

# Development of Lipoprotein(a) siRNAs for Mechanism of Action Studies in Non-Human Primate Models of Atherosclerosis

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**Abstract** Lipoprotein(a) [Lp(a)] has recently been recognized as an independent risk factor for coronary heart disease. While plasma Lp(a) levels are correlated with cardiovascular risk, the mechanism by which this particle contributes to atherosclerosis is largely unknown. Although humanized transgenic mouse model has recently been described to study Lp(a) biology, non-human primates (NHP) are the only preclinical model available that allow study of the role of Lp(a) in atherosclerosis in an innate setting. We describe targeting of *LPA* using lipid nanoparticle formulated short interfering RNAs (siRNAs) in lean rhesus macaque monkeys. We show >90 % *LPA* mRNA lowering in the liver and >95 % Lp(a) plasma reduction for over 3 weeks after a single siRNA dose.

Given the potency of *LPA* siRNAs, siRNA approach may enable chronic reduction of Lp(a) in atherosclerotic NHP and help to unmask the role for Lp(a) in the genesis and progression of atherosclerosis in man.

**Keywords** Apo(a) · Lp(a) · NHP models · LDL-c lowering · Atherosclerosis · siRNA · LNP · RNAi

## Abbreviations

apo(a)	apolipoprotein a
Lp(a)	lipoprotein (a)
NHP	non-human primate
LDL-c	low density lipoprotein cholesterol

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**Clinical Relevance** Lipoprotein(a) [Lp(a)] has recently been recognized as an independent risk factor for coronary heart disease. The application of siRNAs (whose development and characterization are described here) for *LPA* gene silencing in a relevant model of atherosclerosis could define a causal role for Lp(a) in atherosclerosis and offer a potential path to novel therapeutics to combat cardiovascular disease.

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siRNA short interfering RNA  
 LNP lipid nanoparticle  
 RNAi RNA interference

## Introduction

Lipoprotein(a) [Lp(a)] is a low density lipoprotein (LDL)-like particle covalently bound to apolipoprotein (a) [apo(a)]. Although its function is largely unknown, multiple lines of evidence, including epidemiology and Mendelian randomization, support Lp(a) as a possible causal risk factor for coronary heart disease [1–3]. As such, Lp(a) represents a possible target for therapeutic intervention in coronary heart disease.

Apo(a) is encoded by the *LPA* gene located on chromosome 6q26-27. The *LPA* locus is derived from a duplication of the neighboring plasminogen gene [4]. Apo(a) is made up of repeating units known as kringles (KIV and KV) and an inactive protease domain. KIV is composed of 10 subunits of which KIV type 2 is present in multiple copies ranging from 3 to greater than 40 [5]. Lp(a) levels can vary by more than 1,000-fold in humans [6] and are predominantly determined by variation in the *LPA* gene. The genetic factors which determine Lp(a) levels include copy number variants of KIV type 2, a pentanucleotide repeat in the *LPA* promoter that modulates gene expression, variations in RNA splicing, and several single nucleotide polymorphisms located within structural and functional domains [3, 4, 6, 7]. *LPA* has arisen recently in mammalian evolution and is only present in humans, apes, and old world monkeys making the non-human primate (NHP) the only preclinical model that allows for the study of endogenous Lp(a) biology [8].

RNA interference (RNAi) provides means for modulation of gene expression. RNAi is an evolutionary conserved mechanism of gene silencing that uses complementary double-stranded RNA molecules to direct sequence-specific cleavage of target mRNA [9]. RNAi has been successfully used for hepatic and extra-hepatic gene knockdown following both local and systemic administration [10–14]. Recent advancements in siRNA delivery platforms, specifically with regard to improvements in efficacy and tolerability, enabled the use of these reagents in NHP models and in the clinic [15–17]. siRNAs can be used for acute and chronic inhibition of mRNA expression that can be fine-tuned using dose titrations, and enable quick assessment of single or multiple target inhibition.

We describe the development of siRNA tools for hepatic *LPA* silencing in the rhesus macaque. We have identified a potent and well-tolerated siRNA oligo that results in >90 % inhibition of Lp(a) with a dosing of once a month. The development of the siRNA tools and the characterization of serum

lipid changes following *LPA* silencing in the lean NHP are the first steps in understanding the role Lp(a) plays in the development of atherosclerosis.

## Methods

### Lp(a) Measurements

All plasma samples were collected following an overnight fast. Lp(a) levels were measured on a clinical chemical analyzer using a commercially available immunoturbidity assay (Polymedco Inc., Cortlandt Manor, NY). Analytes were reported in nmol/L. The distribution of Lp(a) was measured in a random sampling of 30 humans, 90 cynomolgus, 56 rhesus, and 14 African Green monkeys. The same immunoturbidimetric assay was used to measure plasma Lp(a) levels in the NHP experiment detailed below.

### Oligo Design and Synthesis

siRNA oligos were designed and synthesized as previously described [12, 18]. Apo(a) and non-targeting (nt) siRNAs that were used in these experiments are listed in Table 1. siRNAs contained the following chemical modifications added to the 2' position of the ribose sugar: deoxy (d), ribo (r), 2' fluoro (flu), and 2' O-methyl (ome); and phosphothioate linkage were indicated (s). iB stands for an inverted abasic nucleotide. ApoB siRNA used was described previously [16].

### Generation of Luciferase Reporter Clones

siRNA screening was done using siCHECK dual luciferase reporter vector in Hepa 1-6 cells. All luciferase reporter plasmids used were derived from psiCHECK2 vector (Promega, Madison, WI) through de novo synthesis. The rhesus *LPA* insert sequence was XM\_001098061 nucleotides 1-3987 cloned downstream of *Renilla* reporter gene to generate genetic fusion.

### Transfection for Luciferase Reporter Silencing

Hepa1-6 cells were seeded in 96-well plates at a density of 10,000 cells per well and incubated at 37 °C overnight. The siRNAs and luciferase reporter plasmids were cotransfected using Lipofectamine 2000 (Life Technologies, Grand Island, NY). For primary screens, siRNAs were transfected at 10 nM final concentration and luciferase activity was measured 48 h later. For 12-point dose response experiments, the siRNA series are threefold or fourfold serial dilutions starting at 40 nM; whereas, plasmid concentration remained constant.

**Table 1** List of siRNAs used for in vitro and in vivo studies (5' to 3')

siRNA	Guide strand	Passenger strand
apo(a) Seq1	fluA;fluU;omeA;fluA;omeC;rU;fluC;rU;omeG;fluU;omeC;omeC; omeA;fluU;rU;rA;omeC;omeC;fluA;omeUs;omeU	iB;omeU;rG;rG;omeU;rA;rA;omeU;rG;rG;rA;omeC; rA;rG;rA;rG;omeU;omeU;rA;omeU;omeUs;omeU;iB
apo(a) Seq2	rU;omeG;rA;omeG;fluC;fluC;fluU;fluC;omeG;omeA;fluU; omeA;omeA;fluC;fluU;fluC;fluU;omeG;fluU;omeUs;omeU	iB;dA;fluC;dA;dG;dA;dG;fluU;fluU;dA;fluU;fluC; dG;dA;dG;dG;fluC;fluU;fluC;dA;dTs;dT;iB
ApoB	omeU;omeU;rG;rG;rA;rU;omeC;rA;rA;rA;omeU;rA;omeU; rA;rA;rG;rA;omeU;rU;rC;omeCs;omeCs;rU	rG;rG;rA;rA;rU;rC;omeU;omeU;rA;omeU;rA;omeU; omeU;omeU;rG;rA;rU;rC;omeC;rAs;rA
Non-targeting (nt) control	rU;rA;rU;fluC;omeG;omeA;fluC;omeG;fluU;omeG;fluU; fluC;fluC;omeA;omeG;fluC;fluU;omeA;omeG;omeU;omeU	iB;fluC;fluU;dA;dG;fluC;fluU;dG;dG;dA;fluC;dA; fluC;dG;fluU;fluC;dG;dA;fluU;dA;dT;dT;iB

### Lipid Nanoparticle (LNP) Preparation and Characterization

1,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1, 3]-dioxolane (DLinKC2-DMA) was synthesized by MRL according to established methods [19]. 1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were obtained from Avanti (Alabaster, AL) and Sigma-Aldrich Co (St. Louis, MO). 2-Dimyristoyl-sn-glycerol methoxypolyethylene (PEG 2000-DMG) was purchased from NOF Corporation (White Plains, NY). All purchased lipids were used as received. Ethanol was obtained from Sigma-Aldrich (St. Louis, MO), SYBR gold was obtained from Molecular Probes (Eugene, OR). Dulbecco's phosphate buffered saline solution (PBS) was obtained from HyClone laboratories (Logan, UT). All other buffers were prepared from the acid or sodium salt form of the components.

siRNA LNP were prepared by mixing appropriate volumes of lipids stock solution with an aqueous buffer containing siRNA duplexes. Lipid stock solution was prepared by dissolving desired amounts of DLinKC2-DMA, cholesterol, DSPC, and PEG-DMG in a mixed ethanol/aqueous citrate buffer system. siRNA duplexes were dissolved in an aqueous citrate buffer (pH 5). Mixing of the lipid and siRNA solutions was performed using an impingement jets T-mixer as previously described [19]. Equal volumes of lipid in ethanol and the siRNA in buffer were combined in the T-mixer using a dual syringe pump (Harvard Apparatus PHD 2000, Holliston, MA). The mixed material was sequentially diluted into equal volumes of citrate and PBS buffers upon leaving the mixer outlet in a multi-stage mixing process. The resulting LNPs were purified from free siRNA via anion exchange chromatography and were solvent exchanged into PBS via ultrafiltration. Nanoparticle size distribution was determined by dynamic light scattering using a DynaPro particle sizer (Wyatt Technology, Santa Barbara, CA). Particle size was calculated from the time based fluctuation in the light scattering intensity, quantified by a second order correlation function. The particle diameter for all LNP formulations was 65–75 nm. The concentration of the four lipids in LNP (DLinKC2-DMA, DSPC, cholesterol, and

PEG 2000-DMG) was determined by gradient reverse-phase ultrahigh-performance liquid chromatography methods. The siRNA concentration in LNPs was determined by a gradient strong anion-exchange method. Encapsulation efficiency of siRNA was greater than 90 % for all LNP formulations, as measured by SYBR gold fluorimetric method.

### NHP Experiments

All NHP studies were conducted at New Iberia Research Center (NIRC) with the approval by NIRC's and Merck's Institutional Animal Care and Use Committees (IACUC). Sexually mature male and female lean rhesus macaque were randomized into groups of 6 (for apo(a) and nt siRNAs) and 3 (for vehicle and ApoB controls) based on pre-study body weight and serum lipid levels (TC: median 141 mg/dl, range 109–223 mg/dl; LDL-c: median 60 mg/dl, range 31–93 mg/dl; HDL-c: median 73 mg/dl, range 52–117 mg/dl). TC, LDL-c, and HDL-c were measured directly from plasma using the Siemens Dimension assays for Clinical Chemistry Analyzer: CHOL, ALDL, and AHDL respectively (Siemens, Princeton, NJ). Animals were dosed intravenously (i.v.) with a single rate controlled 2-minute infusion of either 13.35 mg/m<sup>2</sup> dose (ApoB) or 26.7 mg/m<sup>2</sup> dose (apo(a), nt control) of LNP-encapsulated siRNAs. Blood samples were taken in conscious monkeys on days 7, 0, 3, 5, 7, 10, 14, 21, 28, and 35. Percutaneous liver biopsies were performed under sedation on days 7, 7, and 14. Serum analysis was performed at each blood collection time point to determine different clinical chemistry, hematology, and coagulation parameters. Plasma Lp(a) levels were determined on days 0, 3, 7, 14, 21, 28, and 35 (median: 52 mg/dl; range 3.5–126 mg/dl). Plasma ApoB levels were determined on days 0, 7, 14, 21, 28, and 35 (median: 40 mg/dl; range 22–123 mg/dl). Total RNA was isolated from liver tissue using the RNeasy 96 Tissue Kit (Qiagen, Valencia, CA). Taqman qPCR was done on an ABI 7900 Real-Time PCR System using the commercially obtained primers from ABI: Rh02789278\_m1 (apo(a)), MmuAPOBMGB1 (ApoB), and Rh02621745\_g1 (GAPDH).

## Statistical Analysis

Longitudinal analysis was done by repeated measure linear modeling to assess significant changes in plasma Lp(a) protein and serum lipids between treatment groups using a defined pharmacodynamics (PD) window of days 14–35. For each measurement, the percent relative to each animal's pre-dose baseline was calculated and then divided by the median of the nt control at each time point for double normalization. Normality tests were performed and log normal data was used to obtain Gaussian distribution for repeated measure linear modeling.

Single time-point analysis was done on each time point separately to assess significant changes in gene expression as there were too few biopsy time points to perform longitudinal analysis for mRNA knockdown. Parametric one-way ANOVA was performed on the ddCt values at days 7 and 14, and Tukey multiple comparison test was used to assess significant differences between groups.

## Results

### Distribution of Lp(a) in Human and Different NHP Models

By comparing plasma Lp(a) levels between lean healthy rhesus macaque, cynomolgus, and African Green monkeys and lean healthy humans, we found that while Lp(a) plasma levels in NHP models are slightly higher than in humans, the distribution of Lp(a) is similar across different NHP species tested (Supplemental Figure 1). Rhesus macaque was selected as the model for these studies based on animal availability.

### In Vitro siRNA Screening

One hundred sixty-eight siRNAs were designed against a conserved portion of the rhesus *LPA* (XM\_001098061) and were screened using a luciferase reporter. This portion of rhesus *LPA* shares 99 % sequence identity with cyno transcript and >90 % identity with human *LPA* gene (data not shown). Screening scheme for apo(a) siRNA lead identification is shown in Fig. 1a. Over 20 sequences were identified with greater than 85 % in vitro knockdown and 18 of these were further characterized in dose response experiments (Fig. 1b). Top six siRNAs from the initial dose response studies were confirmed in a second round of dose response experiments (Fig. 1c). Based on maximum observed knockdown and calculated IC<sub>50</sub> values, apo(a) Seq1 and apo(a) Seq2 siRNAs were selected for testing in NHP (Table 2). These two oligos have either the best observed maximum knockdown (apo(a) Seq2) or lowest IC<sub>50</sub> value (apo(a) Seq1). They also target two

distinct regions of *LPA* mRNA to preclude the possibility that any phenotypic effect observed in vivo is a result of off-target activity.

### NHP Testing

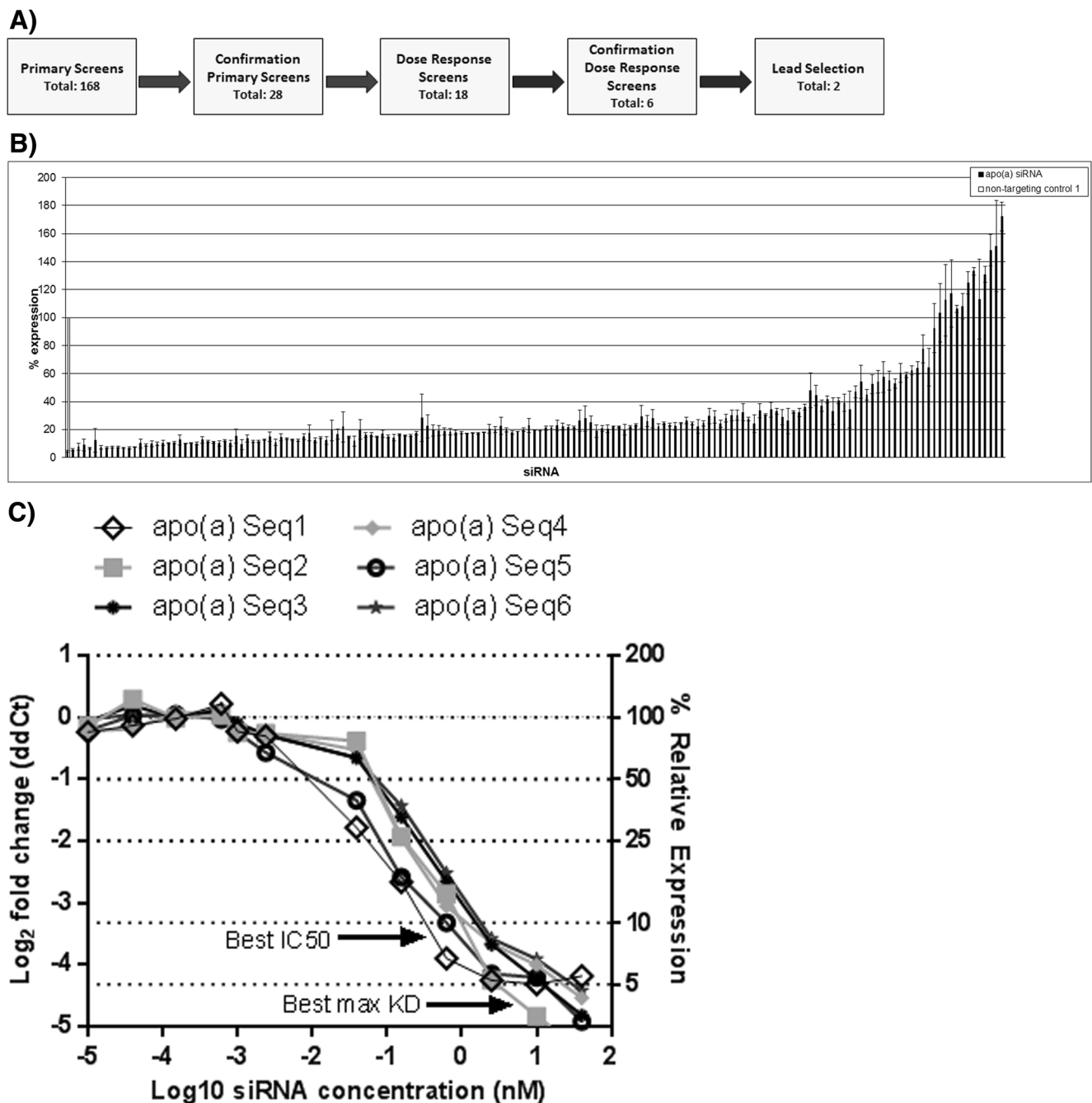
apo(a) Seq1 and apo(a) Seq2 siRNAs were formulated into LNP for in vivo delivery. Potency of the formulated material was confirmed in dose response experiments in vitro using *LPA* luciferase reporter prior to testing in rhesus monkeys (Supplemental Figure 2).

Both sequences showed >90 % *LPA* knockdown and sub-nanomolar IC<sub>50</sub> values. LNP-formulated siRNAs were then qualified in lean rhesus. Study schematic is shown in Fig. 2. Animals received a single dose of apo(a)-LNP, non-targeting (nt)-LNP, ApoB-LNP or vehicle. Apo(a)-LNP administration resulted in significant and sustained reductions in hepatic *LPA* mRNA levels (Fig. 3a). Both siRNAs showed >90 % mRNA knockdown at day 7. In agreement with in vitro potency, as assessed by calculation of IC<sub>50</sub>, apo(a) Seq1 showed superior in vivo efficacy. This siRNA showed a maximum target knockdown of ~95 % at day 7 that was maintained for over 2 weeks. For both siRNAs, *LPA* mRNA knockdown was accompanied with significant lowering in plasma Lp(a) levels (Fig. 3b). Maximum plasma Lp(a) reduction for apo(a) Seq2 was around 80 % trending back to baseline after 2 weeks while apo(a) Seq1 showed maximum plasma Lp(a) reduction of >95 % which was maintained for over a month. The effect of apo(a) Seq1 siRNA on circulating Lp(a) was uniform in all animals tested regardless of the pre-treatment Lp(a) levels (Fig. 3b, Supplemental Figure 3).

Interestingly, only a modest Lp(a) plasma lowering was observed in ApoB siRNA group despite significant ApoB mRNA knockdown (Fig. 3c). No significant effects on ApoB mRNA levels were observed with either apo(a) siRNA. We also found no effect on plasminogen mRNA levels suggesting high specificity of apo(a) siRNAs (data not shown). Some fluctuation in ApoB protein levels (Fig. 3d) was observed in all groups; however, no significant effect on ApoB protein was observed following *LPA* knockdown. Pre-dose levels of Lp(a) and ApoB are provided in Supplemental Figure 3. Based on liver mRNA knockdown and associated reductions in plasma Lp(a) levels, apo(a) Seq1 was selected as the lead siRNA for future experiments.

### Serum Chemistry Changes Following *LPA* mRNA Knockdown in the Liver

Serum lipid levels were measured over a time course starting prior to LNP-siRNA dosing through day 35. As expected, ApoB siRNA showed significant lowering of plasma LDL-c levels (Fig. 4). We also observed LDL-c lowering in apo(a)



**Fig. 1** **a** In vitro screening scheme for apo(a) siRNA lead identification. One hundred sixty-eight siRNAs were designed against a conserved portion of the rhesus *LPA* (XM\_001098061) and screened using a luciferase reporter. Twenty-eight top sequences were confirmed in a second round of screening, and the best performing oligos were further evaluated in

dose response studies. At the end of the selection process top two performing siRNAs were selected for scale up and in vivo testing. **b** Results from primary screen of 168 oligos. **c** Dose response results from the best performing six siRNAs

siRNA groups while there was no effect on serum lipids in nt control siRNA and vehicle groups. In agreement with mRNA and plasma Lp(a) data, apo(a) Seq1 showed a more robust effect on LDL-c than apo(a) Seq2 (Fig. 4). There was no significant effect on the plasma levels of TC or HDL-c (Table 3). LNP treatment was found to be well tolerated

without significant increases in aminotransferase levels (Fig. 5a, b), kidney function tests or any of the coagulation and hematology parameters tested (data not shown). Animal body weights also remained stable following LNP treatment (Fig. 5c) and no changes in food intake were observed in any of the animals (data not shown).

**Table 2** Summary of in vitro target knockdown and IC<sub>50</sub> values for top 6 apo(a) sequences

siRNA sequence	Maximum target knockdown (ddCt)		IC <sub>50</sub> (nM)
	ddCt	%knockdown	
<b>apo(a) Seq1</b>	<b>3.87</b>	<b>93</b>	<b>0.017</b>
<b>apo(a) Seq2</b>	<b>4.25</b>	<b>95</b>	<b>0.065</b>
apo(a) Seq3	3.97	94	0.067
apo(a) Seq4	3.17	89	0.045
apo(a) Seq5	4.18	94	0.026
apo(a) Seq6	3.81	93	0.020

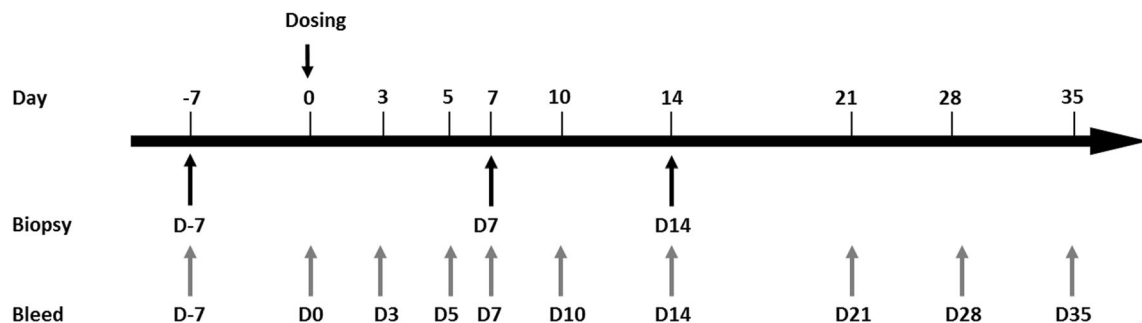
Two siRNA leads selected for in vivo studies are shown in bold

**Discussion**

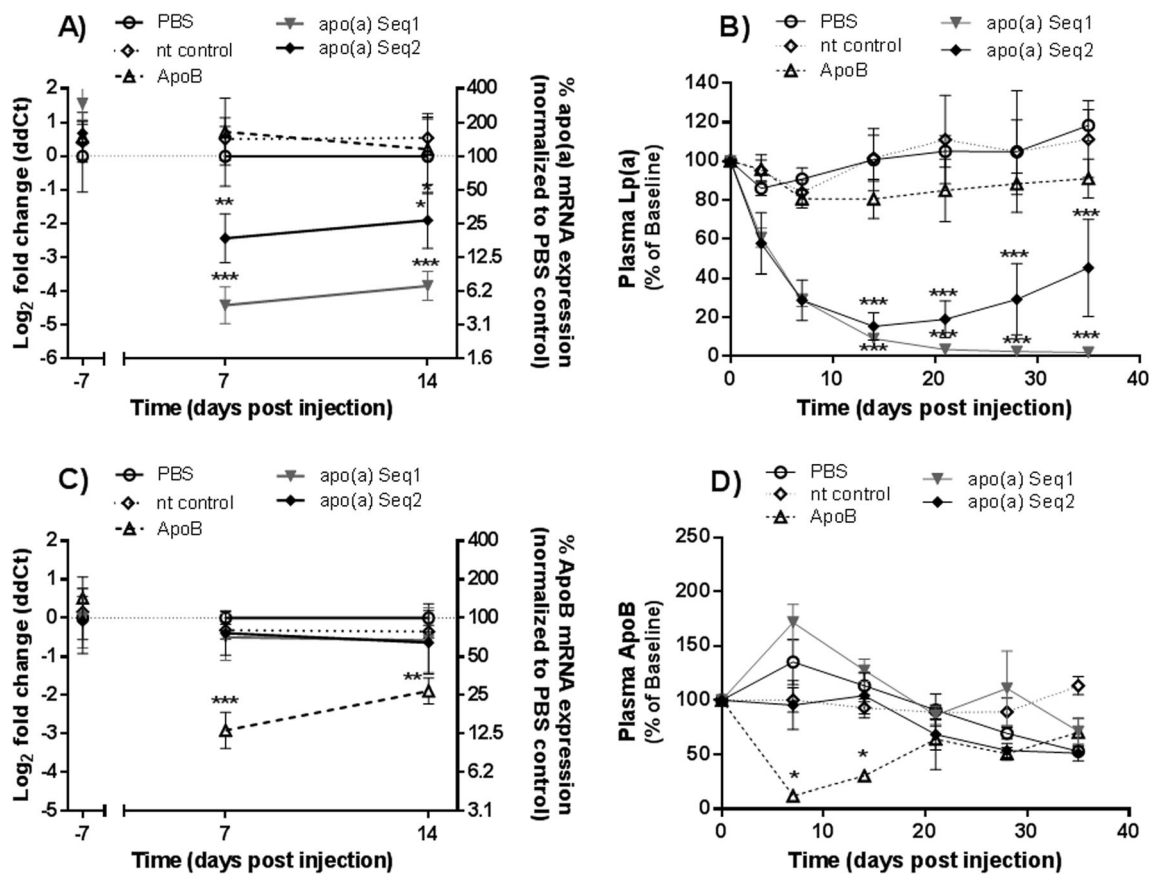
Because Lp(a) represents a possible causal factor in the development and progression of coronary heart disease [1–3, 20], the pursuit of Lp(a) as a target for cardiovascular therapeutic intervention requires a suitable preclinical model to understand the link between Lp(a) and disease. The purpose of the present study was to develop selective and potent siRNAs and to qualify these in NHP model for efficacy of hepatic *LPA* mRNA knockdown and effects on circulating plasma Lp(a). siRNAs offer high target specificity and selectivity and have been successfully used for gene knockdown across preclinical species [12, 16, 21–23]. We designed chemically modified siRNAs and used in vitro screening assays to select highly potent siRNAs against rhesus *LPA*. siRNAs were encapsulated in cationic LNP optimized for hepatic delivery and well-tolerated in cynomolgus monkeys without the necessity for dexamethasone pretreatment [24, 25]. We and the others have demonstrated that LNP- siRNAs are effective tools for knockdown of target genes in the liver across a number of preclinical species [12, 16, 26]. These tools can be developed faster than conventional genetic animal models and allow targeting of genes that may be considered “undruggable” via traditional approaches.

Our lead *LPA* siRNA shows >90 % mRNA knockdown for over 2 weeks and >95 % Lp(a) lowering in the plasma for over 35 days. siRNA treatment was well tolerated, and results were confirmed with two independent oligos verifying that the effect is *LPA*-specific and not due to off-target activity. We also used a previously published ApoB siRNA [16] as a process control. In agreement with existing data for this siRNA in NHP, we observed significant knockdown of ApoB mRNA (~90 % max), and significant lowering in plasma ApoB (~88 % max) and LDL-c (~82 % max). Interestingly, *ApoB* mRNA knockdown in the liver was accompanied by only a modest reduction in Lp(a) plasma levels suggesting that the main mechanism for plasma Lp(a) lowering following apo(a) siRNA treatment is related to *LPA* mRNA synthesis. This is in contrast to ApoB anti-sense oligonucleotide (ASO) findings published by ISIS Pharmaceuticals, both in transgenic mice carrying human ApoB-100 and human *LPA* [8] and in patients with heterozygous familial hypercholesterolemia (FH) [27], where administration of ApoB ASO resulted in robust lowering of plasma Lp(a). The possible disparity between the two findings may be due to the duration of treatment. In patients with FH receiving ApoB ASO treatment, statistically significant effects on plasma Lp(a) levels were not evident until 13 weeks of dosing suggesting over a month of *ApoB* inhibition is required to result in significant changes in circulating Lp(a).

ISIS Pharmaceuticals reported development of *LPA* ASO, ISIS 144367 [28]. In transgenic mice carrying a copy of human *LPA* gene (8K-Lp(a) mice) ~27 % plasma Lp(a) lowering was observed after 6 weeks of therapy. In cynomolgus monkeys treated with the ISIS apo(a) ASO for 12 weeks [29], plasma apo(a) and hepatic mRNA were reduced > 80 % with no change in plasminogen mRNA, and no effect on plasma lipid and circulating ApoB levels, similar to our study. Contrary to our study, there was no effect on LDL-c. It is possible that the difference between the two findings is due to difference in maximum observed *LPA* mRNA knockdown (>80 % mRNA and 86 % plasma Lp(a) reduction with ASO vs. >95 % mRNA and >95 % plasma Lp(a) reductions with siRNA). Follow-up studies with chronic administration of



**Fig. 2** Study schematic for in vivo qualification of apo(a) siRNAs. Animals were dosed and blood and liver samples collected as described in the “Methods” section. n=6 (for apo(a) and nt siRNAs); n=3 (for vehicle and ApoB controls)



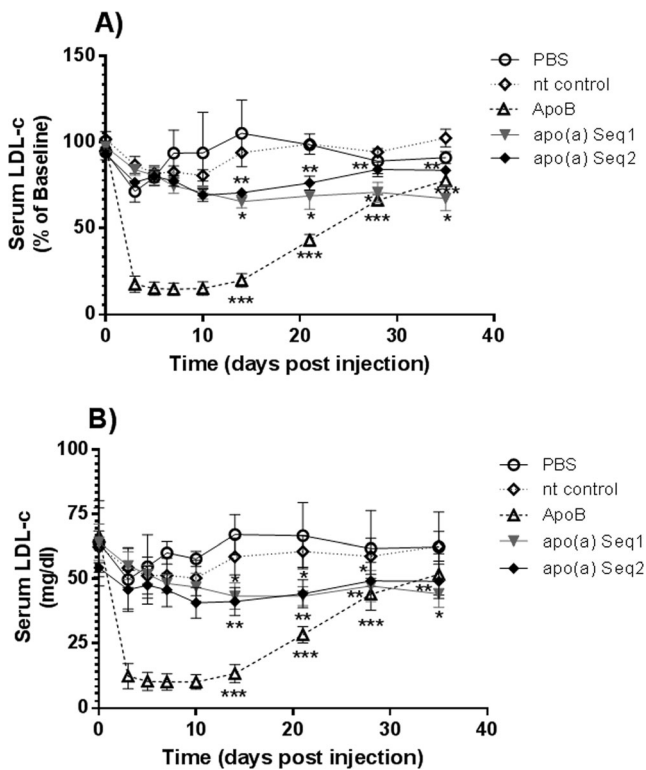
**Fig. 3** In vivo qualification of apo(a) siRNAs. **a** Liver *LPA* mRNA levels and **b** plasma Lp(a) protein levels. Administration of apo(a) siRNAs did not significantly affect **c** ApoB mRNA or **d** ApoB protein levels. Group

sizes are as follows: PBS,  $n=3$ ; ApoB,  $n=3$ ; nt control,  $n=6$ ; apo(a) Seq1,  $n=6$ ; and apo(a) Seq2,  $n=6$ . Group means $\pm$ SD are shown. \* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$

siRNA are required to better understand the differences between the two platforms. The observation that apo(a) siRNA treatment did not result in detectable change in plasma ApoB despite the observed LDL-c lowering may be due to the method used for LDL-c measurement. In the current study, the direct LDL assay selectively solubilizes apoB particles [30] followed by biochemical determination of cholesterol in supernatants. Because Lp(a) particles carry cholesterol and apoB, if they are also solubilized as part of the LDL-c detection method, a large reduction in circulating Lp(a) could affect LDL-c readouts. Lp(a)-cholesterol can contribute up to 10–15 % of the TC or LDL-c value, depending on the nature of the samples being studied (normal vs dyslipidemic, etc.) [31, 32]. Therefore, methods of LDL-c determination that do not rely on apoB particle solubilization may be more appropriate for measuring lipoprotein-associated cholesterol when Lp(a) levels are changing.

While a specific causal mechanism for Lp(a)'s contribution to atherosclerosis has yet to be identified, several hypotheses have been studied recently. Lp(a) can deliver oxidized phospholipids to the atherosclerotic plaque, activating resident macrophages, and resulting in rupture of coronary plaques

[33]. Lp(a) may also carry chemokines like MCP-1, which activates CCR2 and CCR4, and could lead to increased recruitment of monocytes to the atherosclerotic plaque [34, 35]. Because *LPA* resembles plasminogen but inhibits fibrinolysis in vitro, Lp(a) could be considered a prothrombotic factor [36, 37]. Finally, Lp(a) is also an ApoB-containing, cholesterol-rich lipoprotein, so Lp(a) may also deliver cholesterol to the arterial wall, like other ApoB particles [38]. Therefore, Lp(a) could be considered an atherothrombotic factor, and the ability to reduce circulating Lp(a) levels in NHP opens the door to studies which could determine the role of Lp(a) in the progression of atherosclerosis. In order to study chronic knockdown of Lp(a) in NHP, it will be important to determine whether chronic knockdown of Lp(a) presents any potential safety risk. Several years ago, Ogorelkova et al. reported a variant of apo(a) which is associated with congenital deficiency of Lp(a) in plasma [39]. These individuals appear healthy, suggesting that chronic Lp(a) knockdown in primates is well tolerated. Whether this deficiency in Lp(a) results in improvements in long-term cardiovascular outcomes remains to be determined and requires additional investigation. Another aspect of this model lies in the natural variation in Lp(a) levels in



**Fig. 4** Serum LDL-c changes following hepatic *LPA* and *ApoB* knock-down. **a** Percent LDL-c relative to pre-dose baseline. **b** Absolute values of serum LDL-c. Group sizes are as follows: PBS, *n*=3; ApoB, *n*=3; nt control, *n*=6; apo(a) Seq1, *n*=6; and apo(a) Seq2, *n*=6. Group means ±SD are shown. \**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001

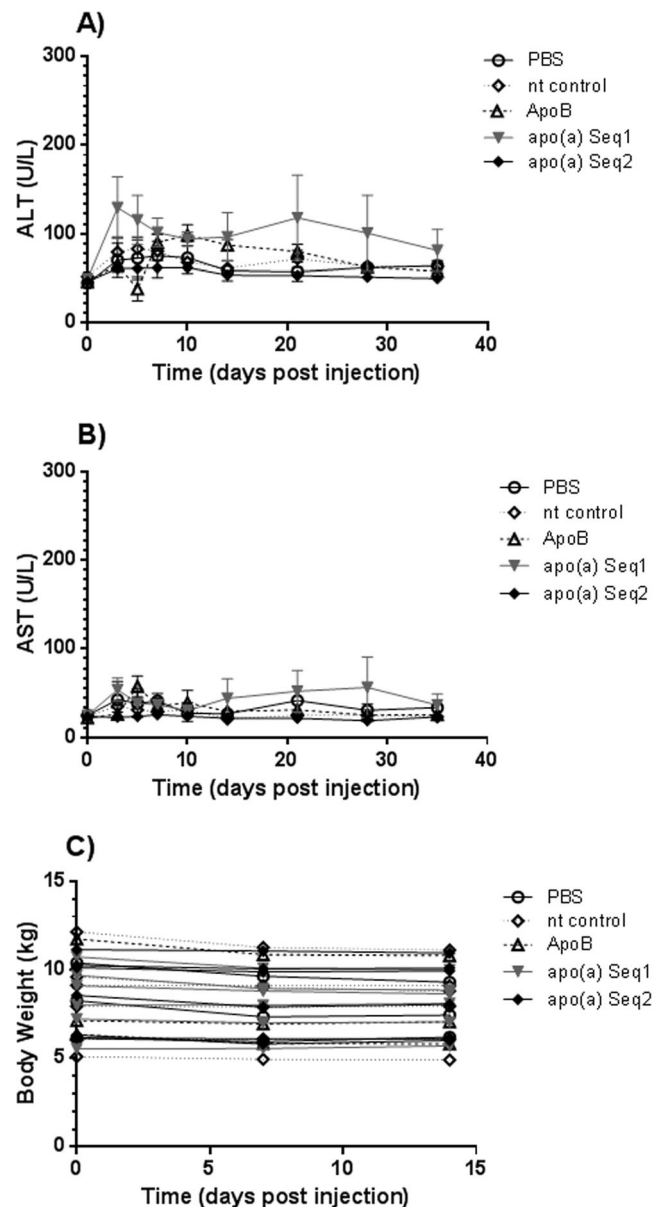
NHP. The presence of a spectrum of animals with high and low Lp(a) renders this model ideal to study the effect of naturally occurring levels of Lp(a) on susceptibility to atherosclerosis. Studies in transgenic mice suggest that overexpression of apo(a) in animals expressing human apoB may increase sensitivity to atherogenic diets [40]; however, results in multiple studies with this particular model are somewhat

**Table 3** Serum lipid changes following apo(a) and ApoB siRNA dosing. Statistical analysis was done using linear regression modeling

Comparator: nt control siRNA	apo(a) Seq1	apo(a) Seq2	ApoB
Lipoprotein (a)	-96 %***	-73 %***	-14 % <sup>ns</sup>
LDL Cholesterol	-32 %*	-21 %**	-48 %***
Total Cholesterol	-9 % <sup>ns</sup>	-5 % <sup>ns</sup>	-25 %***
HDL Cholesterol	+3 % <sup>ns</sup>	+7 % <sup>ns</sup>	+2 %**

Values shown are mean change across pharmacodynamic window used for statistical analysis, days 14–35

\**p*<0.05; \*\**p*<0.01; \*\*\**p*<0.001



**Fig. 5** In vivo safety data following a single 26.7 mg/m<sup>2</sup> dose of LNP-siRNA in lean rhesus. No elevations in liver function tests were observed. Group sizes are as follows: PBS, *n*=3; ApoB, *n*=3; nt control, *n*=6; apo(a) Seq1, *n*=6; and apo(a) Seq2: *n*=6. Group means ±SD are shown for **a** absolute values of serum ALT and **b** absolute values of serum AST. **c** Animal body weights were collected on days of sedation. Body weights remained stable following LNP-siRNA administration

conflicting [41, 42]. Studies in transgenic rabbits (which express endogenous CETP) have been more consistent, showing increased atherosclerosis in rabbits overexpressing human apo(a) [43]. Therefore, the NHP, which also expresses CETP and displays human-like coronary atherosclerosis, represents an ideal model to study the importance of Lp(a) in atherosclerosis.



The present study demonstrated, for the first time, a robust reduction in circulating Lp(a) levels by siRNA-mediated knockdown of *LPA* in the lean rhesus macaque. The application of this gene silencing tool in a relevant model of atherosclerosis, could define a causal role for Lp(a) in atherosclerosis and offer a potential path to novel therapeutics to combat cardiovascular disease.

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