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Title:

Cell penetrating peptide-mediated systemic siRNA delivery to the liver

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Abstract

The Cell-penetrating peptide (CPP) is one of the most attractive tools for efficiently delivering biomolecules to a target organnelle. Here, we describe the use of octaarginine (R8)-modified lipid nanoparticles for the efficient and targeted in vivo delivery of siRNA to the liver. In this study, SR-BI (a scavenger receptor class B, member 1) was targeted by this nanoparticle. Our results demonstrate that R8-modified lipid nanoparticles can be used for the efficient and targeted delivery of liver siRNA to induce the specific knock-down of an endogenous gene with minimum liver toxicity and immune response, and that this CPP based technology holds considerable promise for further in vivo biological applications of siRNA.

Keywords:

Cell penetrating peptide , octaarginine , siRNA delivery , liver

1. **Introduction**

Since the first report of the cell-penetrating peptide (CPP) in 1994 (Derossi D et al., 1994), many types of CPPs have shown to improve the intracellular delivery of various biologically active molecules into living cells, with a high degree of efficiency (El-Andaloussi S et al., 2005; Deshayes S et al., 2005), and have more recently been applied to the in vivo delivery of siRNA (Kumar P et al., 2007; Eguchi A et al., 2009; Crombez L et al., 2009; Jarver P et al., 2010). Many kinds of systemic siRNA delivery techniques have been successfully achieved by conjugating compounds such as cholesterol (Soutschek J et al., 2004), polymers (Rozema DB et al., 2007), and targeted ligands (Nishina K et al., 2008) to siRNA, by forming stable nucleic acid-lipid nanoparticles (SNALP) of siRNA (Zimmermann TS et al., 2006; Semple SC et al., 2010), and by assembling lipid-siRNA complexes (Santel A et al., 2006). Carbon nanotubes have also been used to systemically deliver siRNA (McCarrol J et al., 2010). Despite this progress, CPP mediated systemic siRNA delivery have not been uniformly successful, except for delivery to the brain (Kumar P et al., 2007) and tumors (Crombez L et al., 2009), because most of the CPP mediated in vivo siRNA delivery studies have used a peptide-siRNA complex system, which was only partly protected from degradation in the serum. Therefore, the development of a CPP-mediated systemic siRNA delivery system which fully encapsulates siRNA, remains a major challenge for in vivo applications.

CPPs have unique pharmacokinetic properties as well as cell membrame penetrating properties. Tat (the transduction domain derived from the HIV Tat protein) and the NLS (nuclear localization signal) peptide rapidly accumulate in the kidney, while the nona-arginine (R9) peptide, an arginine-rich peptide, accumulates at high levels in the liver compared to ten of the most commonly used CPPs (Sarko D et al., 2010). We previously showed that octa-arginine (R8) peptide modified liposomes largely accumulated in the liver after intravenous administration and that this depends on the density of the R8 peptide on the liposomal surface (Mudhakir D et al., 2005). We further demonstrated that liposomes modified with a high density

of R8 peptide are taken up mainly through macropinocytosis, a process that is less susceptible to lysosomal degradation, and that this results in high gene expression in vitro (Khalil IA et al., 2006; Khalil IA et al., 2007). Therefore, we hypothesized that R8 peptide could be used as both a targeting peptide to the liver and to improve intracellular trafficking. We previously showed that R8-modified lipid nanoparticles, in which nucleic acid is condensed with a polycation followed by encapsulation by a lipid envelope, can efficiently encapsulate siRNA and silence the luciferase gene without any detectable cytotoxicity (Nakamura Y et al., 2007; Akita H et al., 2010). In this report, we report on the development of a targeted in vivo siRNA delivery system using R8-modified lipid nanoparticles. The findings indicate that the liver targeting ability of R8-modified lipid nanoparticles causes the specific knock-down of the endogenous gene in primary hepatocytes and in the liver.

2. Material and Methods

2.1 Reagent

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids (Alabaster, AL, U.S.A.). Stearylated-R8 (STR-R8) was purchased from NeoMPS Industries (San Diego, CA, U.S.A.). Cholesteryl-GALA (Cho-GALA) was purchased from Kurabo Industries, Ltd. (Osaka, Japan). Polyethyleneimine (PEI) (average MW 10000) was purchased from Wako Pure Chemicals (Osaka, Japan). [cholesteryl-1,2-3H(N)]-cholesteryl hexadecyl ether ([^{3}H]CHE) was purchased from PerkinElmer Life Science Japan (Tokyo, Japan). Anti-SR-BI siRNA (21-mer, 5'-GUCGCAUGGCUCAGAGAGUTT-3' , 5'-ACUCUCUGAGCCAUGCGACTT-3'), Cy3-labeled anti-SR-BI siRNA (21-mer, 5'-GUCGCAUGGCUCAGAGAGUTT-3' , 5'-Cy3-ACUCUCUGAGCCAUGCGACTT-3'), and anti-lucferase siRNA (21-mer, 5'-GCGCUGCUGGUGCCAACCCTT-3' , 5'-GGGUUGGCACCAGCAGCGCTT -3') were obtained from Hokkaido System Science (Sapporo, Japan). PolyI:C was obtained from SIGMA-Aldrich Co. (St.Louis, MO, USA). All other reagents were commercially available reagent-grade products.

2.2 Animals

Male ICR mice (4-5 weeks old) and female C57BL/6 mice (6-7 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The experimental protocols were reviewed and approved by the Hokkaido University Animal Care Committee in accordance with the guidelines for the care and use of laboratory animals.

2.3 Preparation of R8-modified lipid nanoparticles

R8-modified lipid nanoparticles were prepared by the hydration method as described previously (Nakamura Y et al., 2007). In brief, a PEI solution (1mg/mL) was added to the siRNA solution (2mg/mL) under vortexing at a nitrogen/phosphate (N/P) ratio of 1.8. A lipid

film was then formed by evaporating an ethanol solution (312.5 nmol total lipids), composed of STR-R8, DOPE, and Cho-GALA (molar ratio of STR-R8/DOPE/Cho-GALA: 18:77.5:4.5). The siRNA/PEI complex (50 μg siRNA) was applied to the lipid film, followed by incubation for 5-10 min at room temperature to hydrate the lipids. To coat the siRNA/PEI complex with the lipid, the lipid film was sonicated for approximately 1 min in a bath-type sonicator (AU-25, AIWA Co., Tokyo, Japan). The average diameter and zeta-potential of the nanoparticles were determined using a Zetasizer Nano ZS instrument (Worchestershire, U.K.).

2.4 Pharmacokinetics and biodistribution experiment in mice

R8-modified lipid nanoparticles were prepared with a non-exchangeable lipid label [^{3}H]CHE incorporated. The labeled nanoparticles containing 80 μg of siRNA were injected via the tail vein of ICR mice. At the indicated times, the mice were sacrificed and blood, liver, lung, splen, and kidney were collected. After weighing the tissue samples, approximately 0.2 g of tissue samples (in the case of blood samples, 20 μL) were solubilized in 2 mL of Soluene-350 overnight at 55 ºC. All samples were decolorized by treatment with H_2O_2. The radioactivity was determined by liquid scintillation counting (LSC-6100; ALOKA CO, Ltd., Tokyo, Japan) after adding 10 mL of scintillation fluid (Hionic fluor; Perkin Elmer, Inc., MA, U.S.A.) to the scintillation vials. Blood concentration and tissue accumulation is represented as the percent of the initially injected dose (%ID/mL and %ID/tissue, respectively).

2.5 Primary hepatocyte culture and R8 peptide-mediated siRNA transfection study

Primary hepatocytes were collected from ICR mice 7 to 8 weeks of age, as described previously (Ukawa M et al., 2010). After checking for cell viability and cell numbers, 5×10^5 cells were seeded on a 100 mm plate in 10 mL of culture medium (William's E medium with antibiotics, 10 % serum, 1 nM insulin and 1 nM dexamethazone) for 3 h. The cells were washed in phosphate-buffered saline (PBS) to remove dead cells. The indicated dose of siRNA

containing nanoparticles was then added to the cell culture medium (the concentration of FBS was fixed 10% in all samples) and the suspension then incubated in a CO_2 incubator for 24 hours.

2.6 R8 peptide-mediated siRNA delivery in mice

C57BL/6 mice (6-7 weeks old) were treated with 100 μg or 50 μg or 25 μg siRNA in a 500 μL injection volume by intravenous injection. The mice were killed 24 h after the treatment, and blood and liver were collected. Liver samples were stored in RNAlater solution.

2.7 Measurement of serum biochemical value and cytokine

Blood samples were centrifuged at 10000 rpm, 4 °C, 10 min to separate serum. GPT and GOT levels in serum were measured using a transaminase C II test kit (Wako Pure Chemicals, Osaka, Japan) and IL-6 and IFN-gamma levels in serum were determined with ELISA kits (R&D systems, Inc.Minneapolis, USA), according to manufacturer's instructions.

2.8 Real time RT-PCR analysis

Total RNA was extracted from primary hepatocytes and liver tissue with the TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's recommended procedures. The cDNA was synthesized using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA, USA). Amplification of the SR-BI fragment was performed with the forward primer (5'-TCCAGCCTGACAAGTCGCATGG-3') and the reverse primer (5'-GCTTGCTCTCCATCAATATCGAGCC-3'), and beta-actin as an internal control was performed with the forward primer (5'-GAAGGAGATTACTGCTCTGG-3') and the reverse primer (5'-ACACAGAGTACTTGCGCTCA-3') using the SYBR Green Realtime PCR Master Mix instrument (TOYOBO, Osaka, Japan). PCR was performed using the Mx3005P Real-time QPCR system (Agilent, Foster City, CA, U.S.A.) : a 10-min pre-incubation at 95 ºC, followed

by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Data were analyzed using the comparative ΔΔCt method.

2.8 Analysis of lysosomal localization

To examine the intracellular trafficking of R8-modified lipid nanoparticles, 1×10^5 primary hepatocyte cells were seeded on a 35 mm glass base dish (IWAKI, Osaka, Japan) in 2 ml of culture medium. Cy3-labeled siRNA loaded R8-modified lipid nanoparticles (100nM) were incubated with cells in 1ml of culture medium for 1.5 h at 37 °C, 5% CO_2. To stain the nuclei and endosomes, the cells were incubated with culture medium containing 5 μg/ml Hoechst 33342 and 2 μg/ml Lysotracker Green for 30 min at 37 °C, 5% CO_2. After washing the cells 3 times with PBS supplemented with heparin (40 Unit/ml) to remove nanoparticles that were bound to the cell membrane, the medium was changed to observation buffer (135 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 1.8mM $CaCl_2$, 5 mM HEPES, 10 mM Glucose, pH 7.3) and the cells were observed by confocal laser scanning microscopy (Nikon A1; Nikon Co, Ltd., Tokyo, Japan)

2.9 Statistical analysis

Statistical comparisons were performed by one-way ANOVA, followed by Student's t-test for the two groups.

3. Results

3.1. Physicochemical characterization of R8-modified lipid nanoparticles

The physical characteristics of the R8-modified lipid nanoparticles used in this study are shown in Table 1. The initially prepared siRNA core had a particle size of 90 nm and a zeta-potential of - 30 mV. After coating the particle with lipids, the particle size increased to approximately 160 nm and the zeta-potential was inverted to + 40mV. This suggests that the siRNA core was encapsulated by the lipid envelope.

3.2. Blood clearance and biodistribution studies

To determine the in vivo distribution of R8-modified lipid nanoparticles, the mice were injected with [3H]-CHE-labeled nanoparticles via a tail vein injection. The resulting clearance property is described in Fig.1 (a). The plasma half-life value, using a one compartmental model, was caluculated as 6.3 min. On the other hand, R8-modified lipid nanoparticles rapidly accumulated in the liver (44 ± 2 % injected dose per organ) at 10 min (Fig.1 (b)). The biodistribution of liver, lung, spleen, kidney, tissues that are well-known as first-pass organs, 1 h after administration is shown in Fig.1 (c). The accumulation in the liver was significantly higher compared to other tissues, indicating that R8-modified lipid nanoparticle system has a high selectivity for the liver.

3.3. R8 peptide-mediated delivery of siRNA in primary hepatocyte

Dose-response experiments performed on primary hepatocytes revealed that the R8 peptide-mediated delivery of SR-BI siRNA induced a significant reduction in SR-BI mRNA at a dose of more than 25 nM (36 % reduction compared to non-treatment) (Fig.2). In contrast, no significant silencing was observed in the case of 100 nM of luc siRNA compared to non-treatment although there was slight reduction in SR-BI mRNA (23 % reduction compared

to non-treatment). To investigate the intracellular trafficking fate of siRNA, their colocalization with the lysosomal compartment was evaluated (Fig.3). Most of the Cy3-labeled siRNA (Red-dots) was not colocalized with the lysosomal compartment (Green-dots) although a few dots were colocalized (yellow-dots), demonstrating that R8-modified lipid nanoparticles can be used for the efficient delivery of siRNA to the cytosol.

3.4. In vivo R8 peptide-mediated SR-BI siRNA delivery

To assess the ability of R8 peptide-mediated siRNA delivery in vivo, R8-modified lipid nanoparticles containing SR-BI siRNA were injected into mice by using a single injection via the tail vein. As shown in Fig.4 (a), a dose-dependent reduction in SR-BI mRNA was observed (45 % silencing observed at 25 μg of siRNA, and 75 % silencing achieved at 100 μg of siRNA). In contrast, no significantly detectable reduction was observed with only HEPES buffer or luc siRNA loaded nanoparticles (Fig.4 (b)), indicating that the silencing is specific to the SR-BI gene.

3.5 Evaluation of toxicity

GPT and GOT levels in mouse serum were determined 24 h after R8 peptide-mediated siRNA delivery. GPT and GOT are respectively regarded as reliable indicators of liver tissue damage and more general, systemic tissue damage. As shown in Table 2, the levels of both proteins in the R8 peptide-modified nanoparticle treated mice were not significantly increased compared to non-treatment or Hepes buffer treated mice. Furthermore, the IL-6 and IFN-gamma levels in mouse serum were measured 6h and 24h (Table 3). When mice were treated with R8-modified lipid nanoparticles containing 100 μg of siRNA, these IL-6 levels were slightly increased at 6h, but these numbers were significantly lower compared to the treatment of poly IC ($p<0.001$). This level was back to almost normal level at 24h. On the other hand, the production of IFN-gamma was no detectable in either case. Therefore, the R8 peptide-mediated siRNA

delivery system can be assumed to be well tolerated for immune response as well as liver toxicity.

4. Discussion

The findings reported herein show that R8-modified lipid nanoparticles may enable the deliver siRNA to the liver. When siRNA is delivered in this way, it is capable of silencing the expression of SR-BI in both either primary hepatocytes and the liver with minimum liver toxicity. The key features of these R8-modified lipid nanoparticles are as follows: (i) this system has ability to target in the liver rapidly within 10-20 min (Fig.1 (b) and (c)). This liver targeting ability can be attributed to the high density of the R8 peptide (more than 10 % of modification of total lipids) on the surface of the nanoparticles (Mudhakir D et al., 2005). (ii) the system delivers siRNA to the cytosol quite efficiently. Imaginig data revealed that most of the siRNAs were not colocalized in the lysosomal compartment (Fig.3), which is consistent with previous reports indicating that a high density of R8 peptide on the surface of nanoparticle facilitates the induction of macropinocytosis, which is an advantageous route for efficient intracellular trafficking, and which stimulates efficient endosomal escape (Khalil IA et al., 2006; El-Sayed A et al., 2008). (iii) there is no need to use chemically modified siRNA (Braasch DA et al., 2003) to protect against degradation in the serum because siRNA is efficiently encapsulated within a nanoparticle (Nakamura Y et al., 2007).

Delivery of the SR-BI siRNA caused significant knockdown, which was dependent on the amount of nanoparticles employed (Fig.4). A Cy3-siRNA signal was detected in isolated hepatocytes after injection via the tail vein (data not shown). Although SR-BI is mainly expressed in hepatocytes (Hoekstra M et al., 2003), the contribution of endothelial cells and Kupffer cells was also take into consideration because this type of nanoparticle is highly positively charged (Table 1). Further experiments will be needed to investigate the knockdown contribution of hepatocytes and non-parenchymal cells, and to refine the intrahepatic selective delivery by using a target ligand or biodegradable polyethyleneglycol (PEG) to avoid recognition by kupffer cells.

The hepatotoxicity and innate immune response of R8-modified lipid nanoparticles were

assessed. It has been reported that intravenous injection of cationic nanoparticles encapsulating pDNA without PEG modification induced proinflammatory cytokines as well as hepatic toxicity (Hatakeyama H et al., 2011). In contrast, in the case of siRNA systems, IL-6 and IFN-gamma responses were greatly reduced (Table 3). In particular, the level of IFN-gamma was not detectable when delivering R8-modified lipid nanoparticles. This phenomenon is very surprising because the production of IFN-gamma is usually detected on the intravenous injection of liposome/siRNA complex (Sato A et al., 2007) or siRNA encapsulated nanoparticles (Zimmermann TS et al., 2006). These observations suggest that siRNA may be safer than pDNA even when the cationic nanoparticle systems without PEG modification are used and R8-modified lipid nanoparticles may be tolerated by the immune response. Further studies will be needed to completely inhibit these toxicity parameters for the safe siRNA delivery systems. The modification of PEG or a biocompatible material is one such example.

In conclusion, we report on the development of an R8 peptide-mediated siRNA delivery system that targets the liver. The system significantly silences endogenous genes at low effective concentrations of siRNA (25 μg / mouse) in only a single treatment, and no evidence was found for liver toxicity. These results provide the first evidence to demonstrate that R8-modified lipid nanoparticles can be safely and effectively used to systemically deliver siRNA to the liver, and opens the way to the development of more efficient CPP-mediated siRNA delivery systems for widespread applications, in both experimental biology and molecular medicine.

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Table 1

Physicochemical properties of the R8-modified lipid nanoparticles

	size (nm)	z-potential (mV)	PdI
siRNA/ PEI core	89 ± 10	-27 ± 3	0.22 ± 0.01
R8-modified lipid nanoparticles	162 ± 8	38 ± 4	0.21 ± 0.04

Data are presented as the mean ± SD for four independent experiments.

Table 2

GPT and GOT levels in mouse serum 24 h after R8 peptide-mediated siRNA (100 μg) delivery

	GPT (IU/L)	GOT (IU/L)
Non-treatment	4.3±0.9	6.1±2.8
Hepes buffer	6.5±2.6	8.9±4.8
luc siRNA	9.9±1.3	20.9±12.9
SR-BI siRNA	5.7±2.4	18.2±9.3

Data are presented as the mean ± SD (n=3).

Table 3

IL-6 and IFN-gamma levels for the R8-modified lipid nanoparticles (100 μg siRNA)- or poly IC (10 μg)-treated mice.

Treatment	Cytokine type	Time	pg/mL
MEND	IL-6	0h	N.D.
		6h	119±51
		24h	11＋4
	IFN-gamma	0h	N.D.
		6h	N.D.
		24h	N.D.
poly IC	IL-6	6h	1508±417

Data are presented as the mean± SD (n=4).

Figure legends

Fig.1 Pharmacokinetics and biodistribution of R8-modified lipid nanoparticles in mice.

(a) Plasma clearance and (b) liver biodistribution of ^{3}H-CHE-labeled nanoparticles in mice. Each mouse was injected with nanoparticles containing 80 μg of siRNA via the tail vein. (c) Tissue distribution was analyzed 1 h after treatment. Data are expressed as mean ± SD (n=3, ** P<0.01).

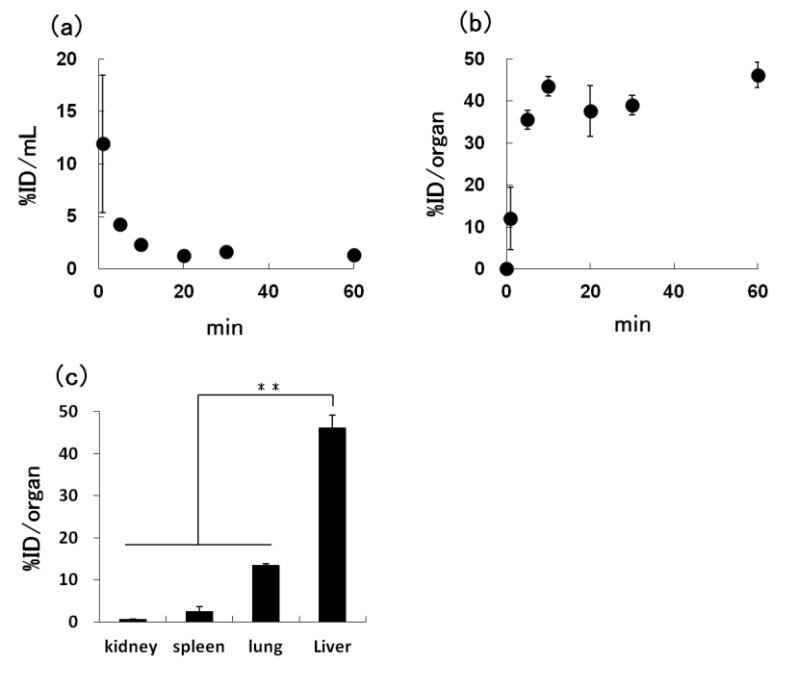


Fig.2 R8 peptide-mediated gene-silencing in mouse primary hepatocyte.

Primary hepatocytes were treated with nanoparticles containing SR-BI siRNA for 24 h. SR-BI mRNA levels normalized to beta-actin mRNA measured 24 h after transfection. Data are shown as mean ± SD (n=3).

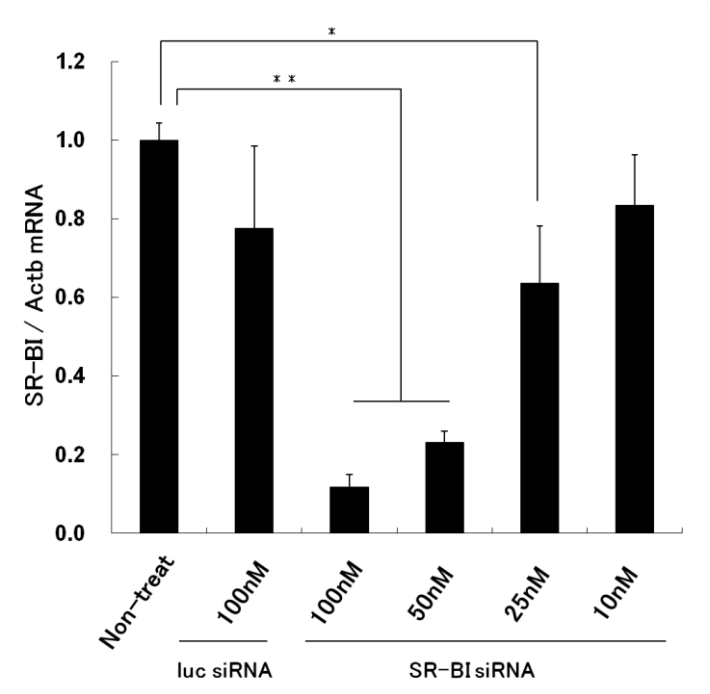


Fig.3 Lysosomal localization of R8-modified lipid nanoparticles.

Primary hepatocytes were incubated with Cy3-labeled siRNA loaded nanoparticles for 1.5 h. The lysosomal localization of siRNA was analyzed by means of confocal microscopy. The lysosomal compartment were stained with Lysotracker Green and nuclei were stained with Hoechst 33342. Bar indicates 50 μm.

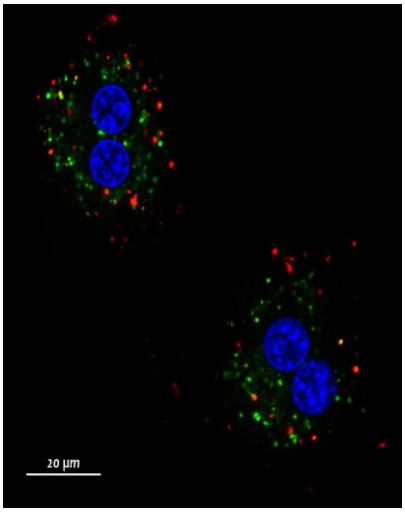
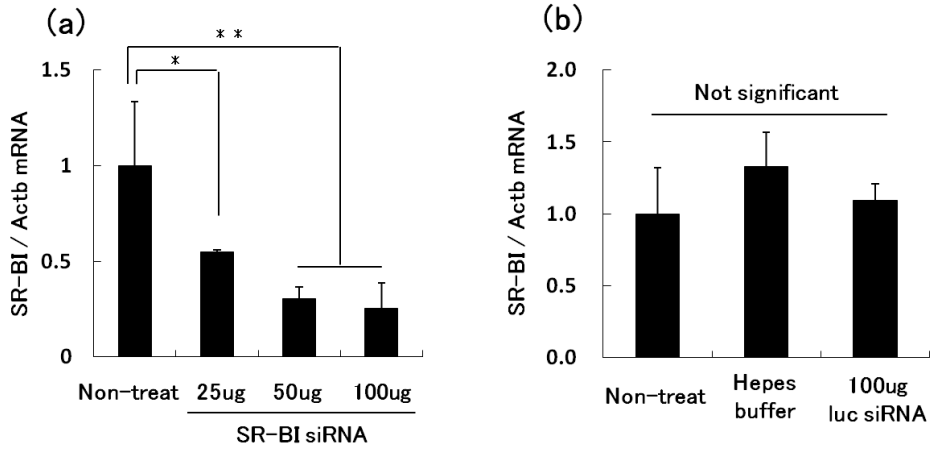


Fig.4 R8 peptide-mediated silencing of SR-BI in mice.

Liver SR-BI mRNA levels normalized to beta-actin mRNA measured 24 h after single intravenous injections. (a) dose-dependent and (b) gene-specific silencing were observed. Data are expressed as mean ± SD (n=3, $^{*}P<0.05$,$^{**}P<0.01$).



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