TAT Peptides Mediated Small Interfering RNA Delivery to Huh-7 Cells and Efficiently Inhibited Hepatitis C Virus RNA Replication

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Key Words

Delivery systems • Gene silencing • Hepatitis C virus • HIV-1 • Small-interfering RNA • TAT peptide

Abstract

Objective: To observe whether or not the small-interfering RNA (siRNA) that conjugated with human immunodeficiency virus type 1 (HIV-1) TAT_{47–57} peptides can enter Huh-7 cells and efficiently silence hepatitis C virus (HCV) infection in cell culture. **Methods:** siRNA targeting the highly conserved stem loop IV of the HCV 5' untranslated region (5'UTR) was conjugated to TAT_{47–57} peptides via the crosslinker sulfo-succinimidyl-4-(p-maleimidophenyl)-butyrate, and then the conjugates were added to the Huh-7 cell culture. Firefly luciferase activity and HCV RNA were assessed using a luciferase assay reagent and real-time reverse transcript polymerase chain reaction, respectively. **Results:** The expression of firefly luciferase in HCV replicons and the concentration of HCV RNA were downregulated by siRNA-TAT_{47–57}, and siRNA-TAT_{47–57} mediated RNA interfering activity which was directly correlated with increasing concentrations of the siRNA-TAT_{47–57} conjugate used. **Conclusion:** Cell-penetrating peptides such as HIV-1 TAT are an effective method for the delivery of siRNA targeted at 5'UTR of HCV in mammalian cells.

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Introduction

Hepatitis C virus (HCV) has infected an estimated 170 million people worldwide and is one of the most common causes of chronic hepatitis, cirrhosis and hepatocellular carcinoma [1–3]. Currently, hepatitis C patients are treated with interferon- α alone or in combination with ribavirin, but it is estimated that less than half of the patients respond effectively to currently available agents [4]. Therefore, developing a new therapy for chronic HCV is a major public health concern.

Since gene silencing by RNA interference (RNAi) became possible using small-interfering RNA (siRNA) <30 bp in mammalian cells [5], siRNA has become the tool for gene function studies [6–11]. The replication cycle of HCV occurs in the cytoplasm of infected cells making an excellent target for siRNA-based antiviral development [12–14].

However, owing to its relatively large size and anionic charge, one of the key problems in utilizing siRNA as a therapeutic tool is the availability of effective delivery systems [11]. A number of approaches for delivering siRNA have recently been explored [15–18]. One approach is DNA- and RNA-based siRNA expression relying on plasmid or viral vectors. This method has potential virus-associated immunogenicity problems. Although retrovirus-mediated RNAi for liver delivery has been reported, the safety of such a vector in a long-term treatment program is always a concern due to the virus

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being integrated into the host cell chromosome [16, 17]. Other employed methods, e.g. electroporation and microinjection, are harsh and impractical to use in vivo because these methods necessitate disruption of the cell membrane before substances can be introduced into the cell and they are restricted to a limited amount of cells [19]. Currently, the most often used method of siRNA delivery is Lipofectamine transfection, but the use of this method is limited to specific cell types, and this procedure could be toxic to cells and animals [20].

Cell-permeable peptides can promote cellular uptake through endocytosis and facilitate the entry of macromolecules, including DNA, lipids and antisense RNA, into many types of cells in culture [19, 21–26]. One of these translocating peptides was derived from the human immunodeficiency virus type 1 (HIV-1) TAT protein. TAT peptides chemically attached to various proteins (horseradish peroxidase, β -galactosidase, nucleic acids and some others) were able to deliver these proteins to various cells and even to several tissues in the mouse, showing high levels in the heart, lung and spleen [22]. Cellular uptake of TAT depends on an 11-amino acid cationic peptide sequence that corresponds to aa 47–57 (YGRKKRRQRRR) of TAT. Chiu et al. [19] conjugated siRNA targeting enhanced green fluorescent protein with TAT_{47–57} peptides via heterobifunctional crosslinker, and the TAT-siRNA conjugates effectively delivered siRNA into Hela cells for specific gene silencing.

Here, we conjugated the most effective siRNAs targeting the highly conserved 5' untranslated region (5'UTR) of HCV with TAT_{47–57} peptides via the crosslinker sulfo-succinimidyl-4-(p-maleimidophenyl)-butyrate (sulfo-SMPB), and then the conjugates were added to Huh-7 cell cultures. We observed that the siRNA can enter the Huh-7 cells and efficiently silence HCV infection in cell culture.

Materials and Methods

siRNA Preparation

The siRNA sequence targeting position 322–340 [27] of the 5'UTR stem loop IV of HCV was chemically synthesized as 2'bis (acetoxymethoxy)methyl ether-protected oligonucleotides by Dharmacon (Lafayette, Colo., USA). The sequence of synthesized siRNA is shown in figure 1. Synthetic oligonucleotides were deprotected, annealed and purified as described by the manufacturer. Duplex siRNAs with fluorescent 5'DY547-modified sense strands were used to determine the uptake efficiency of siRNA, while duplex siRNA with 3' amino modification was used to conjugate siRNA with TAT peptide, as described below. All the siRNAs were stored in diethylpyrocarbonate-treated water at -80° .

DY547-GGUCUCGUAGACCGUGCAC dTdT
N3-dTdTCCAGAGCAUCUGGCACGUG

Fig. 1. Chemically synthesized siRNA modified with DY547. Duplex siRNA with 5'DY547-modified sense strands were used to determine the uptake efficiency of siRNA while duplex siRNA with 3' amino modification were used in conjugating siRNA with TAT peptides.

Conjugating siRNA with TAT_{47–57} Peptide

The TAT_{47–57} peptide (Cys⁴⁷-Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg⁵⁷) was synthesized by solid-phase synthesis (Gima). A cysteine residue was added at the N terminus of the TAT_{47–57} peptide in order to provide a free sulfhydryl group [19]. siRNA with 3'N3 modification at 0.1 mM final concentration in phosphate-buffered saline (PBS; 20 mM sodium phosphate and 150 mM NaCl; pH 7.2) was incubated with 50-fold molar excess of the heterobifunctional crosslinker sulfo-SMPB (Pierce, Rockford, Ill., USA). After shaking for 30 min at room temperature, the reaction products were purified by dialysis using conjugation buffer. The activated siRNA crosslinker was incubated with an equal molar ratio of TAT_{47–57} peptide at room temperature for 30 min. A cysteine residue added to the TAT_{47–57} peptide was used for siRNA conjugation. The reaction was quenched by addition of buffer containing reduced cysteine at a high concentration.

Cell Culture and TAT-Mediated siRNA Delivery

Cells of the human hepatoma cell line Huh-7 containing the HCV luciferase reporter system (kindly provided by R. Bartenschlager, University of Heidelberg, Germany) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 500 μ g/ml G418 (Sigma Aldrich, St. Louis, Mo., USA) at 37° in 5% CO₂. Huh-7 cells containing the HCV luciferase reporter system were plated on 60-mm plates at 70% confluency 20 h before transfection. TAT-siRNAs at amounts of 0.5, 1.0 and 1.5 μ g were added to the medium, and the cells were incubated with the mixture for 24–48 h. Unconjugated TAT and siRNA, as well as TAT alone, were used as negative controls in all experiments. Standard Lipofectamine-mediated transfections were performed as positive control according to the manufacturer's recommendations.

Fluorescent Assay of Cellular Uptake

After transfection, cells were washed three times with PBS (0.01 M, pH 7.4) to remove the siRNA-containing medium and subjected to fluorescence measurements on a fluorescence spectrophotometer. All experiments were carried out at room temperature. Uptake and intracellular distribution were monitored by a fluorescence spectrophotometer using appropriate filters.

Cell Viability Assay Using Trypan Blue Staining

Huh-7 cells (5×10^5 cells) containing siRNA were seeded onto 24-well plates and the cell suspension was prepared at a high concentration. Subsequently, a clean hemocytometer slide was taken and coverslipped, pressing down the grooves and semi-silvered

Fig. 2. Images of cellular uptake of Tat-siRNA analyzed by fluorescence microscopy ($\times 200$). **a** Cellular uptake of Tat-siRNA. **b** Cellular uptake of Lipofectamine-siRNA. **c** The culture medium as negative control. **d** Cellular uptake of unconjugated Tat and siRNA. **e** Lipofectamine 2000 alone as negative control.

counting area, and 100 μ l of cell suspension were mixed with 100 μ l of trypan blue in the tip of a micropipette. After 3–5 min, 20 μ l of the mixture were transferred to the edge of the coverslip and the suspension was run into the counting chamber. The fluid should run to the edges of the grooves and be left for only 1–2 min (not longer). Finally, using a microscope with $\times 10$ objective, the cells were counted [28].

Firefly Luciferase Activity Assay

Firefly luciferase activity was assessed using a luciferase assay reagent (Promega, Madison, Wisc., USA) [29, 30]. Huh-7 cells were harvested after 24 h of transfection and washed three times with PBS. The cells were then incubated with 200 μ l of luciferase lysis buffer for 10 min and the cell lysis solution was harvested. After 15 min of centrifugation at 13,000 g at 4°, 20 μ l of lysate were mixed with 200 μ l of assay buffer. Luciferase activity was measured using a luminometer (Berthold Technologies, Bad Wildbad, Germany). Each experiment was performed in triplicate, and means $\pm$ SD of three independent experiments are given.

Nucleic Acid Extraction and Real-Time Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)

The nucleic acid was extracted from transfected Huh-7 cells (cultured 24–48 h) using Trizol as cell lysis solution according to the manufacturer's instructions (Trizol reagent instructions) [31]. After cell lysing, chloroform, isopropanol and ethanol were added consecutively to elute the nucleic acid. Finally, the extracted nucleic acid was stored in 30 μ l of diethylpyrocarbonate-treated water and then used as a template for real-time RT-PCR.

The real-time RT-PCR was carried out in single-step mode using HCV RNA detection kits (Shanghai KeHua Bioengineering, Shanghai, China). All the operation steps were according to the manual provided by the manufacturer. RT-PCR was conducted in one reaction at a final reaction volume of 25 μ l containing 7 μ l of PCR major reaction solution, 5 μ l of enzyme mixture, 0.5 μ l of probe and 12.5 μ l of extracted RNA. The temperature profile comprised the RT round conducted at 50° for 25 min and the PCR rounds denaturing at 94° for 2 min and then at 93° for 3 s, followed by a 5-cycle program consisting of 93° for 3 s, 55° for 15 s

and 72° for 15 s, and completed by a 42-cycle program at 93° for 3 s and 60° for 45 s. RT-PCR was carried out in a 32-well thermal cycler system (LightCycler; Roche, Penzberg, Germany). Data were then analyzed using graphic interpretation.

Results

Uptake Efficiency of siRNA-TAT Conjugates

siRNA conjugated to TAT peptides was evaluated for siRNA uptake efficiency by fluorescence detection. The cells transfected with siRNA-TAT exhibited efficient siRNA cellular uptake that was almost equal to uptake observed using siRNA-Lipofectamine (fig. 2a, b). The cells transfected with unconjugated siRNA plus free TAT_{47–57} or Lipofectamine only did not show any measurable uptake of siRNA (fig. 2c–e), demonstrating that siRNA uptake did not occur without conjugated TAT_{47–57}. These results indicated that siRNA covalently modified with TAT peptides can be effectively delivered to cells.

Effect of siRNA on the Replication of HCV Replicons

The percentage of viable cells was about 85%. Subsequently, we examined the effects of siRNA on HCV translation using a luciferase reporter assay. The expression of firefly luciferase in HCV replicons was downregulated with decreasing HCV replicons. In this assay, siRNA-TAT_{47–57} at different amounts (0.5, 1.0 and 1.5 μ g) and unconjugated siRNA plus free TAT_{47–57}, Lipofectamine plus 1.0 μ g of siRNA and Lipofectamine alone were transfected into Huh-7 cells; thereafter, firefly luciferase activity was determined 24 or 48 h after transfection. The inhibitory ratio of luciferase in the presence of siRNA was calculated and compared with the control (fig. 3a). We

Fig. 3. Effect of siRNA-TAT on the replication of a subgenomic HCV replicon. Various amounts of siRNA-TAT conjugates can effectively inhibit the expression of firefly luciferase activity (**a**) and concentration of HCV RNA (**b**); the inhibitory effect tends to increase with increasing amount of siRNA. The inhibitory effect of siRNA-TAT (1.0 µg) on firefly luciferase expression was equal to that of Lipofectamine-siRNA (1.0 µg; **b**). Cells treated with 1.5 µg of unconjugated siRNA plus free Tat₄₇₋₅₇ peptides did not affect HCV replication (**a**, **b**). However, treated with Lipofectamine 2000 alone, severe inhibition of HCV replication was observed in Huh-7 cells (**a**).

observed a gene-silencing activity of 96.5% when siRNA-TAT (1.5 µg) was used, demonstrating that siRNA-TAT was functional after being delivered into the cells. The siRNA-TAT-mediated RNAi activity could be directly correlated with increasing concentrations of the siRNA-TAT conjugate used in the experiments. The gene-silencing effect of siRNA-TAT (1.0 µg) was equal to that of Lipofectamine-siRNA (1.0 µg). The cells treated with 1.5 µg of unconjugated siRNA plus free Tat₄₇₋₅₇ peptides showed no effect on HCV translation; however, following Lipofectamine 2000 treatment, the cells demonstrated severe inhibition of HCV translation, leading to the conclusion that Tat₄₇₋₅₇ peptides have less toxic effects on cells than Lipofectamine 2000.

Quantitative Detection of HCV RNA in siRNA-TAT-Treated Cells

Total RNA was extracted from cells 24–48 h after transfection and analyzed for HCV RNA using KHB HCV RNA RT-PCR detection kits. The concentration of HCV RNA decreased with increasing amount of siRNA-TAT, being consistent with the results of the assay of firefly luciferase activity (fig. 3b).

Discussion

In the present study, we succeed in the intracellular delivery of siRNA, targeting position 322–340 of the 5'UTR stem loop IV of the HCV genome, which was chemically conjugated with the HIV-1 TAT peptide, thus efficiently silencing HCV replication in Huh-7 cells. First, siRNA was chemically synthesized and the synthesized sense strands of duplex siRNA were modified with DY547 at the 5' terminus. Then, siRNA was conjugated with Tat₄₇₋₅₇ peptides via the crosslinker sulfo-SMPB and transfected into Huh-7 cells. During conjugation, the N-hydroxysuccinimidyl ester group of the crosslinker reacted with the amino group of siRNA; during this process, the amino group was maleimide activated. The TAT peptide contained a cysteine residue, and the N-hydroxysuccinimidyl ester group of the crosslinker reacted with the sulfhydryl group of the cysteine residue of the TAT peptides in the process of conjugation [19]. The TAT peptides and the siRNA conjugations were incubated with cultured cells at 37° for 24–48 h. Assessing DY547 red fluorescence with a fluorescence microscope, the conclusion was reached that TAT peptides could effectively deliver siRNA intracellularly via covalent modification.

5'UTR and the internal ribosome entry site of HCV have often been used to target RNA since these regions are highly conserved in all subgenomic viruses [27]. In this study, we used two methods to detect the gene-silencing function of TAT₄₇₋₅₇-siRNA conjugates in Huh-7 cells containing HCV replicon. One method is the assay of firefly luciferase. In the HCV replicons, the neomycin genes were placed at the 5'UTR by the gene encoding the luciferase of the firefly *Photinus pyralis*. Since the copy number of this reporter gene should be determined by the replication levels of the corresponding replicon RNAs, the luciferase activity in a lysate of transfected cells could be used to directly monitor the replication of RNA [29]. Firefly luciferase gene expression decreased following inhibition of HCV replication in Huh-7 cells. As shown in figure 2a, transfection of the siRNA-TAT₄₇₋₅₇ conjugates inhibited firefly luciferase gene expression in a dose-dependent manner. We observed the maximum inhibition when 1.5 µg of the siRNA-TAT conjugate was used, and the gene-silencing effect of 1.0 µg of the siRNA-TAT conjugate was equal to that of Lipofectamine-siRNA (1.0 µg). Unconjugated siRNA plus free TAT₄₇₋₅₇ peptides showed no effect on HCV replication. However, cells treated with Lipofectamine 2000 alone demonstrated severe inhibition of HCV replication (fig. 3a). Apparently, TAT₄₇₋₅₇ peptides had less toxic effects on cells compared with Lipofectamine 2000.

The other method is to detect HCV RNA concentration by real-time RT-PCR. Total RNA was extracted from transfected cells and detected with KHB HCV RNA detection kits. The results were consistent with those of the firefly luciferase assay, i.e. the concentration of HCV RNA decreased with increasing amount of conjugated siRNA.

TAT₄₇₋₅₇ peptides offer several advantages over conventional delivery systems (e.g. electroporation and Lipofectamine). First, they can rapidly translocate siRNA into the cell interior and give rise to efficient RNAi activities. TAT₄₇₋₅₇ peptides have a high cationic charge, cross cellular membranes freely and are able to transport relatively large molecules, e.g. 200-nm liposomes, into the cytoplasm. Second, compared with Lipofectamine 2000, using TAT₄₇₋₅₇ peptides, transfection was fairly efficient, and TAT₄₇₋₅₇ peptides did not confer toxic effects to cells or induce specific immune responses. Third, some transfection reagents, e.g. lipofectin, could not be used in vivo; however, numerous studies have shown that the TAT peptides were effective not only for in vitro but also for in vivo delivery [32, 33]. Fourth, TAT₄₇₋₅₇ peptides had no cell type specificity but a high yield of delivery, and induced efficient RNAi activities. TAT₄₇₋₅₇ peptide conjugates promote cellular uptake through endocytosis [34, 35] and facilitate the entry of macromolecules into many types of cells cultured [2, 36, 37]. The approach described here demonstrated that HIV-1 TAT peptides were effective for the delivery of siRNA targeting at HCV in mammalian cells.

In conclusion, HIV-1 TAT peptides can effectively deliver siRNA targeting 5'UTR of HCV in Huh-7 cells and block HCV replication in the cells. Hopefully, this approach may be used in the future to inhibit HCV replications in vivo.

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