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A Novel p19 Fusion Protein as a Delivery Agent for Short-interfering RNAs

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RNA interference (RNAi) is the biological mechanism that allows targeted gene knockdown through the addition of exogenous short-interfering RNAs (siRNAs) to cells and organisms. RNAi has revolutionized cell biology and holds enormous potential for human therapy. One of the major challenges facing RNAi as a therapy is achieving efficient and nontoxic delivery of siRNAs into the cell cytoplasm, since their highly anionic character precludes their passage across the cell membrane unaided. Herein, we report a novel fusion protein between the tombusviral p19 protein, which binds siRNAs with picomolar affinity, and the "TAT" peptide (RKKRRQRRRR), which is derived from the transactivator of transcription (TAT) protein of the human immunodeficiency virus and acts as a cell-penetrating peptide. We demonstrate that this fusion protein, 2x-p19-TAT, delivers siRNAs into the cytoplasm of human hepatoma cells where they elicit potent and sustained gene knockdown activity without toxic effects.

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Introduction

RNA interference (RNAi) is a gene silencing mechanism critical for endogenous gene regulation and host–pathogen interactions in a variety of organisms.[1] In this process, small double-stranded RNA molecules inhibit gene expression post-transcriptionally in a sequence-specific manner. Short-interfering RNAs (siRNAs) are 19-bp duplexes with 3′ 2-nucleotide overhangs and 5′ phosphates which associate with cellular Argonaute proteins to form the RNA-induced silencing complex, which then targets complementary RNA sequences for inhibition of gene expression.[1]

RNAi is currently an indispensable tool in biotechnology as siRNAs can be made synthetically, designed to target any gene of interest, and added exogenously to cells or organisms to knockdown target gene expression. siRNAs are also promising as therapeutics, as they are potentially able to knockdown expression of any disease-causing or viral genes.[2] siRNA-based therapeutics have been hindered because they are highly anionic molecules that do not readily cross the cell membrane unaided.[2–4] Effective delivery agents for siRNAs are being pursued for applications in therapy and biotechnology. The standard method of transfecting siRNA into cells *in vitro* involves lipofection (*i.e.*, Lipofectamine, RNAiMAX); however, these reagents are associated with substantial cytotoxicity. For therapeutic applications, viral vectors and variety of nonviral approaches are being investigated; including lipid–siRNA conjugation, stable nucleic acid lipid particles, polymers, aptamers, nanoparticles, and antibodies.[3]

There is an emerging field of cell penetrating peptide (CPP)-mediated siRNA delivery for both *in vitro* and *in vivo* applications.[5–7] CPPs are highly cationic short peptides that facilitate cell entry of attached cargo through association with cell surface glycans and uptake via macropinocytosis, a type of fluid-phase endocytosis used by all cell types.[8,9] CPPs can have a range of sequences. Peptides consisting of eight or nine arginine residues ("R8, R9") have been reported and widely used for cell entry technologies.[10] CPPs can also be derived from natural sequences such as the "TAT" CPP, derived from amino acids 49–57 of the HIV-1 TAT protein (RKKRRQRRR).[11,12] The TAT peptide has been widely used for mediating cell entry of fusion proteins and other cargos with high efficiency and without cytotoxicity.[13,14] One caveat of CPP-mediated cell entry is that the material can be trapped in the endosome, precluding full delivery to the cytoplasm unless an endosomolytic agent is applied simultaneously.[15] With this in mind, these peptide sequences have still been widely applied to allow cell entry of a variety of cargo and hold therapeutic potential.[10,16]

In this work, we report a novel fusion protein between a CPP and a siRNA-binding protein, p19, to function as a siRNA delivery agent. The 19-kDa protein, p19, is expressed by tombusviruses to bind viral-derived siRNAs and prevent their incorporation into the RNA-induced silencing complex, thereby suppressing the RNA silencing pathway in the host plant.[17] The p19 protein exhibits the highest reported affinity for siRNA duplexes (K_d ~ 0.2 nmol/l) and is able to bind siRNAs of any sequence, as its binding specificity is based on the length of the duplex.[18] The reported crystal structures of p19 in complex with siRNA demonstrate how p19 is able to act as a "molecular calliper" for siRNA.[18,19] As a dimer, p19 interacts with the sugar phosphate backbone of the siRNA and two N-terminal tryptophan residues on each monomer

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provide end-capping interactions with the terminal nucleotide of a 19-bp duplex, allowing for size-specific binding. Importantly, p19 does not bind DNA or single-stranded RNA.[18]

Recombinant p19 proteins with novel properties have been described, such as a p19-linked dimer fusion protein where two monomers are fused by a semirigid linker sequence, which displays improved affinity for siRNA and higher thermostability than wild-type p19.[20] Herein, we have fused a CPP sequence "TAT" to this linked dimer construct to allow cell entry of the p19:siRNA complex. In this work, we observe that the fusion protein 2x-p19-TAT is an effective delivery agent for siRNA, allowing for potent gene knockdown in human cells without cytotoxic effects. In addition, we investigate the role of endosome entrapment in the delivery process, and conclude that the 2x-p19-TAT approach would be more potent with the addition of an endosomolytic agent.

Results

Designing p19 as a siRNA delivery agent

In designing the p19 protein as a siRNA delivery agent (**Figure 1**), we built upon a p19 construct previously described where two p19 monomers are connected by a semirigid linker, resulting in improved thermostability and higher affinity for siRNA than wild-type p19.[20] To allow cell entry of the linked p19 dimer, we fused the CPP "TAT" to the C-terminus of the construct. This "2x-p19-TAT" construct also contains a C-terminal 8-histidine tag for affinity purification. The fusion protein 2x-p19-TAT expressed well in *Escherichia coli* and was purified by nickel affinity and size exclusion chromatography.

p19 fusion protein binds siRNA with high affinity

We hypothesized that p19's high affinity for siRNA would be an asset for its development as a siRNA delivery agent. Therefore, following expression and purification of the 2x-p19-TAT fusion protein, we analyzed its ability to bind siRNAs by fluorescence-based electrophoretic mobility shift assay (**Figure 2a**). We observed that the addition of the TAT domain to the 2x-p19 construct did not interfere with its ability to bind siRNA with high affinity. We determined the dissociation constant (K_d) of 2x-p19-TAT for fluorescently labelled siRNA as 0.44 ± 0.16 nmol/l (**Figure 2d**). We also designed a fusion protein to serve as a negative control, which would distinguish between delivery of p19-bound siRNAs and siRNAs that are electrostatically associated with the cationic TAT peptide. We have previously described the p19-Y73S mutation which completely abrogates siRNA binding.[21] We linked Y73S monomers into a linked dimer construct (2x-Y73S) in the same manner as the 2x-p19 construct and confirmed that the linked Y73S dimer remains incapable of binding siRNA (**Figure 2b**). When the TAT peptide is added to the C-terminus (**Figure 2c**), the fusion construct is able to bind siRNA with low affinity (K_d = 1.1 μmol/l) (**Figure 2e**).

p19 fusion protein delivers siRNA in human hepatoma cells

To characterize the ability of 2x-p19-TAT to deliver siRNA, we first used live-cell fluorescence microscopy to monitor the entry of fluorescently labeled siRNA complexed with 2x-p19-TAT into human hepatoma (Huh7) cells (**Figure 3**). The cells treated with 2x-p19-TAT:Cy3-siRNA complexes for 5 hours display cytoplasmic Cy3-fluorescence (**Figure 3a**) that is not present in control samples where Cy3-siRNA alone was added to the cells (**Figure 3b**). We confirm that this treatment maintains cytoplasmic fluorescence after 48 hours (**Figure 3c**). This indicates that 2x-p19-TAT is allowing cell entry of the Cy3-siRNA. The cell entry is mediated specifically by the TAT domain, as when cells are treated with 2x-p19:Cy3-siRNA complex, we do not see any internalized fluorescence signal (**Figure 3d**) Importantly, we observed this cytoplasmic fluorescence in all of the cells treated, thus 2x-p19-TAT delivers siRNA with high transfection efficiency.

To demonstrate that 2x-p19-TAT-mediated siRNA delivery results in gene knockdown, we employ a luciferase reporter assay. We delivered siRNA targeting the firefly luciferase mRNA to Huh7 cells expressing a dual-luciferase reporter vector (psiCHECK-2) and monitored the response via dual luciferase assays (**Figure 4**). The results show that 2x-p19-TAT complexed with luc2 siRNA allows for knockdown of firefly luciferase expression, where after 24 hours the normalized firefly/renilla luciferase signal is reduced to ~40% of the mock samples and after 72 hours show a further reduction to ~4% of the mock signal. Adding the fusion protein alone, without

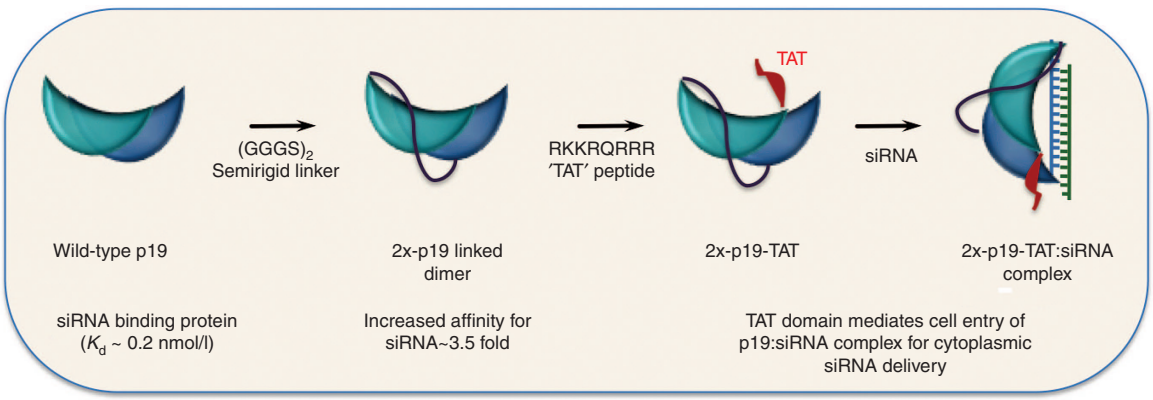

Figure 1. Developing the 2x-p19-TAT fusion protein for siRNA delivery. Linking p19 monomers with a semirigid linker to give a linked p19 dimer construct provides improved thermostability and higher affinity for siRNA.[20] For siRNA delivery, a fusion with the cationic TAT peptide on the C-terminus of the linked dimer allows cell entry of the p19:siRNA complex. siRNA, short-interfering RNA; TAT, transactivator of transcription.

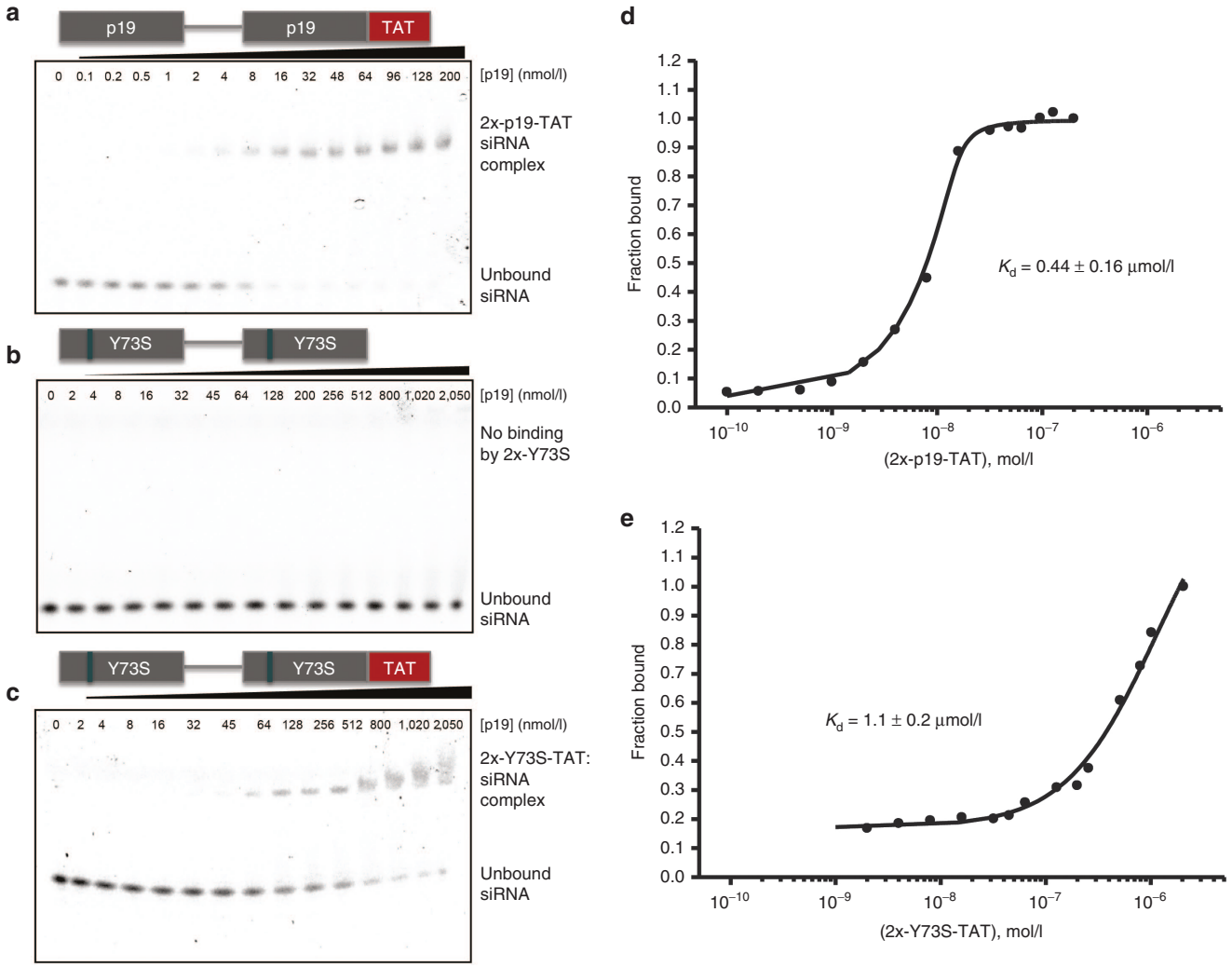

Figure 2. Binding affinity determination of p19 fusion proteins by electrophoretic mobility shift assay. (**a**) Diagram of the p19-based siRNA delivery construct, consisting of a linked p19 dimer with C-terminal TAT domain. EMSA between 2x-p19-TAT and fluorescently labelled (Cy3) siRNA. Cy3-siRNA (2 nmol/l) is incubated with increasing concentrations of 2x-p19-TAT (0–0.2 µmol/l). As the concentration of p19 fusion protein increases, the siRNA mobility is altered due to p19 binding. (**b**) Construct diagram of 2x-Y73S. The Y73S mutation has been shown to prevent p19 from binding siRNA.[21] EMSA assay between the linked dimer construct 2x-Y73S (0–0.2 µmol/l) and Cy3-siRNA (2 nmol/l) indicates that the linked Y73S dimer remains unable to bind siRNA. (**c**) Construct diagram of 2x-Y73S-TAT, where a C-terminal TAT domain has been added to the linked Y73S dimer from **b**. EMSA between 2x-Y73S-TAT (0–0.2 µmol/l) and Cy3-siRNA (2 nmol/l) indicates low-affinity binding between the construct and RNA, attributable to electrostatic interactions between the TAT domain and the siRNA. (**d**) Binding affinity determination through plotting fraction of RNA bound versus molar concentration gives a dissociation constant (K_d) of 0.44 nmol/l of 2x-p19-TAT for siRNA. (**e**) Binding affinity determination of the low-affinity interaction between 2x-Y73S-TAT and siRNA gives a K_d = 1.1 µmol/l. EMSA, electrophoretic mobility shift assay; siRNA, short-interfering RNA; TAT, transactivator of transcription.

siRNA treatment, does not result in a substantial effect on the normalized luciferase signal. We also examine the specificity of the knockdown response by treating with the fusion protein and a negative control, nontargeting siRNA. Importantly, we observe no knockdown response when we perform the same assay with the 2x-p19-Y3S-TAT construct complexed with luc2 siRNA and applied to the cells for 72 hours (**Figure 4b**). Given that the Y73S mutation to p19's binding site abrogates its ability to bind siRNA, this result suggests that low-affinity interactions between the TAT domain and siRNA are not sufficient to allow gene knockdown activity, and the high-affinity interactions between p19 and siRNA are required for this activity. These results demonstrate that 2x-p19-TAT functions as a siRNA delivery agent as it allows for potent and sustained gene knockdown in human cells.

p19 fusion protein is a noncytotoxic delivery agent

As cytotoxicity is a problem with many siRNA delivery agents, we sought to investigate whether siRNA delivery using the 2x-p19-TAT fusion protein is associated with any cytotoxicity. We utilized MTT assays to report on cell viability over the course of a 72-hour treatment (**Figure 5**). These assays demonstrate that the p19 fusion protein is noncytotoxic, as the viability of the cells is comparable to the untreated control sample even after 72 hours of treatment. In contrast, transfecting the siRNA using a cationic

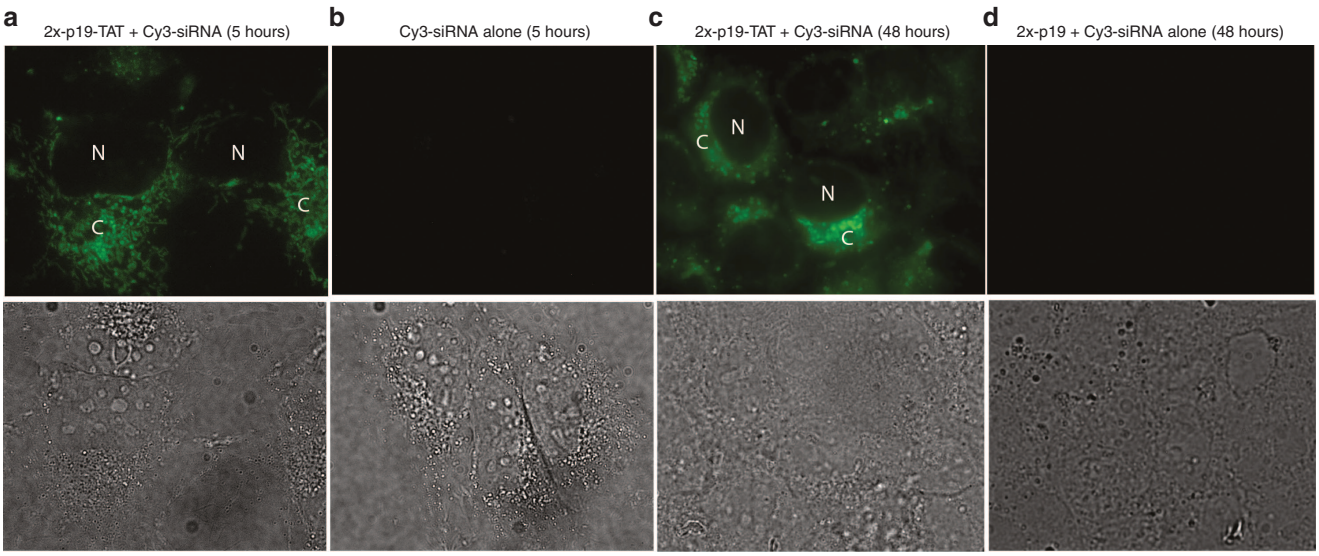

Figure 3. Live-cell fluorescence imaging of 2x-p19-TAT delivery of Cy3-siRNA in Huh7 cells. (**a**) Cells treated with 2x-p19-TAT:Cy3-siRNA for 5 hours show cytoplasmic fluorescence indicating cell entry of Cy3-siRNA. (**b**) Cells treated with the control treatment of Cy3-siRNA alone do not show any fluorescence signal, indicating no cell entry of the naked siRNA. (**c**) 2x-p19-TAT + Cy3-siRNA for 48 hours display cytoplasmic fluorescence. (**d**) Cells treated with the control treatment of 2x-p19 + Cy3-siRNA for 48 hours do not show any fluorescence, indicating that the TAT domain fusion protein is required for cell entry of the complex. siRNA, short-interfering RNA; TAT, transactivator of transcription.

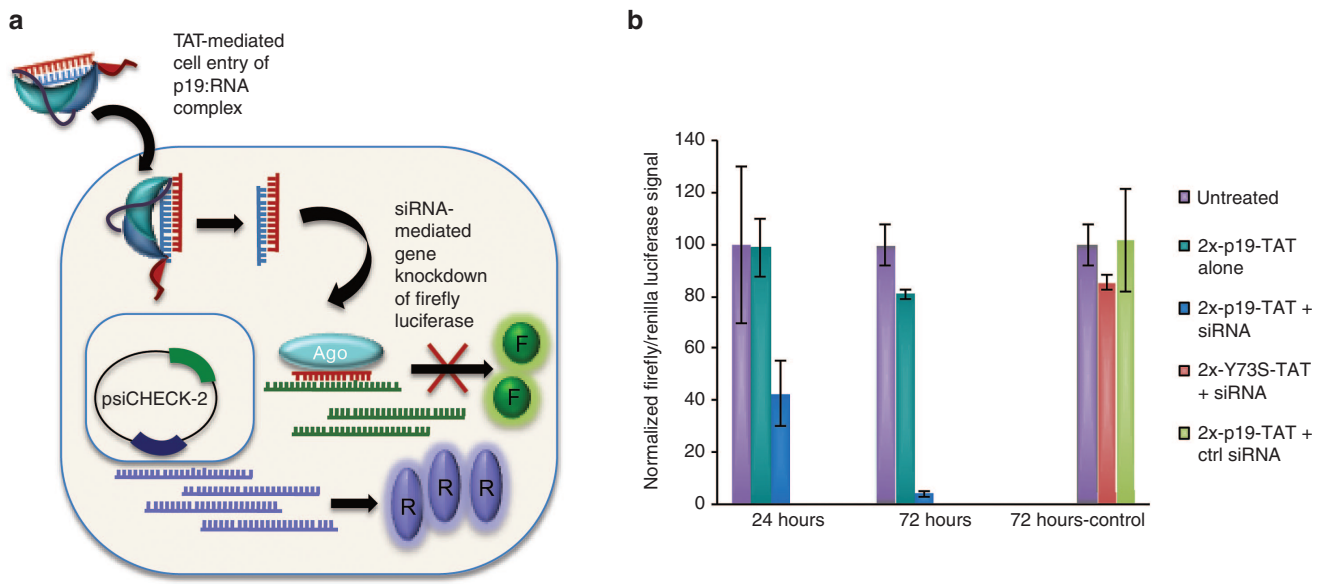

Figure 4. 2x-p19-TAT delivers siRNA for gene knockdown in human cell culture. (**a**) Schematic depicting the experimental design, where 2x-p19-TAT-mediated delivery of siRNA targeting firefly luciferase into Huh7 cells expressing a dual luciferase reporter vector (psiCHECK-2). (**b**) Treatments of 2x-p19-TAT alone or 2x-p19-TAT:siRNA complexes were applied for 24 or 72 hours until cells were harvested for dual luciferase assays, which assessed the level of siRNA-mediated knockdown of firefly luciferase as compared with the renilla control. Treatments indicate potent and sustained gene knockdown activity of 2x-p19-TAT in human cells. Further control treatments assess the specificity of gene knockdown after 72 hours. The wild-type p19 delivery construct, 2x-p19-TAT, is complexed with a nontargeting control siRNA ("ctrl siRNA") and does not show knockdown activity. To delineate the role of p19's high affinity binding for siRNA versus electrostatic interactions between the TAT domain and siRNA, we employ a control fusion protein (2x-Y73S-TAT), where p19 is incapable of binding siRNA but which maintains low-affinity interactions between TAT and siRNA. We apply 2x-Y73S-TAT with siRNA targeting firefly luciferase for a 72-hour treatment and do not observe knockdown activity. siRNA, short-interfering RNA; TAT, transactivator of transcription.

lipid-based transfection reagent RNAiMAX (Invitrogen, Burlington, Ontario, Canada) is associated with substantial cytotoxicity after 72 hours, resulting in nearly 80% decrease in cell viability.

Enhancing delivery efficiency through endosomolysis

It has been reported that cell-penetrating peptide-based methods of cell delivery are limited by endosomal entrapment after cellular uptake.[15] Endosomal entrapment can be overcome by

applying cell-penetrating, endosomolytic peptides as a cotreatment, which has been applied to enhance cytoplasmic delivery of proteins to live cells.[8,22,23] In response to the shift to acidic pH which occurs with endosome maturation, peptides derived from the N-terminal 20 residues of influenza hemagglutinin-2 undergo a conformational change that allows a central hinge region to become amphipathic and insert into the membrane, thus disrupting the endosomal membrane and allowing pore formation.[24] The E5 peptide is derived from influenza hemagglutinin-2, but has been optimized for solubility and endosomolytic activity[25,26] and the E5-TAT peptide cotreatment has been demonstrated to be an effective and nontoxic cell treatment.[22] Thus, we applied the E5-TAT peptide as a cotreatment to our delivery assay to enhance 2x-p19-TAT-mediated siRNA delivery to the cytoplasm (**Figure 6a**). In this assay, we apply 2x-p19-TAT complexed with luc2 siRNA targeting firefly luciferase to cells expressing the dual luciferase reporter vector (psiCHECK-2) as performed previously, but in this assay we add 10 µmol/l E5-TAT to the cell culture media. After a 24-hour treatment, we assay the gene knockdown activity via dual luciferase assays. With the E5-TAT cotreatment, we observe an enhanced potency of gene knockdown activity of the firefly luciferase reporter relative to the renilla control, after a 24-hour treatment (**Figure 6b**). We observe that the 24-hour treatment of 2x-p19-TAT with siRNA displays a range of gene knockdown activity, as there is a variable level of gene knockdown seen at the 24-hour point between **Figures 6b** and **4b**. We observe consistent and robust knockdown at the 72-hour time point. We thus observe that the addition of the E5-TAT peptide allows for a more efficient gene knockdown activity, as the extent of knockdown at 24 hours with the E5-TAT peptide is as robust as the 72-hour activity observed in **Figure 4b** without the addition of the peptide.

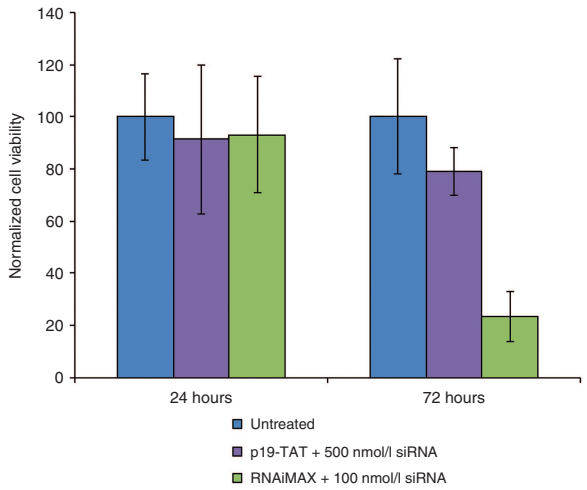

Figure 5. Cell viability assays indicate 2x-p19-TAT mediated siRNA delivery is non-toxic. MTT assays reporting on cell viability following 2x-p19-TAT or RNAiMAX-mediated siRNA delivery for 24 and 72 hours. The 2x-p19-TAT:siRNA treatment does not cause any substantial decreases in cell viability whereas RNAiMAX shows substantially reduced cell viability after 72 hours. RNAi, RNA interference; siRNA, short-interfering RNA; TAT, transactivator of transcription.

Discussion

In this work, we report a novel fusion protein between the p19 protein and the TAT peptide for use as a siRNA delivery agent. The addition of the cationic "TAT" peptide to the linked dimer construct, 2x-p19, does not alter the protein's ability to

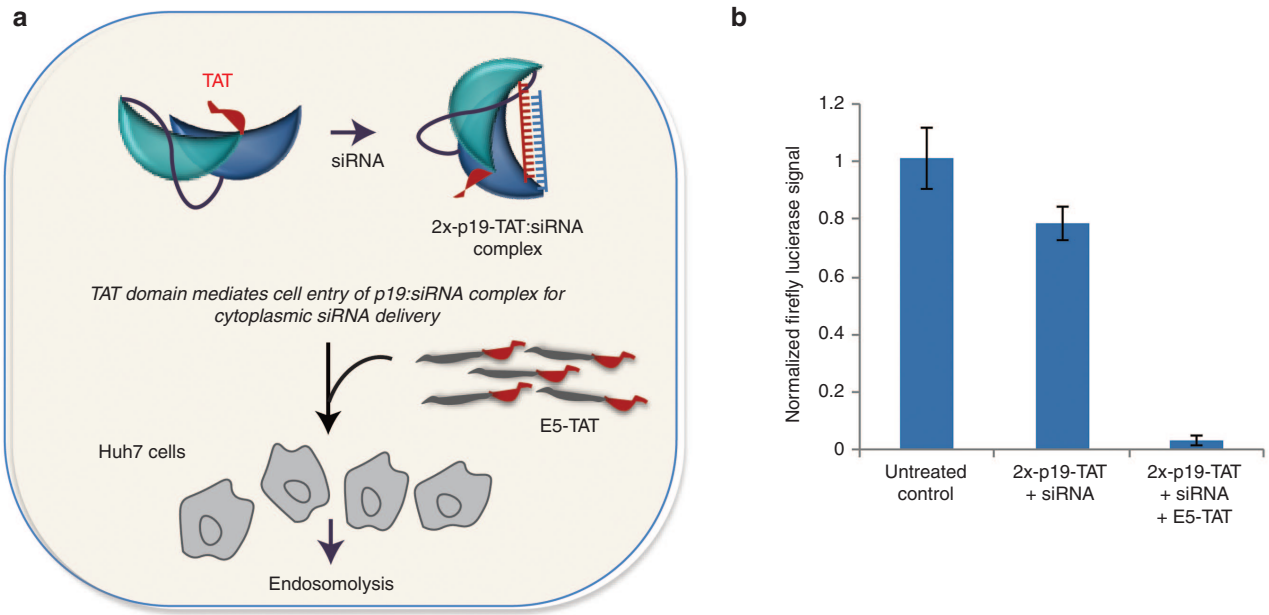

Figure 6. 2x-p19-TAT mediated siRNA delivery is improved by co-incubating endosomolytic peptide. (**a**) Experimental approach for coincubating cells with endosomolytic peptides (E5-TAT) and p19:siRNA complexes to enhance cytoplasmic delivery of siRNA. (**b**) Gene knockdown reporter assay via dual luciferase assays assesses the effect of enhanced endosomolysis on the gene knockdown activity of 2x-p19-TAT treatment in Huh7 cells expressing a dual luciferase reporter vector (psiCHECK-2). A 24-hour treatment of 2x-p19-TAT in complex with siRNA targeting the firefly luciferase gene is performed with and without the coincubation of 10 µmol/l E5-TAT peptide. The additional treatment with the E5-TAT peptide enhances gene knockdown activity of the 2x-p19-TAT treatment. siRNA, short-interfering RNA; TAT, transactivator of transcription.

bind siRNA with high affinity (K_d = 0.44 nmol/l, **Figure 2a**,**d**). This dissociation constant is only slightly altered from that of wild-type p19 (K_d = 0.2 nmol/l).[18,21,27] We demonstrate that 2x-p19-TAT is able to deliver siRNA into Huh7 cells through the use of fluorescence microscopy and genetic reporter assays. Imaging the delivery of fluorescently labelled siRNA demonstrates that 2x-p19-TAT facilitates entry of siRNA into the cell (**Figure 3**). Furthermore, this fluorescence was observed in all of the cells treated, reflecting that 2x-p19-TAT allows siRNA delivery with high transfection efficiency. Here, we demonstrate the effectiveness of 2x-p19-TAT in Huh7 cells, but we hypothesize that it will also be effective in a variety of cell types, including primary cells; just as other TAT-fusion proteins have been demonstrated to be noncell type dependent.[14] The genetic reporter assays demonstrate 2x-p19-TAT-mediated siRNA delivery results in potent gene knockdown that is sustained over 72 hours (**Figure 4**). The level of knockdown observed is comparable to that of standard cationic lipid transfection reagents. Importantly, 2x-p19-TAT achieves this potent gene knockdown activity without toxicity, in contrast to the stark toxicity observed with lipofection (**Figure 5**). This observation is in agreement with other approaches for TAT-mediated siRNA delivery that are also nontoxic.[28] We also explore the role of endosome entrapment of the p19:siRNA complex, and observe that knockdown activity is improved through coincubating the p19:siRNA complex with the E5-TAT endosomolytic peptide. This observation supports previous reports that TAT-mediated protein transduction to the cytoplasm is limited by endosome entrapment,[15] and so potency and efficiency of this technology would thus greatly benefit from targeting this limiting step in the delivery process.

The p19 protein has been employed in several systems for investigating small RNA biology, including human cells.[21,29–31] The utility of p19 has been further enhanced through mutations and protein fusions.[21,32–35] As reported previously, linking two p19 monomers with a flexible linker gives the protein a higher thermostability and a higher affinity for its bound siRNAs, which are generally beneficial properties for p19's development as a biotechnology tool.[29,32]The linked dimer construct, 2x-p19, gives the opportunity to genetically fuse a single tag or fusion protein to the functional dimer unit, giving unique opportunities for p19-based technology. For example, a cyan-fluorescent protein fused to the linked dimer functions as Forster resonance energy transfer reporter of Cy3-labeled siRNAs.[32] In this study, we generate a dimer construct with one fused TAT domain on the C-terminus of the linked p19 dimer. We demonstrate that this one domain is sufficient to allow cell entry of p19 with its bound RNA and have thus avoided unnecessarily employing two cell-entry domains. Furthermore, the addition of a single tag to the functional dimer unit avoids potential deleterious effects on protein stability from having two tags per dimer. As described by the available crystal structures, p19 forms a tail-to-tail homodimer, making the C-termini of the monomers closely associated. The addition of a highly charged domain such as the TAT peptide to this terminus of the monomer construct would likely result in electrostatic clashes, which could negatively impact p19 folding or the dimerization activity. Therefore, the base construct 2x-p19 employed in this study affords unique opportunities in p19's development as a biotechnology tool and as an siRNA delivery agent.

The first description of a protein-based siRNA delivery approach was a fusion protein between a dsRNA binding domain and three consecutive TAT domains, allowing for potent and noncytotoxic siRNA delivery *in situ* and *in vivo*.[28,36] The novelty of this approach was that fusing a CPP to a RNA-binding moiety circumvented the charge neutralization that has negatively impacted other CPP-mediated siRNA delivery strategies.[16] Additionally, the TAT-dsRNA binding domain fusion protein binds siRNAs of any sequence and thus is broadly applicable. Our approach using the p19 protein shares these two assets with TAT-dsRNA binding domain, and may also benefit from p19's unique binding characteristics for small RNAs. p19's high affinity and its mechanism of binding siRNA are likely advantages that will aid in developing p19 as an *in vivo* siRNA delivery agent.

Two other approaches have been described that also apply recombinant p19 fusion proteins for siRNA delivery into human cells. The first employs p19 linked to capsid proteins derived from the Hepatitis B virus, to create a macromolecular siRNA carrier with many p19 molecules on the inside of the larger capsid structure. Integrin binding peptides are fused to the capsid proteins, allowing targeting to cancer cells and subsequent cytoplasmic delivery of siRNA.[37,38] A second approach employs p19 fused to a ephrin mimetic peptide (YSA), allowing targeting of p19:siRNA complexes to EphA2 receptors on cancer cells,[39] and subsequent cytoplasmic delivery of siRNA. Our work is the first to employ the linked p19 dimer construct for siRNA delivery, which serves as a useful approach as p19 exists as a functional dimer, and by recombinantly linking the two proteins we observe enhanced properties including higher thermal stability and higher affinity for siRNA.[20] Working with the recombinantly linked dimer construct affords the ability to make mutations or alterations to a single site of the functional dimer unit. These reports along with this study add to a growing body of applications of p19 for siRNA delivery.

Due to p19's very high affinity for siRNA, the question arises of how it would release the siRNA upon cell entry to allow the siRNA to associate with the RNA-induced silencing complex. This is especially interesting given that p19's endogenous function during viral infection is largely attributed to its ability to sequester siRNAs away from Argonaute proteins. There are two explanations for the release of siRNA by p19 upon cell entry. Firstly, we hypothesize that in the context of siRNA delivery the equilibrium will be shifted in favor of Argonaute proteins. During viral infection, p19 is expressed at high levels to outcompete Argonaute, but it cannot deprogram an already loaded RNA-induced silencing complex.[40] As a siRNA delivery agent, there will eventually be less p19 than Argonaute proteins in the cytoplasm, allowing Argonaute proteins to outcompete p19 for siRNA. Furthermore, kinetic studies have demonstrated that p19 exhibits highly reversible binding of siRNA, characterized by rapid binding and marked dissociation.[27] Secondly, TAT-mediated transduction involves an initial trapping of the material in intracellular endosomes that undergo acidification.[16] We have previously reported that p19's binding of siRNAs is pH dependent and is optimal at neutral pH,[41] therefore it is likely that the drop in pH in the endosome promotes dissociation of siRNA from p19.

In this study, we have engineered the p19 protein, which has naturally evolved to bind siRNAs with extremely high affinity, for use as a siRNA delivery agent through fusion with a TAT peptide. This novel fusion protein can be applied to deliver any siRNA of interest and has advantages over current strategies for *in situ* gene knockdown and awaits further development for *in vivo* siRNA delivery. Fusion proteins have only begun to be explored for siRNA delivery agents; however, it is clear they offer unique advantages over other delivery technologies including small size of the complex, simple generation, and opportunity for fusions with cell-targeting peptide domains.[42] The technology reported here adds to the growing field of creative strategies for overcoming the largest challenge facing siRNA therapeutics.

MATERIALS AND METHODS

DNA oligonucleotides and siRNAs. All DNA oligonucleotides used in the cloning experiments were purchased from Sigma GenoSys (Oakville, Ontario, Canada) and purified by reverse-phase cartridge purification. All RNAs were synthesized by Dharmacon (Lafayette, CO), purified by polyacrylamide gel electrophoresis, and desalted using reverse-phase high pressure liquid chromatography. The purity was demonstrated to be >95% according to the manufacturer's specifications. The duplexed siRNAs used in the electrophoretic mobility shift assay have the following sense and antisense sequences respectively: Cy3-CSK siRNA (21-mer) 5′-Cy3-CUACCGCAUCAUGUA CCAUdTdT-3′ and 5′-AUGGUACAUGAUGCG GUAGdT dT-3′. The siRNA used for monitoring gene knockdown via luciferase assays "luc2" siRNA, targeting the synthetic firefly luciferase gene in the psiCHECK-2 vector (Promega, Madison, WI): 5′-GGACGAGGACGAGCACUU-3′ and 5′-GAA GUGCUCGUCCUCGUCCUU-3′. Controls for gene knockdown studies employed negative control siRNA (*Silencer* Negative Control No.1 siRNA (AM4635, ThermoFisher Scientific).

Cloning 2x-p19-TAT fusion protein. Cloning of the Carnation Italian Ringspot Virus p19 protein as a linked dimer fusion in the pTriEx plasmid was described previously.[20] The 2x-p19-TAT fusion protein was created by PCR amplification of the p19 monomer with an N-terminal linker and C-terminal TAT sequence with by generating PCR primers to contain the TAT sequence at the C-terminus of the linker-p19 monomer, with a terminal 8-histidine tag flanked by Xho1 restriction sites. The forward primer used was: TTAG CTC GAG GGC GGC GGC GGC TCC GGC GGC GGC GGC TCT ATG GAA CGC GCT ATC CAA G and the reverse primer used was: TTAG CTC GAG GCG GCG GCG CTG GCG GCG TTT CTT GCG CTC GCT TTC TTT CTT GAA G. The PCR product was then digested with the Xho1 restriction enzyme and inserted into pTriEx vector containing the p19 monomer. The resulting construct "2x-p19-TAT" was confirmed by sequencing. The nonbinding p19-Y73S control construct was previously described[21] and the 2x-Y373S-TAT construct was cloned using the same primers and restriction enzymes as stated above for the wild-type 2x-p19-TAT construct.

Protein expression and purification. Bacterial expression of 2x-p19-TAT was performed using *E. coli* strain BL21 (DE3), which were grown at 37 °C in LB + 100 µg/ml ampicillin until an OD_{600} of 0.5–0.6, induced with 1mmol/l IPTG, grown for an additional 3 hours at 25 °C. After harvesting, bacterial pellets were resuspended in 50 mmol/l Tris, 300 mmol/l NaCl, 10 mmol/l imidazole, 1 mmol/l dithiothreitol and lysed by sonication on ice bath. Cell lysate was then centrifuged at 20,000×*g* for 20 minutes at 4 °C. The proteins were purified by gravity affinity chromatography using Ni-NTA Fast Flow (GE Healthcare, Piscataway, NJ), the resin washed with 10 column volumes of 50 mmol/l Tris, 300 mmol/l NaCl, 80 mmol/l imidazole, pH 7.0, and then eluted with 5 CV of 50 mmol/l Tris, 300 mmol/l NaCl, 250 mmol/l imidazole, pH 7.0, with 1 mmol/l of dithiothreitol added immediately to the eluate. The eluate was then purified via a Superdex200 size exclusion column (GE Healthcare, Piscataway, NJ). Fractions containing the desired p19 proteins, as determined by sodium dodecyl sulfate polyacrylamide electrophoresis analyses, were pooled and stored at 4 °C for subsequent assays.

Electrophoretic mobility shift assay and data analysis. For electrophoretic mobility shift assay binding experiments samples were prepared by incubating 2 nmol/l Cy3-labelled siRNAs with 0–2 µmol/l of purified 2x-p19-TAT in 20 mmol/l Tris, 100 mmol/l NaCl, 1 mmol/l EDTA, 0.02% v/v TritonX-100, 2 mmol/l dithiothreitol, pH 7 for 1 hour at room temperature. The samples were then analyzed by electrophoresis, where 5× Tris/Borate/EDTA (TBE) sample buffer (90 mmol/l Tris, 90 mmol/l boric acid, 2 mmol/l EDTA, 15% Ficoll type 400, 0.02% xylene cyanol) was diluted to 1× in the binding reaction and then 10 µl applied to a 6% TBE gel and electrophoresed at a constant voltage of 100 V for 50 minutes in 0.5× TBE buffer (Novex, Invitrogen Waltham, MA). The gels were imaged with Fluorescent Method Bio Image Analyzer III (Hitachi, Japan) and densitometry performed with ImageJ software (NIH, United States). The fraction of RNA bound by p19 was determined by dividing the band intensity of p19-bound RNA by the sum of the band intensities from the complex and unbound RNA. The data were analyzed by plotting the fraction bound values against p19 concentration and fitted using GraphPad Prism 4 according to the following equation (Eq. 1):

$$\Delta P = \Delta P_{\text{Max}} \left(\frac{K_d + np + x}{2np} - \sqrt{\left(\frac{K_d + np + x}{2np} \right)^2 - \frac{x}{np}} \right) \quad (1)$$

where ΔP denotes the change in fluorescence intensity, ΔP_{Max} is the maximal change in fluorescence intensity, K_d is the dissociation constant, n is the number of equivalent sites on the p19 dimer, p is the concentration of labelled small RNA, and x is the concentration of the p19 dimer.

Cell culture. Huh7 cells were grown at 37 °C with 5% CO_2, in DMEM (Gibco-Invitrogen) with 10% fetal bovine serum, 100 nmol/l nonessential amino acids, 50 U/ml penicillin, and 50 µg/ml streptomycin.

Gene knockdown in human cell culture. Purified 2x-p19-TAT was concentrated using Amicon Ultra 10-kDa MWCO

centrifugal filter device (Millipore, Concord, MA) to 100 µmol/l as determined by the DC protein assay (Bio-Rad, Hercules, CA) and then complexed with luc2 siRNA (Thermo Scientific Dharmacon) with the sequence 5′-gga cga gga cga gca cuu cuu-3′,in a 10:1 molar ratio for 45 minutes at room temperature and applied to cells in reduced-serum transfection medium (Optimem, Invitrogen) at a final siRNA concentration of 500 nmol/l. After 4 hours, the cells were recovered by adding DMEM with 20% fetal bovine serum. For the treatments coincubated with the E5-TAT peptide (GLFEAIAEFIENGWEGLIEGWYGGRKKRRQRRR) (GenScript), the 45-minute incubated treatment mixtures were supplemented with 10 µmol/l E5-TAT and the treatments were maintained for 24 hours.

Imaging delivery of fluorescently labelled siRNA. Huh7 cells were seeded at 60% confluency in glass-bottom chamber slides and after 24h, the treatments were applied as described above and incubated for 5h or 48 hours. The cells were then washed twice with phosphate buffered saline and imaged live cell in phenol red-free media. The fluorescence imaging was performed using a 100× oil objective on an Olympus 1X81 fluorescence microscope equipped with appropriate filter for Cy3 excitation and emission and a Photometrics (Coolsnap ES) camera, taken with 1-second exposure times. The images were false coloured with ImageJ and the same brightness and contrast applied to all images of samples within the same time point.

Luciferase assays. To monitor the gene knockdown activity of 2x-p19-TAT, Huh7 cells were seeded into a 100 mmol/l dish and after 24 hours and at 70% confluency were transfected with a dual luciferase reporter vector, psiCHECK-2 (Promega, Madison, WI) using Lipofectamine2000 (Invitrogen, Burlington, Ontario, Canada) as per manufacturer's instructions. After 24 hours, the psiCHECK-2 expressing Huh7 cells were reseeded into a 96-well plate. After another 24 hours, the cells were treated with 2x-p19-TAT:luc2 siRNA complexes as discussed above and after 24 or 72 hours, the level of knockdown was determined via dual luciferase assays (Promega, Madison, WI) using a Lmax luminometer microplate reader (Molecular Devices, Sunnyvale, CA). Percentage of gene knockdown was determined by calculating firefly/renilla signal and normalizing to the untreated control.

Cell viability assays. Huh7 cells were seeded in 96-well plates and after 24 hours and at 70% confluency were treated with protein:siRNA complexes as outlined above or treated with siRNA:RNAiMAX (Invitrogen, Burlington, Ontario, Canada) complexes. Cells were treated for 24 and 72 hours, the media was removed and 50 µl of a solution of 2.5 mg/ml MTT in phosphate buffered saline was added to each well. The cells were incubated with the reagent for 3 hours, aspirated and the formazan crystals were dissolved in 150 µl of dimethyl sulfoxide. Absorbance at 570 nmol/l was measured using a Spectra Max M2 (Molecular Devices, Sunnyvale, CA) and recorded using Softmax Pro 4.7 software. The data were recorded in triplicates and normalized to the untreated control samples to reflect percent cell viability.

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