Published as: *Adv Drug Deliv Rev.* 2008 March 1; 60(4-5): 530–536.

Enhancing the cellular uptake of siRNA duplexes following noncovalent packaging with protein transduction domain peptides

Bryan R. Meade and Steven F. Dowdy *

Howard Hughes Medical Institute, and Department of Cellular & Molecular Medicine, UCSD School of Medicine, 9500 Gilman Drive, La Jolla, CA 92093-0686, USA

Abstract

The major limitation in utilizing information rich macromolecules for basic science and therapeutic applications is the inability of these large molecules to readily diffuse across the cellular membrane. While this restriction represents an efficient defense system against cellular penetration of unwanted foreign molecules and thus a crucial component of cell survival, overcoming this cellular characteristic for the intracellular delivery of macromolecules has been the focus of a large number of research groups worldwide. Recently, with the discovery of RNA interference, many of these groups have redirected their attention and have applied previously characterized cell delivery methodologies to synthetic short interfering RNA duplexes (siRNA). Protein transduction domain and cell penetrating peptides have been shown to enhance the delivery of multiple types of macromolecular cargo including peptides, proteins and antisense oligonucleotides and are now being utilized to enhance the cellular uptake of siRNA molecules. The dense cationic charge of these peptides that is critical for interaction with cell membrane components prior to internalization has also been shown to readily package siRNA molecules into stable nanoparticles that are capable of traversing the cell membrane. This review discusses the recent advances in noncovalent packaging of siRNA molecules with cationic peptides and the potential for the resulting complexes to successfully induce RNA interference within both *in vitro* and *in vivo* settings.

Keywords

RNA Interference; Peptide Transduction Domain; Cell penetrating Peptide; siRNA Delivery; Macromolecular Delivery; HIV-1 TAT peptide

1. Introduction

Peptide transduction domain (PTD), also called cell penetrating peptides (CPPs) are best described as a class of small cationic peptides of approximately 10–30 amino acids in length that have been shown to engage the anionic cell surface through electrostatic interactions and rapidly induce their own cellular internalization through various forms of endocytosis [1–5]. After being internalized within endocytic bodies, PTD/CPPs are capable of endocytic vesicle escape and gain access to the intracellular environment through an as yet undefined mechanism

*Corresponding author: sdowdy@ucsd.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

[6–8]. Some of the most well characterized PTD/CPPs thus far are TAT peptide [9], penetratin [10], transportan [11], poly-arginine [12] and MPG [13]. The power of the cellular delivery properties of these PTD/CPPs resides within the dense cationic charged motifs that are present within the large majority of all PTD/CPPs discovered to date. Importantly, these cationic peptides have also been shown to enhance the cellular uptake of covalently coupled cargo, making them attractive candidates for applications where the intracellular delivery of large macromolecules is desirable [14].

Since the discovery that the intracellular administration of short, synthetic double stranded RNA duplexes can lead to the downregulation of specific mRNA targets [15], the race has been on to invent efficient delivery systems to increase the ability of these molecules to cross the cell membrane. Unaided, siRNA molecules have no ability to cross the phospholipid bilayer due to the strong anionic charge of the siRNA phosphodiester backbone as well as an approximate size of 14,000 Da. However, the field of siRNA delivery has advanced rapidly due in part to the utilization of many delivery systems that have previously been shown to have success in the delivery of nucleic acids for gene therapy. Not surprisingly, many studies have also attempted to utilize the cellular internalization efficiency of PTD/CPPs to increase the ability of siRNA molecules to gain access to the intracellular environment.

The most obvious method for enhancing the cellular uptake of siRNA molecules with cationic peptides is through covalent conjugation of a siRNA duplex to a PTD/CPP prior to cellular treatment. Our last review on PTD/CPP-mediated siRNA delivery summarized the current position of the field with an emphasis on the difficulties that arise following the covalent attachment of cationic peptides to anionic siRNA cargo [16]. The most widely used covalent attachment methodology employed is a disulfide bond formation between PTD/CPP and siRNA cargo post-synthesis. While three published studies have shown successful siRNA delivery following covalent PTD/CPP/siRNA conjugation [17–19], our own unpublished experimental observations have shown the difficulty with successfully inducing siRNA uptake in this manner.

In our hands, after extensive purification of PTD/CPP/siRNA conjugates from the excess cationic peptide in the conjugation reaction, no enhanced cellular internalization of siRNA is observed after cellular treatment with micromolar concentrations by either assays for RNA interference or flow cytometry analysis of cells treated with fluorescently-labeled PTD/CPP/siRNA conjugates. However, in the absence of PTD/CPP/siRNA conjugate purification, excess peptide in solution leads to insoluble complex formation upon dilution to physiologic salt concentrations during cellular treatment. While this noncovalent complexing is sufficient to induce cellular uptake in some instances, it should be noted that the soluble PTD/CPP/siRNA conjugate is not the active component of siRNA delivery. We believe the three initial published studies that observed successful siRNA uptake with PTD/CPP/siRNA conjugates were not assessing soluble PTD/CPP/siRNA conjugate uptake but rather noncovalent packaging of siRNA with excess cationic peptide in solution.

The dense cationic charged motifs present within PTD/CPPs are crucial for enhancing cellular uptake by engaging the necessary components on the cell surface. Unfortunately, upon conjugation of cationic PTD/CPPs to anionic cargo, no cellular internalization is seen due to charge neutralization of the critical cationic residues [20]. We conclude that this is also the case with anionic siRNA cargo; the ~40 negatively-charged phosphodiester linkages are in excess enough to neutralize the ~8 positively charged residues of the cationic PTD/CPP necessary to mediate cellular uptake. We are confident with this conclusion due to the rigorous experimentation we have done employing multiple types of PTD/CPP/siRNA conjugate treatments to multiple cell lines in culture. In further support of our unpublished observations

are two recent publications that support the idea that PTD/CPP/siRNA covalent conjugates are inefficient at delivering functional siRNA to cells in the absence of noncovalent packaging.

The first study analyzed the ability of multiple PTD/CPPs including TAT, penetratin and transportan to enhance the cellular uptake of siRNA after covalent conjugation through a disulfide linkage followed by extensive purification of PTD/CPP/siRNA conjugates from the conjugation reaction solutions [21]. In HeLa cells expressing a luciferase reporter gene, no RNA interference against luciferase was seen with 500 nM of any PTD/CPP/siRNA conjugate in comparison to siRNA cationic lipofection that resulted in an IC_{50} value of 50 nM for inhibition of luciferase expression. Curious that the lack of RNAi against luciferase was due to the high expression levels of the reporter gene, the authors attempted to analyze RNAi against an endogenous gene with PTD/CPP/siRNA conjugates. In HeLa cells, p38 kinase mRNA levels were assessed by quantitative RT-PCR following treatment with PTD/CPP/siRNA conjugates or cationic lipofection of siRNA. After incubation of HeLa cells for 24 hours with 10 μ M PTD/CPP/siRNA conjugates, a 60% knockdown of p38 kinase mRNA levels was achieved with all PTD/CPPs tested. However, the concentrations used were 1000-fold or greater than that required for cationic lipofection that displayed an IC_{50} of 1 nM in the same p38 kinase mRNA knockdown assay. Importantly, the concentration of PTD/CPP/siRNA conjugates used to obtain this moderate knockdown is considerably higher than the previously published studies using PTD/CPP/siRNA conjugates in the absence of extensive conjugate purification [17–19].

The second study analyzed the ability of TAT and penetratin peptides to induce the cellular uptake of siRNA following covalent conjugation after peptide/siRNA conjugate formation within both *in vitro* and *in vivo* settings [22]. Within murine L929 cells, a 24-hour treatment of 10 μ M PTD/CPP/siRNA conjugates resulted in only a small (20–40%) inhibition of p38 kinase mRNA levels by RT PCR. In comparison to cationic lipofection, these concentrations of PTD/CPP/siRNA conjugates are greater than 100,000-fold the concentration of cationic lipoplexed siRNA needed for 50% inhibitory activity. Though negative results were obtained within cultured murine cell lines, the authors sought to determine whether or not these PTD/CPP/siRNA conjugates could function as efficient siRNA delivery molecules within an *in vivo* setting. Intratracheal administration of PTD/CPP/siRNA conjugates to the lungs of live mice resulted in no gene knockdown when compared to administration of PTD/CPP only treated mice.

These two studies, as well as our own unpublished observations, highlight the difficulties associated with enhancing the delivery of siRNA duplexes through covalent conjugation to PTD/CPPs. Nonetheless, the successes with PTD/CPP-mediated siRNA delivery have been realized by using an alternative noncovalent complexing strategy that utilizes the dense cationic charged motifs within PTD/CPPs to package siRNA molecules into complexes capable of traversing the cellular membrane. This review will discuss these current noncovalent siRNA packaging schemes and their application to induction of RNA interference within both *in vivo* and *in vitro* settings.

2. siRNA Delivery—An Overview

The observation that long double-stranded RNA molecules (dsRNA) can lead to gene specific silencing in *C. elegans* was a breakthrough observation, altering the paradigm of gene expression regulation and catapulting the idea that endogenously expressed dsRNA molecules play a functional role in gene regulation [23]. In mammalian cells, it has since been shown that endogenously expressed long dsRNA molecules are processed to shorter dsRNA molecules by the protein Dicer and are then capable of becoming effector molecules for RNA interference

(RNAi) [reviewed in 24]. These small dsRNA duplexes, termed siRNA, are approximately 19–23 base pairs in length and contain two nucleotide 3' overhangs.

After nuclear export, these siRNA become incorporated in and activate a multiprotein complex called the RNA induced silencing complex or RISC [25]. Upon incorporation into RISC, one of the RNA strands is cleaved (the passenger strand) while the remaining single stranded RNA molecule (the guide strand) remains incorporated and directs RISC-mediated gene specific downregulation through complementation to target mRNA. The catalytic component of the RISC machinery is Argonaute 2 (Ago2), a protein that confers slicing activity to the complementing mRNA strand [26]. After guide strand directed complementation to mRNA, Ago2 down regulates gene expression by cleaving mRNA molecules and thus inhibiting translation of a specific mRNA. Following mRNA cleavage, guide strand-loaded RISC is capable of multiple rounds of mRNA cleavage making RISC-mediated regulation of gene expression a powerful catalytic event.

Importantly, it was shown that the introduction of synthetic siRNA molecules into the cytoplasm of mammalian cells mimics Dicer processing and results in activation of RISC and subsequent induction of RNAi against target gene expression [15]. This observation was monumental and paved the way for synthetic siRNA duplexes to become crucial tools for the analysis of gene function determination, validation of previous basic science observations and for gene-specific silencing in therapeutic applications. What makes siRNA molecules so attractive for therapeutic applications is the exquisite selectivity for target mRNA downregulation and importantly, with an IC₅₀ value in the low picomolar range, siRNA molecules hold great promise for the therapeutic intervention of human disease processes.

The main limitation of siRNA technology is the inability of naked siRNA to successfully cross the cell membrane and induce an RNAi response. Multiple delivery systems have been investigated in order to circumvent this problem including cationic lipids [27], cationic polymers [28], antibody-protamine fusions [29] and RNA aptamers [30]. While few studies exist, cell penetrating peptides remain an attractive candidate for siRNA delivery given their previously validated functionality in delivering a large number of macromolecules [14]. This review will discuss the few published studies that have used cell penetrating peptides as noncovalent packaging molecules to help aid the passage of information rich siRNA duplexes across the cellular membrane.

3. Noncovalent complexing of siRNA with cationic cell penetrating peptides

3.1 MPG Peptide

The amphipathic MPG peptide was originally designed to enhance the cellular uptake of oligonucleotides for antisense technologies and DNA vectors for gene therapy applications [13]. Since the discovery of RNAi, MPG has been translated into an efficient siRNA delivery peptide and has become the most well characterized PTD/CPP in regards to siRNA delivery potential [reviewed in 31]. MPG is a 27 amino acid amphipathic peptide composed of a basic domain from the nuclear localization signal (NLS) of SV40 large T antigen and a hydrophobic domain derived from HIV-1 gp41 (GALFLGLGAAGSTMGAWSQPKKRKV). The basic domain aids in the electrostatic packaging of siRNA molecules as well as the interaction with hydrophilic moieties on the cell membrane while the hydrophobic sequence enhances cellular delivery through membrane destabilization following interaction with hydrophobic lipid moieties on the cell surface.

In the original report analyzing the potential for MPG to enhance the cellular uptake of siRNA duplexes, the authors report a slight alteration within the MPG peptide sequence to enhance the effectiveness of the delivery system. By changing a single lysine residue to serine within

the basic NLS domain of MPG (PKSKRKV), the subcellular localization of introduced siRNA changed from the nucleus to the cytoplasm as assayed by microscopy, resulting in a stronger RNAi response due to the cytoplasmic localization of the RISC machinery [32]. This altered peptide, termed MPG Δ^{NLS} , has been the peptide of choice in all subsequent studies of MPG-mediated siRNA delivery studies.

Initially, the mode of PTD/CPP-mediated internalization into cells was thought to be an uptake process that was separate from traditional forms of endocytosis. Cell membrane translocation was shown to be unaffected by low temperatures, depletion of intracellular ATP or by inhibitors of endocytosis [9,10]. However, these conclusions were eventually shown to be artifacts of cellular fixation and misinterpretation of flow cytometry analysis of cells treated with fluorescently labeled peptides [33,34]. Thus, it was surprising to find the initial report describing the method of internalization of MPG/siRNA complexes was shown to be endocytosis independent. Cellular internalization was shown to occur at 4°C and MPG-mediated delivery was not effected in the presence of the endocytic inhibitors cytochalasin B and bafilomycin A and was not enhanced following the addition of the endosomolytic reagent chloroquine [32]. Because the readout in these assays was phenotypic, it was thought that the initial misinterpretations of PTD/CPP-mediated uptake did not hold true with MPG and MPG/siRNA complexes were capable of traversing the cell membrane without the aid of endocytic processes.

However, a recent study has challenged this initial report and has reevaluated the uptake mechanism of MPG/siRNA complexes [35]. Veldhoen et al. have recently published a report arguing the case that MPG/siRNA complexes are indeed internalized in endocytic processes. To begin their analysis, two cell lines (HTOL and ECV304) were stably integrated with luciferase reporter gene constructs as a read out for RNAi. MPG-mediated delivery of siRNA was shown to be as efficient as previously reported, resulting in 80–90% knockdown of target gene expression with an IC₅₀ value of ~0.8 nM. However, the induction of RNAi against luciferase was completely abolished with treatments of MPG/siRNA complexes at 4°C. Additionally, the endocytotic inhibitors cytochalasin B, nystatin, wortmannin and okadaic acid all reduced MPG/siRNA-mediated downregulation of luciferase gene expression while the endocytic enhancer chloroquine enhanced the induction of RNAi against luciferase. In further support of an endocytosis-dependent mode of uptake, microscopy analysis of cells treated with fluorescently labeled MPG/siRNA complexes displayed punctate, vesicular localization indicative of endocytosis.

In addition to showing a requirement for endocytotic processes in the uptake of MPG/siRNA complexes, Veldhoen et al. also used a sensitive liquid hybridization protocol to determine the amount of siRNA taken up by cells treated with MPG/siRNA complexes compared to Lipofectamine/siRNA complexes in a side by side experiment. In order to efficiently quantitate the amount of internalized siRNA, the authors utilized a thorough cell washing step prior to quantitation with a mixture of trypsin and heparin washes to rule out any siRNA complexes that may be bound to the extracellular membrane and confuse results obtained. Following a thorough washing procedure, the authors found that at the concentration needed to obtain a half maximal response for Lipofectamine/siRNA complexes there were only 300 siRNA molecules per cell. However, in the case of MPG/siRNA treated cells, the number of siRNA molecules per cell was considerably higher at 10,000 siRNA molecules per cell. These results, along with the observation that the endosomolytic reagent chloroquine enhanced MPG/siRNA-mediated RNAi, led the authors to conclude that the major bottleneck in MPG/siRNA delivery appears to be endosomal entrapment as opposed to delivery efficiency or complex decomposition in the cytoplasm.

The reason for these experimental differences is unclear and perhaps additional experimentation by another outside group will help in further understanding the method in which MPG/siRNA complexes gain access to the intracellular environment. Nonetheless, what is clear is that MPG peptide remains a very powerful siRNA delivery tool with effective concentrations low enough to have potential utility within *in vivo* applications. In addition to its low inhibitory concentration, MPG complexation of siRNA is a technically simplistic approach and offers a relatively low cost way in which efficient siRNA delivery can be obtained in a wide variety of cells.

3.2 Poly-arginine fusion peptides

When it was determined early on that the process of PTD/CPP-mediated internalization was driven primarily by the dense cationic residues within the PTD/CPP sequence, synthetic poly-arginine peptides were synthesized as potential candidates for cell delivery peptides. Today, many studies utilize poly-arginine peptides of ~8–10 residues instead of traditional PTD/CPPs such as TAT or penetratin and in comparative studies have shown an equal and sometimes enhanced ability for these peptides to translocate across the cellular membrane [36]. Similar to other densely cationic PTD/CPPs, poly-arginine peptides have a strong propensity to electrostatically complex siRNA when mixed at high enough molar ratios. Utilizing the packaging properties of poly-arginine, two groups have created poly-arginine fusions to either cholesterol moieties or cell targeting peptides to increase the efficiency of siRNA uptake into cells in culture and tissues *in vivo*.

The first study utilized a cholesteryl oligo-D-arginine (Chol-R9) peptide composed of nine arginine residues fused to an N-terminal cholesteryl group [37]. While the domain composed of nine arginines offers efficient siRNA packaging and some cell surface interaction, the cholesterol moiety is capable of engaging the hydrophobic residues of the extracellular membrane surface and enhances the deliverability of the complexed siRNA duplexes. By using gel electrophoresis, the authors were able to determine at what ratio the cationic nitrogen groups (N) were sufficient to complex the negatively charged phosphate groups (P). The N/P ratio that was shown to successfully remove all free siRNA from solution was 40 and this ratio was subsequently shown to be the favorable ratio for successful siRNA delivery. Importantly, at this N/P ratio no cellular toxicity was seen; however, it is important to note that cell viability was compromised at N/P ratios of ~72.

Using N/P ratios of 10, 20 and 40, Chol-R9 peptide versus R9 complexed VEGF siRNA were added to CT-26 cells for 4 hours and assayed for VEGF secretion by ELISA 24 hours later. After 24 hours, VEGF production was suppressed by 55% for the Chol-R9/siRNA complexes formed from an N/P ratio of 40 compared to the untreated control and the unmodified R9/siRNA complex that displayed no VEGF inhibition. This observation further supports the argument that the cholesterol moiety plays a functional role in the delivery of these peptide/siRNA complexes. The authors then sought to validate this siRNA delivery system within an *in vivo* model system for RNA interference. CT-26 cells were injected into the flank of mice and allowed to form established tumors over a two-week period. After two weeks, intratumoral administration of Chol-R9/VEGF siRNA complexes versus Chol-R9/scrambled VEGF siRNA complexes was performed with 50 micrograms of siRNA per mouse every 4 days for a total of 20 days. Tumor volume was measured throughout the duration of the experiment and showed an ~7-fold reduction in tumor volume by day 17 after the start of siRNA treatments for the Chol-R9/VEGF siRNA compared to the Chol-R9/scrambled VEGF siRNA control. Importantly, no differences were seen with the untreated animals or the Chol-R9 packaged scrambled VEGF siRNA sequence. Additionally, the intratumoral concentration of VEGF was decreased by ~60% as assayed for by ELISA at day 17.

While this siRNA delivery methodology shows successful siRNA delivery *in vitro* and functional induction of RNAi within an *in vivo* model system, the likelihood that this technology will be utilized for anything other than localized delivery is highly unlikely. Cholesterol sequestration by LDL and HDL particles in circulation and subsequent shuttling to the liver will most likely restrict this delivery system from applications of systemic administration [38]. Additionally, intravenous administration of cholesterol-conjugated siRNA duplexes has already been documented and is currently being utilized for *in vivo* targeting to the liver [39]. While knockdown of liver genes may be important for certain applications, administration of Chol-R9/siRNA complexes will most likely have insufficient biodistribution as a systemic siRNA delivery system to anything but the liver. However, this article is a clear demonstration that these Chol-R9/siRNA particles can deliver siRNA *in vitro* and in a locally delivered *in vivo* model system.

Perhaps the most intriguing PTD/CPP packaging scheme to have been documented is the recent use of a cell targeting peptide fused to a poly-arginine peptide motif [40]. Kumar et al. fused a short peptide derived from rabies virus glycoprotein (RVG) to a 9 arginine siRNA packaging peptide (RVG-9R) to specifically target neuronal cells expressing the nicotinic acetylcholine receptor (AChR). Using a peptide to siRNA molar ratio of 10:1, efficient siRNA packaging was obtained from the poly-arginine sequence as observed by loss of free siRNA by gel electrophoresis. When comparing RVG-9R with a control peptide RV-MAT-9R following siRNA complexation, RVG-9R specifically delivered FITC-labelled siRNA to Neuro2a cells while not to HeLa cells. RV-MAT-9R complexed siRNA delivered to neither cell type while Lipofectamine packaged siRNA was successful at delivering to both cell types as determined by flow cytometry analysis of FITC-siRNA uptake. Additionally, the above examples of FITC-labelled siRNA delivery were corroborated by flow cytometry assessment of eGFP knockdown for both HeLa and Neuro2a cells stably expressing eGFP. The RVG-9R peptide not only showed delivery of functional GFP siRNA molecules across the cell membrane, it also did it in a neuronal cell type-specific manner.

To test brain-specific gene silencing *in vivo*, GFP transgenic mice were injected with GFP siRNA complexed with either RVG-9R peptide or RV-MAT-9R control peptide on three consecutive days. Two days after the final treatment, the brain, liver and spleen cells were harvested from the mice and assayed for GFP knockdown by flow cytometry analysis. Surprisingly, RVG-9R complexed GFP siRNA led to significant knockdown of GFP in the brain while not effecting GFP levels in the liver or spleen. Importantly, the control RV-MAT-9R peptide complexed to GFP siRNA displayed no GFP knockdown in any organs assayed. The authors further validated this approach by systemically delivering antiviral siRNA duplexes complexed with RVG-9R to protect mice from encephalitis induced by Japanese encephalitis virus (JEV) infection.

As far as we know, this study is the first published observation showing the systemic administration of siRNA duplexes that can lead to passage across the blood brain barrier and specifically induce an RNAi response in the brain of adult mice. This is also the first example of a cell-specific targeting peptide fused to a cationic siRNA packaging peptide to enhance the delivery of siRNA duplexes. This study shows the utility of PTD/CPPs in siRNA delivery and the efficiency in which they can package siRNA duplexes for *in vivo* delivery applications. This methodology is easily amendable to other cell-targeting peptides and should be pursued to enhance the cell-specific delivery of siRNA within *in vivo* systems.

3.3 Endosomolytic peptides

The main limitation in PTD/CPP-mediated delivery of biologically active molecules is the sequestration and entrapment of the majority of internalized material within endocytic vesicles. In order to circumvent this problem, endosomolytic reagents such as chloroquine can be used

to aid in the rupturing of endosomes and lead to an enhanced effect for PTD/CPP-mediated delivery [1]. Unfortunately, while efficient for cells in culture, this approach is not practical for *in vivo* administration of PTD/CPP-coupled cargo. Importantly, our lab has shown the ability of the endosomolytic peptide HA2 covalently synthesized with TAT peptide to induce release of TAT-coupled cargo from endocytic bodies and help exert a more significant biological effect at lower concentrations [1]. Lundberg et al. have attempted a similar strategy to noncovalent packaging of siRNA duplexes with an endosomolytic peptide that is potentially capable of enhancing the effectiveness of PTD/CPP/siRNA complex delivery by inducing release from endosomes [41].

The published study is a comparative analysis of the ability of multiple PTD/CPPs to noncovalently package siRNA molecules in a manner sufficient to induce cellular internalization and elicit a functional RNAi response. The PTD/CPPs used in the study include bPrPp, MPG, penetratin, TP-10 and the designed endosomolytic EB1 peptide. EB1 peptide is a modified penetratin peptide that has specifically placed histidine insertions that theoretically induce an alpha helical formation upon protonation in the acidic endosome environment. This conformation change could theoretically lead to endosomal disruption and consequently, enhanced endosomal release of functional siRNA cargo.

In order to determine whether or not efficient siRNA delivery was seen with the various PTD/CPPs after noncovalent packaging, FITC-labeled siRNA duplexes were mixed with varying molar ratios of PTD/CPP to determine what ratio was most favorable for cellular uptake. Using an electrophoretic gel shift assay, it was determined that for all PTD/CPPs except for penetratin a 20-fold peptide/siRNA molar ratio was sufficient to completely complex all free siRNA in solution. Penetratin was shown to package all siRNA successfully with a 10-fold peptide/siRNA molar ratio. Upon treatment of HeLa cells with PTD/CPP-packaged siRNA, both bPrPp and penetratin peptide were able to deliver significant amounts of FITC-labeled siRNA to cells in comparison to MPG, TP-10 and the Lipofectamine control. In an *in vitro* system for RNA interference, HeLa cells were transiently transfected with a luciferase gene construct and cells were treated with varying concentrations of PTD/CPP/siRNA noncovalent complexes. While penetratin peptide was shown to deliver the largest amount of FITC-labeled siRNA, these complexes were insufficient to induce RNAi even at the highest concentration of 100 nM as assayed for by decrease in luciferase intensity. For all other peptides tested including MPG, bPrPp and EB1, 100 nM of packaged siRNA was capable of eliciting an RNAi response comparable to 10 nM siRNA complexed with Lipofectamine.

Because the modified penetratin peptide EB1 caused a higher induction of RNAi against luciferase it was hypothesized that the increase in RNAi effectiveness compared to unmodified penetratin is due to enhance endosomal release. However, after a more thorough investigation, it was shown that EB1 has a higher ability to package and deliver siRNA molecules across the cell membrane so it is more likely that the increased efficiency has little to do with enhanced endosomal escape. However, the low efficiency of penetratin-packaged siRNA molecules to induce an RNAi response was enhanced by coinubation with a penetratin-HA2 peptide during cellular treatment. This result is similar to our results with TAT-HA2 peptide enhancing the endosomal escape of TAT-Cre protein [1]. This observation offers hope that efficient endosomolytic peptides can be utilized to increase the ability of noncovalent PTD/CPP/siRNA complexes to successfully deliver biologically active siRNA duplexes across the endosomal membrane.

4. Conclusions

Since the discovery of RNA interference, research groups worldwide have sought to invent efficient delivery systems to enhance the ability of siRNA duplexes to cross the cell membrane

and elicit a biological response. The most widely used siRNA delivery methodologies consist of cationic lipids and cationic polymers that package siRNA into stable nanoparticles capable of translocating across the cellular membrane [27,28]. While only a few studies exist, the noncovalent packaging of siRNA molecules with cationic PTD/CPPs has also been shown to help aid the passage of siRNA duplexes across the cell membrane. The benefit of utilizing PTD/CPPs for siRNA delivery lies within their proven potential to deliver a wide range of macromolecules into a large number of cultured cells and their capacity to facilitate the uptake of macromolecular cargo within *in vivo* applications.

At the correct peptide/siRNA molar ratios, noncovalent complexing of siRNA with PTD/CPPs results in stable nanoparticles that have the capacity to traverse the cellular membrane. This characteristic has been extended upon by attachment of hydrophobic domains (as with MPG), cholesterol and cell-specific targeting ligands, and endosomolytic peptide motifs. While all appear as promising approaches, much is yet to be done to establish PTD/CPPs as efficient siRNA delivery methodologies.

Perhaps the most important issue in the utilization of PTD/CPPs for the intracellular delivery of siRNA molecules is the sequestration of the majority of siRNA cargo within endocytic compartments. Surprisingly, only a couple of attempts have been made to promote efficient PTD/CPP cargo release from endocytic compartments in order to realize the full potential of PTD/CPP delivery systems [1,41]. While the most success has been from the utilization of PTD/CPP-HA2 peptides, there most likely exist multiple technologies that will also be able to show as efficient or better release of endosome-sequestered PTD/CPP cargo.

Along these same lines are the contradictory observations of the internalization mechanism of MPG/siRNA complexes. Initially thought to enter cells in an endocytosis-independent mode of uptake [32], this interpretation has recently been challenged and it has been proposed that MPG/siRNA complexes utilize traditional endocytosis routes in order to gain access to the intracellular environment [35]. Due to the low half maximal inhibitory concentration of MPG/siRNA at ~0.8 nM, it seemed plausible to agree with the conclusion that endocytosis was not a mode of uptake and endosomal sequestration did not play a role in decreasing the effectiveness of MPG-mediated delivery of siRNA. However, with the recent quantification by Veldhoen et al., it now appears that many siRNA molecules have hindered bioavailability when delivered with MPG peptides. The amount of siRNA molecules per cell for MPG-mediated delivery at 10,000 was substantially higher than lipofection of siRNA at 300. With this experimental observation, in addition to vesicular localization by microscopy and inhibition of RNAi by endocytosis inhibitors, it may be that MPG/siRNA complexes indeed traffic into cells through more traditional routes of endocytosis. Unfortunately this conclusion would place MPG peptide in the same category as the majority of other PTD/CPPs, that being intense endosomal sequestration has a significant inhibitory effect on the delivery potential.

Arguably, the most significant methodology that has been developed utilizing the noncovalent siRNA packaging properties of PTD/CPPs is the synthesis of the cell-specific targeting ligand RVG with a poly-arginine motif for siRNA complexing (RVG-9R) [40]. Systemic administration of RVG-9R/siRNA complexes to GFP transgenic mice resulted in siRNA passage across the blood brain barrier and knockdown of GFP within the brains of adult mice while not affecting GFP expression in other organ systems. This simple yet specific siRNA delivery approach could prove to be valuable in multiple other tissue-specific targeting attempts if the cell-specific targeting properties of other peptide ligands could be utilized.

While much work still needs to be done, PTD/CPPs remain potent candidates for the intracellular delivery of siRNA duplexes. With the proven ability to mediate cargo internalization into multiple types of cells *in vitro* and *in vivo* in addition to having routinely

shown internalization into 100% of cells within a given population, PTD/CPPs represent a promising delivery methodology that can have a major impact on the realization of siRNA therapeutics as the field of siRNA delivery matures.

References

1. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat Med* 2004;10:310–315. [PubMed: 14770178]
2. Foerg C, Ziegler U, Fernandez-Carneado J, Giralt E, Rennert R, Beck-Sickinge AG, Merkle HP. Decoding the entry of two novel cell-penetrating peptides in HeLa cells: lipid raft-mediated endocytosis and endosomal escape. *Biochemistry* 2005;44:72–81. [PubMed: 15628847]
3. Lundberg M, Wikstrom S, Johansson M. Cell surface adherence and endocytosis of protein transduction domains. *Mol Ther* 2003;8:143–150. [PubMed: 12842437]
4. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol Ther* 2003;8:284–294. [PubMed: 12907151]
5. Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, Takehashi M, Tanaka S, Ueda K, Simpson JC, Jones AT, Sugiura Y, Futaki S. Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. *Mol Ther* 2004;10:1011–1022. [PubMed: 15564133]
6. Kawamura KS, Sung M, Bolewska-Pedyczak E, Garipey J. Probing the impact of valency on the routing of arginine-rich peptides into eukaryotic cells. *Biochemistry* 2006;45:1116–1127. [PubMed: 16430208]
7. Fischer R, Kohler K, Fotin-Mleczek M, Brock R. A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides. *J Biol Chem* 2004;279:12625–12635. [PubMed: 14707144]
8. Rothbard JB, Jessop TC, Wender PA. Adaptive translocation: the role of hydrogen bonding and membrane potential in the uptake of guanidinium-rich transporters into cells. *Adv Drug Del Rev* 2005;57:495–504.
9. Vives E, Brodin P, Lebleu B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J Biol Chem* 1997;272:16010–16017. [PubMed: 9188504]
10. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. *J Biol Chem* 1994;269:10444–10450. [PubMed: 8144628]
11. Pooga M, Kut C, Kihlmark M, Hallbrink M, Fernaeus S, Raid R, Land T, Hallberg E, Bartfai T, Langel U. Cellular translocation of proteins by transportan. *FASEB J* 2001;15:1451–1453. [PubMed: 11387254]
12. Futaki S, Suzuki T, Ohashi W, Yagami T, Tanaka S, Ueda K, Sugiura Y. Arginine-rich peptides. An abundant source of membrane-permeable peptides having potential as carriers for intracellular protein delivery. *J Biol Chem* 2001;276:5836–5840. [PubMed: 11084031]
13. Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res* 1997;25:2730–2736. [PubMed: 9207018]
14. Wadia JS, Dowdy SF. Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. *Adv Drug Deliv Rev* 2005;57:579–596. [PubMed: 15722165]
15. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001;411:494–498. [PubMed: 11373684]
16. Meade BR, Dowdy SF. Exogenous siRNA delivery using peptide transduction domains/cell penetrating peptides. *Adv Drug Deliv Rev* 2007;59:134–140.
17. Muratovska A, Eccles MR. Conjugate for efficient delivery of short interfering RNA (siRNA) into mammalian cells. *FEBS Lett* 2004;558:63–68. [PubMed: 14759517]
18. Chiu YL, Ali A, Chu C, Cao H, Rana TM. Visualizing a correlation between siRNA localization, cellular uptake and RNAi in living cells. *Chem Biol* 2004;11:1165–1175. [PubMed: 15324818]

19. Davidson TJ, Harel S, Arboleda VA, Prunell GF, Shelanski ML, Greene LA, Troy CM. Highly efficient small interfering RNA delivery to primary mammalian neurons induces microrna-like effects before mRNA degradation. *J Neurosci* 2004;24:10040–10046. [PubMed: 15537872]
20. Jiang T, Olson ES, Nguyen QT, Roy M, Jennings PA, Tsien RY. Tumor imaging by means of proteolytic activation of cell-penetrating peptides. *Proc Natl Acad Sci* 2004;101:17867–17872. [PubMed: 15601762]
21. Turner JJ, Jones S, Fabani MM, Ivanova G, Arzumanov AA, Gait MJ. RNA targeting with peptide conjugates of oligonucleotides, siRNA and PNA, Blood Cells. *Mol Dis* 2007;38:1–7.
22. Moschos SA, Jones SW, Perry MM, Williams AE, Erjefalt JS, Turner JJ, Barnes PJ, Sproat BS, Gait MJ, Lindsay MA. Lung delivery studies using siRNA conjugated to TAT(48–60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjug Chem* 2007;18:1450–1459. [PubMed: 17711319]
23. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998;391:806–811. [PubMed: 9486653]
24. Sontheimer EJ. Assembly and function of RNA silencing complexes. *Nat Rev Mol Cell Biol* 2005;6:127–138. [PubMed: 15654322]
25. Sen GL, Blau HM. Argonaute 2/RISC resides in sites of mammalian decay known as cytoplasmic bodies. *Nat Cell Biol* 2005;7:633–636. [PubMed: 15908945]
26. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004;15:185–197. [PubMed: 15260970]
27. Sioud M, Sorensen DR. Cationic liposome-mediated delivery of siRNAs in adult mice. *Biochem Biophys Res Commun* 2003;312:1220–1225. [PubMed: 14652004]
28. Urban-Klein B, Werth S, Abuharheid S, Czubayko F, Aigner A. RNAi-mediated gene-targeting through systemic application of polyethylenimine (PEI)-complexed siRNA in vivo. *Gene Ther* 2005;12:461–466. [PubMed: 15616603]
29. Song E, Zhu P, Lee SK, Chowdhury D, Kussman S, Dykxhoom DM, Feng Y, Palliser D, Weiner DB, Shanker P, Marasco WA, Lieberman J. Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* 2005;23:709–717. [PubMed: 15908939]
30. McNamara JO, Andrechek ER, Wang Y, Viles KD, Rempel RE, Gilboa E, Sullenger BA, Giangrande PH. Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol* 2006;24:1005–1015. [PubMed: 16823371]
31. Crombez L, Charnet A, Morris MC, Aldrian-Herrada G, Heitz F, Divita G. A non-covalent peptide-based strategy for siRNA delivery. *Biochem Soc Trans* 2007;35:44–46. [PubMed: 17233597]
32. Simeoni F, Morris MC, Heitz F, Divita G. Insight into the mechanism of peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. *Nuc Acids Res* 2003;31:2717–2724.
33. Richard JP, Melikov K, Vives E, Ramos C, Verbeure B, Gait MJ, Chernomordik LV, Lebleu B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J Biol Chem* 2003;278:585–590. [PubMed: 12411431]
34. Lundberg M, Johansson M. Is VP22 nuclear homing an artifact? *Nat Biotechnology* 2001;19:713–714.
35. Veldhoen S, Laufer SD, Trampe A, Restle T. Cellular delivery of small interfering RNA by a non-covalently attached cell-penetrating peptide: quantitative analysis of uptake and biological effect. *Nucleic Acids Res* 2006;34:6561–6573. [PubMed: 17135188]
36. Jones SW, Christison R, Bundell K, Joyce CJ, Brockbank SMV, Newham P, Lindsay MA. Characterization of cell-penetrating peptide-mediated peptide delivery. *Br J Pharmacol* 2005;145:1093–1102. [PubMed: 15937518]
37. Kim WJ, Christensen LV, Jo S, Yockman JW, Jeong JH, Kim YH, Kim SW. Cholesteryl oligoarginine delivering vascular endothelial growth factor siRNA effectively inhibits tumor growth in colon adenocarcinoma. *Mol Ther* 2006;14:343–350. [PubMed: 16765648]
38. Wolfrum C, Shi S, Jayaprakash KN, Jayaraman M, Wang G, Pandley RK, Rajeev KG, Nakayama T, Charrise K, Ndungo EM, Zimmerman T, Koteliensky V, Manoharan M, Stoffel M. Mechanisms and

- optimization of in vivo delivery of lipophilic siRNAs. *Nat Biotech.* September 16;2007 (Epub ahead of print)
39. Soutschek J, Akinc A, Bramlage B, Charisse K, Constien R, Donoghue M, Elbashir S, Geick A, Hadwiger P, Harborth J, John M, Kesavan V, Lavine G, Pandey RK, Racie T, Rajeev KG, Rohl I, Toudjarska I, Wang G, Wuschko S, Burncrot D, Koteliensky V, Limmer S, Manoharan M, Vornlocher HP. Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 2004;432:173–178. [PubMed: 15538359]
40. Kumar P, Wu H, McBride JL, Jung KE, Kim MH, Davidson BL, Lee SK, Shankar P, Manjunath N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* 2007;448:39–43. [PubMed: 17572664]
41. Lundberg P, El-Andaloussi S, Sutlu R, Johansson H, Langel U. Delivery of short interfering RNA using endosomal cell-penetrating peptides. *FASEB J* 2007;21:2664–2671. [PubMed: 17463227]