Targeted delivery of siRNA against hepatitis B virus by preS1 peptide molecular ligand

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Aim: For chronic hepatitis B virus (HBV) infection, the effects of current therapies are limited. RNA interference of virus-specific genes has emerged as a potential antiviral mechanism. However, a suitable delivery vector is still to be developed. We studied a novel vector transferring siRNA targeting hepatic cells in vivo and in vitro in order to find a new way to cure HBV-related liver diseases.

Methods: The preS1-9Arg ligand was used to deliver siRNA to HepG2 and to HepG2 2.2.15 cells. To validate the antiviral efficacy in vivo, a HBV viremic animal model was established by s.c. inoculation of HepG2 2.2.15 tumor cells in nude mice. The minimal retardation effect on the migration of siRNA was detected by gel electrophoresis to confirm the combination and the optimal ratio. Hepatitis B surface antigen (HBsAg) levels were detected by semiquantitatively enzyme-linked immunosorbent assay RNA levels were quantified with quantitative real-time polymerase chain reaction and protein levels were determined with immunoblots and immunohistochemistry.

Results: PreS1-9Arg peptide strongly combined and transferred siRNA into HepG2 cells. PreS1-9Arg-siRNA molecular conjugate effectively reduced the production of HBsAg and HBV DNA without liver toxicity in vitro and in vivo.

Conclusion: The results indicated that preS1-9Arg may be a potential novel vector to deliver siRNA targeting liver cells.

Key words: hepatitis B virus, molecular ligand, preS1, RNA interference

INTRODUCTION

VIRAL HEPATITIS CAUSED by hepatitis B virus (HBV) infection is common in Asia, Africa and the Middle East. China has one of the highest carrier prevalence rates of HBV in the world with an infection rate nearly 10% of the general population. It is well known that chronic HBV infection leads to the development of liver cirrhosis and hepatocellular carcinoma in a significant proportion of patients. Attempts at treatment of chronic infections have had only limited success. Currently, chronic HBV infection is generally treated with interferon or nucleoside analogs such as lamivudine, entecavir and adefovir dipivoxil. However, these treatments have some drawbacks, including side-effects in the case of interferon, and development of escape mutants after a long period of lamivudine treatment. Studies have shown that RNA interference (RNAi), which can be induced in mammalian cells by siRNA, induces post-transcriptional silencing and thus inhibits the replication of HBV. Nevertheless, there are significant hurdles that need to be overcome, including optimizing dose control, limiting off-target effects, improving delivery of RNAi effectors. To obtain a non-toxic and highly efficient delivery system is vital for transferring specific siRNA to the target tissues and cells. To date, kinds of different delivery approaches have demonstrated success ranging from the relative simplicity of direct local administration of saline-formulated siRNA, to liposome- and polymer-based nanoparticle approaches, to conjugation and complexation approaches. The surface proteins of HBV include large protein (L), middle protein (M) and small protein (S), all of which have the same domains of preS1. PreS1 was reported to play a key role in mediating HBV particles attaching to liver cells as a ligand binding to specific membrane receptors. The N-terminal is the principal binding domain of preS1 and the 21–47 a.a. may be the pivotal sequence in the binding step. This 27-mer preS1 peptide (21–47 a.a.) was showed binding to HepG2 cells in a dose-dependent manner with high affinity. Based on this characteristic, the preS1 peptide may be applied as a
vector to deliver biomolecules targeting liver cells. Arginine-containing membrane translocational signals, such as Tat and VP22, and synthetic arginine-rich peptides have been reported to be efficient carriers for transporting various types of biomolecules translocate through the cell membranes and accumulate in the nucleus of living cells. Among those arginine-containing membrane translocational signals, synthetic 9-mer arginine peptide (9Arg) was proved to be the most economical and efficient. In this report, 9-mer arginine peptide was fused to the C-terminal of preS1 peptide to make a different molecular ligand (preS1-9Arg). Strong binding and internalization of this fusion peptide was observed in hepatic cell lines. This molecular ligand exhibited a strong ability to combine and transfer siRNA into HepG2 cells. Furthermore, the preS1-siRNA molecular conjugate suppressed the expression and replication of HBV in HepG2 2.2.15 cell line and on a HBV viremic animal model.

**METHODS**

**Synthesis of peptide and siRNA**

The peptide of 21–47 a.a. of preS1 fused with 9-mer arginine (preS1-9Arg, PAFGANSNPDPWDGNPNK DQWPAANQVGGGRRRRRRRRRR) was synthesized at Invitrogen (Shanghai, China). The 9-mer arginine residues were D-arginine. Three effective siRNA targeting HBV gene X, S and P were chemically synthesized by Ruibo (Guangzhou, China). The sense sequences were 5′-CCGACCUUGAGGCCUACUU-3′, 5′-GCAAGGUAGGAGCUGGAGCAUU-3′ (forward) and 5′-GCCAAGUGCUGUACAGCAUC-3′ (reverse), respectively. An siRNA targeting firefly luciferase (siLUC) was used as irrelevant control: the sense sequence was 5′-CUGAUAGGGUGCUUGCGAGUU-3′. Fluorescein isothiocyanate (FITC)-labeled peptide and cy3 labeled or unlabeled siRNA were synthesized and the antisense strands were also synthesized at the same time (Ruibo). Before transfection, the sense and antisense fragments were mixed and annealed to produce double-strand siRNA.

**Cell lines and culture**

HeLa, HepG2, HepG2x and HepG2 2.2.15 cells were grown in minimum essential medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (HyClone, Logan, UT, USA). A final concentration 200 mg/mL of G418 (Gibco) was added into the cultures of HepG2x and HepG2 2.2.15 cells. HepG2x is a cell line stably expressing hepatitis B x protein (HBx), which was constructed by transfecting HepG2 with pcDNA3.1-HBx and selected with G418.

**Preparation of preS1-9Arg peptide and siRNA conjugation**

PreS1-9Arg and cy3 labeled or unlabeled siRNA were mixed gently, respectively, in phosphate-buffered saline (PBS) at different ratios and incubated at 37°C for 15 min to form peptide-siRNA molecular conjugate. A mixture containing 100 pmol siRNA and various volume of peptide was analyzed by 1.5% agarose gel electrophoresis to observe the minimal retardation effect on the migration of siRNA, in order to confirm the combination and the optimal ratio. The optimal ratio of siRNA and peptide based on the above experiment was used in transfection. To enhance the efficacy of RNAi, the three siRNA targeting different region of HBV were mixed with equimolar ratio to assemble peptide-siRNA molecular conjugates.

**PreS1-9Arg peptide delivers siRNA to HepG2 cells**

To test attachment of synthesized peptide to liver cells, 100 μmol/L FITC-preS1-9Arg peptide and was added into HeLa and HepG2 cells and incubated at 37°C for 20 min, followed by paraformaldehyde fixation and three washes. The cell nucleus was stained with 6-diamidino-2-phenylindole (DAPI; Roche, Mannheim, Germany) and analyzed using a fluorescent microscope (Olympus, Tokyo, Japan). To test the efficacy of preS1-9Arg transfection, peptide and cy3-siRNA conjugate was added to the HepG2 cell culture medium and observed 24 h post-transfection to confirm the enrollment of siRNA.

**Assessment of preS1-9Arg-siRNA in HepG2x and HepG2 2.2.15 cells**

To verify the RNAi effect of peptide-siRNA complex, the peptide was used to transfet siHBx into HepG2x. The RNAi effect was analyzed by reverse transcription polymerase chain reaction (RT–PCR) and western blot of HBx. The primers for HBx were 5′-ACCGACCTTGGCCTACTT-3′ (forward) and 5′-GCTTGGCAGAGGTGAAAAAG-3′ (reverse). The primers for glyceraldehyde 3-phosphate dehydrogenase were 5′-CTGGGACGGACAGGTGTAAGAAG-3′ (forward) and 5′-GGTACGTGCTGCCAGAGG-3′ (reverse). Total RNA was extracted by Trizol reagent (Roche) and RT was taken out by a reverse system transcription (Promega, Madison, WI, USA). Routine PCR with the Bio-Rad S1000 Thermal Cycler system (Bio-Rad, Richmond, CA, USA) was run with a...
denaturing step at 95 °C for 3 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s, then a final extension at 72 °C for 10 min. The PCR product was analyzed by gel electrophoresis. Western blotting was carried out to analyze the expression level of HBx. Anti-HBx and anti-tubulin (Sigma-Aldrich, St Louis, MO, USA) was used as the primary antibody. Horseradish peroxidase-conjugated second antibody (Zhongshan, Beijing, China) was used to detect the bound antigens with SuperSignalWest Pico Chemiluminescent Substrate Kits (Pierce, Rockford, IL, USA) according to the manufacturer’s instructions.

Then, HepG2 2.215 was used to assess the efficiency of RNAi on HBV. To do so, PreS1-9Arg and siRNA were mixed gently, respectively, in PBS at different ratios and incubated at 37 °C for 15 min to form peptide-siRNA molecular conjugate. Cells grown to 80% confluence were then overlaid with these preformed complexes. After 30 min incubation at 37 °C, fresh Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum was added to the HepG2 2.215 cells, without removing the overlay of carrier/siRNA. Control transfection experiments were performed using Lipofectamine 2000 (Roche Corp, Mannheim, Germany). Hepatitis B surface antigen (HBsAg) secretion into the culture supernatants was measured by enzyme-linked immunoassay (ELISA; Lizhu, Zhuhai, China). The ELISA system was aimed to measure HBsAg but not preS1 domain, so that it did not react with the preS1 21–47 a.a. peptide. HBV DNA was assayed by quantitative real-time PCR. Controls included Lipofectamine 2000 and unrelated siRNA.

Animal model of HBV viremic

Nude mice aged 4–5 weeks were purchased from the experimental animal center of Chongqing Medical University. The establishment of a HBV viremic animal model was performed as with a previous report. In brief, 10 nude mice were divided into two groups: HepG2 (n = 5) and HepG2 2.2.15 (n = 5). The mice were injected s.c. with 10⁷ HepG2 or HepG2 2.2.15 cells in 100 μL PBS into the left flank of the armpit. Tumors were allowed to grow to day 15, and blood was withdrawn before drug treatments. For drug treatments, three daily consecutive i.v. injections of preS1-9Arg-siRNA poly-conjugate (siRNA 1.0 mg/kg per day, preS1-9Arg: siRNA = 10:1) were carried out for the test group. For the control group, 1.0 mg/kg per day of free siRNA, 10 mg/kg per day of preS1-9Arg or PBS was administrated to each mouse. On day 21, blood was collected by retro-orbital sampling. On day 30, all animals were killed and blood from injected mice was collected.

Assessment of HBsAg and HBV DNA

Hepatitis B surface antigen levels in cell culture and blood samples were determined by semiquantitative ELISA. HBV DNA in cell culture or blood were extracted with the QIAamp DNA Blood Mini Kit (Invitrogen, San Diego, CA, USA), HBV DNA was quantitated with the BIO-RAD CFX 96 (Bio-Rad) system using the Syber Green Real-Time PCR Kit (Roche) with the primers 5′-CCTAGTAGTCAGTTATGTCAAC-3′ (forward) and 5′-TCATAAAGCTGAGGAGTTCGA-3′ (reverse). Concisely, PCR was performed with a primary denaturing step at 95 °C for 3 min, followed by 35 cycles at 94 °C for 15 s and annealing at 55 °C for 30 s. Then, the data were collected and real-time analysis was carried out.

Experimental toxicity

Mouse liver function was assessed based on the levels of alanine aminotransferase (ALT). The activity of serum ALT was determined with an automatic biochemical analyzer (Hitachi, Tokyo, Japan) in accordance with protocols. Cytokine induction and toxicities associated with siRNA were assayed by interferon (IFN)-α. Mouse IFN-α was quantified using sandwich ELISA kits (Huamei, Wuhan, China). Tumor sections from mice were fixed with formalin, dehydrated with graded alcohol, cut into 5-μm serial sections, embedded in paraffin, and detected by staining with hematoxylin to visualize cell outlines.

Statistical analysis

One-way ANOVA was used to analyze variance, and significant differences were performed by Dunnett’s test for multiple comparisons. Wilcoxon rank sum test was

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used to calculate significant differences at different time points in the same mouse. A \( P < 0.05 \) was considered statistically significant.

RESULTS

PreS1-9Arg binding and transfer to siRNA into HepG2 cells

FIRST, WE CONFIRMED the ability of fusion peptide preS1-9Arg binding to HepG2 cells by cell attachment test. When incubating cells with FITC-preS1-9Arg, strong green fluorescence was observed in the membrane and cytosol of HepG2 cells (Fig. 1A,a–c). Green fluorescence was also observed in HeLa cells (Fig. 1A,d–f). However, it was weaker than that of HepG2. The results demonstrated a specific ability of preS1-9Arg binding to HepG2 cells. Then, the ability of peptide combining with siRNA was confirmed by agarose gel electrophoresis. PreS1-9Arg and siRNA were mixed in various ratios. According to the results of gel electrophoresis, siRNA was completely trapped by the peptide with a slow migration speed at a ratio of 10:1 (Fig. 1B), so this ratio was regarded as the optimal ratio of peptide and siRNA to produce peptide-siRNA conjugate in the

![Figure 1](image-url)
later experiments. In the transfection experiments, red fluorescence in the cells indicated that the peptide did transfer siRNA into cells just as liposome did when the peptide and cy3-siRNA complex were incubated with HepG2 (Fig. 1C). The transfection efficiency was nearly the same as Lipofectamine 2000.

Inhibitory effect of preS1-9Arg-siRNA conjugate on HBx

To test the inhibitory effect of the preS-9Arg-siRNA conjugate on the expression of the target gene, mRNA level and protein level of HepG2x were analyzed by RT–PCR (Fig. 2a) and western blot (Fig. 2b) at 48 h post-transfection, respectively. The mRNA level was decreased significantly by 51% whereas the positive control was decreased by 64% (lip-siRNA) to the negative group (unrelated) (Fig. 2c). For protein examination, the expression of HBx was decreased by 52% for the test group whereas the positive control was decreased by 70% compared to unrelated siRNA control (Fig. 2d). The results showed a good inhibitory effect of preS1-9Arg-siRNA conjugate on the endogenous gene

Antiviral efficacy of preS1-9Arg-siRNA conjugate in vitro

To assess the antiviral efficacy of preS1-9Arg-siRNA conjugate in cell culture, the conjugate composed of three anti-HBV siRNA was added into the HepG2.2.215 cell culture. At 72 h post-transfection, HBsAg in supernatants of cell cultures in the test group were decreased significantly compared with those of the unrelated siRNA control group (Fig. 3a). Real-time PCR showed that the HBV DNA was decreased by 60% compared with that of control siRNA (Fig. 3b).

Animal model established by HepG2 2.2.15 cells

We first identified the viability of this model. HepG2 and HepG2 2.2.15 were inoculated s.c. into the flank of nude mice and the volumes of tumors were measured as tumor size (Fig. 4a). Sera from tumor-bearing mice were collected and assayed for HBsAg and HBV DNA at a specific time after tumor cell inoculation. A significant difference was observed in the HBsAg level between the HepG2 2.2.15 and the HepG2 tumor-bearing mice (Fig. 4b). To expand the ability of producing HBV particles by the model, we performed quantitative real-time PCR to analyze HBV DNA copies in the sera. In the HepG2 2.2.15 group, serum HBV DNA copies increased from $6.31 \times 10^4$ to $5.46 \times 10^6$ copies/mL at different time points whereas the HepG2 tumor-bearing mice did not have a similar result (Fig. 4c). Tumor size reached 4600–4900 mm$^3$ within 30 days (Fig. 4d). The results suggested that HBV particles were produced in nude mice and the conjugate composed of three anti-HBV siRNA was effective in inhibiting the expression of HBx.
animals that were injected with HepG2 2.2.15 cells. Otherwise, serum HBV DNA copies were related to the size of the HepG2 2.2.15 group. Thus, the HepG2 2.2.15 nude mouse model would be suitable for further study of HBV therapy.

Antiviral efficacy of preS1-9Arg-siRNA conjugates in vivo

In an animal model, anti-HBV efficacy of preS1-9Arg-siRNA conjugate was assessed by HBsAg and HBV DNA. Blood was withdrawn from mice before and after the i.v. injection of preS1-9Arg-siRNA molecular conjugate. On day 21 post-inoculation, compared with that of day 15, significant differences of HBsAg and HBV DNA were observed before and after preS1-siRNA conjugate treatment. In the preS1-9Arg-siRNA group, the serum level of HBsAg was decreased by 46% (0.41 to 0.22). On day 28, a diminishing trend still could be observed (Fig. 5a). On day 21, the serum HBV DNA copies were diminished significantly in the preS1-9Arg-siRNA group (5.0 × 10^5 to 6.3 × 10^3). On day 28, the HBV DNA copies could hardly be detected (see below for manufacturers’ details). For the siLUC, preS1-9Arg and PBS groups, there were no significant differences between two time points (Fig. 5b).

Toxicities associated with preS1-9Arg-siRNA conjugate

Potential toxic side-effects of preS1-9Arg-siRNA molecular conjugate were assessed experimentally by ALT. IFN response was assessed by IFN-α in vivo. Cell outlines were examined by immunohistochemistry. No significant change in the liver enzyme levels were observed in mice treated with preS1-9Arg-siRNA polyconjugate compared to free siRNA, preS1-9Arg and PBS (Fig. 6a). IFN-α were assayed by ELISA before and after drug treatment. There was no significant difference between the test group and control group, which revealed that the preS1-9Arg-siRNA molecular conjugate would not induce significant activation of the IFN response genes (Fig. 6b). At histological examination, no significant changes were found in tumors from the preS1-9Arg-siRNA molecular conjugate group compared with the other three groups. These data suggested that these animals responded biochemically to the implanted tumors; drugs caused no acute toxic effects in liver (Fig. 6c).

DISCUSSION

The application of RNAi to developing new HBV treatment is very promising. The inadequate target specificity and non-toxicity of most approaches to hepatocytes has limited their suitability for clinical use. Although viral vectors have been used successfully to deliver expressed siRNA sequences, non-viral vectors are believed to be the preferred class for therapeutic use. Thus far, a number of non-viral vector approaches have been employed to deliver siRNA to the liver by systemic injections. These include use of cholesterol conjugation, apolipoprotein AI complexes, α-tocopherol conjugates, phage fusion proteins, targeted exosomes and vitamin
A-coupled liposomes. In this report, we developed a new transfer vector using a 27-a.a. fragment of HBV preS1 for its ability to bind to hepatic cells.

PreS1 is believed to play a key role in the attachment and enrollment into hepatic cells in the course of HBV infection via binding to a putative viral receptor on hepatocytes. Also, myristoylated preS1 was reported to have the ability of binding to liver cells including PHH. Especially, the residue of 21–47 a.a. was found to be the essential sequence for its ligand binding. This ability of the preS1 21–47-a.a. peptide may help realize targeting transfer of siRNA to hepatic cells. In order to achieve RNA binding ability and facilitate peptide-siRNA conjugate being taken up into cells, a 9-mer arginine peptide was added to the C-terminal of preS1 peptide. The 9-mer arginine polymer has been reported to be an efficient carrier for transporting various types of biomolecules into living cells including nucleic acids, peptide, proteins and even nanoparticles, although the detailed mechanism was still not clear. Some researchers proved it different from the classical endocytosis pathway because it even happened at 4 °C and without receptors involved. Although the preS1 peptide has the ability to bind hepatic cells and enter the cytoplasm, it does not bind nucleic acids and therefore cannot be used to transport siRNA. However, a positively charged cell-penetrating peptide, 9-mer arginine polymer, can bind negatively charged nucleic acids by charge interaction and facilitate the cellular uptake of the nucleic acids. In addition, the combination could protect siRNA from degradation by endogenous RNase in vivo. Thus, we constructed a novel vector by fusing 9Arg to preS1 peptide and tested its ability to transfer siRNA into hepatic cells such as HepG2 and inhibit the expres-

Figure 4 HepG2 and HepG2 2.2.15 cells (10⁷) were inoculated s.c. into the flank of nude mice. (a) Two groups of mice were grown with tumors, successfully. (b) On days 15 and 30, sera were collected from mice, the level of hepatitis B surface antigen (HBsAg) was measured by enzyme-linked immunoassay on days 15 and 30. Data are shown as means. (c) Serum hepatitis B virus (HBV) DNA was measured by quantitative real-time polymerase chain reaction. Data are shown as means. (d) Tumor size was calculated on days 15, 20, 25 and 30 after inoculation. 15th day; 30th day; HepG2 2.2.15; HepG2.
sion of the target gene. We found that preS1-9Arg were able to combine siRNA and transport them into HepG2 cells, resulting in efficient gene silencing. In the HBV cell model, preS1-9Arg effectively inhibited the expression and replication of HBV. A recent publication also verified the ability of preS1 to target transfer drugs into human liver cells.28 The researchers synthesized preS1 to make a nanocage carrying small heat-shock protein and found that preS1-carrying nanocages showed lower cytotoxicity and significantly higher specificity for human hepatocyte cell lines than other cell lines in vitro.

Some researchers reported that N-terminal myristoylation of preS1 was very important for its biological activity. Our preS1-based molecular conjugate showed similar ability. Some researchers reported that N-terminal myristoylation of preS1 was very important for its biological activity.
binding to receptors on the membrane of hepatic cells.\textsuperscript{25} But some researchers also got controversial results and found that naked preS1 also had the ability to recognize receptors.\textsuperscript{29} In this paper, we used the naked preS1 peptide without myristoylation modification and achieved a satisfying result. Whether myristoylation of preS1 21–47 a.a. is more efficient in transferring siRNA to liver cells needs to be studied in the future.

Animal models are an obstacle in HBV research involving the natural infection course. Extensive efforts have been put forth in this field.\textsuperscript{29} The chimpanzee is a good research model for HBV, but its broad range of application is limited by the high cost and resource. The scid/Alb-uPA mouse with chimeric human liver cells may be an ideal small animal model for studying the basic biology of human hepatitis viruses, but it is difficult to construct the model because resource limitation and complex procedure.\textsuperscript{30,31} Transgenic mice carrying HBV are not suitable for research involving the entry of HBV particles into liver cells because of the species difference.\textsuperscript{32} Chang et al. reported a simple HBV viremic animal model by s.c. inoculating the HBV-producing cell line in SCID mice and observing the efficacy of anti-HBV drugs in this model. This model is not a liver transplanting model, but just a hepatic cell tumor model. HBV exists and produces continuously in the tumor other than in the mouse liver for virus DNA integrated in the genome DNA of HepG2 cells. Because the environment of virus replication is human source cells (HepG2), it is a suitable alternate for studying HBV biology and therapy. In this study, we constructed a HBV viremic animal model by s.c. inoculating HepG2 2.2.15 cells in nude mice. HBsAg and HBV DNA were also detected in the circulation of HepG2 2.2.15 tumor-bearing mice. In this model, serum HBsAg and HBV DNA titers were suppressed significantly by administration of preS1-9Arg-siRNA conjugate. The HBsAg titer reduced 56% and HBV DNA decreased 69%. Consistent with \textit{in vitro} study in cell lines, the results demonstrated that preS1-9Arg conjugate could effectively deliver siRNA to target tissue and inhibit the expression and replication of HBV \textit{in vivo}.

To evaluate the toxicity of preS1-9Arg-siRNA conjugate, serum IFN-\textalpha, ALT and liver histology were examined. The levels of IFN-\textalpha and ALT were not significantly different between the preS1-9Arg-siRNA group and the controls. Histological examination of the livers of all mice revealed no apparent damages. All these results suggested that our molecular conjugate did not impair the liver cells or induce non-specific innate immune response. The observed inhibitory effects on HBV expression and replication were unlikely to be the result of any unintended toxic immune stimulatory mechanisms, but rather the direct consequence of RNAi-mediated effects.

In summary, preS1-9Arg may be an effective and safe therapeutic option for an siRNA delivering system \textit{in vivo}. Although there remain some challenges to be investigated such as dose optimization, detailed evaluation of non-specific effects, assessment of stability, pharmacokinetic analysis and metabolite profiling, this study represents a potential non-viral siRNA delivery technology targeting human liver to advance hepatitis B therapy.

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