Selected Strategies for the Delivery of siRNA *In Vitro* and *In Vivo*

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Abstract RNA-based therapeutic strategies are considered as a highly promising alternative to conventional drug development. Among the different classes of oligonucleotide-derived prospective drugs, small interfering RNAs (siRNAs) are of particular interest. However, cellular uptake and subsequent intracellular trafficking to the effector complex (RNA-induced silencing complex; RISC) represent major technical hurdles for the efficacy of these macromolecular drugs. Thus, the development of appropriate delivery systems is an essential requirement to turn these molecules into medicine. In this review, we will focus on two particular auspicious aspects in this context, the phosphorothioate-stimulated uptake of naked siRNA and the use of cell-penetrating peptides as shuttles for a controlled cellular

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uptake. Moreover, we will present some of the most promising recent approaches for siRNA delivery *in vivo*, which may help to pave the road to drugs of the future.

Keywords Argonaute 2 · Caveosomal endocytosis pathway · Cell-penetrating peptides · Cellular uptake · Clinical trials · Delivery · Endocytosis · Endoplasmic reticulum · Endosomal escape · Extracellular RNA · Golgi apparatus · Ilimaquinone · Microinjection · Nanoparticles · Non-covalent · Nonviral delivery systems · Oligonucleotide-based drugs · Phosphorothioate-stimulated uptake · Polycations · Protein transduction domains · RNAi · Signal peptides · siRNAs · siRNA-peptide conjugate

Abbreviations

Ago2	Argonaute 2
AMD	age-related macular degeneration
CPP	cell-penetrating peptide
dsRNA	double-stranded RNA
ER	endoplasmic reticulum
exNA	extracellular nucleic acids
exRNA	extracellular RNA
GFP	green fluorescent protein
gp41	glycoprotein 41
HA	hemagglutinin
HIV	human immunodeficiency virus
IL	interleukin
IFN	interferon
JEV	Japanese encephalitis virus
LF2000	Lipofectamine™ 2000
MEND	multifunctional envelope-type nano device
miRNA	microRNA
NLS	nuclear localization sequence
PCI	photochemical internalization
PCR	polymerase chain reaction
PEG	polyethylene glycol
PEI	polyethyleneimine
PLL	Poly-L-Lysine
PS-ON	phosphorothioate-modified oligonucleotides
PTD	protein transduction domain
PTGS	posttranscriptional gene silencing
R8/R9	oligoarginines
RBD	RNA-binding domain
RISC	RNA-induced silencing complex
RNAi	RNA interference

RVG	rabies virus glycoprotein
siRNA	small interfering RNA
shRNA	short hairpin RNA
ssDNA	single-stranded DNA
STR-R8	stearyl-R8
TLR	Toll-like receptor
TNF	tumor necrosis factor
TP10	transportan 10
VEGF	vascular endothelial growth factor

1 Introduction

In recent years, RNA interference (RNAi) has gained a lot of interest as a tool for functional genomics and probably equally important as a promising therapeutic approach for the treatment of various diseases (Bumcrot et al. 2006; Castanotto and Rossi 2009; de Fougerolles et al. 2007). However, despite these bright prospects, a major impediment to the development of siRNA-based strategies for treatment and prevention of diseases is the relatively inefficient means to effectively deliver these macromolecules into the desired target cells or tissues. Although viral vectors have been widely used to transfer genetic material into cells (Kootstra and Verma 2003; Verma and Weitzman 2005), they bear an inherent risk for the patient to encounter severe immunological responses or even develop cancer (Check 2005; Hacein-Bey-Abina et al. 2003; Raper et al. 2002, 2003). As a result of these problems, much attention has been paid in recent years to the delivery of naked RNA into a target organ such as the lung or eye and the development of nonviral delivery systems. Accumulating experimental evidence suggests that naked oligonucleotide-based drugs including siRNA may be taken up by specific cell types in cell culture and *in vivo* where they exert suppression of their target gene expression. Those findings warrant more detailed analyses of this mode of delivery. The conception of nonviral delivery includes an assortment of fairly unrelated approaches yielding various degrees of enhanced cellular uptake of nucleic acids. Currently, liposomes and cationic polymers are used as a standard tool to transfect cells *in vitro*. However, these procedures are characterized by a significant lack of efficiency accompanied by a high level of toxicity rendering them mostly inadequate for *in vivo* applications. In this context, cell-penetrating peptides (CPPs) represent an interesting alternative as they generally are less toxic than liposomes or cationic polymers. Moreover, they are commonly better suited to transfer cargo into different cell types such as nonadherent cells and primary cells, which are hard to transfect using commercially available standard protocols. The most advanced approaches in the field are complex carrier systems combining vantages of assorted strategies to generate nanoparticles with better defined properties aimed toward enhanced uptake as well as intracellular trafficking in combination with cell-specific functionalities.

In this chapter, we will report about particular aspects of siRNA delivery *in vitro* and *in vivo*, with special emphasis on naked and CPP-mediated cellular delivery of these macromolecules. Additionally, we will present and briefly discuss selected recent examples of promising siRNA delivery approaches *in vivo*.

2 Mechanism of RNA Interference

RNAi is a highly evolutionally conserved and specific process of posttranscriptional gene silencing (PTGS) by which double-stranded RNA (dsRNA), when introduced into a cell, causes sequence-specific degradation of homologous mRNA sequences (Fire et al. 1998; Rana 2007). Mechanistically, the process can be divided into two steps. In the initiator step, dsRNA is cleaved by Dicer, a member of the RNase III family, into 21–25 nt long siRNA fragments (Bernstein et al. 2001). In a consecutive step, these fragments are transferred to RISC where one of the strands, the so called guide strand, serves as a molecular template to recognize homologous mRNA that is cleaved by Argonaute 2 (Ago2) (Hammond et al. 2001; Hutvagner and Simard 2008), a protein component of RISC (Fig. 1a). Ago 2 is a protein of ca. 100 kDa and contains four defined domains, N-terminal, PAZ, Mid, and PIWI (Fig. 1b). Current structural knowledge is mainly derived from crystal structures of archaebacterial proteins (Jinek and Doudna 2009). In the binary complex of *Thermus thermophilus* Ago and guide strand, the 3′ end of single-stranded RNA is bound to the PAZ domain and the 5′-phosphate is anchored within a binding pocket in the Mid domain (Wang et al. 2008a). Enzymatic activity is mediated by the C-terminal PIWI domain, which resembles the catalytic triad of three carboxylate groups of RNase H (Song et al. 2004). These amino acid residues coordinate the essential metal and activate water molecules for nucleolytic attack (Wang et al. 2008b). Once the guide strand is bound to RISC, this complex can undergo many rounds of mRNA binding and cleavage (Haley and Zamore 2004). To circumvent application of long double-stranded RNAs, which inevitably trigger an interferon response, it is sufficient to extracellularly supply 21 nt long dsRNAs (Elbashir et al. 2001a, b). Alternatively, siRNAs can be expressed endogenously using DNA vectors that code for short hairpin (sh) RNAs (Leung and Whittaker 2005; Paddison et al. 2002; Yu et al. 2002). These shRNAs are then cleaved by Dicer to siRNAs. Short hairpin RNA constructs have advantages over siRNA because the effects of these constructs can lead to a more stable and long-term result (Rao et al. 2009). However, evidently they can interfere with the endogenous microRNA pathway, thus causing severe side effects (Grimm et al. 2006; Snøve and Rossi 2006).

3 Naked Delivery of siRNA *In Vitro*

3.1 Cellular Uptake of Naked Nucleic Acids

In tissue cell culture of mammalian cells and *in vivo,* nucleic acids including single-stranded RNA and double-stranded DNA can be isolated from the extracellular

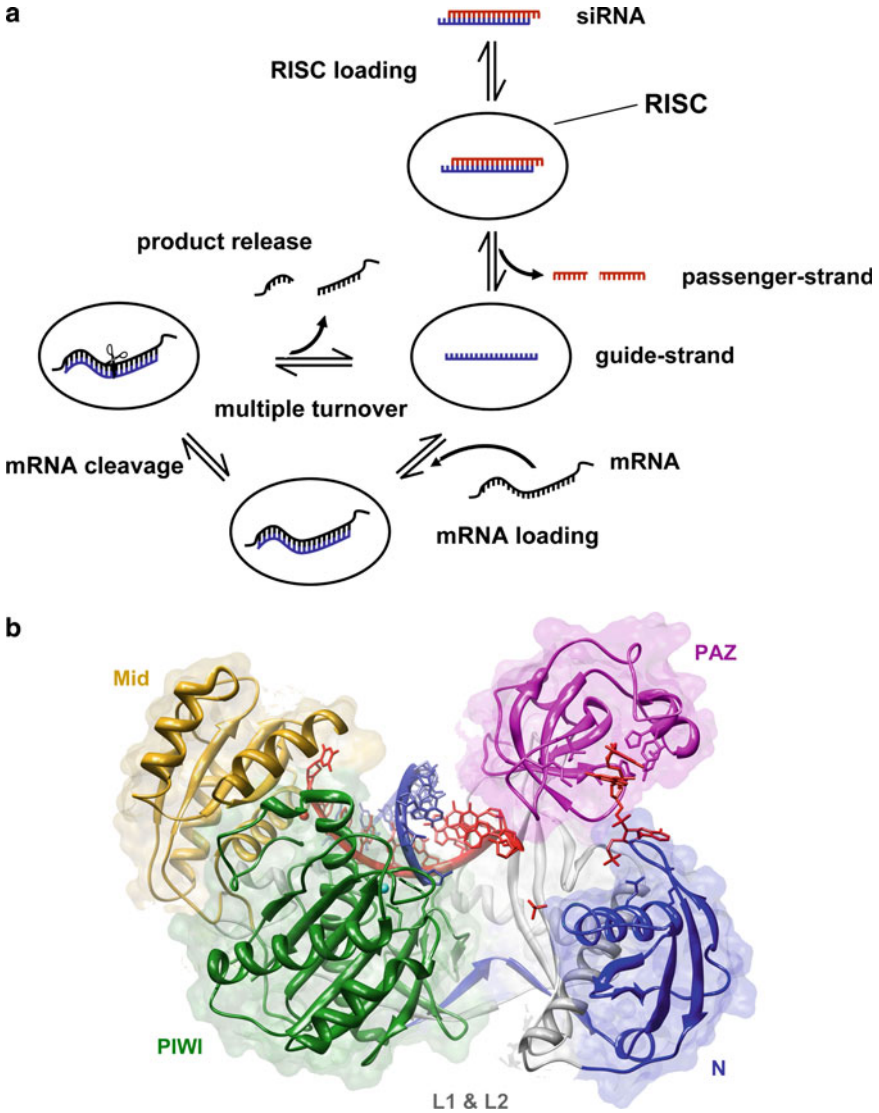

Fig. 1 Mechanistic principles of RNAi and structure of *Thermus thermophilus* Argonaute protein. (**a**) Details are given in the text. (**b**) X-ray structure of the ternary complex of *T. thermophilus* Argonaute bound to a 21-nucleotide guide DNA and a 20-nucleotide target RNA (pdb file: 3F73). The protein contains four defined domains, N-terminal, PAZ, Mid, and PIWI, which are color coded *blue*, *magenta*, *gold*, and *green*, respectively. Additionally, two linker regions are shown in *grey*. Guide DNA is shown in *red* and target RNA in *blue*. The coordinated Mg^{2+} within the active site (amino acids: D478, D546 and D660) is shown in *cyan*

environment. In higher mammals, this includes different body fluids such as blood, serum, and urine.

Extracellular nucleic acids (exNA) were shown to be released from normal cells and also from tumor cells, which means that one could hypothesize on tumor

cell-specific DNA and RNA in blood. In fact, non-invasive methods of early tumor diagnostics are increasingly based on the analysis of circulating DNA. Even though RNA is thought to be highly instable in blood, when compared to DNA, it was found that extracellular RNA (exRNA) circulates in humans at amounts and integrity that allow isolation, reverse transcription, and quantification by polymerase chain reaction (PCR). The existence of amplifiable tumor-specific RNA in the plasma of melanoma patients (Kopreski et al. 1999) and breast cancer patients (Chen et al. 2000) was discovered despite the fact that the activity of blood RNases is increased in patients with malignancies (Reddi and Holland 1976). Possible sources of cell-free DNA and RNA are apoptotic bodies resulting from somatic cell death [summarized by Garcia-Olmo et al. (2000)] and nutrition (Doerfler et al. 2001). Another endogenous source of cell-free nucleic acids to which cells and organs of mammals are exposed is blood. The circulating blood system contains significant concentrations of cell-free DNA and RNA (Anker and Stroun 2002; Ng et al. 2002). Uptake of exNA by individual cells seems to be possible and may be of biological relevance (Garcia-Olmo et al. 2000).Thus, it is warranted to speculate on a biological role of exNA, which implies their recognition by cell surface molecules and it might even include their cellular uptake.

Little is known about the internalization of cell-free nucleic acids by cells. However, over the past years, Doerfler and colleagues have shown that mice fed with naked DNA may incorporate this DNA in specific subsets of mononuclear cell populations in the bloodstream (Doerfler 1995; Schubbert et al. 1994, 1997). Surprisingly, such DNA is not completely degraded or metabolized, as fragments of 200–400 bp in length of exogenously introduced DNA could be unequivocally detected (Schubbert et al. 1994). For single-stranded DNA (ssDNA), much more is known about the pathways of their cellular uptake (de Diesbach et al. 2000; Laktionov et al. 1999).

Conversely, almost nothing is known about the conceivable cellular uptake of short-chain RNA. By co-incubating a mammalian cell culture set-up with various classes of nucleic acids and short double-stranded DNA competition of uptake was measured quantitatively (Lehmann and Sczakiel 2005). Firstly, these studies suggest that higher mammalian cells do take up nucleic acids measurably. Secondly, cells distinguish between DNA and RNA as well as their characteristics regarding chain length, global structure, and single- versus double-stranded forms. Recent studies have shown that simple co-incubation of certain human cell types with naked siRNA at micromolar and sub-micromolar concentrations leads to their spontaneous cellular uptake within a few hours (Overhoff et al. 2004). Under certain conditions, this is related to siRNA-specific target suppression indicating that critical amounts of siRNA are internalized by the exposed cells in a biologically functional fashion.

3.2 The Phosphorothioate-Stimulated Cellular Delivery of siRNA

Fully phosphorothioate-modified oligonucleotides (PS-ON) enhance the cellular uptake of naked siRNA *in trans* by various mammalian cell types (Overhoff and

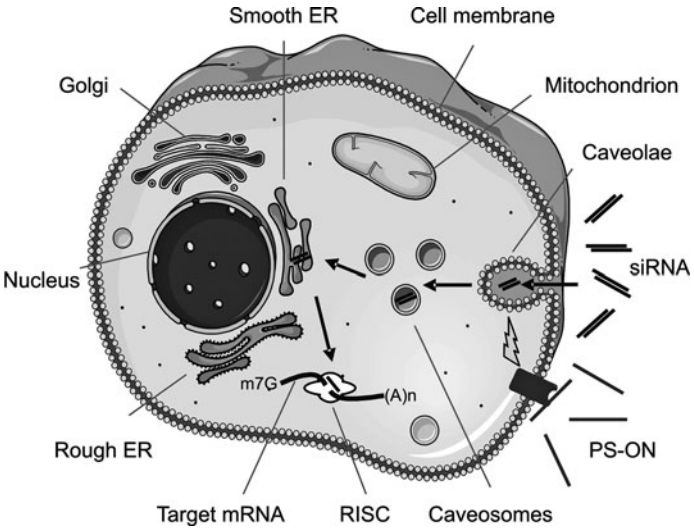

Fig. 2 Model of the PS-stimulated cellular uptake of naked siRNA. Upon stimulation of a yet unknown cell surface molecule by PS-ON, siRNA is taken up via caveolae into caveosomes and transported to the perinuclearly located smooth ER. Since RNAi is thought to be a cytoplasmic process, internalized siRNA needs to be released from the perinuclear compartments to be able to interact with the RISC machinery. This figure was produced using Servier Medical Art

Sczakiel 2005). This means that siRNA and PS-ON are neither complexed nor is there any measurable co-uptake of the PS-ON by the cells. A schematic depiction of the underlying model is shown in Fig. 2. Essentially, one hypothesizes that PS-ON recognize an unknown cell surface molecule that induces a kind of cellular stimulation cascade giving rise to increased apparent uptake of coincubated extracellular naked siRNA. This process is critically dependent on a number of characteristics including the chemistry of the stimulating nucleic acid, its chain length, and its concentration (Fig. 3). More specifically, one hypothesizes that two characteristics of the stimulating nucleic acid are important for its activity, the phosphorothioate internucleotide phosphate and a certain structure of the sugar.

The cellular uptake pathway of the cargo, i.e., siRNA, seems to make use of the caveosomal endocytosis pathway, which is supported by experimental constraints using pathway-specific activators or inhibitors and by fluorescence microscopy (Overhoff and Sczakiel 2005). Present experimental data suggest that siRNA migrates via caveosomes to the smooth endoplasmic reticulum (ER) where it is trapped and only small amounts of siRNA seem to be released, thereby giving rise to suppression of target gene expression (Detzer et al. 2009).

3.3 The siRNA-Peptide Conjugate Approach

A bulk of observations concerning the PS-stimulated delivery of siRNA indicates that siRNA needs to be released from intracellular compartments or vesicles in

a

Modification	Stimulated uptake
DNA	–
PS-DNA	+++
RNA	–
PS-RNA	+
2'OMe	–

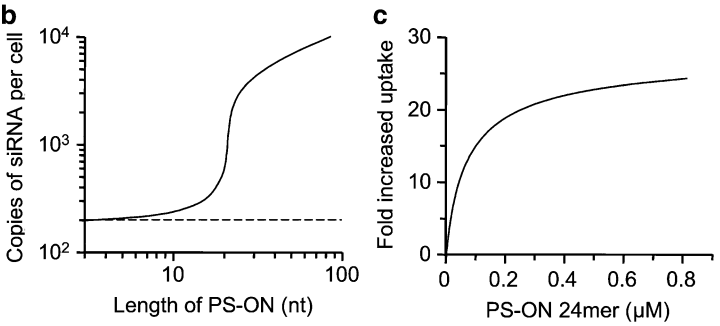

Fig. 3 Key characteristics of the PS-stimulated uptake of siRNA by mammalian cells. (**a**) Only a fully PS-modified DNA backbone is active. (**b**) There is a sharp dependency of the amount of internalized siRNA on the length of the PS-ON. The dotted line represents the detection limit of the used nuclease protection assay. (**c**) The PS-stimulated uptake of siRNA reaches a plateau above a concentration of PS-modified 24 mer of 500 nM

order to become biologically active as a suppressor of target RNA via RNAi. This includes microscopic studies in the use of fluorescently labeled siRNA after its PS-stimulated delivery and the discrepancy between large amounts of intracellular siRNA and surprisingly low effectiveness, i.e., target suppression (Overhoff and Sczakiel 2005). This view is compatible with the finding that ilimaquinone, a substance that transiently disrupts the Golgi apparatus and at higher concentrations also the ER (Takizawa et al. 1993; Wang et al. 1997), is related to increased target suppression (Fig. 4). In particular, the concentration-dependent disruption of these two cellular compartments strongly indicates that capturing of siRNA mainly occurs in the smooth ER (Detzer et al. 2008).

In case of intracellular sorting of proteins, signal peptides serve as promoters of intracellular transport, a process that may include transmembrane translocation steps. Similar transport signals on the level of nucleic acids are not known; however, one might think of a covalent attachment of signal peptides derived from intracellular protein sorting to siRNA in order to facilitate the intracellular release and, hence, to enhance the biological activity of siRNA (Fig. 5). For this reason, the signal peptide TQIENLKEKG, which is thought to facilitate translocation of the catalytic domains of several bacterial protein toxins from transport vesicles into the cytoplasm, was used as a tool to be covalently conjugated to siRNA, thereby bypassing its presumed capturing in the ER (Detzer et al. 2009). This study showed increased RNAi, i.e., siRNA-mediated target suppression.

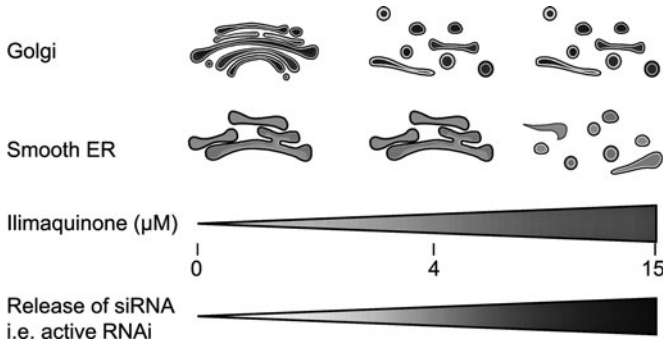

Fig. 4 The progressive disruption of the ER and the Golgi apparatus by ilimaquinone is related to increased intracellular release and biological activity of siRNA. This figure was produced using Servier Medical Art

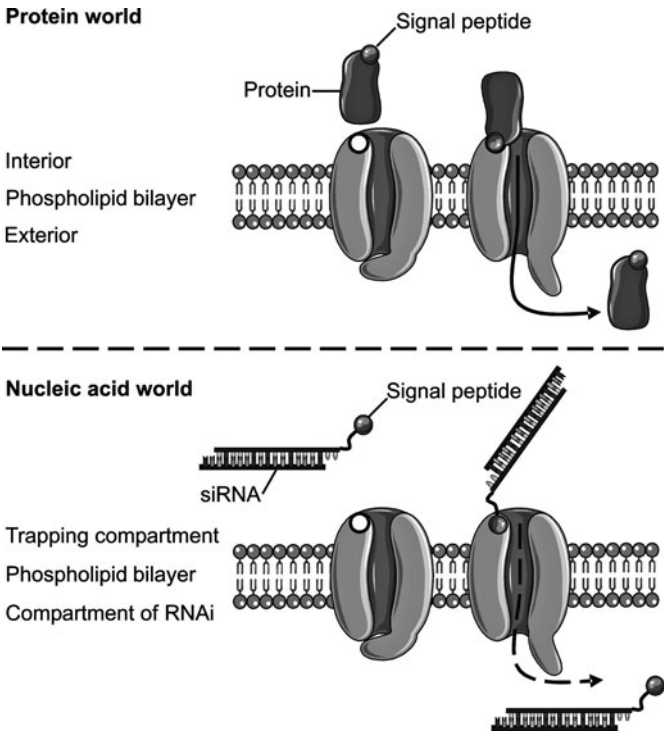

Fig. 5 Signal peptides steer a transmembrane translocation step of polypeptides (*upper panel*). The concept of covalently attaching signal peptides to siRNA in order to enhance its release from capturing in intracellular compartments is depicted in the lower panel. This figure was produced using Servier Medical Art

We hypothesize that this is due to increased intracellular release and bioavailability of this siRNA–peptide conjugate.

3.4 Intracellular Release of siRNA: A Major Hurdle

In past years, cellular delivery of siRNA was regarded as one of the major technical problems for the successful application of oligonucleotide-based drugs in therapeutic settings including antisense oligonucleotides and siRNA. Progressively, it became obvious that physical delivery to mammalian cells could be substantially improved with regard to the percentage of transfected cells as well as the total amounts of internalized oligonucleotide-based drugs. However, in many cases, improved delivery was not reflected by the extent of target suppression. For example, limited biological activity of siRNA was observed in the use of a number of delivery peptides as well as in the use of the PS-stimulated pathway. Hence, it seems to be reasonable to assume that a block of "functional delivery" exists intracellularly. Further, those findings suggest that intracellular transport and intracellular release are crucial for the effectiveness of a variety of delivery modes of siRNA (Fig. 6). A comparison of the mode of delivery of siRNA, the amount of intracellular

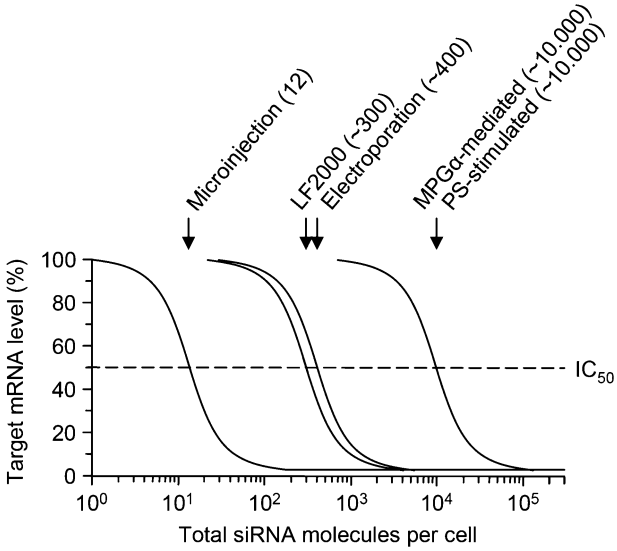

Fig. 6 Schematic depiction of the dose–response relationship of siRNA delivered by different delivery modes as indicated at the top panel. Target gene expression is indicated by the Y-axis, where half maximal inhibition is indicated by the IC_{50} value (dotted line). The concentration of the drug, i.e., the siRNA is shown here as siRNA molecules per cell rather than using the usual dimensions. This figure shows, for example, that MPGα-mediated delivery gives rise to approximately 10,000 copies per cell at half maximal target suppression. Conversely, LF2000 or electroporation require the amount of approximately 300 or 400 siRNA molecules per cell in order to achieve a similar extent of target suppression

siRNA molecules, and the extent of siRNA-mediated target suppression indicates that intracellular transport and/or release is a major obstacle to the application of siRNA as, for example, microinjection of less than 20 copies of siRNA per cell leads to a similar extent of target inhibition as the delivery of several hundred copies and even up to more than 10,000 copies of siRNA by using the delivery technologies indicated in Fig. 6 (Laufer and Restle 2008; Mescalchin et al. 2007).

As a consequence, this suggests exploring new strategies for steered intracellular trafficking and biological activation of siRNA via subcellular release in order to increase its biological effectiveness. One of such approaches might be the use of siRNA–peptide conjugates as described above.

4 CPP-Mediated siRNA Delivery

4.1 Cell-Penetrating Peptides

The idea of using peptides as carriers for a controlled cellular delivery of siRNA represents a promising concept to bypass the problem of poor bioavailability and clinical efficacy of these nucleic acids. Twenty years ago, it was discovered that the HIV-1 transactivating protein Tat is taken up by mammalian cells (Frankel and Pabo 1988; Green and Loewenstein 1988), and a few years later, the Antennapedia homeodomain of *Drosophila melanogaster* was shown to act similarly (Joliot et al. 1991). Later on, it could be shown that peptides derived from Tat and Antennapedia, i.e., $Tat^{48–60}$ and penetratin, as well as other proteins are capable of transporting macromolecular cargo molecules into cells (Allinquant et al. 1995; Fawell et al. 1994; Schwarze et al. 1999). Based on such promising results, a rapidly expanding field focusing on the so-called cell-penetrating peptides (CPPs), also referred to as protein transduction domains (PTDs), began to develop. Since the first reports about Tat, a large number of naturally occurring as well as engineered CPPs have been described (Foged and Nielsen 2008; Heitz et al. 2009; Langel 2006; Lindgren et al. 2000; Morris et al. 2008; Patel et al. 2007; Veldhoen et al. 2008; Zorko and Langel 2005). In addition to Tat and penetratin, well-known examples include transportan, a chimeric peptide composed of galanin and mastoparan (Pooga et al. 1998), and oligoarginines (Futaki et al. 2001; Futaki 2006). Generally, CPPs are short polycationic sequences of less than 30 amino acids that are able to translocate different cargoes (e.g., nucleic acids, peptides, and even entire proteins) into cells. The only common characteristic of these peptides appears to be that they are net positively charged at physiological pH. Table 1 gives an overview of selected “classical” CPPs. In the majority of cases, the cargo is covalently attached to the CPP, which can be achieved by expression as a fusion construct or by chemical coupling [for a review see, Zatsepin et al. (2005)]. In particular cases, cargo and carrier bind each other non-covalently through mainly ionic interactions (Crombez et al. 2008; Deshayes et al. 2008; Laufer and Restle 2008; Morris et al. 2008). Depending on the nature of both binding partners, the assembly of nanoparticles may occur.

Table 1 Selected examples of "classical" CPPs

Peptide	Sequence	References
$Tat^{48–60}$	GRKKRRQRRRPPQ	Vives et al. (1997)
Penetratin ($Antp^{43–58}$)	RQIKIWFQNRRMKWKK	Derossi et al. (1994)
Transportan	GWTLNSAGYLLGKINLKALAALAKKIL	Pooga et al. (1998)
TP10	AGYLLGKINLKALAALAKKIL	Soomets et al. (2000)
Oligoarginine (R8)	RRRRRRRR	Futaki et al. (2001)
MAP	KLALKLALKALKAALKLA	Oehlke et al. (1998)
MPG	GALFLGFLGAAGSTMGAWSQPKKKRKV	Morris et al. (1997)
MPGα	GALFLAFLAAALSLMGLWSQPKKKRKV	Deshayes et al. (2004)

Despite the widespread interest in using peptides as carriers, the mechanisms underlying the cellular translocation of CPPs are still not completely understood. Early work relied upon fluorescence imaging or flow cytometry analysis of chemically fixed cells to examine the intracellular localization of fluorescently labeled peptides in the absence or presence of cargo. From these experiments, it was concluded that CPPs penetrate cell membranes by an energy-independent mechanism as they appeared to be internalized very rapidly within minutes even at 4°C (Derossi et al. 1996; Futaki et al. 2001; Morris et al. 1997; Schwarze and Dowdy 2000; Vives et al. 1997). Despite some reports that certain fixation procedures may cause artifacts leading to an overestimation of cellular uptake rates (Lundberg and Johansson 2001; Lundberg and Johansson 2002; Pichon et al. 1999), the whole extent of this problem was not commonly recognized until a detailed side by side comparison was performed by Richard et al. (2003). The authors demonstrated that the distribution pattern of $Tat^{48–60}$ and R9 as well as their conjugates was completely different in living versus fixed cells and that an important source of misinterpretation is caused by difficulties to distinguish cell surface-associated CPPs from internalized CPPs. Above all, it could be shown that endocytotic transport is significantly involved in the internalization process (Richard et al. 2003). Prior to endocytosis, CPPs interact electrostatically with the extracellular matrix of the cell surface mostly through binding to negatively charged glycosaminoglycans, i.e., heparan sulfate proteoglycans (Console et al. 2003; Tyagi et al. 2001). Based on the findings described above, many groups reexamined their data, and in most cases, endocytosis was suggested as the main route of internalization (Fig. 7), although substantial difficulties are encountered in identifying the exact-pathway of CPP uptake ((Veldhoen et al. 2006; Veldhoen et al. 2008) and references therein). As a consequence, retention in endosomes is one of the major rate-limiting steps for cellular delivery of macromolecules via cationic lipids, polyplexes, and especially CPPs. Endosomal release can be increased by endosome-disrupting substances (i.e., chloroquine, calcium, or sucrose), by coadministration of photosensitive substances [so-called photochemical internalization (PCI) (Berg et al. 2007; Bøe et al. 2007; Bonsted et al. 2008; Folini et al. 2007; Oliveira et al. 2007a, 2008)] or viral fusogenic peptides (Epand 2003; Futaki et al. 2005; Haque et al. 2005; Kwon et al. 2008; Michiue et al. 2005; Oliveira et al. 2007b; Plank et al. 1998; Tu and Kim 2008).

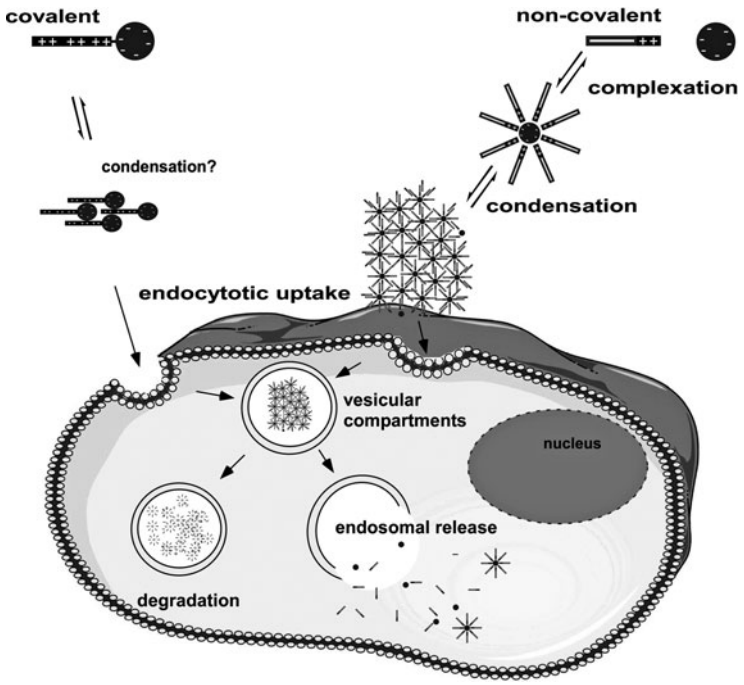

Fig. 7 Principles of peptide-based nucleic acid delivery systems. Interaction of CPP and cargo is either achieved by covalent attachment or by non-covalent complexation through mainly ionic interactions. In case of non-covalent complex formation, a further assembly of cargo/carrier complexes occurs, leading to the formation of large nan-oparticles. In case of covalently joined molecules, a similar scenario is less likely, yet cannot be excluded. Prior to the translocation process, the particles attach to the cell surface by ionic interactions of positively charged CPP residues with negatively charged membrane components. Subsequently, complexes are taken up via an endocytotic pathway. Although less likely, direct penetration cannot be excluded and may occur simultaneously. Once inside the cell, the cargo has to escape from vesicular compartments; otherwise, it eventually gets degraded in the lysosome. This figure was produced using Servier Medical Art

In summary, the precise mechanism of internalization remains elusive and strongly depends on the properties of both CPP and cargo as well as on the transfection conditions and the cell lines used (De Coupade et al. 2005; Edenhofer 2008; El-Andaloussi et al. 2007; Fittipaldi et al. 2003; Maiolo et al. 2005; Mano et al. 2005; Richard et al. 2003, 2005; Rothbard et al. 2004; Wadia et al. 2004).

4.2 Selected Examples of CPP-Mediated siRNA Delivery

As described above, siRNAs represent a valuable tool to inhibit the expression of a target gene in a sequence-specific manner. In the following section, selected

examples of CPP-mediated siRNA delivery will be presented, which are summarized in Table 2.

Only a few studies describe the covalent attachment of nucleic acid cargo and peptide carrier (confer Table 2). In one approach, simple mixing of siRNA targeted against GFP or CDK9 and Tat peptide did not generate any measurable RNAi effect, whereas cross-linked siRNA-Tat$^{47–57}$ led to a significant downregulation of the target proteins (Chiu et al. 2004). Tat$^{47–57}$-mediated transfection of siRNA resulted in a perinuclear localization of the nucleic acid. In contrast, fluorescently labeled Tat$^{47–57}$ without cargo was mainly found in the nucleolus, suggesting that interactions with RISC influence subcellular localization. In another approach, significant uptake of siRNAs targeted against luciferase or GFP could be observed after coupling the 5′-end of the sense strand via a disulfide bond to penetratin or transportan (Muratovska and Eccles 2004). Davidson et al. (2004) similarly used disulfide coupling to conjugate siRNAs directed against different endogenous proteins, e.g., several caspases, to penetratin. After transfection into neuronal cells, a remarkably strong downregulation of the target proteins in these hard to transfect primary cells was observed, implying that peptide-mediated siRNA delivery was far more effective in comparison to LF2000. Concerning the *in vivo* delivery of Tat$^{48–60}$- or penetratin-siRNA conjugates, Moschos et al. showed that intratracheal administration did not lead to any intensification of the knockdown of the target gene p38 mitogen-activated protein kinase in mouse lungs in comparison to unmodified nonformulated siRNA (Moschos et al. 2007). Strikingly, it was found that the peptides alone triggered a detectable decrease in target gene expression and that the penetratin-conjugate induced elevated levels of the immune markers IFN-α, TNF-α, and IL-12p40 in lung tissue.

Although CPPs are able to deliver a wide variety of cargo into cells, technical difficulties arise especially from the syntheses of conjugates consisting of short cationic or hydrophobic peptides and highly negatively charged siRNAs. Moreover, Dowdy and his group (Meade and Dowdy 2008) present a rather critical point of view referring to previous studies with CPP-siRNA-conjugates. They claim that the successful delivery described therein is solely the result of excess free peptide, which leads to additional complexation, and thereby cellular import of the siRNA. This is in accordance with Turner et al. (2005), who were the first to observe that careful purification of CPP-antisense oligonucleotide-conjugates abrogates their biological effect. Among other things, this might be the reason why most of the studies reporting on successful peptide-mediated delivery of siRNAs use a noncovalent complexation approach (confer Table 2).

As described above, direct conjugation of anionic siRNAs to cationic peptides, in this paragraph called PTDs, results in charge neutralization followed by aggregation and thereby inactivation of the PTD. To avoid this problem, Eguchi et al. (2009) fused Tat with a double-stranded RNA-binding domain (DRBD), which binds to siRNA and masks its negative charge. The resulting complex consists of a single RNA surrounded by four PTD-DRBDs and was used to deliver siRNAs against GFP or GAPDH. Efficient gene silencing without cytotoxicity or off-target effects could be shown even in difficult-to-transfect primary cells as well as in a reporter mouse model *in vivo*.

Table 2 Examples for peptide-mediated delivery of siRNA

CPP/delivery system	Mode of linkage[a]	Target	Cell line	References
$Tat^{47–57}$, Tat-derived oligocarbamate	c	EGFP, CDK9	HeLa	Chiu et al. (2004)
Penetratin, transportan	c	luciferase, GFP	Cos-7, C166, EOMA, CHO-AA8	Muratovska and Eccles (2004)
Penetratin	c	Cu-ZN SOD-1, Caspase-3/-8/-9	Primary rat hippocampal or sympathetic neurons	Davidson et al. (2004)
$Tat^{48–60}$, penetratin	c	p38 MAP kinase	L929 (mouse fibroblasts), mouse lung (intratracheal)	Moschos et al. (2007)
PTD-DRBD	n-c	dGFP, GAPDH	H1299, Jurkat, HUVEC, human embryonic stem cells	Eguchi et al. (2009)
MPG, $MPG\Delta^{NLS}$	n-c	Luciferase, GAPDH	HeLa, Cos-7, HS-68	Simeoni et al. (2003)
MPGα	n-c	Luciferase	HeLa, ECV 304	Veldhoen et al. (2006)
CADY	n-c	GAPDH, p53	U_2OS, THP1, HUVEC, 3T3C	Crombez et al. (2009a)
MPG-8	n-c	cyclin B1	HeLa, HS68, MCF-7, PC3, SKBr3-HER, mouse (intravenously, intratumoral)	Crombez et al. (2009b)
Chol-R9	n-c	VEGF	CT-26, mouse (intratumoral)	Kim et al. (2006)
H3K8b, H3K8b(+RGD)	n-c	β-Gal, luciferase	SVR-bag4, MDA-MB-435, C6	Leng et al. (2005)
POD	n-c	EGFP	HER 911	Johnson et al. (2008)
EB1, $MPG\Delta^{NLS}$, bPrPp	n-c	Luciferase	HeLa, HepG2	Lundberg et al. (2007)
TatU1A	n-c	EGFP, EGFR	CHO, A431,	Endoh et al. (2008)
stearyl-R8	n-c	EGFP, MAP2B	Primary rat hippocampal neurons	Tönges et al. (2006)
R8-MEND (siRNA/stearyl-R8 core)	n-c	Luciferase	HeLa	Nakamura et al. (2007)
R8/GALA-MEND	n-c	Luciferase	HeLa	Sakurai et al. (2009)
Chol-R9	n-c	VEGF	CT-26, mouse	Kim et al. (2006)
YSA-nanogel	n-c	EGFR	Hey, BG-1	Blackburn et al. (2009)
DMMAn-Mel	n-c	Luciferase	Neuro 2A-eGFPLuc	Meyer et al. (2008)

[a]c = covalent/n-c = non-covalent

Simeoni et al. (2003) were the first who non-covalently complexed siRNA with the peptide MPG. MPG is a 27 amino acid peptide composed of a hydrophobic domain derived from the N-terminal fusion sequence of the HIV-1 glycoprotein 41 and a hydrophilic domain derived from the nuclear localization sequence (NLS) of the SV40 large T-antigen, which are linked by a 3 amino acid spacer (Morris et al. 1997). At a 1:10 ratio of negative nucleic acid to positive peptide charges, a decrease in luciferase activity of about 80% was detectable in HeLa or Cos-7 cells. This effect was further enhanced to about 90% downregulation by a mutation in the NLS sequence of the carrier peptide ($MPG\Delta^{NLS}$), presumably due to an increased delivery to the cytoplasm, where RISC is localized.

In the following, Veldhoen et al. (2006) used a derivative of the MPG peptide for the delivery of siRNA. This variant, termed MPGα, differs from MPG by five amino acids in the hydrophobic part. These changes result in an alteration of the overall structure of the peptide towards a higher tendency of adopting a helical conformation (Deshayes et al. 2004). MPGα forms highly stable non-covalent complexes with nucleic acids through ionic interactions of the positively charged NLS sequence and negative charges of the cargo. Furthermore, hydrophobic peptide/peptide interactions lead to the formation of nanoparticles. Using a luciferase-targeted siRNA as cargo, reporter gene activity could be inhibited up to 90% with an IC_{50} value in the subnanomolar range. Confocal microscopy studies as well as transfections in the presence of inhibitors of different endocytotic pathways strongly indicate that endocytosis is involved in the cellular uptake of peptide/siRNA complexes. As a key issue, the authors quantified the intracellular number of siRNA molecules after MPGα-mediated transfection and compared it to the amount of extracellularly applied RNA. Together with data from microinjection experiments (Laufer and Restle 2008), this comparison yields the percentage of internalized molecules that are biologically active. In the case of MPGα-mediated siRNA delivery, only 0.1% of internalized oligonucleotides are biologically active whereas more than 99% are probably retained in endosomes (confer Fig. 6).

Recently, Crombez et al. (2009a) designed a similar secondary amphipathic peptide, called CADY, which adopts a helical conformation within cell membranes, exposing cationic arginine residues on one side and aromatic tryptophan groups on the other. CADY forms stable complexes with siRNAs already at a molar ratio of 5:1–10:1 (peptide:siRNA), whereas for protection from serum nucleases, optimal cellular uptake and significant target knockdown higher molar ratios (>20:1) are required. Cellular uptake and the associated biological response were hardly affected in the presence of different inhibitors of endocytosis; therefore, the authors concluded that the entry mechanism of CADY/siRNA complexes is independent of the endosomal pathway.

The same group (Crombez et al. 2009b) shortened the original MPG peptide by six residues and mutated two residues to tryptophan, yielding a 21 amino acid peptide called MPG-8. In addition to the cysteamide group at the C-terminus, a β-alanine was added at the N-terminus to allow further functionalization of the peptide. Concerning siRNA delivery, the optimal molar ratio was determined to be 20:1 (peptide: siRNA), and under these conditions, MPG-8 exhibited a

significantly higher gene silencing activity than the parent peptide MPGΔ^{NLS}. In addition to target downregulation on the mRNA- as well as the protein-level, MPG-8-mediated delivery of anticyclin B1 siRNA induced G2 arrest and blocked cell proliferation specifically in cancer cells. Using a xenograft tumor mouse model, local intratumoral administration but not intravenous injection of 0.25 mg/kg MPG-8/siRNA particles prevented tumor growth completely. To improve systemic delivery, MPG-8/siRNA particles were functionalized with a cholesterol moiety through activation of the N-terminal β-alanine group. This modification increased the distribution level of anti-cyclin B1 siRNA and blocked tumor growth upon systemic intravenous administration in a xenograft human prostate as well as human lung cancer mouse model without activation of the innate immune system.

Similar synergistic effects had already been shown by Kim et al. (2006), who combined oligoarginine with cholesterol (Chol-R9) for the non-covalent complexation of an anti-VEGF siRNA. Chol-R9/siVEGF complexes suppressed VEGF production *in vitro* in CT-26 cells as well as in an *in vivo* mouse model after local administration to a subcutaneous tumor. Here, the lowered VEGF level was accompanied by decreased tumor growth, which was probably due to the anti-angiogenetic effect on tumor vascularization.

As briefly outlined above, the major rate-limiting step for most delivery approaches is endosomal entrapment of the nucleic acids. Thus, many groups try to improve their systems with the aim to increase endosomal escape of siRNA after peptide-mediated delivery. Lundberg et al. (2007) rationally modified penetratin to form a CPP (termed EB1) with improved endosomolytic properties. They achieved a pH-dependent conformational change of the peptide to a higher degree of helicity by the replacement of two basic amino acids with histidines and the N-terminal addition of six amino acids. In this study, several CPPs were compared in a non-covalent approach by measuring the overall cellular uptake via fluorescence and the biological effect of siRNA targeted to luciferase mRNA. Penetratin- as well as TP10-mediated transfection did not lead to any silencing of luciferase gene expression, despite high amounts of intracellular siRNA (Lundberg et al. 2007) in contrast to previous reports using siRNA–penetratin-conjugates (Davidson et al. 2004) or TP10/DNA-complexes (El-Andaloussi et al. 2005). EB1-mediated delivery of 100 nM siRNA led to approximately 50% reduction of luciferase activity. This silencing effect was slightly better than for bPrPp and in the same range as for MPGΔ^{NLS}. As it was described earlier that addition of a pH-sensitive peptide derived from hemagglutinin (HA2) can promote endosomal escape (Wadia et al. 2004), the authors linked HA2 to penetratin (Lundberg et al. 2007). It turned out that although HA2-penetratin improved the silencing effect when coincubated with penetratin, EB1 was more potent than this combination of peptides. Together with confocal microscopy studies, the authors concluded that the lack of biological effect after penetratin-mediated siRNA delivery is due to a lack of endosomal escape and that EB1 has a superior endosomolytic activity in comparison to HA2-penetratin.

Endoh et al. (2007, 2008) and Endoh and Ohtsuki (2009) recently presented an innovative strategy, called CLIP-RNAi (i.e., CPP-linked RBP-mediated RNA

internalization and photoinduced RNAi), combining delivery of a specific RNA sequence with enhanced photoinduced release of RNA from endosomes. This goal was accomplished by fusing the U1A RNA-binding domain (RBD) to the Tat peptide and extending the siRNA with a short stretch of nucleotides specifically recognized by this RBD. These complexes were efficiently internalized but exhibited a punctate cytoplasmic localization pattern, indicative of endosomal entrapment. However, photostimulation of a fluorophore attached to the peptide led to a redistribution of complexes into the cytosol followed by efficient RNAi-mediated gene silencing.

In addition to "simple" CPP-based delivery systems composed of single peptides, there is a trend to develop systems of higher complexity. A main goal of such approaches is to generate nanoparticles with defined properties (e.g., size and charge distribution) as well as to provide cell-specific functionalities, which are especially important for *in vivo* use. Since a comprehensive description of recent developments would be far beyond the scope of this article, we can only give a few examples with respect to successful siRNA delivery. In general, there are attempts to combine peptides with cationic liposomes (Futaki et al. 2005; Hyndman et al. 2004; Preuss et al. 2003; Read et al. 2003, 2005; Torchilin et al. 2001, 2003) or polyethyleneimine (PEI) (Kilk et al. 2005). Other applications are aimed towards the synthesis of high or low molecular weight branched polymers and/or peptides (Chen et al. 2001; Fattal and Barratt 2009; Leng et al. 2005; Liu et al. 2005; Midoux and Monsigny 1999; Read et al. 2003; Ritter et al. 2003) or dendrimers (Bayele et al. 2005, 2006; Kang et al. 2005). Recent developments of even more complex systems are particularly promising with respect to *in vivo* delivery (Kale and Torchilin 2007a, b; Khalil et al. 2007; Rahbek et al. 2008; Soundara Manickam and Oupický 2006).

One example is a recently developed novel packaging approach, a multifunctional envelope-type nano device (MEND), which allows the assembly of multiple devices in a single delivery system (Kogure et al. 2008). In principle, nucleic acids are condensed using a polycation to form a core particle, followed by encapsulation in a lipid envelope. For the delivery of siRNA, further modifications were included into MEND (Nakamura et al. 2007; Sakurai et al. 2009). First, the addition of stearylated octaarginine (STR-R8) on the lipid envelope led to efficient cellular uptake by macropinocytosis. Second, dioleoylphosphatidyl ethanolamine (DOPE) and phosphatidic acid (PA) were added because of their high fusogenic activity, which improved silencing activity through increased release of functional siRNA into the cytosol. Third, the pH-sensitive fusogenic peptide GALA, additionally conjugated with cholesterol, also enhanced endosomal release of encapsulated nucleic acids. Finally, the STR-R8-MEND, prepared with a lipid composition of DOPE/PA plus Chol-GALA, was modified with a cleavable PEG–peptide–DOPE conjugate (PPD) to enhance *in vivo* tumor targeting. So far, this system has already been used for efficient gene silencing in HeLa cells and appears to be a promising new carrier for siRNA into tumor cells.

Several groups have developed drug delivery approaches using synthetic hydrogel nanoparticles (nanogels). These core/shell particles physically segregate the function of cell and drug binding (=shell) from the function of endosomal

disruption (=core) (Hu et al. 2009). Furthermore, they assemble into stable and well-defined complexes with a high payload capacity and can be selectively surface-functionalized to enable cell type-specific targeting. For example, Blackburn et al. (2009) used the 12 amino acid peptide YSA for the delivery of anti-EGFR siRNA to ovarian cancer cells via ligand-receptor binding mediated endocytosis.

Polycations, like PEI or PLL alone, can promote significant plasmid DNA transfer efficiency but show only modest siRNA delivery activity. Therefore, Meyer et al. (2008, 2009) functionalized these polycations with polyethylene glycol (PEG) and a pH-responsive endosomolytic melittin peptide from bee venom (Ogris et al. 2001). To minimize lytic activity in the extracellular environment, melittin was further modified with dimethylmaleic anhydride (DMMAn), which is cleaved in the endosome and therefore restores lytic activity in the intracellular compartment. Modification of PEI or PLL with DMMAn-Mel greatly enhanced siRNA-mediated luciferase gene knockdown (Meyer et al. 2009).

5 Selected Examples of siRNA Delivery *In Vivo*

When it comes to *in vivo* delivery of siRNA, the situation gets much more complicated than described above on a cellular level. In principle, the nucleic acid molecules can be administered topically or locally to, for example, the eye, skin, mucus membranes, and local tumors, or systemically through the blood stream. Especially in the latter case, besides cellular uptake, there are many more additional hurdles to consider like serum stability, aggregation with serum proteins, uptake by phagocytes, and clearance by the kidneys (Alexis et al. 2008; Xie et al. 2006). Moreover, a significant challenge for siRNA delivery to many tissues represents migration from the bloodstream across the vascular endothelium and subsequently diffusion through the extracellular matrix, a dense network of polysaccharides and fibrous proteins.

There are a variety of techniques described to deliver siRNA *in vivo*. The simplest option is the application of naked RNA into a target organ either non-modified or chemically modified (e.g., 2′-O-methyl modifications). For systemic delivery, siRNA can be conjugated for example with PEG, cholesterol, or small peptides or alternatively complexed with peptides, lipids, polymers, polycations, or even complex nanoparticles, in certain cases, in combination with antibodies or cell surface-specific ligands for targeted delivery [for a review see, Jeong et al. (2009)]. To cover all of the different approaches described in the literature would be far beyond the scope of this article. Therefore, we will focus on some of the most promising examples described in recent years. A summary of these experiments can be found in Table 3. For a more comprehensive coverage, the reader is referred to these recent reviews (Aigner 2008; Castanotto and Rossi 2009; Whitehead et al. 2009) and references therein.

The first successful downregulation of a target mRNA by siRNA in mammals was shown by McCaffrey et al. (2002). In this study, the authors showed that

Table 3 Selected examples of nonviral siRNA delivery *in vivo*

Formulation of siRNA	Mode of administration	Target protein	Observed effect	References
Naked	Hydrodynamic transfection	Firefly luciferase	Reduction of target gene expression	McCaffrey et al. (2002)
Naked	Intravenous injection	Fas (also known as TNFRSF6)	Protected mice from liver fibrosis	Song et al. (2003)
Naked/TransIT-TKO (polyamine)	Intranasal	RSV-P, PIV-P	Protection from respiratory infection	Bitko et al. (2005)
Protamine–antibody fusion protein	Intratumoral or intravenous injection	c-myc, MDM2, VEGF	Inhibition of s.c. melanoma xenograft growth	Song et al. (2005)
Aptamer/siRNA chimeras	Intratumoral injection	PLK1 (A10-Plk1), BCL2 (A10-Bcl2)	Triggering of apoptosis, growth inhibition, and tumor regression in mouse xenograft model	McNamara et al. (2006)
Rabies virus glycoprotein peptide/r9/siRNA	Intravenous injection	Japanese encephalitis virus (JEV)	Protection against fatal viral encephalitis	Kumar et al. (2007)
β1,3-D-glucan-encapsulated siRNA particles	Oral gavage	Tumor necrosis factor α (TNF-α), mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4)	Protection from lipopolysaccharide (LPS)-induced lethality	Aouadi et al. (2009)

transgene firefly luciferase expression can be suppressed in adult mice by synthetic siRNAs injected to the liver. The first therapeutic application was reported just 1 year later by Song et al. (2003). In this study, mice could be protected from Fas-mediated liver fibrosis by downregulation of Fas. In a fulminant hepatitis induced by injecting agonistic Fas-specific antibody, 82% of mice treated with siRNA that effectively silenced Fas survived for 10 days of observation, whereas all control mice died within 3 days. This was a first promising example of the therapeutic potential of RNAi *in vivo*. In 2005, Bitko et al. (2005) reported that individual as well as joint infection by respiratory syncytial virus (RSV) and parainfluenza virus (PIV) can be specifically prevented and inhibited by siRNAs, instilled intranasally in the mouse, with or without transfection reagents. Their results suggested for the first time that, if properly designed, low dosages of inhaled siRNA might offer a fast, potent, and easily administrable antiviral regimen against respiratory viral diseases in humans.

One of the first examples of targeted delivery, i.e., cell type-specific delivery, was a study by Song et al. (2005). Here, the authors used a protamine/antibody fusion protein to deliver siRNAs specifically to cells expressing the HIV-1 envelope protein. The positively charged protamine served as binding partner for the negatively charged siRNA, whereas a heavy-chain antigen-binding region (Fab) permitted

specific interaction with surface exposed gp160 molecules followed by internalization and eventual release of the siRNA cargo. Intratumoral or intravenous injection of Fab/protamine-complexed siRNAs into mice targeted HIV envelope-expressing B16 melanoma cells, but not normal tissue or envelope-negative B16 cells. Using siRNAs against c-myc, MDM2 or VEGF envelope-expressing subcutaneous B16 tumors could be inhibited. Another technology for cell type-specific delivery is based on aptamer/siRNA chimeras (McNamara et al. 2006). Aptamers are small (25–60 nucleotides) oligonucleotide ligands (either DNA or RNA) derived from an *in vitro* evolution process called SELEX (systematic evolution of ligands by exponential enrichment) (Ellington and Szostak 1990; Robertson and Joyce 1990; Tuerk and Gold 1990). Such nucleic acid ligands do bind with high affinity and specificity to their target molecules. In the present case, an aptamer selected against the cell-surface receptor PSMA (prostate specific membrane antigen) was used, linked to either polo-like kinase 1 (PLK1) or B-cell lymphoma 2 (BCL2)-specific siRNAs. Intratumoral injection of these conjugates into a mouse xenograft model resulted in triggering of apoptosis, growth inhibition, and tumor regression. A third example of targeted delivery is the use of a short peptide derived from rabies virus glycoprotein (RVG), which enabled transvascular delivery of siRNAs directed against Japanese encephalitis virus (JEV) to the brain (Kumar et al. 2007). The 29-amino-acid RVG peptide specifically binds to the acetylcholine receptor expressed by neuronal cells. This peptide was fused with R9 to permit siRNA binding. Intravenous treatment with RVG-9R-bound antiviral siRNA let to a robust protection against fatal viral encephalitis in mice.

An interesting additional delivery route for siRNAs was recently published by Aouadi et al. (2009). Here, orally delivered siRNA targeting macrophage mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) suppressed systemic inflammation in mice. As vehicle hollow, porous 2–4 μm-sized shells composed primarily of β1,3-D-glucan were prepared by treating baker's yeast with a series of alkaline, acid and solvent extractions to remove cytoplasm and other cell wall polysaccharides. The anionic siRNA is bound within these particles between cationic polyethylenimine layers through electrostatic interactions. The orally administered particles are then phagocytosed by macrophages and dendritic cells in the gut-associated lymphatic tissue. Moreover, the authors speculate that these cells may traffic away from the gut and infiltrate other reticuloendothelial system tissues, so that, over time, total body macrophages contain siRNAs. Lipopolysaccharide (LPS)/D-galactosamine (DGalN) challenged mice could be protected from inflammatory cytokine toxicity by oral gavage of Map4k4-siRNA-containing particles through inhibition of tumor necrosis factor α (TNF-α) and Interleukin-1β (IL-1β) production in macrophages. Interestingly, *in vivo* potency of these siRNAs was 5–250 times greater than that in previous studies reporting systemic delivery (Filleur et al. 2003; McCaffrey et al. 2002; Peer et al. 2007; Song et al. 2005; Sorensen et al. 2003; Soutschek et al. 2004; Wesche-Soldato et al. 2005; Zimmermann et al. 2006).

Currently, there are several ongoing clinical trials for siRNA therapeutics (Table 4). Several of the more advanced trials are targeted at age-related macular

Table 4 Selected examples of current clinical trials for siRNA therapeutics

siRNA	Company	Disease	Mode of administration	Status
ALN-RSV01	Alnylam Pharmaceuticals	Respiratory syncytial virus	Local	II
CALAA-01	Calando Pharmaceuticals	Solid tumors	Systemic/intravenous	I
Sirna-027	Sirna Therapeutics	Age-related macular degeneration	Topical/intravitreal	I
TD101	TransDerm	Pachyonychia congenita	Topical/foot	I
I5NP	Quark Pharmaceuticals	Acute kidney injury after cardiac bypass surgery	Systemic/intravenous	I
Bevasiranib	Opko Health	Age related macular degeneration	Topical/intravitreal	III, discontinued

degeneration (AMD), which is a leading cause of blindness. This disease arises from excessive blood-vessel growth and rupture within the cornea. In terms of drug delivery, a treatment of AMD is less challenging than other diseases since the molecules of interest can be administered intravitreally, a procedure which avoids many of the problems with *in vivo* delivery briefly described above. The siRNAs under investigation are targeted to vascular endothelial growth factor (VEGF) and its receptor (VEGFR). Although initially the results obtained were quite encouraging, a recent study by Kleinman et al. (2008) reported about sequence-independent angiogenesis suppression by siRNA via nonspecific stimulation of the Toll-like receptor 3 (TLR3) pathway. While this study questions the AMD-related clinical trials, it does not explain the therapeutic effects of other trials where appropriate controls have been performed.

6 Conclusions and Future Prospects

Currently, the development of effective and safe delivery systems for therapeutic oligonucleotides like siRNA is crucial to one day bring these molecules to the clinic. Besides the development of viral vectors as delivery vehicles, there is a highly diverse and constantly increasing number of nonviral systems evolving. However, at present, even the most advanced systems either lack the efficiencies required for downstream drug development or do show a substantial degree of toxicity or both. Of the many factors that limit their use, cellular uptake of the cargo/carrier complexes and particularly subsequent intracellular trafficking to reach the target site are the most important. Moreover, for *in vivo* use, various additional obstacles are to be taken into account like serum stability, pharmacokinetic considerations, and tissue barriers as well as target cell specificity. In spite of these somewhat sobering insights, there is noticeable progress especially in recent years. While most of the underlying problems are meanwhile identified, the answers to these problems remain challenging. Most likely, there will be no magic bullet but individual solutions for any given application.

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