LETTER

ACS chemical biology

Design and Creation of New Nanomaterials for Therapeutic RNAi

Huricha Baigude†, Joshua McCarroll†, Chao-shun Yang†, Pamela M. Swain, and Tariq M. Rana*

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01605. †These authors contributed equally to this work.

ABSTRACT RNA interference is an evolutionarily conserved gene-silencing phenomenon that shows great promise for developing new therapies. However, the development of small interfering RNA (siRNA)-based therapies needs to overcome two barriers and be able to (i) identify chemically stable and effective siRNA sequences and (ii) efficiently silence target genes with siRNA doses that will be clinically feasible in humans. Here, we report the design and creation of interfering nanoparticles (iNOPs) as new systemic gene-silencing agents. iNOPs have two subunits: (i) a well-defined functionalized lipid nanoparticle as a delivery agent and (ii) a chemically modified siRNA for sustained silencing *in vivo*. When we injected iNOPs containing only 1−5 mg kg^{-1} siRNA into mice, an endogenous gene for apolipoprotein B (apoB) was silenced in liver, plasma levels of apoB decreased, and total plasma cholesterol was lowered. iNOP treatment was nontoxic and did not induce an immune response. Our results show that these iNOPs can silence disease-related endogenous genes in clinically acceptable and therapeutically affordable doses.

RNA interference (RNAi), an evolutionarily conserved process whereby double-stranded RNA (dsRNA) induces the sequence-specific degradation of homologous messenger (mRNA) (*1*), has been widely used to identify gene functions and holds great potential to provide a new class of therapeutics (*2*). During RNAi, long dsRNA is processed by Dicer into short interfering RNAs (siRNAs) and incorporated into the RNA-induced silencing complex (RISC) (*3*), a multiturnover enzyme complex that cleaves the target mRNA. The RNAi machinery can also be programmed in cells by introducing duplexes of siRNAs (*4, 5*) that are assembled into siRISC containing Dicer, Argonautes, and other proteins (*6*). Therefore, new siRNA-based therapeutic agents could be designed to treat diseases by lowering concentrations of disease-causing gene products.

However, development of siRNA-based therapies faces two challenges: (i) identification of chemically stable and effective siRNA sequences and (ii) efficient delivery of these sequences to tissue-specific targets *in vivo* with siRNA amounts that can be translated to clinically feasible doses for humans. Recent advances in understanding the rules for chemically modifying siRNA sequences without compromising their gene-silencing efficiency (*6*–*9*) have allowed the design and synthesis of therapeutically effective siRNA molecules that can silence target genes *in vivo* (*10, 11*). Furthermore, the *in vivo* delivery of siRNAs and inhibition of various gene functions have recently been achieved by conjugating cholesterol to siRNA (*11*) or to oligonucleotide inhibitors of micro RNA (*12*), by forming stable nucleic acid-lipid particles (SNALP) of siRNA (*10, 13*), and by assembling lipid–siRNA complexes (*14, 15*). In addition, a protamine–antibody fusion protein has been used to deliver siRNAs to HIV-infected cells (*16*).

Despite much recent progress, new chemistry and delivery approaches are greatly needed to systematically silence disease-causing genes in a tissue-specific manner with high efficiencies and at clinically achievable doses. Here we describe the design and synthesis of new well-defined nanoparticles functionalized with lipids. We used these nanoparticle–siRNA assemblies to systemically silence a target gene in mice at siRNA doses of ~1 mg kg^{-1}. Furthermore, this treatment did not induce an immune response and showed favorable pharmacokinetics.

We reasoned that an ideal delivery vehicle for siRNA should have at least three functions: (i) to efficiently assemble siRNA, (ii) to be non-immunogenic, and (iii) to provide functional groups for covalent attachment of tissue-specific moieties and for modulation of pharmacological properties for future investigations. Thus, we decided to construct natural amino acid-based nanoparticles that fulfilled all of the three criteria outlined above. Here, we report the design and synthesis of interfering nanoparticles (iNOPs) as new siRNA delivery

*Corresponding author, tariq.rana@umassmed.edu.

Received for review March 14, 2007 and accepted March 28, 2007.

Published online April 13, 2007

10.1021/cb7000582 CCC: $37.00

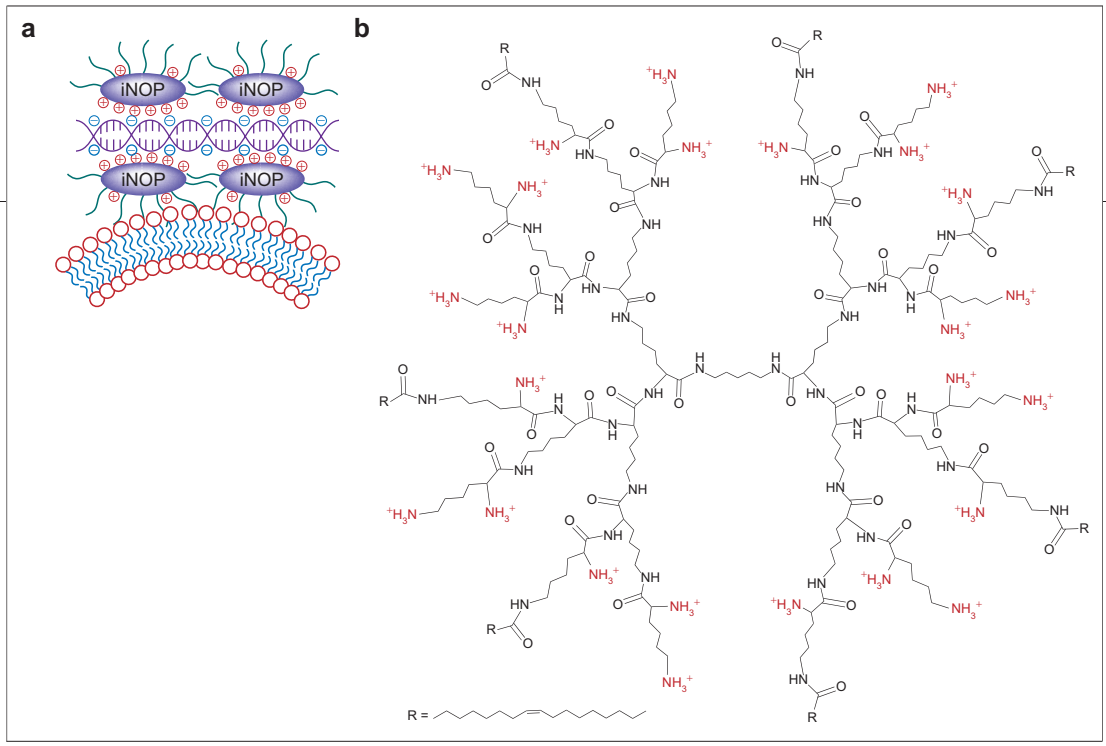

Figure 1. Structure of iNOP-7. a) Schematic illustration of nanoparticle–siRNA complex (iNOP-7) initiating contacts with cellular membrane for entry. b) Chemical structure of the nanoparticle used to construct iNOP-7.

agents containing a chemically modified siRNA that therapeutically silences an endogenous gene in mice, apolipoprotein B (apoB). ApoB is a large protein that exists in two forms, apoB100 and apoB48 (*17*), is a ligand for the low-density lipoprotein receptor, and is involved in cholesterol metabolism. Mouse apoB contains 4515 amino acids and is predominantly expressed in the liver and intestine. In mice, both apoB100 and apoB48 are expressed in the liver, whereas apoB48 is predominantly expressed in the intestine. Previous studies showed that heterozygous knockout mouse models for apoB had a 20% decrease in serum cholesterol levels and a resistance to diet-induced hypercholesterolemia (*18*). Serum levels of apoB in humans have also been strongly correlated with increased risk of coronary artery disease (*17, 19*). Because apoB is a large protein for which no 3D structural data are available, it is not a suitable target for small-molecule drug development. As an alternative therapy for hypercholesterolemia, apoB silencing by RNAi has recently been explored in a mouse model treated with siRNA chemically modified by conjugation to cholesterol (*11, 20*). These studies were very encouraging in providing proof of the concept for developing RNAi-based therapeutics (*11*), but the amounts of siRNA used (50 mg kg^{-1}) would translate to unfeasible doses for clinical application in humans (*20*). Furthermore, the delivery of siRNA required cholesterol conjugation, which might increase risk factors in patients who cannot tolerate higher levels of cholesterol. Recently, lower doses of siRNAs have been used with SNALP encapsulation to efficiently silence apoB in nonhuman primates (*13*), but this approach requires a sophisticated SNALP assembly. We planned to develop new nanomaterial that can be used to easily assemble siRNA for systemic delivery. To test our strategy for siRNA delivery and to achieve apoB silencing with siRNA doses in animals that are clinically relevant for RNAi-based therapy in humans, we chose apoB silencing in mice as an animal model system to investigate the efficacy of our siRNA chemical modifications and iNOPs.

To deliver functional siRNA to cells, we recently tested a generation-four polyamidoamine dendrimer-based nanoparticle (NP-45) (*21*) with 64 surface groups and a diameter of 45 Å (*22*). Interestingly, the efficiency of siRNA uptake using 20–40 μg/ml NP-45 was almost equal to that for 20 μg mL^{-1} Lipofectamine (*21*). However, only a narrow range of nontoxic NP-45 concentrations had efficient RNAi activities in cells. Therefore, we reasoned that nanoparticles composed of natural amino acids would be less toxic and non-immunogenic to cells, thus providing the molecular architecture to develop new nanomedicines. In addition, amino acid-based synthesis of nanoparticles allows better control over specific synthetic steps to modulate physical and pharmacological properties of the nanomaterials. To enhance *in vivo* uptake, we synthesized lysine-containing nanoparticles and modified the surface functional groups with lipid chains (Figure 1). The stability of the siRNA component of the iNOPs was enhanced by using chemically modified siRNA sequences targeting apoB according to modification rules established in our previous studies (*6, 8, 9*).

To determine whether iNOP-7 could deliver active siRNA to its target and silence apoB mRNA levels in FL83B cells, we treated cells with iNOP-7 and analyzed the decrease in apoB mRNA levels by quantitative polymerase chain reaction (qPCR). iNOP-7 containing unmodified apoB siRNA almost completely silenced apoB mRNA expression (>90%) in FL83B cells when compared to controls or cells treated with iNOP-7 containing mismatched siRNA (Figure 2, panel a). Notably, the efficiency of apoB mRNA silencing using iNOP-7 as an siRNA transporter was similar to that of cells transfected with Lipofectamine 2000 complexed to unmodified siRNA (data not shown). More importantly, iNOP-7 containing our chemically modified siRNA directed against apoB was more efficient in silencing apoB mRNA than iNOP-7 containing unmodified apoB siRNA

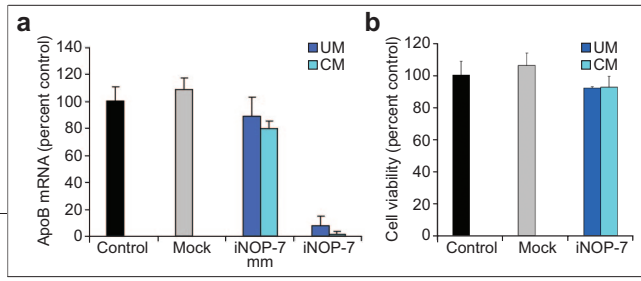

Figure 2. *In vitro* silencing of apoB by iNOP-7. a) iNOP-7 containing siRNA specifically silences apoB in FL83B cells. Cells were treated for 2.5 h with iNOP-7 without siRNA (Mock) or with iNOP-7 containing unmodified (UM) siRNA, chemically modified (CM) siRNA, or their respective mismatches (mm). ApoB mRNA levels are expressed as percent of control (no transfection). Each value represents the mean ± standard deviation (SD) of duplicate cultures from two representative experiments. b) FL83B cells remain viable 24 h after treatment with iNOP-7 containing siRNA. Cells were treated with iNOP-7 without siRNA (Mock) or iNOP-7 with unmodified (UM) or chemically modified (CM) siRNA. Cell toxicity levels are expressed as percent of control (no transfection). Each value represents the mean ± SD of duplicate cultures from two representative experiments.

(Figure 2, panel a). These results therefore show that our modifications to siRNA did not negatively influence its RNAi activity (Figure 2, panel a). These reduced levels of apoB mRNA levels in FL83B cells were not due to iNOP-7-induced cell toxicity, as confirmed by phase contrast microscopy (results not shown) and by a modified MTS cell viability assay (Figure 2, panel b). Taken together, these results demonstrate that our nanoparticle (iNOP-7, Figure 1) is nontoxic and efficiently transports siRNA into cells.

We next determined the ability of iNOP-7 to deliver siRNA to its target and silence apoB expression in mice. Consistent with a previous report (*11*), we did not observe a significant knockdown of apoB expression in mice injected with cholesterol-conjugated unmodified siRNA (data not shown). Therefore, our *in vivo* experiments focused on iNOP-7 containing chemically modified siRNA. Mice were injected *via* tail vein with iNOP-7 complexed to either chemically modified siRNA or its mismatch, and samples of liver and plasma were analyzed. To quantify mRNA silencing throughout the liver tissue, three separate regions were analyzed for apoB mRNA levels. ApoB mRNA was significantly lower in liver tissue from mice treated with 1.25, 2.5, or 5 mg kg^{-1} iNOP-7 containing chemically modified siRNA (51 ± 3%, 51 ± 3%, and 47 ± 3%, respectively, $n = 3–4$ animals) than in livers from control mice and mice treated with iNOP-7 containing mismatched siRNA (Figure 3, panel a). The maximum silencing effect of iNOP-7 was reached at 1 mg kg^{-1}, and increasing the siRNA dose did not enhance the *in vivo* RNAi efficiency (Figure 3, panel a; Supplementary Figure 1). It was not clear why higher concentrations of siRNA did not increase RNAi efficiency. Nonetheless, these observations show a clear correlation between *in vivo* RNAi efficiency and the dose of siRNA (0.25–1 mg kg^{-1}) used in these experiments.

We next determined the presence of the guide strand of ApoB siRNA in mice liver by performing Northern blot analysis of total RNA isolated from mice liver treated with iNOP-7. Our results showed that the guide strand was still present in the liver of animals after 24 h of final iNOP-7 injections (Figure 3, panel b). Interestingly, a strong signal for the guide strand was observed when iNOP-7 contained the chemically modified siRNA as compared with iNOP-7 assembled with unmodified siRNA (Figure 3, panel b). These results correlate with the findings that unmodified siRNA did not efficiently silence apoB mRNA *in vivo* (*11*). No detectable amount of siRNA was found in mice liver when unmodified or chemically modified siRNA duplexes were injected without the nanoparticle (NOP-7). Consistent with the siRNA guide strand Northern blot results, we did not obtain significant knockdown of apoB mRNA in mice liver when unmodified or chemically modified siRNAs were injected without nanoparticles (Supplementary Figure 2). These results collectively indicate that chemically stabilized RNA and the nanoparticle were essential for efficient delivery of siRNA *in vivo*. Tissue distribution analysis of the guide strand showed that iNOP-7 delivered the siRNA to mainly liver and spleen, and a minor amount of the guide strand was detected in lung (Supplementary Figure 3).

To determine if silencing of apoB mRNA correlated with reduced plasma levels of apoB protein, we measured apoB100 and apoB48 levels by immunoblot. We found that injecting 1.25-5 mg kg^{-1} iNOP-7 containing chemically modified siRNA decreased both apoB100 and apoB48 serum levels to >70% of control (Figure 3, panels c and d), whereas fibronectin levels were unaffected. These results show that iNOP-7 complexed to chemically modified siRNA efficiently silenced apoB expression *in vivo*. Remarkably, these iNOP-7-mediated silencing activities required only 1.25 mg kg^{-1} siRNA, a clinically feasible dose for RNAi therapeutic applications.

To investigate the physiological effects of apoB mRNA silencing on cholesterol metabolism, we measured total plasma cholesterol levels in mice 24 h after the final injection of iNOP-7. As shown in Figure 4, panel a, iNOP-7-mediated silencing of apoB expression in liver and plasma samples was correlated with a reduction of total cholesterol (34.4 ± 7%). Cholesterol levels were unchanged in mice receiving control treatments or treated with iNOP-7 containing chemically modified, mismatched siRNA (Figure 4, panel a). Together, these findings demonstrate that iNOP-7-mediated targeting of apoB could provide a clinically significant new approach to reducing cholesterol levels in patients with hypercholesterolemia.

siRNA-based therapies have two major side effects: (i) activation of an immune response (*23, 24*) and (ii) toxic effects. To address the concern of eliciting a nonspecific immune response by injecting animals with iNOP-7 containing chemically modified siRNA, liver tissue RNA was assessed by qPCR for the induction of the interferon (IFN)-inducible genes IFIT-1 and STAT 1, and plasma IFN-α levels were measured by ELISA. Our results showed that injecting mice with iNOP-7 alone or with iNOP-7 con-

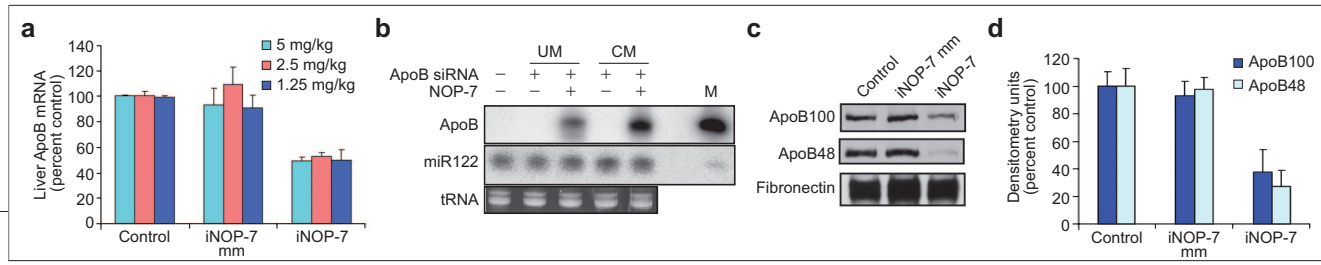

Figure 3. *In vivo* silencing of apoB by iNOP-7. a) ApoB is specifically silenced in livers of mice injected with iNOP-7 containing siRNA. ApoB mRNA levels were reduced in liver 24 h after injecting mice with 1.25, 2.5, or 5 mg kg^{-1} of iNOP-7 containing either CM siRNA or its mm. Values represent the mean ± SD of tissue samples from 3 liver regions (3 or 4 animals). Data are expressed as percent of control. b) Detection of ApoB siRNA delivered by nanoparticles in mice liver. Total RNA from liver of mice treated with UM or CM siRNA with and without the nanoparticle (iNOP-7) was isolated and detected by Northern blots. A representative Northern blot shows the presence of the ApoB guide strand siRNA in mice liver 24 h after the final treatment. Detection of miR-122 and transfer RNA (tRNA) was used as a control. c) ApoB protein levels are reduced in plasma of mice injected with iNOP-7. ApoB100 and apoB48 protein expression levels were measured by Western blot 24 h after the injection of 5 mg kg^{-1} of iNOP-7 containing CM siRNA or its mm. Total protein loading was confirmed by assessing plasma fibronectin levels. d) Quantification of reduced ApoB plasma levels after iNOP-7–siRNA treatment. Western blots for mouse plasma levels of ApoB100 and ApoB48 (panel c) were analyzed by densitometry. Data are expressed as percent of control (3 or 4 animals).

taining chemically modified siRNA did not alter the expression of IFIT-1 and STAT 1 genes in the liver (data not shown), nor did it induce the release of IFN-α in plasma relative to controls (Figure 4, panel b). To address concerns about iNOP toxicity, all mice were monitored daily for overall health, food intake, and weight changes. At the end of iNOP-7 treatment, mice were sacrificed and necropsied. Histological sections of liver, our target tissue for apoB silencing, were prepared and independently examined for toxic effects by a board-certified animal pathologist. No histological differences were noted between tissues from control (no treatment or nanoparticle only) mice and from those treated with iNOP-7 containing chemically modified siRNA (Supplementary Figure 4). These results demonstrate that iNOP-7 treatment did not induce an immune response in animals and caused no apparent toxic effects.

One concern about applying nanotechnology in biology and medicine is its safety. Nanomaterials used to deliver a drug could cause undesired effects by inhibiting the drug's biodegradation and clearance, thus prolonging its half-life *in vivo*. To address this question, we determined the *in vivo* pharmacokinetics of iNOP-7 by injecting mice with 1 mg kg^{-1} of iNOP-7–siRNA *via* tail vein, isolating liver tissues at various times after injection, and analyzing different regions of liver for ApoB mRNA levels. The maximum knockdown of ApoB mRNA was observed 48 h after injecting iNOP-7–siRNA, and the ApoB mRNA levels increased to ~80% after 72 h and reached 100% after 120 h (Figure 4, panel c). These results show that iNOP treatment displays favorable pharmacokinetics and suggest that siRNA was not trapped in iNOP-7 complex in liver and was cleared quite rapidly from the body.

In summary, our results show that the newly designed nanoparticles can be used to deliver siRNA to silence disease-related endogenous genes in clinically acceptable and therapeutically affordable doses. When we injected iNOPs containing only 1 mg kg^{-1} siRNA into animals, apoB mRNA and protein levels were silenced in liver, plasma levels of apoB decreased, and total plasma cholesterol was lowered. In addition, iNOP treatment was nontoxic and did not induce an immune response. The low dose of siRNA (1 mg kg^{-1}) needed in our studies to achieve therapeutic effects provides great hope for developing RNAi-based therapies in the near future to cure diseases caused by proteins that cannot be targeted by conventional drugs. Furthermore, these nanoparticles can be modified to target specific tissues and to modulate pharmacological properties of the RNAi-based therapeutics.

METHODS

Preparation of iNOP-7. All siRNAs used in these studies were chemically synthesized using silyl ethers to protect 5′-hydroxyls and acid-labile orthoesters to protect 2′-hydroxyls (2′-ACE) (Dharmacon, Lafayette, CO). After deprotection and purification, siRNA strands were annealed as described previously (*8*): apoB siRNA (ORF position 10049–10071), UM sense 5′-GUCAUCACACU GAAUACCAAU-3′, antisense 5′-AUUGGUAU UCAGUGUGAUGACAC-3′; CM, sense 5′-G^{S} U^{F}C^{F}AU^{F}C^{F}ACACUGAA UAC^{FS}C^{F}AA^{S}U^{F}-propyl-amine-3′, antisense 5′-PAU^{F}U^{F}GGUAUUCA GUGUGAU GAC^{FS}A^{S}C; UM mm siRNA, sense 5′-GUGAUCAGACUC AAUACGAAU propyl-amine-3′, antisense 5-′AUUCGUAUUGAGU CUGAUCACAC-3′; CM mm, sense 5′-G^{S}U^{F} GAU^{F}C^{F}AGACUCAA UAC^{F}GAA^{S}U^{F} propyl-amine-3′, antisense 5′-AU^{F}U^{F}CGUAUUGAG UCUGAU^{F}CAC^{FS}A^{S}C.

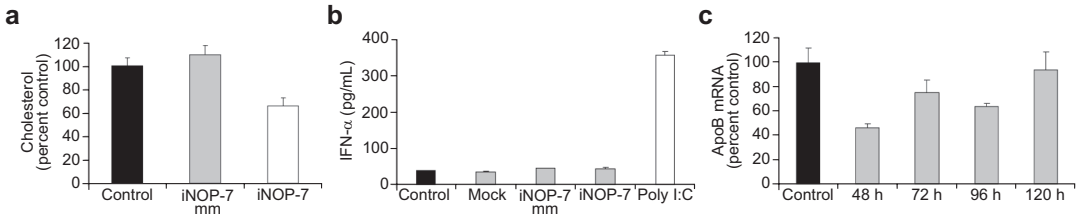

Figure 4. Therapeutic effects, immune response, and pharmacokinetics of iNOP-7. a) iNOP-7 therapeutically reduces cholesterol levels. Plasma cholesterol levels 24 h after the injection of 5 mg kg^{-1} iNOP-7. b) iNOP-7 treatment does not induce interferon response in mice. IFN-α plasma levels 24 h after the treatment with iNOP-7 without siRNA (Mock) or with 5 mg kg^{-1} iNOP-7 containing CM siRNA or its mm. As a positive control, animals were injected with 250 μg of Poly IC, and plasma levels of IFN-α were assessed. Each value represents the mean ± SD of pooled plasma samples from each treatment group. c) Pharmacokinetics of iNOP-7–siRNA. Mice were injected with 1.25 mg kg^{-1} of iNOP-7–siRNA, and ApoB mRNA levels were measured in liver at various times after injection (as described in Figure 3).

The superscript letters F and S represent 2′-O-F and HS backbone modifications, respectively. Nanoparticle-7 (Figure 1) was chemically synthesized, labeled with lipids, purified, and characterized by NMR and mass spectrometry (*25, 26*). iNOPs were prepared by mixing siRNA and nanoparticle-7 at a ratio of 1:2–1:8 (w/w) in HEPES saline or Opti-MEM culture medium (Invitrogen) and incubating at RT for 20 min (see below).

***In Vitro* RNAi Activity of iNOP-7.** FL83B (mouse hepatocytes) cells were maintained at 37 °C with 5% CO_2 in F12 Khangians modified culture medium (ATCC, Manassas, VA) supplemented with 10% fetal bovine serum, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin. Cells were regularly passaged and plated in 96- and 6-well culture plates for 16 h before transfection at 70% confluency. Cells were transfected with 1 mL $well^{-1}$ of complex (siRNA–nanoparticle-7) for 2.5 h at 37 °C. Efficiency of RNAi and cellular toxicity were determined as described previously (*11, 21*).

***In Vivo* Silencing.** Six- to eight-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were maintained under a 12 h dark cycle in a pathogen-free animal facility. Mice were injected on three consecutive days *via* the lateral tail vein with phosphate buffered saline pH 7.4 (PBS) or iNOP-7 complexes of CM apoB siRNA or its mm siRNA. Daily dosages of 0.25–5 mg kg^{-1} siRNA were delivered in a final volume of 0.15 mL. Twenty-four hours after the final injection, liver tissue levels of apoB mRNA, plasma levels of apoB protein, and total plasma cholesterol were measured.

Measurement of apoB mRNA and Protein Levels *in Vivo*. To determine apoB mRNA levels in liver tissue after siRNA treatment, small uniform tissue samples were collected from three regions of the liver. Total RNA was extracted with Trizol and treated with DNase I before quantification. ApoB mRNA levels were determined by qPCR as described above. ApoB protein levels were determined by Western blot using a polyclonal goat anti-apoB100/48 antibody (Santa Cruz Biotechnology, Santa Cruz, CA). ApoB protein levels were then detected by enhanced chemiluminescence (PerkinElmer Life Sciences, Waltham, MA). As a control, fibronectin was visualized by immunoblot using a polyclonal rabbit anti-fibronectin antibody (Sigma, St. Louis, MO).

Measurement of Total Cholesterol Levels in Plasma. Plasma cholesterol was measured by a commercial enzyme assay according to the manufacturer's instructions (Biodesign International, Saco, ME).

***In Vivo* Interferon Induction.** To assess for any nonspecific immune response to injected iNOP-containing siRNA, mouse liver tissue RNA was analyzed for expression of the IFN-inducible genes by qPCR and quantification of plasma IFN-α levels (*23*).

Acknowledgment: We thank C. Mello and members of the Rana lab for helpful discussions and David Garlick for histological examination of slides. We also thank Jochen Weiss and his laboratory, the Department of Food Biophysics and Nanotechnology at University of Massachusetts Amherst, for their advice and help in dynamic light scattering experiments. This work was supported in part by institutional funds and a grant from the National Institutes of Health to T.M.R.

Competing Interests Statement: The authors declare competing financial interests.

Supporting Information Available: This material is available free of charge *via* the Internet.

REFERENCES

1. Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E., and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans, *Nature 391*, 806–811.
2. Hannon, G. J., and Rossi, J. J. (2004) Unlocking the potential of the human genome with RNA interference, *Nature 431*, 371–378.
3. Hammond, S. M., Bernstein, E., Beach, D., and Hannon, G. J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells, *Nature 404*, 293–296.
4. Caplen, N. J., Parrish, S., Imani, F., Fire, A., and Morgan, R. A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems, *Proc. Natl. Acad. Sci. U.S.A. 98*, 9742–9747.
5. Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., and Tuschl, T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells, *Nature 411*, 494–498.
6. Rana, T. M. (2007) Illuminating the silence: understanding the structure and function of small RNAs, *Nat. Rev. Mol. Cell Biol. 8*, 23–36.
7. Amarzguioui, M., Holen, T., Babaie, E., and Prydz, H. (2003) Tolerance for mutations and chemical modifications in a siRNA, *Nucleic Acids Res. 31*, 589–595.
8. Chiu, Y. L., and Rana, T. M. (2003) siRNA function in RNAi: a chemical modification analysis, *RNA 9*, 1034–1048.
9. Chiu, Y. L., and Rana, T. M. (2002) RNAi in human cells: basic structural and functional features of small interfering RNA, *Mol. Cell 10*, 549–561.
10. Morrissey, D. V., Lockridge, J. A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C. S., Jeffs, L. B., Judge, A., MacLachlan, I., and Polisky, B. (2005) Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs, *Nat. Biotechnol. 23*, 1002–1007.
11. Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R. K., Racie, T., Rajeev, K. G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M., and Vornlocher, H. P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs, *Nature 432*, 173–178.
12. Krutzfeldt, J., Rajewsky, N., Braich, R., Rajeev, K. G., Tuschl, T., Manoharan, M., and Stoffel, M. (2005) Silencing of microRNAs in vivo with 'antagomirs', *Nature 438*, 685–689.
13. Zimmermann, T. S., Lee, A. C., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., Harborth, J., Heyes, J. A., Jeffs, L. B., John, M., Judge, A. D., Lam, K., McClintock, K., Nechev, L. V., Palmer, L. R., Racie, T., Rohl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J., Toudjarska, I., Wheat, A. J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H. P., and MacLachlan, I. (2006) RNAi-mediated gene silencing in non-human primates, *Nature 441*, 111–114.
14. Palliser, D., Chowdhury, D., Wang, Q. Y., Lee, S. J., Bronson, R. T., Knipe, D. M., and Lieberman, J. (2006) An siRNA-based microbicide protects mice from lethal herpes simplex virus 2 infection, *Nature 439*, 89–94.
15. Santel, A., Aleku, M., Keil, O., Endruschat, J., Esche, V., Fisch, G., Dames, S., Loffler, K., Fechtner, M., Arnold, W., Giese, K., Klippel, A., and Kaufmann, J. (2006) A novel siRNA-lipoplex technology for RNA interference in the mouse vascular endothelium, *Gene Ther. 13*, 1222–1234.
16. Song, E., Zhu, P., Lee, S. K., Chowdhury, D., Kussman, S., Dykxhoorn, D. M., Feng, Y., Palliser, D., Weiner, D. B., Shankar, P., Marasco, W. A., and Lieberman, J. (2005) Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors, *Nat. Biotechnol. 23*, 709–717.
17. Olofsson, S. O., and Boren, J. (2005) Apolipoprotein B: a clinically important apolipoprotein which assembles atherogenic lipoproteins and promotes the development of atherosclerosis, *J. Intern. Med. 258*, 395–410.
18. Farese, R. V., Jr., Veniant, M. M., Cham, C. M., Flynn, L. M., Pierotti, V., Loring, J. F., Traber, M., Ruland, S., Stokowski, R. S., Huszar, D., and Young, S. G. (1996) Phenotypic analysis of mice expressing exclusively apolipoprotein B48 or apolipoprotein B100, *Proc. Natl. Acad. Sci. U.S.A. 93*, 6393–6398.
19. Brown, M. S., and Goldstein, J. L. (1986) A receptor-mediated pathway for cholesterol homeostasis, *Science 232*, 34–47.
20. Rossi, J. J. (2004) Medicine: a cholesterol connection in RNAi, *Nature 432*, 155–156.
21. Chiu, Y. L., Ali, A., Chu, C. Y., Cao, H., and Rana, T. M. (2004) Visualizing a correlation between siRNA localization, cellular uptake, and RNAi in living cells, *Chem. Biol. 11*, 1165–1175.
22. Dufes, C., Uchegbu, I. F., and Schatzlein, A. G. (2005) Dendrimers in gene delivery, *Adv. Drug Delivery Rev. 57*, 2177–2202.
23. Marques, J. T., and Williams, B. R. (2005) Activation of the mammalian immune system by siRNAs, *Nat. Biotechnol. 23*, 1399–1405.
24. Robbins, M. A., and Rossi, J. J. (2005) Sensing the danger in RNA, *Nat. Med. 11*, 250–251.
25. Baigude, H., Katsuraya, K., Okuyama, K., Tokunaga, S., and Uryu, T. (2003) Synthesis of sphere-type monodispersed oligosaccharide-polypeptide dendrimers, *Macromolecules 36*, 7100–7106.
26. Shao, J., and Tam, J. P. (1995) Unprotected peptides as building blocks for the synthesis of peptide dendrimers with oxime, hydrazone, and thiazolidine linkages, *J. Am. Chem. Soc. 117*, 3893–3899.