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Title:

Non-linear pharmacokinetics of octaarginine-modified lipid nanoparticles : Barriers from in vitro to in vivo

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Abstract

A rational development of an efficient siRNA delivery system is important for streamlining the RNAi-based drug development process. However, a huge gap frequently exists between *in vitro* and *in vivo* activity, which is the rate limiting step for developing versatile nanoparticles. We report herein on a remarkable non-linearity in the pharmacokinetics (PK), but not the pharmacodynamics (PD) using octaarginine (R8) modified lipid nanoparticles in mice. A quantitative study of siRNA molecules between cultured cells and mouse liver revealed a high correlation between intracellular siRNA molecules and their RNAi activities, indicating that there was no significant difference in the efficiency in PD. In contrast, a remarkable difference was observed in the non-linearity in PK. Quantitative analysis of the time profile for siRNA showed that the percentage of siRNA accumulation in mice was severely decreased with decreasing input dose compared to in vitro data. These unexpected data reveal an important clue to bridging the gap between *in vitro* and *in vivo* activity.

1. Introduction

A remarkable difference in nanoparticle activity between *in vitro* and *in vivo* systems is the most troublesome issue in the development of compatible nanoparticle-mediated nucleic acid delivery systems. Commercially available transfection reagents such as lipofectamine^{TM} 2000 and lipofectamine^{TM} RNAiMAX deliver pDNA or siRNA into cultured cells, resulting in efficient transfection activities, but they are not recommended for *in vivo* use because of their poor *in vivo* transfection efficiency. In a previous study, we also demonstrated that an octaarginine (R8) modified multifunctional envelope type nano device (R8-MEND) achieved a comparable transfection efficiency (10^8 RLU/mg protein) comparable to adenovirus in cultured cells [1], however, the *in vivo* transfection efficiency was determined to be 100 times less than *in vitro* [2]. In addition, some lipidoid-siRNA complex systems that have the most efficient siRNA knockdown activity in cultured cells fail to show the highest *in vivo* knockdown activity [3,4]. One possible explanation for these discrepancies is that our understanding of the rate limiting step between *in vitro* and *in vivo* is incomplete.

A quantitative evaluation of the intracellular pharmacokinetics (PK) and pharmacodynamics (PD) could shed some light on bridging the gap between *in vitro* and *in vivo* activity. The importance of controlling the intracellular PK was proposed for the rational development of an effective non-viral gene delivery method [5,6]. The intracellular PK process, including cellular uptake, endosomal escape, and nuclear localization can have a substantial effect on transfection efficiency. On the other hand, the prominence of PD has been exhibited in gene delivery systems [7]. In a previous study, we reported on a remarkable non-linearity between the number of pDNA molecules delivered to the nucleus and their gene expression activity in the case of LipofectAMINE and the PLUS reagent [8,9] and R8-MEND [10]. In addition, Wei J et al. demonstrated that a saturated PD effect existed in the case of an siRNA delivery system [11]. These results suggest that PD rather than PK is a determining factor that governs transfection efficiency, however, the rate limiting step for creating a huge gap between *in vitro* and *in vivo* still remains unclear.

In this study, comparative analyses between the *in vitro* and *in vivo* siRNA accumulation, intracellular trafficking, and silencing activity were carried out using R8-modified lipid nanoparticles that have already been reported to silence endogenous genes in cultured cells and in mouse liver [12]. Since a great need exists for a nucleic acid delivery system to exert silencing activity at a lower dose, the influence of dose-dependency on PK and PD between *in vitro* and *in vivo* situations was examined.

2. Materials and methods

2.1. Materials

2.1.1. Chemicals

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-shGALA (Cho-WEAALAEALAEALAEHLAEALA) was purchased from Sigma (St. Louis, MO, USA) [13]. Polyethyleneimine (PEI) (average MW 10000) was purchased from Wako Pure Chemicals (Osaka, Japan). Anti-SR-BI siRNA (21-mer, 5'-GUCGCAUGGCUCAGAGAGUTT-3', 5'-ACUCUCUGAGCCAUGCGACTT-3') and anti-lucferase siRNA (21-mer, 5'-GCGCUGCUGGUGCCAACCCTT-3', 5'-GGGUUGGCACCAGCAGCGCTT -3') were obtained from Hokkaido System Science (Sapporo, Japan). All other reagents were commercially available reagent-grade products.

2.1.2. Animals

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.

2.1.3. Cell lines

Hepa1c1c7 (CRL-2026) was purchased from ATCC (Manassas, VA, USA). These cells were cultured in alpha-MEM (Life technologies Japan Ltd, Tokyo, Japan) containing 100U/ml penicillin-streptomycin and 10% fatal bovine serum in a 5% CO_2 incubator.

2.2. Methods

2.2.1. Preparation of R8-modified lipid nanoparticles.

R8-modified lipid nanoparticles were prepared by the hydration method as described previously [12]. 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-shGALA (molar ratio of STR-R8/DOPE/Cho-shGALA: 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 characterization of R8-modified lipid nanoparticles was determined using a Zetasizer Nano ZS instrument (Worchestershire, U.K.). The average diameter and zeta-potential of the nanoparticles were determined to be 161 ± 17 nm and 41 ± 4 mV, respectively.

2.2.2 Evaluation of siRNA silencing activity in vitro and in vivo.

The dose dependent gene silencing activity of R8-modified lipid nanoparticles was evaluated in *in vitro* and *in vivo* as follows. In an *in vitro* experiment, 1.5×10^5 cells were seeded on a 6 well plate in 2 mL of culture medium 1 day before the start of the experiment. R8-modified lipid nanoparticles containing SR-BI siRNA were transfected with these cells in 2 mL of culture medium with or without 10% serum for the indicated times at 37 ℃, 5% CO_2 incubator. The transfection medium was then removed and culture medium was added to these cells, which were then collected 24 h after the transfection. In *in vivo* experiments, mice were treated with R8-modified lipid nanoparticles in a

500 μL injection volume by intravenous injection. The mice were killed 24 h after the treatment, and livers were collected. The liver samples were stored in an RNAlater solution. Total RNA was extracted from Hepa1c1c7 cells and liver tissue with the TRIzol Reagent (Invitrogen, CA, USA) according to the manufacturer's recommended procedures. siRNA silencing activity was evaluated as described previously [12].

2.2.3. Quantification of siRNA molecules *in vitro* and *in vivo*

Both the *in vitro* and *in vivo* experimental protocols were the same as described in the above section, with the following exceptions. In the *in vitro* experiment, R8-modified lipid nanoparticles that were bound to the cell surface were washed 3 times with PBS supplemented with heparin (40 Unit/mL). The number of cells was counted just before sample collection to calculate the number of siRNA molecules per cell. In the *in vivo* experiment, 5-10 mL of a heparin containing PBS (40 units/mL) solution was perfused via the portal vein to remove R8-modified lipid nanoparticles that had become bound to cells on the surface of the liver. The weights of the livers were determined for each mouse and were converted into the number of liver cells using the relationship (1.5×10^8 cells/g of liver tissue) [14]. The number of siRNA molecules were calculated by a heating-in-Triton quantitative reverse transcription polymerase chain reaction method as described previously [15]. In a typical run, 1 mL of 0.25 % Triton X-100 PBS solution was added directly to siRNA transfected cells or 100 mg of liver tissues. After adding 250 pmol of siRNA to non-treatment cells or naive liver tissues, they were used as standard samples. After homogenizing these samples, they were incubated for 10 min at 95 ℃. All lysates were then cooled on ice for 10 min and the suspensions then centrifuged at 20,000 g for 20 min at 4 ℃ and the supernatants were collected and diluted with 0.25 % Triton PBS. To detect intact SR-BI antisense-siRNA strands, cDNA was synthesized using a Tagman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). A Custom TagMan (R) RNA assays kit (Applied Biosystems) and TagMan Gene Expression Master Mix (Applied Biosystems) were used to amplify cDNA fragment corresponding to the SR-BI antisense-siRNA. PCR was performed using the Mx3005P Real-time QPCR system (Agilent, Foster City, CA, USA) : a 10-min pre-incubation at 95 ℃, followed by 40 cycles of 95 ℃ for 15 s and 60 ℃ for 1 min. The number of siRNA molecules in each experimental sample was calculated using a standard sample curve. A lower limit of detection of 48 ± 6 and 51 ± 33 siRNA molecules per cell was calculated from the non-treated samples in *in vitro* and *in vivo*, respectively.

2.2.4 Statistical analysis

Comparisons between multiple treatments were made using one-way analysis of variance (ANOVA), followed by the Dunnett's test. Linear regression method was used for assessing an in vitro and in vivo correlation between siRNA amount and gene silencing activity. A *P*-value of < 0.05 was considered significant.

3. Results

3.1. Comparison of Gene Silencing Activity between *in vitro* and *in vivo*

We recently reported that the intravenously administered R8 modified lipid nanoparticles can silence an endogenous gene in the liver [12]. To assess the ability of R8 modified lipid nanoparticles both *in vitro* and *in vivo*, dose-response experiments were performed. We used siRNAs for targeting scavenger receptor class B, member 1 (SR-BI) gene, which is highly expressed in hepatocytes, as an endogenous gene. Dose dependent gene silencing activity was observed with or without 10% serum (**Figure 1a**). Significant gene silencing was detected at concentrations as low as 2 nM without serum, and the concentration of siRNA required to knock down 50 % of the SR-BI gene (i.e. ED_{50}) was about 13 nM. In contrast, in the presence of 10 % serum, ED_{50} shifted from 13 nM to 78 nM. A similar dose dependent gene silencing activity was

Figure1 In vitro and in vivo silencing of SR-BI expression by R8-modified lipid nanoparticles. (a) SR-BI silencing in vitro. (b) SR-BI silencing in vivo.
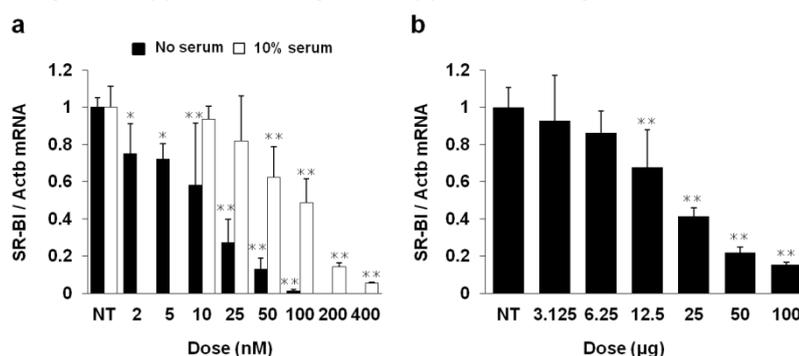
also found in mouse liver (**Figure 1b**). Considering the body weight of the mice used in this study (16.9±0.5 g), the ED_{50} was calculated to be 1.2 mg/kg.

3.2. Analysis of a PD between *in vitro* and *in vivo*

We considered two factors in analyzing the rate-limiting step between *in vitro* and *in vivo*, namely, intracellular PK and PD (**Figure 2**). We initially focused on the PD, which can be analyzed based on the relationship between the number of cytosolic siRNA molecules and its gene silencing activity, because previous reports suggested that PD contributed to the non-linear relationship in both pDNA and siRNA delivery systems [8-11]. In this study, the number of siRNA

Figure2 Schematic image of the intracellular PK and PD of siRNA loaded by R8-modified lipid nanoparticles.
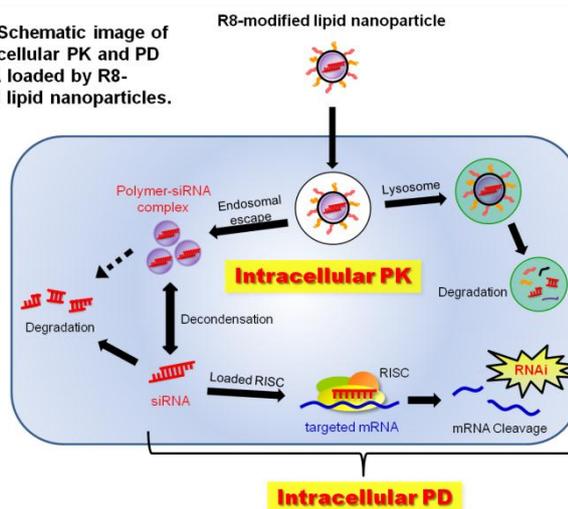
molecules in the cells was used to estimate PD of siRNA. The methodology used to determine the number of cells in both *in vitro* and *in vivo* experiments is described in the Materials and Methods section. Strong correlations between gene silencing activity and the number of siRNA molecules per cell were observed both *in vitro* ($R^2 = 0.78$, n = 24) (**Figure 3a**) and *in vivo* data ($R^2 = 0.80$, n = 19) (**Figure 3b**). Independent of the administered dose, 5.0×10^4 and 4.4×10^4 siRNA molecules per cell were required to achieve an ED_{50} for SR-BI gene silencing in both *in vitro* and *in vivo* experiments, respectively. Furthermore, a statistical analysis of the two regression lines revealed that
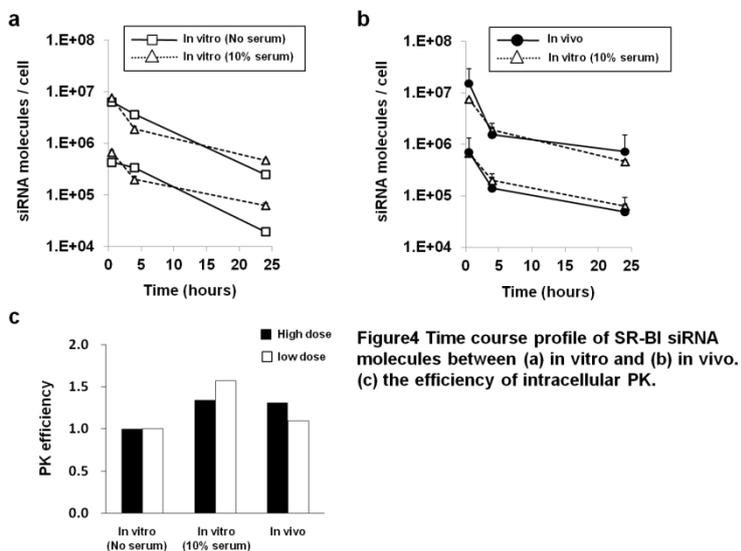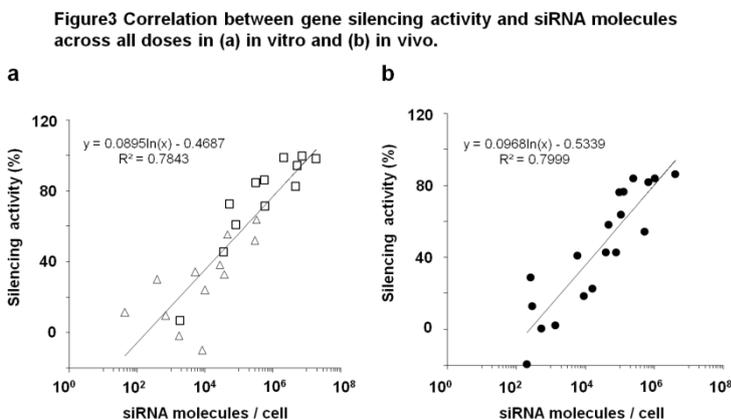
the P value for the slope and y-intercept were not significantly different ($P_{(slope)}$=0.64, and $P_{(y-intercept)}$=0.71), indicating that PD are not the critical factor for the discrepancy between *in vitro* and *in vivo* activity.

3.3. Quantification of an Intracellular PK, *in vitro* and *in vivo*

We next evaluated the intracellular distribution of siRNA molecules delivered via R8-modified lipid nanoparticles. We compared data showing similar values at 30 minutes after the R8-modified lipid nanoparticle treatment, to exclude a possibility that the difference in the amount of siRNA could affect the subsequent intracellular fate of siRNA. We first analyzed the effect of serum under in vitro transfection conditions. Two different phases appeared in the presence of 10% serum; the first phase (from 30 min to 4 hours) involved the rapid degradation of siRNA, and the rate of degradation was milder in the second phase (from 4 hours to 24 hours), while one phase was observed in the absence of serum (**Figure 4a**). The same tendency was seen in two different dose sets (high dose and low dose), indicating that serum affects the subsequent intracellular siRNA degradation, regardless of input dose. Possible interpretations of this phenomena are discussed below. We next, compared the *in vitro* and *in vivo* data. Similar two phase patterns were evident for both in vitro transfections with 10% serum and in mouse liver at two different dose sets (**Figure 4b**). We then estimated the efficiency of intracellular PK by calculating the ratio of the number of siRNA molecules at 24 hours to 30 minutes. The relative intracellular PK efficiency was not greatly different between the *in vitro* and *in vivo* situations (**Figure 4c**).

Figure3 Correlation between gene silencing activity and siRNA molecules across all doses in (a) in vitro and (b) in vivo.

Figure4 Time course profile of SR-BI siRNA molecules between (a) in vitro and (b) in vivo. (c) the efficiency of intracellular PK.

3.4. Comparison of siRNA accumulation (% of input dose) between *in vitro* and *in vivo*

We examined siRNA accumulation (% of input dose) at 30 minutes to compare the initial disposition of siRNA. In the case of in vitro experiments, the accumulation process simply reflects cellular uptake, while in case of in vivo experiments, siRNA accumulation (% of input dose) involves circulating blood, interaction with blood proteins, tissue uptake by the liver as well as other

reticuloendothelial system (RES) such as the spleen (**Figure 5a**). In this study, we focused on a pattern of non-linearity observed both *in vitro* and *in vivo*, and compared the obtained data with the dose examination, although the precise process does not match perfectly. Based on the data, we conclude that this was a sufficient time for most of the R8-modified lipid nanoparticles to reach the liver [12]. When the input dose was decreased, the number of siRNA molecules in the

Figure5 Quantification of SR-BI siRNA in vitro and in vivo 30 min after the treatment of R8-modified lipid nanoparticles. (a) Schematic image of siRNA accumulation between *in vitro* and *in vivo*. (b) The number of intact siRNA molecules per cell. (c) Accumulation efficiency at different doses.
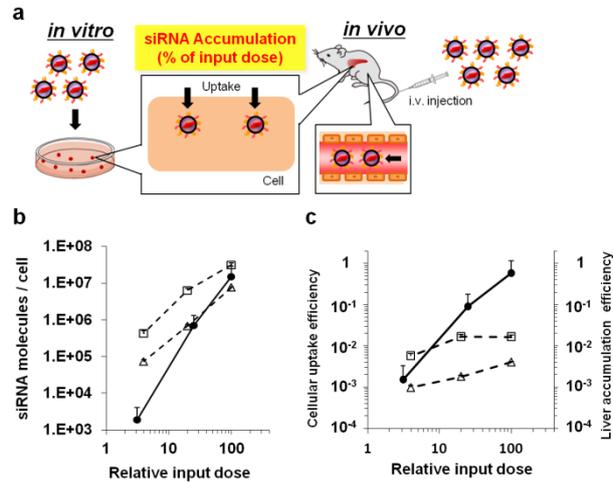
case of in vitro transfection without and with 10 % serum decreased from 3.1×10^7 to 4.3×10^5 (72 folds) and 7.7×10^6 to 7.5×10^4 (103 folds), respectively (**Figure 5b**). In contrast, a sharp reduction from 1.5×10^7 to 1.9×10^3 (8094 fold) was observed in mouse liver. A remarkable non-linearity in accumulation efficiency was found between the *in vitro* and *in vivo* situations (**Figure 5c**). The method used to calculate the accumulation efficiency is described in the Figure legend. The degree of non-linearity defined as the slope in **Figure 5c** was 253 for the case of *in vivo*, and 4.1 and 2.9 in the *in vitro* case, with and without serum, respectively. These results demonstrate that siRNA accumulation (% of input dose), and not intracellular trafficking and siRNA potency in the cytoplasm, is a critical factor for the observed gap between *in vitro* and *in vivo* activity.

4. Discussion

In vitro and *in vivo* PK and PD analysis is provided useful insights in understanding the huge gap between *in vitro* and *in vivo* activity. We divided this into two processes: intracellular PD and PK as described in **Figure 2**. A strong *in vitro* and *in vivo* correlation was seen in the relationship between the number of siRNA molecules per cell and mRNA knockdown (**Figure 3a, b**). Although these results were different from the fact that the Argonaute 2 (Ago2)-bound siRNA fraction, but not the total amount of siRNA found in the liver were correlated with target mRNA inhibition [11], we speculate that the capacity of siRNA to bind Ago2 was not saturated at our doses. The other possible explanation may be that most of the siRNAs in the cytosol are present in condensed form. Since R8-modified lipid nanoparticles contain condensed siRNA particles formed by the presence of polyethylenimine (PEI) in the nanoparticle [12], it is likely that the buffering capacity of the condensed siRNA particles prevents them from releasing excessive free siRNA into the cytosol, which prevents acute saturation with Ago2. Indeed, the findings indicate that about 12.5 % of the total siRNA molecules were detected as a free form released from siRNA/PEI complexes by the Trizol extraction method (data not shown). Furthermore, considering the fact that approximately 500 siRNA molecules per cell are required to achieve a 50% target reduction [15], the active siRNA that can bind Ago2 would be much less than the total siRNA amount detected. On the other hand, we compared the relative amount of SR-BI mRNA between Hepa1c1c7 and mouse liver. The relative SR-BI mRNA per cell in mouse liver was 57.7 times higher than that in Hepa1c1c7 (data not shown). Considering the fact that comparable siRNA numbers per cell were required to produce similar gene silencing activity (**Figure 3**), gene silencing activity would not depend on the amount of target mRNA in the cells. However, we also conclude that the number of siRNA molecules per cell as shown in Figure 3 are apparent values which include both free siRNAs and condensed siRNA with PEI in whole cells. The amount of free siRNA in the cytoplasm should be measured both in vitro and in vivo in order to further investigate whether or not the gene silencing effect is also dependent on the amount of target mRNA in the cells.

Interestingly, although *in vitro* intracellular PK was different between in the presence and absence of 10 % serum, the *in vivo* intracellular PK was similar to that found for the *in vitro* experiment with 10 % serum (**Figure 4a, b**). Since serum protein adsorption greatly affects cellular uptake and mechanism as well as nanoparticle size, aggregation state [16], it is likely that the siRNA degradation seen in the early phase (0-4 hours) in the presence of serum may be a result of rapid degradation via lysosomes. In contrast, the rate of degradation was higher in the absence of serum from 4 to 24 hours. Possibly, some as yet unidentified serum protein might inhibit the degradation of siRNA by becoming attached to the surface of nanoparticles than non-serum coated nanoparticles, however, studies of this phenomena will be needed to confirm this. On the other hand, we found a remarkable non-linear relationship between siRNA dose and accumulation ratio, i.e., the accumulation efficiency exponentially decreased with a decrease in the dose of siRNA (**Figure 5**). Though many steps are involved in the *in vitro* and *in vivo* accumulation of siRNA (% of input dose), the severe non-linearity seen in *in vivo* could be partially explained by the interaction of blood proteins. It is generally accepted that the binding of serum proteins on the surface of nanoparticles plays a critical role in the liposome elimination by cells of the mononuclear phagocyte system [17, 18]. Since the total amount of blood proteins is limited, the number of protein molecules that bind per nanoparticle would sharply decrease with an increase in the injected dose, which might cause the non-linearity. Indeed, Oja CD et al. reported that decreased blood proteins associated per liposome at higher liposome doses resulted in an improved liposome clearance [19]. Because the degree of non-linearity

seen in *in vitro* with and without serum was not greatly different (**Figure 5c**), we speculate that activated serum protein or a blood component might be responsible for the non-linearity phenomena. Further study will be

Table1 The relative value of non-linearity between in vitro and in vivo in each process

	in vitro (-)	in vitro (+)	in vivo
siRNA accumulation (% of input dose)	1	1.4	88
Intracellular PK	1	1.5	1.2
PD	1	1.0	1.2

The relative non-linearity of siRNA accumulation (% of input dose) and PD was estimated by calculating the ratio of the slope of in vitro with serum data or in vivo data to that of in vitro without serum data, as shown in Figure 3 and Figure 5. The relative non-linearity of intracellular PK was the average of the value seen in Figure 4c.

required to determine this. Finally, we summarized the degree of non-linearity in siRNA accumulation (% of input dose), intracellular PK, and PD (Table 1). Contrary to our expectations, siRNA accumulation (% of input dose), and not intracellular PK and PD, was involved in causing the huge gap between *in vitro* and *in vivo*.

In conclusion, we discovered that the pharmacokinetics of octaarginine-modified lipid nanoparticles between *in vitro* and *in vivo* are non-linear by quantitative PK and PD analysis. The finding of this *in vivo* non-linearity should be examined further in order to translate *in vitro* efficacy to *in vivo* utility.

Acknowledgments

Figure Legends

Figure 1 *In vitro* and *in vivo* silencing of SR-BI expression by R8-modified lipid nanoparticles. (a) SR-BI silencing in *in vitro*. Hepa1c1c7 cells were treated with R8-modified lipid nanoparticles containing SR-BI siRNA for 4 hours at the indicated concentrations (2-400 nM). Black bars and white bars represent SR-BI expression level in the absence and presence of 10% serum, respectively. (b) SR-BI silencing in *in vivo*. Mice were treated with R8-modified lipid nanoparticles containing SR-BI siRNA at the indicated dose (3.125-100 μg per each mouse). SR-BI mRNA expression was measured 24 hours after the treatment in both *in vitro* and *in vivo*. SR-BI expression levels were normalized to beta-actin expression levels measured 24 hours after the treatment. Values are the mean ± SD (n=3-6). (*P<0.05,**P<0.01).

Figure 2 Schematic image of the intracellular PK and PD of siRNA loaded in R8-modified lipid nanoparticles. Once the siRNA loaded R8-modified lipid nanoparticles are internalized by cells, they undergo endocytosis. Some fraction of the siRNAs escape from the endosomes into the cytosol, while others are transported into lysosomes, resulting in siRNA degradation. The condensed siRNAs are then release from polycations or nanoparticles, allowing them to diffuse in the cytosol. We define these intracellular processes as intracellular PK. Finally, siRNAs delivered into cytosols bind with RNA-induced silencing complex (RISC), leading to target mRNA degradation (We define this process as PD).

Figure 3 Correlation between gene silencing activity and siRNA molecules across all doses in (a) *in vitro* and (b) *in vivo*. The number of intact siRNA 24 hours after the R8-modified lipid nanoparticle treatment in Hepa1c1c7 and mouse liver was quantified by stem-loop RT-PCR. Detected siRNA molecules were normalized by number of cells. R8-modified lipid nanoparticles were treated with Hepa1c1c7 cells at the indicated concentrations (10, 25, 50, and 100 nM) in *in vitro* and treated with mice at the indicated doses (3.125, 6.25, 12.5, 25, 50 and 100 μg per each mouse) in *in vivo*. White squares, and white triangles, and black circles represent in vitro with no serum, in vitro with 10% serum, and in vivo, respectively. The line represents the regression curve.

Figure 4 Time course profile of SR-BI siRNA molecules between *in vitro* and *in vivo*. R8-modified lipid nanoparticles were treated with (a) Hepa1c1c7 cells for 30 min at the indicated doses (10 and 50 nM (○)) without serum and (50 and 250 nM (△)) with 10% serum and with (b) mice at the indicated doses (25 (▲) and 100 (●) μg). The number of intact siRNA was quantified at 30 min, 4 hours, and 24 hours by stem-loop RT-PCR. Heparin washing was carried out to remove R8-modified lipid nanoparticles both in vitro and in vivo. Values are the mean ± SD (n=3). (c) the efficiency of intracellular PK.

Figure 5 Quantification of SR-BI siRNA *in vitro* and *in vivo* 30 min after the treatment of R8-modified lipid nanoparticles. (a) Schematic image of siRNA accumulation between *in vitro* and *in vivo*. siRNAs were taken up by cultured cells after the R8-modified lipid nanoparticle treatment. On the other hand, R8-modified lipid nanoparticles targets to liver tissue after tail vein injection in mice, and were then taken up by liver cells. We define these two processes as siRNA accumulation (% of input dose). (b) The number of intact siRNA molecules per cell. R8-modified lipid nanoparticles were treated with Hepa1c1c7 cells for 30 min at the indicated concentrations (10, 50, and 250 nM) in *in vitro* and treated with mice at the indicated doses (3.125, 25, and 100 μg per each

11

mouse) in *in vivo*. The number of intact siRNA was quantified by stem-loop RT-PCR. The X axis shows the relative input dose, for which the maximum dose in each condition was assumed to be 100. (c) Accumulation efficiency at different doses. Cellular uptake and liver accumulation efficiency were estimated by calculating the ratio of siRNA numbers detected in all cultured cells or whole liver tissue to treated siRNA numbers. Black circles, white squares, and white triangles represent in vivo, in vitro with no serum, in vitro with 10% serum, respectively. Values are the mean ± SD (n=3).

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