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Research article

Targeting neuronal nitric oxide synthase by a cell penetrating peptide Tat-LK15/siRNA bioconjugate

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HIGHLIGHTS

- Tat-LK15 was used as a siRNA delivery to target nNOS expression.
- Tat-LK15/siRNA improved the stability of siRNA in serum.
- Tat-LK15/siRNA complex is efficient to knock-down nNOS expression in cells.

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ABSTRACT

We developed a cell penetrating peptide (CPP) Tat-LK15, as a siRNA carrier to target nNOS. The feasibility, stability, efficiency and selectivity of this peptide-siRNA complex were evaluated in rat neuronal cells. We also compared the new method with conventional siRNA carrier Lipofectamine™. It was found that the CPP Tat-LK15 effectively and specifically delivered nNOS-siRNA into Rat retinal ganglia (RGC-5) cells and silenced the expression of nNOS. The CPP Tat-LK15 can conjugate with siRNA to form stable complex at a ratio of 2:1 (peptide/siRNA, w/w), which maintained stable in serum for as long as 4 h. The CPP Tat-LK15 was low-toxicity to cells, as the apoptosis rate of treat cells was not increased significantly when the used peptide lower than 10 μg/mL. Moreover, the cellular uptake of nNOS siRNA by Rat Neurons-dorsal spinal cord (RNdsc) cells was also significantly more than naked siRNA by RNdsc cells. The CPP Tat-LK15 was an efficient and stable, and non-cytotoxic siRNA delivery to neurons and effectively silenced the nNOS expression. The CPP Tat-LK15 mediated siRNA delivery was a potential tool to treat neuropathic diseases involving NO or nNOS neurotoxic cascades.

1. Introduction

Recently, a series of evidence have demonstrated up-regulated nitric oxide (NO) and neural nitric oxide synthase (nNOS) are considered to play a key role in generating acute nociception and central sensitization of persistent pathological pain including neuropathic and inflammatory pain [1,2]. In the nervous system, NO is synthesized from the terminal guanidino nitrogen of arginine by the action of nNOS, then mediates modulation of synaptic plasticity in a spatially constrained fashion, and initiates or contributes to neuronal cell death [3,4]. Therefore, silencing nNOS expression by siRNA might be a potent tool to treat neuropathic pain.

Along with providing mechanistic insight into gene silencing, siRNA has recently emerged as a powerful tool in genomic function research and has been broadly applied in the treatment of diseases [5,6]. However, siRNA-based therapeutics have faced a big challenge regarding of safety and efficiency of delivery *in vivo* [7]. Especially, previous studies showed low siRNA transfection effi-

Abbreviations: NO, nitric oxide; nNOS, neuronal nitric oxide synthase; CPP, cell penetrating peptide; CNS, central neural system; RGC-5, Rat retinal ganglia cell; RNdsc, Rat Neurons-dorsal spinal cord; BBB, blood-brain barrier; FBS, Fetal Bovine Serum.

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ciency was observed in neural cells and nervous tissue due to the blood-brain barrier (BBB) in the central neural system (CNS) [8]. Therefore, development of an efficient method for siRNA delivery in neural cells would be benefit for therapy of neural diseases. CPPs exhibited the promising advantage of freely trafficking across the BBB. Therapeutic molecules conjugated to cell-penetrating peptides (CPPs), which were relatively short cationic and/or amphipathic peptides, can pass through the plasma membrane and increase tissue bio-distribution [9].

CPPs were an alternative to viral vectors due to low cytotoxicity [10]. One of the most promising CPPs was the HIV-1 transactivator of transcription (Tat) peptide [11]. Tat peptide can be efficiently combined to a wide range of different molecules, such as small molecules and antibodies, peptides, liposomes, siRNAs, plasmid DNA, and bioactive agents [12–14]. CPPs can electrostatically bind with the phosphate backbone of the nucleic acids to form a stable complex and protect nucleic acids from degradation [15]. Hence, it is believed that Tat can be used as siRNA delivery to target nNOS in neural cells.

To improve the transfection efficiency, amino acids 49–57 of the Tat peptide was fused to the cationic membrane active peptide LK15 to form a new CPP molecule Tat-LK15 [16]. This modified CPP fusion Tat-LK15 increased the transfection efficiency by two orders of magnitude compared to the Tat peptide alone, and exhibited low cytotoxicity in human colorectal adenocarcinoma cells, HT29, and human connective tissue fibrosarcoma cells, HT1080 [16,17].

Although the wide use of siRNA delivery by modified Tat, there were very few reports concerning in the CNS. Giving that nNOS was the main cause of neuropathic pain, we aimed to knock down the expression of nNOS using the powerful CPP based delivery system describe above in this study. We prepared a siRNA targeting nNOS, and fused it to Tat-LK15 peptide, defined as Tat/LK15-siRNA. Our data showed that Tat/LK15-siRNA efficiently delivers siRNA into Rat retinal ganglia (RGC-5) cells and Rat Neurons-dorsal spinal cord (RNdsc) cells, and our strategy potentiated the therapeutic effect of nNOS siRNA on rat neuropathic pain *in vitro*.

2. Materials and methods

2.1. Peptide and siRNA

The CPP Tat-LK15 was synthesized by Boxin Company (Xiamen, China) with a purity >86.59%, in which HIV-Tat (49–57, RKKRRQRRR) was linked with membrane active peptide LK15 (KLLKLLLKLLLKLLK) via a linker GGG. The rat nNOS sequence was obtained from Genbank, and the siRNA targeting nNOS and negative control siRNA were designed and synthesized by RayBio Company (Guangzhou, China). The sequences of used siRNA were shown as below:

nNOS siRNA-1 Sense: 5′-GUCAUUAGCAGUAGACAGAdTdT-3′ and Antisense 5′-UCUGUCUACUGCUAAUGACdTdT-3′;

nNOS siRNA-2 Sense: 5′-CAGAAUACAGGCUGACGAUdTdT-3′ and Antisense 5′-AUCGUCAGCCUGUAUUCUDdTdT-3′

nNOS siRNA-3 Sense: 5′-CAAGUUCCGCCUCACGUAUdTdT-3′ and Antisense: 5′-AUACGUGAGGCGGAACUUGdTdT-3′

NCsiRNA Sense: 5′-UUCUCCGAACGUGUCACGUTT-3′ and Antisense: 5′-ACGUGACACGUUCGGAGAATT-3′.

Tat-LK15 peptide: RKKRRQRRRGGGKLLKLLLKLLLKLLK.

2.2. Preparation of complex

The siRNA and Tat-LK15 peptide powder were dissolved separately in HEPES-buffered saline (10 mM HEPES, pH7.4) for stock. In preparation of complex, the peptide solution was mixed with siRNA solution according to different weight ratios (Tat-LK15/siRNA ranged from 1:3 to 3:1), and incubated at room temperature for 30 min. Final siRNA concentration was 50 nM for each well.

2.3. Gel electrophoresis

To determine whether siRNA was conjugated with Tat-LK15, the complexes (w/w, the Tat-LK15/siRNA proportion of 1:3–3:1) were dissolved and mixed with HEPES-buffered saline. The complexes were electrophoresed on 20% non-denaturing polyacrylamide gel (PAGE) for 90 min at 1.8 v/cm. The gel was stained with ethidium bromide (0.5 μg/mL) for 30 min and exposed using a Gel Imaging System (Doc Gel 2000, Biorad) to detect the conjugated siRNA in the complexes.

2.4. Cell culture and siRNA delivery

Rat retinal ganglia (RGC-5) cells were purchased from Land Biocompany (Guangzhou, China). RGC-5 cells were grown in DEME medium (high glucose, Hyclone) supplemented with 10% Fetal Bovine Serum (FBS) (Hyclone, Australia) and 1% penicillin/streptomycin solution (Hyclone, Australia) at 37 °C in a humidified air atmosphere containing 5% CO_2. The RGC-5 cells (1×10^5) were seeded on 6-well plates and incubated under standard conditions for 24 h. Medium was changed to Opti-MEM without FBS. The Tat-LK15/siRNA of 200 μL was added into the cell culture with a final siRNA concentration of 50 nM per well. According to the manufacturer's instruction, 5 μL of Lipofectamine™ RNAiMAX (Invitrogen, USA) were used to deliver siRNA into cells, as a control group. After incubation for 4 h, the medium was replaced with 2 mL of fresh complete medium and the cells were further incubated for an additional 24 h. Then, the cells were observed by a fluorescence microscope (Leica DMI6000B).

2.5. Flow cytometry analysis

Flow cytometric analysis was conducted to quantitatively examine the transfection efficiency of Tat-LK15 siRNA complexes. The FAM 6-carboxyfluorescein was modified to the 5′-siRNA by Genepharma Company (Shanghai, China) for fluorescence analysis. After RGC-5 cells were transfected with Tat-LK FAM-siRNA complex, the cells were washed twice with PBS, trypsinized using 0.1% v/v trypsin/EDTA in PBS, and centrifuged at 900 × *g* for 5 min. After discarding the supernatant, the cell pellet was resuspended in ice-cold 500 μL PBS at a minimum of 10,000 events/sample. Flow cytometry analysis was performed immediately using fluorescence activated cell sorting (FACS, Becton Dickinson, UK) set up to detect green fluorescence signal (Ex 480 nm, Em 520 nm).

2.6. Evaluation of cytotoxicity

To evaluate the cytotoxicity of Tat-LK15, the apoptosis of cells treated with Tat-LK15 was determined. 1×10^5 cells/well was plated in a 6 well plate 1 day before Tat-LK15 incubation. The RGC-5 cells were incubated with the Tat-LK15 (0.5, 1.25, 2.5, 5, and 10 μg/mL) for 24 h. After rinsing twice with PBS and trypsin addition, the cells were centrifuged at 900 × *g* for 5 min. The supernatant was discarded and the cells was resuspended in 500 μL 1 × binding buffer at a density of 1.0×10^6 cells per mL, and then stained with 1.25 μL Annexin V-FITC and 10 μL propidium iodide (PI) for 15 min at room temperature in the dark. Finally, the samples were analyzed by FACS (Becton Dickinson). Cell viability was determined based on gated live cells on forward and sideward scatter plots.