Supplementary Information: Methods

“RNAi-mediated Gene Silencing in Non-Human Primates”

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Supplementary Methods

Synthesis of siRNAs. The siRNAs used in this study each consisted of a 21 nucleotide (nt) sense strand and a 23 nt antisense strand resulting in a single 2 nt overhang at the 3'-end of the antisense strand of the annealed duplex. siApoB-1 (position 10167-10187, NM_000384) sense: 5’-GUCAUCACACUGAAUACCAAU-3’, antisense: 5’-AUUGGUAUUCAGUGAUAGACAC-3’, siApoB-MM sense: 5’-GUGAUCAGACUCAAUCGAAU-3’, antisense: 5’-AUUCGUAAUGUGUGAGUGACAC-3’. The sequence and synthesis of Chol-siApoB-1 are as previously described1. siApoB-2 (position 2098-2118, NM_000384) sense: 5’-GGAAUCuuAuAuuGAUCcA*A-3’, antisense: 5’-uuGGAUcAAAuAuAAGAuUCc*e*U-3’. 2’O-Methyl modified nucleotides are in lower case and phosphorothioate linkages are represented by asterisks. Sense and antisense strands for siApoB-2 were synthesized as described previously1 except that N4-Acetylcytidine phosphoramidites were used for cytidine residues. These oligonucleotides were characterized by ESMS and anion-exchange HPLC. siRNAs were generated by annealing equimolar amounts of complementary sense and antisense strands.

Encapsulation of siRNA. siRNAs were encapsulated by an adaptation of the method of Jeffs et al2. The SNALP formulation contained the lipids PEG-C-DMA:DLinDMA:DSPC:Cholesterol (2:40:10:48 molar percent). 3-N-[(ω-Methoxy poly(ethylene glycol)2000)carbamoyl]-1,2-dimyristyloxy-propylamine (PEG-C-DMA) MW 2524 and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) MW 790 was obtained from Avanti Polar Lipids (Alabaster, AL), and cholesterol MW 387 was obtained from Sigma (Oakville, ON). The particle sizes of the SNALP samples used in this study were 77-83 nm with a polydispersity range of 0.09-
Nucleic acid encapsulation efficiencies were 92-97%. Particle size was determined using a Malvern Instruments Zetasizer 3000HSA (Malvern, UK) and nucleic acid encapsulation efficiency was determined using the Ribogreen assay as described elsewhere. \(^3\)

**In vivo rodent experiments.** All siRNAs were administered via tail vein injection under normal pressure and at a dosing volume of 0.01 ml/g. Female 8-10 week old C57BL/6 mice (Charles River Laboratories, MA) were used for the Chol-siApoB-1 and SNALP siApoB-1 dose response experiments. For the Chol-siApoB-1 experiment mice received either saline or Chol-siApoB-1 at doses of 100, 50, 25 or 12.5 mg/kg and for the SNALP siApoB-1 dose response mice received either saline or SNALP siApoB-1 at siRNA doses of 1, 0.5, 0.25 or 0.1 mg/kg. Liver mRNA levels were assessed 72 h after injection. For determination of specificity of apoB knockdown, five week old female BALB/c mice (Harlan Labs, IN) received either saline, SNALP siApoB-1 or SNALP siApoB-MM at a siRNA dose of 1 mg/kg or empty SNALP vehicle at an equivalent lipid dosage (25 mg/kg). Animals were sacrificed two days after treatment for liver mRNA and serum apoB-100 protein measures. For the dose response and duration of effect experiments, eight week old female C57BL/6 mice (Charles River Laboratories, MA) received either saline or SNALP siApoB-2. Liver mRNA levels were determined 72 h after injection of 5, 2.5, 1 or 0.5 mg/kg SNALP siApoB-2 for the dose response experiment. For the duration of effect experiment, animals received either saline or 2.5 mg/kg SNALP siApoB-2 and were anesthetized using isofluorine for collection of serum samples by retro-orbital bleed pre-dose (d0) and 3, 6, 9 and 13 days post-dose for measurement of apoB-100 protein. All procedures used in animal studies conducted at Alnylam were approved by the Institutional Animal Care and Use Committee (IACUC) and were consistent with local, state, and federal regulations as applicable. Animal studies conducted at Protiva were performed under the oversight of Protiva’s
Institutional Animal Care and Use Committee (IACUC) in accordance with the Canadian Council on Animal Care guidelines.

**In vivo non-human primate experiment.** For determination of the rate of plasma clearance of SNALP siApoB-2 in cynomolgus monkeys, two animals received 2.5 mg/kg SNALP siApoB-2 via bolus i.v. injection in the saphenous vein and plasma samples were collected 0.25, 0.5, 1, 2, 4, 6, 8, 12 and 24 h post-dose. In addition, blood samples were taken pre-dose, 6 and 24 h post-dose for Interferon-γ and IL-6 measures and liver samples (three punch biopsies per lobe, twelve total) were collected 24 h post-dose for determination of siRNA distribution and apoB mRNA levels. Eight jejunum sections were isolated from animals treated with saline, 1 or 2.5 mg/kg SNALP siApoB-2 48 h \((n = 4)\) or 264 h \((n = 2)\) post-dose for mRNA measurements. Plasma samples from animals treated with saline, 1 or 2.5 mg/kg SNALP siApoB-2 were collected pre-dose, 12, 24 and 48 h post-dose for all animals and 72, 96, 144, 192 and 264 h post-dose for two animals per group for apoB-100 protein measurements. Animals were fasted for 16 h prior to blood sampling for total serum cholesterol and lipoprotein collections pre-dose, 24 and 48 h post-dose for all animals and 144 and 264 h post-dose for two animals per group. Blood samples were collected pre-dose, 0.25 and 48 h post-dose for determination of complement Bb, CH50 and activated partial thromboplastin time (APTT) \((n = 6\) per treatment group) and pre-dose, 24, 48 \((n = 6\) per treatment group), 144 and 264 h post-dose \((n = 2\) per treatment group) for determination of alanine aminotransferase (ALT), aspartate aminotransferase (AST), bilirubin and urea nitrogen levels. All procedures using cynomolgus monkeys were conducted by a certified contract research organization using protocols consistent with local, state, and federal regulations as applicable and approved by the Institutional Animal Care and Use Committee (IACUC).
**In vivo bioanalytical methods for non-human primate experiments.** For determination of the circulation half-life of SNALP siApoB-2 in primates, 30 µl plasma (one per animal and time point) was incubated at 42°C for 20 min in a buffer containing 1.7 mg/ml proteinase K, 0.1 M Tris-Cl pH 7.5, 12.2 mM EDTA, 0.15 M NaCl, 1% SDS, 4.2 µM 40mer RNA internal standard in a 96-well plate. Samples were spin-filtered using a 0.2 µm filter plate and the filter was washed two times with 30 µl water (Varian, CA). Washes were combined with the initial filtrate and analyzed by ion exchange HPLC under denaturing conditions at pH 8 and 80°C with detection at 260 nm. Under these conditions, the siRNA eluted as two well-separated single strands. The internal standard, used for normalization of small differences in recovered filtrate volume, eluted at a later time than the two single strands of the siRNA. The amount of full length sense and antisense strand was determined relative to an external calibration curve derived from SNALP-formulated siApoB-2 and was calibrated over a linear range of 5-200 pmoles RNA. An estimated constant of 40 ml plasma per kg animal weight was used for calculation of the total amount of siRNA and the percent injected dose for each time point was calculated as the ratio of the amount of the sense or antisense strand to the amount of injected siRNA. Each data point represents the group mean ± s.d.

The QuantiGene® assay (Genospectra, CA) was performed to quantitate reduction of apoB mRNA relative to the house keeping gene GAPDH in lysates prepared from mouse liver or cynomolgus liver and jejunum as previously described with minor variations. For detection of cynomolgus mRNA, the apoB probe set was specific to human apoB (positions 13870 to 14110, NM_000384) and cross-reactive to the *Macaca fascicularis* apoB sequence (positions 380 to 615, CO775384) and the GAPDH probe set was specific for human GAPDH (positions 224 to 444, NM_002046) and cross-reactive to *M. fasciularis* GAPDH (positions 142 to 362, AB158631).
siRNA distribution in cynomolgus liver samples was detected using a ribonuclease protection assay with a radiolabeled probe complementary to the antisense strand of siApoB-2 using methods previously described\(^1\). The assay was performed on total RNA isolated from each of the twelve liver biopsies.

5’ RACE analysis was performed using total RNA (5 µg) from pooled liver biopsies (4 biopsies, 1 per lobe) as described previously\(^1\). Ligated RNA was reverse transcribed using a gene specific primer (GSP: 5’-TGGAAAGAAGTTGGTTGCTCATCTGGA-3’). Two rounds of PCR were performed. First round PCR using primers complementary to the RNA adaptor (GeneRacer, Invitrogen, CA) and apoB mRNA (Rev1: 5’-TCTTTTGATAGCCAAAGTGTTGCA-3’). Second round PCR using nested primers complementary to the RNA adaptor and apoB mRNA (Rev2: 5’-AAAGCTTGTGTGACACTGTCTGGGA-3’). The cleavage site at 2108/2109 of the apoB mRNA (NM_000384) was confirmed by sequencing of the PCR products.

Cynomolgus apoB-100 was detected from plasma samples using a sandwich ELISA consisting of a polyclonal goat anti-human apoB capture antibody (Chemicon International, CA) and a horseradish peroxidase-conjugated goat anti-human apoB-100 polyclonal detection antibody (Academy Bio-Medical Company, TX).

**In vivo rodent PK experiment.** Radiolabeled SNALP was prepared for plasma clearance and tissue distribution studies by incorporation of 2.7 µCi/mg total lipid of the non-exchangeable lipid label \(^3\)H-CHE\(^4\). SNALP was administered at a siRNA dose of 2 mg/kg via lateral tail vein injection in eight week old female BALB/c mice (Harlan Labs, IN) and blood was collected via tail vein nick over a 24 h period. At 24 h after injection, mice were euthanized and harvested tissues were homogenized in FastPrep Lysing Matrix Tubes (MP Biomedicals, CA) containing distilled water. Tissue homogenates were assayed for radioactivity by liquid scintillation counting with
Picofluor 40 and whole blood was assayed using Picofluor 15 (Perkin-Elmer, Boston, MA).


