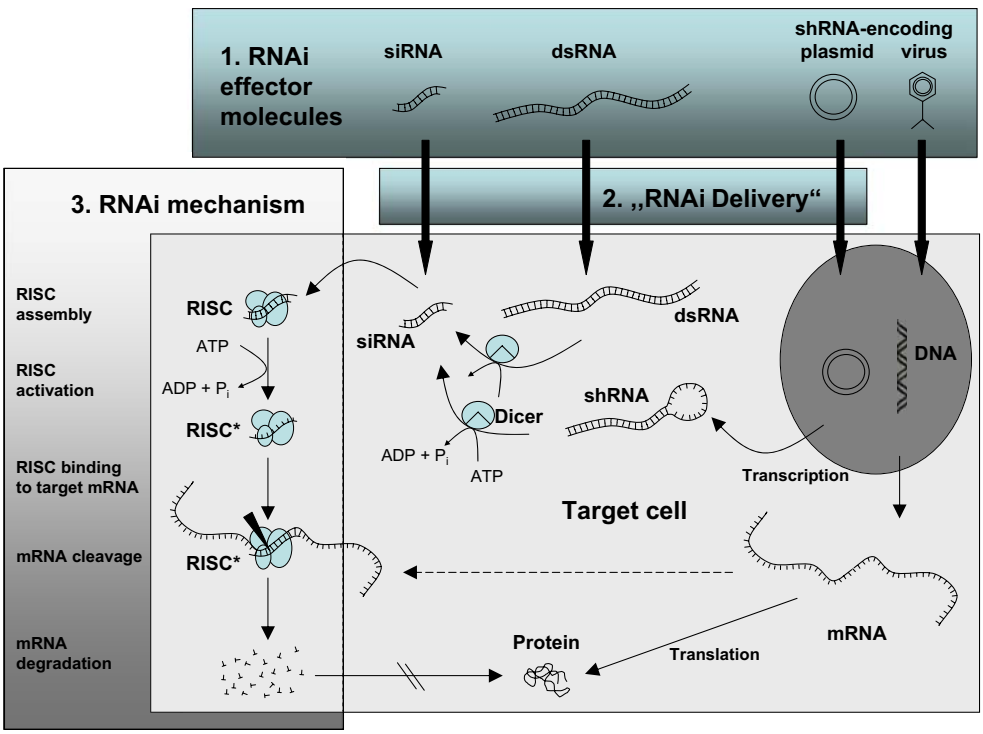

Fig. 1 RNA interference (*RNAi*) is an intracellular mechanism which can be triggered through different effector molecules (*upper part*). Upon their incorporation into the RNA-induced silencing complex (*RISC*), siRNAs are unwinded, and, as single-stranded so-called guidance RNAs, mediate the hybridization of the now activated complex (*RISC**) to its target mRNA. This results in target mRNA cleavage and subsequent degradation, thus emphasizing the pivotal role of siRNAs in the process (*left*). The exploration of RNAi in vivo requires strategies for the intracellular delivery of siRNAs or "upstream" initiation molecules, i.e., molecules that lead to the intracellular formation of siRNAs (*center*)

Design Software (SDS) by the University of Hong Kong which combines algorithms from different companies and is accessible through the internet (http://i.cs.hku.hk/~sirna/software/sirna.php). Nevertheless, any presumably optimal siRNA still requires extensive testing. This refers to a high targeting efficacy, which is, among others, also determined by variations in the accessibility of the target mRNA at different positions, as well as to the absence of any unwanted side effects. In fact, nonspecific silencing of genes due to only partial sequence homology has been described (Jackson et al. 2003). Furthermore, in vivo some siRNA sequences, as well as longer dsRNA molecules, have been shown to activate the innate immune system leading to nonspecific effects due to the stimulation of inflammatory responses (Heil et al. 2004; Judge et al. 2005; Sioud 2005; Sledz et al. 2003). This phenomenon seems to depend on the presence of GU-rich sequences as well as on the formulation and amount of siRNAs (Heidel et al. 2004; Ma et al. 2005; Sioud and Sorensen 2003), and these aspects need to be considered for any therapeutic siRNA application in vivo.

Use of siRNA molecules in vivo

Since the discovery of the pivotal role of siRNAs for inducing RNAi (Elbashir et al. 2001a, b), the direct application of siRNA molecules has been explored in vitro and in vivo. In vitro, several transfection reagents allow the delivery of siRNAs in mammalian cells in the presence or absence of serum. The in vivo application of siRNAs, however, requires the development of more sophisticated formulations and/or the identification of optimal modes of administration (see below).

Several proof-of-principle studies have shown the delivery of fluorophor-labeled siRNA molecules into various organs (see, e.g., Bradley et al. 2005a, b; Pirollo et al. 2006; Sioud and Sorensen 2003). Beyond that, the specific in vivo knockdown of artificially introduced reporter genes like GFP or luciferase, or various endogenous target genes, has been described. The target organ was often the liver, but gene targeting in other organs, in other parts of the body, or in tumor xenografts has been reported as well (Table 1). Taken together, these studies provide valuable insights into the delivery and efficacy of siRNAs for the induction of RNAi.

Therapeutic siRNA molecules

Beyond the detection of the downregulation of an endogenous target gene, the siRNA-mediated RNAi for therapeutical purposes has been explored. Target organs include liver, kidney, lung, eye, ear, heart, pancreas, tumors, blood, as well as the central nervous system, and the peritoneum (see Table 1 for an overview), using locally or systemically administered siRNAs in various formulations.

Cancer

A large body of studies refers to the treatment of cancer with the primary goals being the inhibition of tumor growth. Target molecules usually represent genes that have been shown previously to be relevant or rate-limiting for tumor growth, including growth factors and receptors as well as antiapoptotic or downstream signal transduction proteins in tumor cells. Typically, these studies involve subcutaneous or orthotopic xenografts of different tumor entities in mice, and employ various strategies for local or systemic administration of a wide variety of siRNA formulations (see below). In some cases, the antiangiogenic effect after siRNA delivery to tumor endothelial cells, rather than an inhibitory effect on tumor cells, was explored (Santel et al. 2006a, b), or simultaneous targeting of tumor growth and tumor angiogenesis both contributed to the antitumorigenic effects observed (Grzelinski et al. 2006). Additionally, in some studies the blockage of cancer metastasis, e.g., to the lung, liver or bone has been achieved. Taking into consideration that a large number of cancer patients die from metastases rather than the primary tumor, this represents another very relevant approach in cancer therapy.

Antiviral treatment

Several studies focus on viral gene products, taking into consideration that options for protection or therapy through current antiviral drugs or vaccination strategies are rather limited. Thus, novel RNAi-based approaches to battle viral infections rely on the specific siRNA-mediated knockdown of virus-specific genes. Animal models infected with various viruses including hepatitis virus, influenza virus, respiratory syncytial virus (RSV), SARS corona viruses, or ebola virus have been employed, and primary goals were the reduction in virus titers and protective effects including, when lethal doses were applied, prolonged survival rates upon specific siRNA treatment.

Other targets

Beyond studies related to cancer or viral infection, several target molecules with proven relevance in other pathologies have been selected. Examples include Fas in hepatitis, vascular endothelial growth factor (VEGF) in macular degeneration due to extensive ocular neovascularization or tumor necrosis factor alpha (TNF-α) in arthritis, and these

studies aim at the establishment of novel, improved therapeutic avenues through siRNA-mediated gene silencing. As it can be seen from Table 1, some target genes are relevant in more than one pathology (e.g., Fas in fulminant hepatitis, in renal ischemia-reperfusion injury or in hemorrhagic shock and sepsis in the lung; VEGF in age-related macular degeneration or in tumor growth/tumor angiogenesis; caspase-8 in liver failure or in sepsis), and thus, specific siRNAs may represent drugs applicable for the treatment of different diseases. Some studies also compare siRNA-based therapeutic strategies with already established drugs and discuss decreased side effects and/or higher specificity, or additive effects upon combination of both.

Other papers rather aim at the further elucidation of physiological processes through in vivo knockdown of a certain target gene. Examples include OATC3 in blood-brain barrier transport, V2R in water and sodium homeostasis, or HO-1 in lung ischemia-reperfusion injury (Table 1).

Clinical trials

It should be noted that, although the therapeutic potential of siRNAs has only been explored in the recent years, first siRNA-based drugs are already in clinical trials. This includes ALN-RSV01 (Alnylam Pharmaceuticals) for targeting the human RSV after viral infection, which is the first example of an antivirus siRNA-based therapeutic in a phase I clinical study. Other companies aiming at the development of RNAi therapeutics for viral diseases include Nastech/Galenea, which are expected to start clinical trials in 2007. Benitec, in collaboration with City of Hope in Duarte, California, has developed a multi-RNA therapeutic for treatment of AIDS lymphoma. Furthermore, age-related macular degeneration (AMD) was treated with Sirna-027 (Sirna Therapeutics) targeting the VEGF receptor VEGFR1 (Shen et al. 2006), and resulted in stabilization or even improvement of visual acuity (Whelan 2005). A 24-month phase II study to evaluate multiple doses of Sirna-027 (also termed AGN211745) in the treatment of subfoveal choroidal neovascularization associated with AMD is currently recruiting patients. Targeting the ligand (VEGF) rather than its receptor, Cand5 (Acuity Pharmaceuticals) has been employed for treatment of the same disease, and in the so-called C.A.R.E™ trial showed no adverse effects related to the drug. Cand5, which is now named Bevasiranib, was the first siRNA to enter both phase I and II clinical trials. Recently, SR Pharma plc announced the start of a phase I clinical trial with RTP-801i, an siRNA therapeutic licensed from its subsidiary Atugen AG that targets a gene product involved in the progression of AMD. The same company has most recently announced that the FDA has approved an investigational new drug (IND) application for a second siRNA therapeutic, AKIi-5 being developed for the treatment of acute kidney injury. AKIi-5 is expected to reduce the frequency of postsurgery acute kidney injury in high-risk patients undergoing major cardiovascular surgery. Finally, after successful completion of experiments demonstrating its therapeutic efficacy in animal models of pancreatic cancer, Atu027 is scheduled to enter human clinical trials in 2007. In addition, several other pharmaceutical or biotechnology companies are pursuing collaborative or internal projects for the development of drugs based on RNAi (see, e.g., Behlke 2006 for review).

Strategies for in vivo siRNA delivery

Advantages of the direct application of siRNAs, rather than DNA-based constructs coding for long dsRNA, include the relatively easy chemical synthesis of small RNA molecules, the lower probability of nonspecific side effects (see above) and the safety due to the fact that siRNA delivery is based on nonviral transfer strategies and siRNAs cannot integrate into the genome. On the other hand, successful siRNA-based gene targeting relies on several preconditions: protection of the rather instable siRNA molecules from nucleolytic degradation by serum nucleases, efficient cellular uptake and subsequent intracellular release into the cytoplasm, as well as the absence of intracellular immune responses, in vivo toxicity or rapid elimination in liver or kidney.

Strategies for the in vivo application of siRNA molecules include local as well as systemic modes of administration as detailed in Table 2. However, many studies rely on the use of relatively high amounts of siRNAs. Bearing in mind that intracellular immune responses have been shown to be concentration-dependent, this may increase the risk of nonspecific effects in addition to other side effects and cost considerations. When siRNAs are administered locally, lower doses are sufficient since nonspecific delivery to other organs as well as renal or hepatic elimination are reduced. This approach, however, is invasive and limited to tissues that are sufficiently accessible. With regard to systemic application, several studies rely on the hydrodynamic transfection of siRNAs, i.e., the rapid (~20 s) high-pressure injection of large volumes (up to 2 ml) of siRNA-containing solution. Hydrodynamic injection has led to the efficient induction of RNAi in liver as well as in kidney, lung, pancreas, and spleen and is probably due to the transient enhancement of membrane-permeability. However, in animals, side effects have been observed (Zhang et al. 2004a, b), and in man, this method is not applicable at all.

Many groups have employed different approaches for the formulation of siRNAs in carrier systems (Table 3), which deliver their siRNA "payload" into the target tissue

Table 1 Overview on studies employing siRNA-mediated gene-targeting in vivo

Location/target organ/target mechanism/aim	Targeted gene product	Reference
Proof-of-principle		
Reporter genes		
Peritoneal cavity	GFP	de Jonge et al. 2005
Developing vascular network of chicken embryo	GFP	Bollerot et al. 2006
S.c. HeLa xenograft	GFP	Bertrand et al. 2002
Liver	GFP	Lewis et al. 2002
Muscle	GFP	Golzio et al. 2005
Bronchiole epithelial cells	EGFP	Howard et al. 2006
Liver and limb grafts	DsRed2, GFP	Sato et al. 2005
Liver	Luciferase	McCaffrey et al. 2002
Brain	Luciferase	Hassani et al. 2005
S.c. melanoma xenografts/hepatic metastases	Luciferase	Takahashi et al. 2005
Endogenous genes		
Liver	Fas	Heidel et al. 2004
Pancreas	Ins2	Bradley et al. 2005a, b
Liver	mdr1a/1b	Matsui et al. 2005
Liver	APOB	Zimmermann et al. 2006
Hypothalamus	TRβ1 + 2	Guissouma et al. 2006
Vasculature	CD31, Tie2	Santel et al. 2006a, b
Cancer		
Tumor growth inhibition		
Pancreatic adenocarcinoma xenografts	CEACAM6	Duxbury et al. 2004
Fibrosarcoma xenografts	VEGF	Filleur et al. 2003
S.c. pancreatic carcinoma xenografts	bcl-2	Ocker et al. 2005
Bladder cancer xenografts	Survivin	Hou et al. 2006
Peritoneal cavity	β-catenin	Verma et al. 2003
Bladder cancer	PLK-1	Nogawa et al. 2005
S.c. prostate carcinoma xenografts	bcl-2	Yano et al. 2004
Prostate cancer xenografts	Raf-1	Pal et al. 2005
S.c. breast cancer xenografts	c-raf	Chien et al. 2005
Ovarian cancer xenografts	FAK	Halder et al. 2006
Liver tumor xenografts	PTEN, CD31	Santel et al. 2006a, b
Breast tumor xenografts	Raf-1	Leng and Mixson 2005
S.c. prostate carcinoma xenografts	VEGF	Takei et al. 2004
Orthotopic germ cell tumor xenografts (testes)	HST-1/FGF-4	Minakuchi et al. 2004
S.c. melanoma xenografts	c-myc, MDM2, VEGF	Song et al. 2005
S.c. ovarian carcinoma xenografts	HER-2	Urban-Klein et al. 2005
S.c. N2A neuroblastoma xenografts	VEGF R2	Schiffelers et al. 2004
S.c. breast cancer xenografts	RhoA	Pille et al. 2006
S.c. pancreatic carcinoma xenografts	Mutant K-ras	Zhu et al. 2006
Cervical cancer xenografts	HPV E6 + E7	Fujii et al. 2006
Melanoma xenografts	SOCS1	Yang et al. 2006
Blockage of cancer metastasis		
Metastatic breast cancer cells	CXCR4	Liang et al. 2005
Lung metastasis	Tissue factor	Amarzguioui et al. 2006
Liver metastasis	bcl-2	Yano et al. 2004
Bone-metastatic prostate cancer	EZH2	Takeshita et al. 2005
Others		
Cancer vaccine potency (antigen-presenting cells)	Bak, Bax	Kim et al. 2005
Breast cancer xenografts, induction of tumor apoptosis	HER-2	Hogrefe et al. 2006
S.c. HeLa xenografts, enhancement of cisplatin effect	Rad51	Ito et al. 2005
Vein grafts, attenuation of intimal hyperplasia	Midkine	Banno et al. 2006
Viral infections		
Inhibition of HBV replication	HBsAg	Giladi et al. 2003; Klein et al. 2003
Coxsackieviral cytopathogenicity	CVB 2A	Merl et al. 2005
Influenza virus infections	Nucleoprotein, acidic polymerase	Tompkins et al. 2004

Table 1 (continued)

Location/target organ/target mechanism/aim	Targeted gene product	Reference
Respiratory viral diseases	RSV-P, PIV-P	Bitko et al. 2005
Reduction of plasma viremia levels	ZEBOV L	Geisbert et al. 2006
Reduced serum HBV DNA	HBV, HBsAg	Morrissey et al. 2005a, b
Influenza virus infections	Influenza virus genes	Ge et al. 2004
Respiratory viral diseases	RSV-P, PIV-P	Bitko et al. 2005
Organ-specific effects		
Liver		
Fas-mediated apoptosis/acute liver failure	Caspase-8	Zender et al. 2003
Fulminant hepatitis	Fas	Song et al. 2003
Kidney		
Renal ischemia-reperfusion injury	Fas	Hamar 2004 no. 829
Glomerulonephritis	TGF-β1	Takabatake, 2005 no. 8273
Lung		
Hemorrhagic shock and sepsis (lung)	Fas	Perl et al. 2005
Acute lung injury	KC, MIP-2	Lomas-Neira et al. 2005
Functional analysis in lung ischemia-reperfusion injury	HO-1	Zhang et al. 2004a, b
Increase in lung vascular permeability	Caveolin-1	Miyawaki-Shimizu et al. 2005
Decreased formation of obstructive bronchiolitis	MIF	Fukuyama et al. 2005
CNS		
Reduction of brain-to-blood transport	Organic anion transporter 3	Hino et al. 2006
Chronic neuropathic pain/decreased hyperanalgesia	Pain-related cation channel P2X3	Dorn et al. 2004
Temporal hyperlocomoter response	Dopamine transporter	Thakker et al. 2004
Antidepressant-related behavioural response	Serotonin transporter	Thakker et al. 2005
Antinociception	Delta opioid receptor DELT	Luo et al. 2005
Modulation of pain	NMDA receptor NR2B	Tan et al. 2005
Eye		
Antiapoptosis in retinal ganglion cells	c-Jun, Bax, Apaf-1	Lingor et al. 2005
Ocular neovascularization	VEGF	Reich et al. 2003
Alterations of synaptic function (retina)	APP/APLP2	Herard et al. 2005
Others		
Induction of hypoglycemia and hypertriglyceridemia	PPARα	De Souza et al. 2006
Attenuation of morbidity and mortality in sepsis	Fas, caspase-8	Wesche-Soldato et al. 2005
Collagen-induced arthritis	TNFα	Schiffelers et al. 2005
Cure of collagen-induced arthritis	TNFα	Khoury et al. 2006
Role of V2R in water/sodium homeostasis	V2R	Hassan et al. 2005
Increased metabolic rate/decreased body weight	Agouti-related peptide	Makimura et al. 2002
Inflammation (peritoneum)	IL-12p40	Flynn et al. 2004
Sepsis after lipopolysaccharide injection	TNF-α	Sorensen et al. 2003
Reduction of apoB and total cholesterol	ApoB	Soutschek et al. 2004
Abrogation of HSF-induced cardioprotection	Heat shock factor 1	Yin et al. 2005
Hearing loss	GJBR75W	Maeda et al. 2005

and target cell, some of them already being known as DNA delivery techniques in gene therapy or antisense targeting.

Liposomal formulations

Various liposomes/cationic lipids can be considered as examples of nonviral envelopes that protect siRNAs, thus increasing serum stability, reducing renal excretion and mediating siRNA uptake into the cells through endocytosis. The comparison of neutral versus cationic liposomes also reveals that the biodistribution as well as the uptake into macrophage seems to be dependent on their charge (Landen et al. 2005; Miller et al. 1998), thus emphasizing the need for the further development and analysis of different liposomal particles. SNALPs (stable nucleic acid lipid particles) have been used for siRNA-mediated targeting of an Ebola-virus-specific gene (Geisbert et al. 2006) or ApoB. This is also the first study that describes the systemic efficacy of formulated siRNAs in a nonrodent species (Zimmermann et al. 2006).

Table 2 In vivo application of siRNAs for the induction of RNAi: modes of administration of naked or formulated siRNAs

Modes of administration	Example references
Hydrodynamic transfection	Bradley et al. 2005a, b; Duxbury et al. 2004; Giladi et al. 2003; Hamar et al. 2004; Heidel et al. 2004; Hino et al. 2006; Klein et al. 2003; Lewis et al. 2002; Liang et al. 2005; Matsui et al. 2005; Merl et al. 2005; Sato et al. 2005; Song et al. 2003; Tompkins et al. 2004; Zender et al. 2003
Intravenous (without high pressure)	Bradley et al. 2005a, b; Chien et al. 2005; Ge et al. 2004; Hassan et al. 2005; Miyawaki-Shimizu et al. 2005; Morrissey et al. 2005a, b; Schiffelers et al. 2004; Soutschek et al. 2004; Yano et al. 2004; Takeshita et al. 2005
Intraperitoneal	Filleur et al. 2003; Flynn et al. 2004; de Jonge et al. 2005; Ocker et al. 2005; Sorensen et al. 2003; Verma et al. 2003; Urban-Klein et al. 2005; Yin et al. 2005
Intramuscular	Golzio et al. 2005
Intratracheal	Lomas-Neira et al. 2005; Perl et al. 2005
Intranasal	Bitko et al. 2005; Zhang et al. 2004a, b
Subretinal	Reich et al. 2003
Intraocular	Herard et al. 2005
Intradermal	Kim et al. 2005
Subcutaneous	Yano et al. 2004
Intrathecal	Dorn et al. 2004; Luo et al. 2005
Stereotactic injection to hypothalamus	Makimura et al. 2002
Infusion into the ventricular system (brain)	Hassani et al. 2005; Thakker et al. 2004, 2005
Intrathecal infusion using mini-osmotic pump	Dorn et al. 2004
In situ perfusion/intravenous (pancreatic islet)	Bradley et al. 2005a, b
Intracardiac	Bollerot et al. 2006
Intratumoral	Bertrand et al. 2002; Ito et al. 2005; Leng and Mixson 2005; Takei et al. 2004
Intratumoral+electroporation	Takahashi et al. 2005
Renal artery and electroporation	Takabatake et al. 2005
Transurethral (bladder cancer)	Nogawa et al. 2005
Local (ear, tracheal grafts, liver, optic nerve stump)	Fukuyama et al. 2005; Lingor et al. 2005; Maeda et al. 2005; McCaffrey et al. 2002
Local injection and electroporation (mouse joint)	Schiffelers et al. 2005

Nanoparticles

Another strategy allowing the protection and cellular delivery of siRNAs is the formation of nanoparticles with positively charged macromolecules. Based on electrostatic interactions, complexes are formed with atelocollagen (Banno et al. 2006; Minakuchi et al. 2004; Takei et al. 2004; Takeshita et al. 2005), chitosan (Pille et al. 2006), or polyethylenimine (PEI).

Polyethylenimine

PEIs are synthetic linear or branched polymers available in a wide range of molecular weights (Godbey et al. 1999; Tang and Szoka 1997). Due to the presence of a protonable amino group in every third position, leading to a high cationic charge density at physiological pH, PEIs are able to form noncovalent complexes with DNA as well as small RNA molecules like siRNAs (Urban-Klein et al. 2005) or ribozymes (Aigner et al. 2002). This siRNA complexation results in the complete protection against degradation in the presence of serum or RNase A and allows the efficient cellular uptake of the PEI/siRNA complexes through endocytosis. For any siRNA formulation, the release from endosomes is critical for siRNA delivery. In the case of PEI, the so-called "proton-sponge effect" postulates improved transgene delivery by cationic complexes, which contain H^+-buffering polyamines, based on enhanced endosomal Cl^- accumulation and subsequent osmotic swelling and lysis (Behr 1997; Boussif et al. 1995). This effect may also apply for PEI-mediated siRNA delivery into the cytoplasm. Additionally, to further enhance the efficacy of PEI complexes through membrane-destabilization, the conjugation of melittin analogs to PEI has been described (Boeckle et al. 2005, 2006; Shir et al. 2006). It should be noted, however, that by far not all PEIs are suitable for the transport of nucleic acids like siRNAs (Hassani et al. 2005; Werth et al. 2006).

Table 3 In vivo application of siRNAs for the induction of RNAi: formulations of siRNAs

Formulation	Example references
Unmodified siRNAs, naked	Bradley et al. 2005a, b; Duxbury et al. 2004; Giladi et al. 2003; Heidel et al. 2004; Hino et al. 2006; Klein et al. 2003; Lewis et al. 2002; Liang et al. 2005; Matsui et al. 2005; Merl et al. 2005; Sato et al. 2005; Song et al. 2003; Tompkins et al. 2004; Zender et al. 2003
Chemically modified, naked	Braasch et al. 2004; Elmen et al. 2005; Soutschek et al. 2004
Chemically modified+lipid encapsulation	Morrissey et al. 2005a, b
Coupling to cholesterol	Soutschek et al. 2004
Liposomes	Flynn et al. 2004; Fukuyama et al. 2005; Hassan et al. 2005; Maeda et al. 2005; Miyawaki-Shimizu et al. 2005; Nogawa et al. 2005; Sioud and Sorensen 2003; Sorensen et al. 2003; Verma et al. 2003; Yano et al. 2004
Liposome RPR209120/DOPE	Khoury et al. 2006
Cationic cardiolipin lipsomes	Pal et al. 2005
Cationic cardiolipin analogue	Chien et al. 2005
Cationic lipid (i-Fect)	Luo et al. 2005
Cytofectin GSV	Bertrand et al. 2002
JetSI (+ DOPE)	Hassani et al. 2005
Dioleoylphosphatidylcholine (DOPC)	Landen et al. 2005; Landen et al. 2006
N-[1-(2,3-Dioleoyloxy)]-*N*,*N*,*N*-trimethylammonium propane (DOTAP)	Hassan et al. 2005
Stable nucleic acid lipid particles (SNALP)	Geisbert et al. 2006; Morrissey et al. 2005a, b; Zimmermann et al. 2006
Hybrid siRNA in TfRscFv (anti-transferrin receptor single-chain antibody fragment)-liposome	Hogrefe et al. 2006; Pirollo et al. 2006
Mixture of cationic and fusogenic lipids	Santel et al. 2006a, b
Histidine-lysine complex	Leng and Mixson 2005
Inactivated HVJ (hemagglutinating virus of Japan) suspension	Ito et al. 2005
Protamin-antibody fusion protein	Song et al. 2005
TransIT-TKO (polyamine)	Bitko et al. 2005
Virosomes + cationic lipds	de Jonge et al. 2005
Chitosan/chitosan-coated polyisohexylcyanoacrylate	Maksimenko et al. 2005; Pille et al. 2006
Single-walled carbon nanotubes (SWNTs)	Yang et al. 2006
Atelocollagen	Minakuchi et al. 2004; Takei et al. 2004; Takeshita et al. 2005
Polyamines	Yin et al. 2005
Polyethylenimine (PEI) complexation	Ge et al. 2004; Geisbert et al. 2006; Grzelinski et al. 2006; Urban-Klein et al. 2005
In vivo jetPEI	Hassani et al. 2005
PEI-based nanoplexes (RGD-PEG-PEI)	Schiffelers et al. 2004

In vivo studies in xenografted mice have demonstrated that the i.p. injection of PEI-complexed siRNAs, but not of naked siRNAs, resulted in the delivery of intact siRNA molecules into the subcutaneous tumors, leading to antitumorigenic effects when targeting for example the receptor HER-2 in s.c. ovarian carcinoma xenografts (Urban-Klein et al. 2005) or the growth factors pleiotrophin (PTN, s.c. glioblastoma) (Grzelinski et al. 2006) or VEGF (s.c. prostate carcinoma; Hobel et al., unpublished data). Likewise, intrathecal delivery of PEI-complexed specific siRNAs led to successful PTN targeting and tumor inhibition in an ortotopic glioblastoma mouse model (Grzelinski et al. 2006), or to the knockdown of the *N*-methyl-D-aspartate (NMDA) receptor subunit protein NR2B in a rat model (Tan et al. 2005). PEI complexation of siRNAs has also been employed in antiviral therapy studies (Ge et al. 2004; Geisbert et al. 2006). Furthermore, PEI/siRNA complexes are a good example for the introduction of chemical modifications to enhance tissue specificity and in vivo biocompatibility, to reduce immunogenicity and toxicity and to increase siRNA delivery through improved endocytosis and intracellular siRNA release. This includes full deacetylation of PEI (Thomas et al. 2005), the introduction of novel low molecular weight PEIs (Werth et al. 2006), and the coupling of PEI to other macromolecules like polyethylene glycol (PEG), either alone (Mao et al. 2006) or in combination with a ligand for tissue-specific targeting (RGD peptide for the recognition of tumor vasculature; Schiffelers et al. 2004).

Chemical siRNA modifications

Alternatively, the goals of increased systemic siRNA stability and serum half-life have also been achieved

through extensive chemical modifications of the siRNA strands including the introduction of phosphorothioate (Braasch et al. 2003, 2004), 4′ thioribose (Dande et al. 2006) or methylene linkages between positions 2′ and 4′ (locked nucleic acids, LNAs; Braasch et al. 2003; Elmen et al. 2005), or multiple 2′ modifications (Amarzguioui et al. 2003; Harborth et al. 2003; Holen et al. 2002, 2003). Additionally, chemical conjugation of siRNAs, e.g., to cholesterol (Soutschek et al. 2004) or to a protamin-antibody fusion protein (Song et al. 2005) led to enhanced efficacy and specificity in tissue uptake. For other strategies, refer to Table 3.

Conclusion and outlook

RNAi has already proven to be a very efficient and specific method for the knockdown of physiologically or pathologically relevant genes of interest. Notably, this also applies to so-called "nondruggable" genes, thus opening new therapeutic avenues. Still, the therapeutic applicability and success of siRNAs will largely depend on their efficient and safe in vivo delivery avoiding unwanted side effects. Reflecting the high relevance of RNAi, many studies have been published or are ongoing, which will finally allow to identify optimal strategies based on already promising results. This also refers to the first clinical trials, which are completed or ongoing. Most likely, one major advantage of formulated, modified, or unmodified siRNAs for gene knockdown will be their "double specificity", i.e., the combination of a high target gene specificity through optimal siRNA sequences and an at least somewhat increased target organ specificity through sophisticated delivery vehicles like liganded nanocarriers.

Acknowledgments The author's work reported herein was supported by grants from the Deutsche Forschungsgemeinschaft (AI 24/5-1 and Forschergruppe Nanohale, AI 24/6-1) and by the Deutsche Krebshilfe.

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