Correction

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The authors note that due to a printer’s error, the caption for Fig. 2 did not appear in full. The caption “(B) Luciferase” should instead appear as “(B) Luciferase silencing at low doses of siRNA (s.d., n = 4).” The figure and its corrected legend appear below.

Fig. 2. In vitro screening of lipidoid library. Lipidoids were screened in luciferase-expressing Hela-derived cell line. (A) Antifirefly luciferase siRNA was complexed with lipidoids and incubated with cells in presence of growth media. Relative firefly luciferase expression determined by comparison of detected protein levels in treated groups vs. untreated control. (B) Luciferase silencing at low doses of siRNA (s.d., n = 4).

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Lipid-like materials for low-dose, in vivo gene silencing

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Significant effort has been applied to discover and develop vehicles which can guide small interfering RNAs (siRNA) through the many barriers guarding the interior of target cells. While studies have demonstrated the potential of gene silencing in vivo, improvements in delivery efficacy are required to fulfill the broadest potential of RNA interference therapeutics. Through the combinatorial synthesis and screening of a different class of materials, a formulation has been identified that enables siRNA-directed liver gene silencing in mice at doses below 0.01 mg/kg. This formulation was also shown to specifically inhibit expression of five hepatic genes simultaneously, after a single injection. The potential of this formulation was further validated in nonhuman primates, where high levels of knockdown of the clinically relevant gene transthyretin was observed at doses as low as 0.03 mg/kg. To our knowledge, this formulation facilitates gene silencing at orders-of-magnitude lower doses than required by any previously described siRNA liver delivery system.

Lipidoid | siRNA delivery | multiple gene silencing | primates

Since the discovery of RNA interference (RNAi) by Fire and Mello in 1998 (1) and siRNAs by Tuschl and coworkers in 2001 (2), considerable effort has been directed towards their therapeutic application in humans (3). The most significant challenges to delivery include the relatively large size (~13 kDa) and negative charge of siRNA molecules as well as their susceptibility to enzymatic degradation in vivo (4, 5). In some applications, effective delivery of naked siRNAs, without a carrier, may be possible (6, 7). However, systemic delivery to many tissues, including liver, requires a vehicle to provide protection and transport of siRNA to the cells of interest. To this end, a variety of carrier systems utilizing both natural and synthetic materials have been developed (8–15). Cationic lipids represent one of the most well-studied classes of synthetic materials for siRNA delivery. To date, the most advanced examples of these materials demonstrate the ability to bind and condense siRNA into nanoparticles through electrostatic interactions and to deliver the payload across the cellular membrane into the cytoplasm of target cells (16, 17).

Previously, Akinc et al. (8), reported a high-throughput combinatorial approach to new material synthesis and discovery for siRNA delivery applications (15). Michael addition chemistry was utilized to create a structurally diverse library of amino-alkyl-acrylate and -acrylamide materials termed “lipidoids”, which were then analyzed for their ability to transfect cells both in vitro and in vivo. The lead candidate from the initial study was demonstrated to facilitate sequence-specific knockdown in a variety of cellular targets and animal species, including mice, rats, and nonhuman primates. While promising, delivery with these materials requires siRNA doses greater than 1 mg/kg to achieve high levels of gene silencing in vivo (18). Such doses are comparable to those required by stable nucleic acid lipid particle (SNALP) formulations, another delivery system which has shown utility for siRNA delivery in nonhuman primates (14). To significantly expand the therapeutic potential of lipid-based formulations, different materials with improved efficacy would be of great utility.

Results and Discussion

Synthesis of a Combinatorial Epoxide-Derived Lipidoid Library. In an effort to identify increasingly efficacious delivery materials, we used a unique synthetic strategy to rapidly build a library of lipid-like compounds based on epoxide chemistry. The library is composed of nondegradable amino alcohols consisting of polar amine-containing head groups and nonpolar hydrocarbon tails. Synthesis of the compounds was achieved through efficient ring-opening of epoxides by amine substrates (Fig. 1). This synthetic strategy is particularly well suited to parallel synthesis and high-throughput screening in that reactions can be carried out without solvent, do not require protection/deprotection steps, and resultant materials can be used in cell-based screens without purification. One advantage of this synthetic strategy over the previous scheme (15) is that the reactions were typically complete within 3 d. Using this one-step approach, a library of 126 lipid-like compounds was created and the reaction products were tested in cells without further processing.

Lipidoid-Mediated siRNA Delivery In Vitro. Lipidoid-like materials were screened in vitro in a high-throughput fashion in a luciferase-expressing Hela-derived cell line. These cells are genetically modified to stably express both reporter proteins firefly Photinus pyralis and Renilla reniformis luciferase (15). In these experiments, antifirefly luciferase siRNA was complexed with lipidoid...


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at weight ratios of 2.5:1, 5:1, 10:1, and 15:1 lipidoid:siRNA and incubated with cells in the presence of growth media. Reduction in firefly luciferase expression in the absence of Renilla reduction was considered siRNA-mediated silencing. Renilla expression was monitored as an internal control for lipidoid-related toxicity. Cytotoxicity assays were also performed with no evidence of adverse effects (Fig. S1). In this screen, numerous compounds were identified which facilitated luciferase silencing, including three which silenced greater than 90% (Fig. 2A). For ease of graphical representation, only 5:1 weight ratio data is shown. Interestingly, from these results a number of structure-activity relationships emerged. With respect to tail length, seven of the top 15 performing structures possessed tails 14 carbons in length. Additionally, no compounds with tails less than 12-carbons in length mediated silencing greater than 30%. Regarding amine head groups, amine 113 was present in the top two performing compounds and three of the top 15. While the convergence upon C14 tails and amine 113 is evident, not all compounds containing these structures show silencing activity. For example, neither C8-113 nor C14-116 facilitated gene silencing in vitro, suggesting an optimized combination of amine group and tail length is necessary to impart delivery activity.

To investigate in vitro efficacy at low doses of siRNA, a dose response was conducted in which cells were exposed to titrated concentrations of siRNA-containing lipidoid complexes. siRNA was incubated with cells at doses between 5 and 50 nanogram (ng), with the ratio of lipidoid:siRNA held constant at 5:1 (wt:wt). From these experiments, three compounds were identified which facilitated greater than 70% silencing at a siRNA dose of 5 ng per well (Fig. 2B).

**In Vivo Delivery of siRNA to Hepatocytes in Mice.** While in vitro delivery experiments are useful for identifying compounds with in vivo delivery potential, we find they are not highly predictive for identifying most effective compound for in vivo delivery. To evaluate the utility of the epoxide-based lipidoids in facilitating siRNA delivery in vivo, the mouse Factor VII gene silencing model was employed (15, 18). Lipidoids formulated with siRNA-directed against the blood clotting Factor VII were delivered intravenously. Factor VII is a useful gene target to evaluate hepatocyte-specific delivery in that the protein is produced only in the cells of the liver parenchyma, is secreted into the blood enabling facile protein quantitation, and possesses a relatively short plasma half-life (15).

Twelve of the top-performing lipidoids from the initial in vitro screen were purified and evaluated for in vivo performance. For in vitro experiments, simple complexation of siRNA and lipidoid is sufficient for particle formation and cellular delivery. However, during intravenous administration, barriers such as electrostatic interactions with serum proteins, uptake by cells of the immune system, and aggregation in areas such as the spleen and lung can inhibit delivery to hepatocytes. To improve serum stability of lipidoid particles, distearoyl phosphatidylcholine (DSPC), cholesterol and polyethylene glycol (PEG) were used in the formulations (15). For in vivo screening experiments, lipidoids were formulated at a constant weight ratio of lipidoid:DSPC: cholesterol: PEG and mice were administered a single bolus dose of 3 mg/kg total siRNA via tail vein injection. Mouse body-weight was also monitored over the duration of the experiment, as body-weight loss can indicate toxicity associated with lipidoid particle treatment. Mean particle diameter varied between formulations and ranged from 65 nm to 250 nm. From this screen, three compounds were found to facilitate complete silencing at the administered dose (Fig. 3A). While these results demonstrate the ability of epoxide lipidoids to effectively reduce protein levels in hepatocytes, dose response experiments were conducted with the top three compounds, C16-96, C14-110, and C12-200, to investigate potency of silencing at lower doses (Fig. 3B–D). Dose-dependent gene silencing was achieved with each of the three lipidoids tested, and one compound in particular, C12-200, demonstrated over two orders-of-magnitude higher potency than LNP01 (18), the optimized liver delivery formulation from the previous acrylamide- and acrylate-based library of lipid-like materials. A formulated control siRNA was administered at a dose of 1 mg/kg to confirm the specificity of gene silencing (Fig. 3D).
Fig. 3. In vivo silencing of Factor VII in mice. A) Top-performing lipidoids from in vitro screen were purified, formulated for serum stability and delivered intravenously to C57BL/6 mice. Mice received a single bolus administration of 3 mg/kg total siRNA via tail-vein injection and Factor VII levels were quantified 72 h postinjection. B–D) Dose response experiments with top three performing lipidoids from in vivo screen; C16-96 (B), C14-110 (C), and C12-200 (D). No lipidoid-related toxicity is observed as measured by body-weight loss (E). (s.d., n = 3 or 4, * P < 0.005, **P < 0.001; t-test, single tailed)

Perhaps the most important advantage of low-dose delivery is the significantly reduced amount of carrier material required to transport the siRNA to its target. The several hundredfold improvement in potency of C12-200 over LNP01 translates to the significantly reduced amount of carrier material required to induce silencing, the resulting lipidoid-related tolerability should be greatly increased. This concept is supported by tolerability analysis in mice in which no indication of toxicity was observed as measured by body-weight loss (Fig. 3E, Table 1).

Next, we investigated the durability of gene silencing mediated by C12-200-formulated siRNA. Mice received a single i.v. injection of formulated siRNA at either 0.1 or 1 mg/kg and serum Factor VII levels were monitored for over 40 d (Fig. 4). At both doses, complete silencing was observed at 24 h and protein levels returned to baseline within 20 and 35 d for the 0.1 and 1 mg/kg doses, respectively. These results indicate that larger siRNA doses may lead to a longer duration of effect, further highlighting the potential for dosing at higher multiples of the efficacious dose.

Table 1.

<table>
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<th>Dose (mg/kg)</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
<th>ALP (U/L)</th>
<th>Bilirubin (dL)</th>
</tr>
</thead>
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<td>0</td>
<td>42.3 ± 6.0</td>
<td>90.7 ± 15.0</td>
<td>183.0 ± 8.9</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>0.06</td>
<td>40.7 ± 4.6</td>
<td>74.0 ± 12.8</td>
<td>219.3 ± 35.3</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>0.02</td>
<td>47.3 ± 10.1</td>
<td>103.3 ± 11.8</td>
<td>178.7 ± 46.5</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>0.2</td>
<td>52.0 ± 6.2</td>
<td>161.0 ± 66.2</td>
<td>229.0 ± 46.4</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>0.6</td>
<td>40.7 ± 6.8</td>
<td>102.3 ± 15.1</td>
<td>201.3 ± 37.4</td>
<td>2 ± 0.0</td>
</tr>
<tr>
<td>1</td>
<td>37.0 ± 7.8</td>
<td>77.7 ± 25.5</td>
<td>123.3 ± 25.6</td>
<td>2 ± 0.0</td>
</tr>
</tbody>
</table>

Clinical chemistry parameters following single injection of formulated C12-200 siRNA particles; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase

Low-Dose Efficacy Enables Multiple Gene Silencing in Vivo. As a result of the highly efficient gene silencing achieved by C12-200, we hypothesized that silencing of multiple genes in the liver with a single i.v. administration should be possible while remaining well within the range of tolerability established in previous experiments. It could be envisioned that the ability to regulate multiple genes may provide a powerful therapeutic approach to diseases in which multiple gene targets have already been identified (14, 19). To investigate the feasibility of this approach, siRNA sequences against liver targets of possible therapeutic interest, Factor VII, ApoB, PCSK9, Xbp1, and SORT1, were pooled and formulated with C12-200. ApoB, PCSK9, XBP1 and SORT1 are all genes implicated in metabolic pathways involved in cholesterol homeostasis, and mutations in these genes have been linked to altered cholesterol levels either in knock-out mouse models or in human genetic association studies (20–23). ApoB has a role in cholesterol trafficking from the liver to the plasma, PCSK9 a role in cholesterol clearance from plasma back into the liver, XBP1 has been implicated in cholesterol synthesis, while the mechanistic role of SORT1 is less clear (20–23).

Silencing of these particular five genes is not expected to provide any cooperative therapeutic effect per se; however silencing all five genes simultaneously serves as a proof of principle that multiple genes involved in similar or divergent signaling pathways, could selectively be silenced with a single administration of a single drug product. In this experiment, mice received a single i.v. injection of a C12-200-formulated pool of siRNAs, and at 72 h postadministration, liver tissue was harvested for analysis of mRNA transcript levels. Dose-dependent silencing effects were investigated by titrating dosage of each siRNA from 0.2 to 0.005 mg/kg. Greater than 65% silencing of all five genes was observed at a dose of 0.2 mg/kg per siRNA (1 mg/kg total siRNA dose) (Fig. 5). Consistent with the tolerability studies described above, no adverse side effects were observed.

Fig. 4. In vivo persistence of C12-200-mediated silencing was investigated by monitoring Factor VII protein levels for a period of over 40 days. Mice were administered a single dose of either 1 or 0.1 mg/kg siRNA and blood samples were drawn at varying time points for quantitation of serum protein levels. (s.d., n = 3)
script levels. (s.d., siRNA. 72 h postinjection, liver tissue was harvested for analysis of gene tran-

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Fig. 5. Five hepatocellular gene targets were simultaneously silenced by a single injection of pooled siRNAs formulated with C12-200. Mice we admi-

lished a single dose and dosage was titrated from 0.2 and .005 mg/kg per siRNA. 72 h postinjection, liver tissue was harvested for analysis of gene tran-

hanced by avoidance of lysosomal degradation which is a common problem encountered with drug

To our knowledge, this is the earliest report of the simultaneous siRNA-mediated silencing of five hepatic targets in vivo. Given the potency of C12-200-mediated delivery, we hypothesize that even more genes could be simultaneously silenced by a pooled siRNA product. From a therapeutic standpoint, this could enable more complex therapeutic approaches, where silencing of multiple targets achieves an enhanced therapeutic effect (24). For example, this strategy may be particularly useful in treating viral infections such as Hepatitis C virus (HCV) which rapidly evolving viral genomes have proven elusive to siRNAs of a single sequence. In fact, this idea has been shown previously in vitro utilizing delivery of endoribonuclease-prepared siRNAs and retroviral vectors encoding short hairpin RNAs against multiple regions of the HCV genome (25). This multitarget approach may also allow for different strategies to treat multifactorial diseases such as metabolic syndrome, cancer, or infectious disease where multiple genes and pathways have been implicated.

Investigating the Mechanism of C12-200-Mediated Cellular Delivery.

To investigate the mechanism by which C12-200 particles are internalized, nonspecific antiGFP siRNA labeled with Alexa-Fluor® 647 was delivered to HeLa cells in order to observe siRNA uptake and intracellular trafficking. In these experiments, C12-200-formulated siRNA was incubated with HeLa cells in the presence of labeled cargo known to enter cells by different endocytic pathways. As shown in Fig. 6A, the labeled siRNA colocalized with a fluid phase marker dextran but not with transferrin or Cholera toxin B, markers of clathrin and caveolae mediated endocytosis, respectively. This suggests that C12-200 particles may be internalized through a macropinocytosis mechanism (26, 27). One of the hallmarks of such an uptake pathway is membrane ruffling and actin rearrangement (28), which was observed in HeLa cells within 15 min of the application of the particles (Fig. 6B). Furthermore, the effects of the macropinocytosis inhibitor, 5-N-ethyl-N-isoproamiloride (EIPA), and the actin polymerization inhibitor, Cytochalasin D, on particle uptake were examined with our delivery system (26). Both compounds dose-dependently inhibited siRNA uptake (Figs. 6C,D), which further suggested that the majority of C12-200-formulated siRNA likely entered cells via macropinocytosis. It has been reported that in some cell types the fluid content of macropinosomes does not merge with the degradative pathway (29, 30). We hypothesize that C12-200-facilitated silencing may be enhanced by avoidance of lysosomal degradation which is a common problem encountered with drug delivery vehicles that enter cells through the classical endocytic pathway (31).

Investigating the C12-200-Mediated Silencing in Nonhuman Primates.

To further investigate the potential of these materials, C12-200 was formulated with siRNA specific to transthyretin (TTR), and silencing was evaluated in nonhuman primates. TTR is a serum protein synthesized primarily in hepatocytes. Although amyloidogenic TTR mutations are rare, they are endemic to certain populations and can affect both the peripheral nerves and heart, leading to familial amyloidotic polyneuropathy and familial amyloid cardiomyopathy, respectively. Currently, the only disease modifying therapy is liver transplantation. C12-200-siRNA-mediated silencing of mutant TTR is a potential approach for the treatment of TTR amyloidosis (32). Dosing of nonhuman
primates with C12-200-TTR siRNA resulted in high levels of specific knockdown at 0.3 mg/kg, 0.1 mg/kg, and 0.03 mg/kg (Fig. 7). To our knowledge this formulation provides for the most efficacious knockdown yet reported in primates. We believe that the development of safe and effective siRNA delivery vehicles is an important part of the continued advancement of RNAi-based therapeutics. With the identification of highly efficacious materials such as C12-200, widened therapeutic indices, persistent gene silencing, and multitarget approaches to treatment of disease may be achieved.

Methods

Lipidoid Synthesis. Compounds in the library were synthesized by reacting allyl epoxides with a selection of amines. Substoichiometric amounts of epoxide were added to increase the proportion of products with one less tail than the total possible for a given amine monomer. The amine (1 equiv, typically 1 millimoles (mmol)) and epoxide (N – 1 equiv, where N is the number of secondary amines plus 2x number of primary amines in the amine starting material) were added to a 2 mL glass vial containing a magnetic stir bar. The vial was sealed, and the reaction was heated to 90 °C with stirring for 2.5 h. Selection of crude reaction mixtures were characterized by MALDI-TOF mass spectroscopy (Table S1); the spectra revealed that the mixtures contained predominately N and (N – 1) tailed products, as expected. Crude reaction products were used for in vitro screening: groups of products could be separated by number of lipid tails by chromatography on silica with gradient elution from CH_{2}Cl_{2} to 75:22:3 CH_{2}Cl_{2}/MeOH/NH_{4}OH (aq).

Lipidoid-siRNA Formulations. Lipidoid-siRNA formulations for in vivo screening were made from lipidoid, cholesterol, and a polyethylene glycol modified lipid as previously described (15, 18). Stock solutions of lipidoid, cholesterol (MW 387, Sigma-Aldrich), and MPEG2000-DMG (MW 2660, synthesized by Alnylam) (15) were made in absolute ethanol at concentrations of 100, 20, and 100 mg/mL, respectively. Components were combined to yield weight fractions of 52:20:28. Ethanol mixture was then added to 200 mM sodium acetate buffer (pH 5) while stirring to spontaneously form empty liposomes. siRNA at a concentration of 10 mg/mL in 50 mM sodium acetate was added to empty liposomes at a weight ratio of 10:1 total lipids:siRNA and the mixture was incubated at 37 °C for 30 min. Formulations were then dialedyzied against PBS, 350 MWCO dialysis cassettes (Pierce) for 75 min. Following buffer exchange, a sample of each formulation was used for particle characterization. A modified Ribogreen assay (Invitrogen) was performed to quantify degree of siRNA entrapment (33) and mean particle diameter was measured by dynamic light scattering (ZetaPALS, Brookhaven Instruments).

C12-200-siRNA formulations were prepared using a method adapted from Jeffs et al. (34). Briefly, C12-200, distearoyl phosphatidylcholine (DSPC), cholesterol and MPEG2000-DMG were solubilized in 90% ethanol at a molar ratio of 50:10:38.5:1.5. The siRNA (or pool of siRNAs) was solubilized in 10 mM citrate, pH 3 buffer at a concentration of 0.4 mg/mL. The ethanolic lipid solution and the aqueous siRNA solution were pumped by means of a peristaltic pump fitted with dual pump heads at equivalent volumetric flow rates and mixed in a “T”-junction. Liquids were combined with siRNA at a total lipid to siRNA ratio of 7:1 (wt: wt). The spontaneously formed C12-200-siRNA formulations were dialyzed against PBS (155 mM NaCl, 3 mM Na_{2}HPO_{4}, 1 mM KH_{2}PO_{4}, pH 7.5) to remove ethanol and exchange buffer. This formulation yields a mean particle diameter of 80 nm with approximately 90% siRNA entrapment efficiency.

In Vivo Factor VII and Multiple Gene Silencing in Mice. All procedures used in animal studies were approved by the Institutional Animal Care and Use Committee and were consistent with local, state and federal regulations as applicable. C57BL/6 mice (Charles River Labs) were used for siRNA silencing experiments. Prior to injection, formulations were diluted in PBS at siRNA concentrations such that each mouse was administered a dose of 0.01 mg/g body-weight. Formulations were administered intravenously via tail vein injection. After 48 or 72 h, body-weight gain/loss was measured and mice were anaesthetized by isoflurane inhalation for blood sample collection by retro-orbital eye bleed. Serum was isolated with serum separation tubes (Falcon Becton Dickinson) and Factor VII proteolysis was determined by chromogenic assay (Biophen FVII, Aniara Corporation). A standard curve was constructed using samples from PBS-injected mice and relative Factor VII expression was determined by comparing treated groups to untreated PBS control.

In the multiple gene silencing study Factor VII, ApoB, PCSK9, XBP-1, and SORT1 mRNA levels were assessed in livers harvested from mice treated with C12-200 formulated pool of five siRNAs or control unrelated siRNA targeting experiments. Prior to injection, formulations were diluted in PBS at siRNA concentrations such that each mouse was administered a dose of 0.01 mg/g body-weight. Formulations were administered intravenously via tail vein injection. After 48 or 72 h, body-weight gain/loss was measured and mice were anaesthetized by isoflurane inhalation for blood sample collection by retro-orbital eye bleed. Serum was isolated with serum separation tubes (Falcon Becton Dickinson) and Factor VII proteolysis was determined by chromogenic assay (Biophen FVII, Aniara Corporation). A standard curve was constructed using samples from PBS-injected mice and relative Factor VII expression was determined by comparing treated groups to untreated PBS control.

siRNA Uptake and Microscopy. Hela cells were purchased from ATCC. Alexa-Fluor® 488-labeled dextran, transferrin, cholera toxin, and phallolidin were purchased from Invitrogen. Cells were seeded in 96-well plates (Grenier) overnight, then incubated with C12-200 formulatedAlexa-467 tagged siRNA for durations ranging from 15 min to 3 h. Labeled cargo was added during the final 15 min of nanoparticle incubation prior to nuclear staining with Hoescht. In some experiments, EPA or Cypahlasin D (Sigma-Aldrich) were incorporated with preincubated with cells 1 h prior to incubation with C12-200 particles where drug was continuously present. For examination of actin ruffling, cells were serum starved for 1 h, followed by addition of particles for 15 min in serum-free media. Cells were fixed in 4% paraformaldehyde, permeabilized with 0.1% saponin and stained with Alexa-Fluor® 488 phalloidin. All images were acquired using an Opera spinning disc confocal system (Perkin Elmer), and the data was analyzed using Acapella Software (Perkin Elmer).

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Supporting Information

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Supporting Information corrected 06/08/2010

SI Text

General Methods for Chemical Synthesis and Analysis. Reagents and anhydrous solvents were purchased from commercial sources and were used as received. Epoxide lipid synthesis was performed in capped glass vials under room air; other reactions were performed in oven-dried glassware under argon unless otherwise noted. Flash chromatography was performed using an automated purification system (CombiFlash®, Teledyne Isco) over silica gel (40–63 μm particle size). NMR spectra were obtained on a 600 MHz (1H) spectrometer; chemical shifts are reported in parts per million (ppm) on the δ scale, and were referenced to residual protonated solvent peaks. Spectra obtained in chloroform-d were referenced to CHCl₃ at δH 7.27 and CDCl₃ at δC 77.2.

Mass spectra were obtained on a Bruker Daltonics Omniflex MALDI-TOF mass spectrometer. 2,5-Dihydroxybenzoic acid (2,5-DHB) was used as matrix for the epoxide lipids (20–25 mg of 2,5-DHB into 1 mL of 60:40 MeOH/H₂O). Epoxide lipid stock solutions were prepared by dissolving 1–2 mg of the lipid in 1 mL of MeOH containing approximately 250 μL CH₃Cl; 12 μL of the stock solution was added to 100 μL of matrix solution. Mass spectra were calibrated using an external reference (ProteoMass™ Peptide MALDI-MS Calibration standards, Sigma-Aldrich) with α-cyano-4-hydroxycinnamic acid as matrix.

In Vitro Transfection Assay. 24 h prior to transfection, HeLa cells stably expressing firefly and Renilla luciferase were seeded in opaque white 96-well plates in growth medium. Cells were seeded at a density of 15,000 cells per well and incubated overnight at 37°C in 5% CO₂ to allow cell attachment. For transfection, working dilutions of lipoidoids were prepared in 25 mM sodium acetate (pH 5) at concentrations necessary to yield weight ratios of 2.5:1, 5:1, 10:1, and 15:1, lipoidoids:siRNA. To form lipoidoids:siRNA complexes, 25 μL of lipoidoid solution was mixed with 25 μL of 5ug/ml siRNA in a 96-well plate using a multichannel pipette. Mixtures were allowed 20 minutes incubation at room temperature for complex formation. After incubation, 30 ul of the complexes were then diluted in 200 ul growth media, of which 150 ul was transferred to cells after aspiration of spent media. Transfection with Lipofectamine 2000 was performed as described by the vendor and served as a control. Complexes were allowed 24 h incubation with cells prior to analysis for luciferase expression. Firefly and Renilla luciferase expression was analyzed using the Dual-Glo assay (Promega) as described by the vendor. Luminescence was measured using a Victor3 luminometer (Perkin Elmer).

For data analysis, firefly expression was normalized by Renilla expression and treated groups were compared to untreated cells alone to determine degree of luciferase silencing.

In Vivo Non-Human Primate Experiments. 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 IACUC. Cynomolgus monkeys (n equals 3 per group) received either PBS or 0.03, 0.1, or 0.3 mg/kg siTTR formulated in C12-200 as 15-min intravenous infusions (5 mL/kg) via the cephalic vein. An ultrasound-guided liver biopsy was taken from each animal at 48 h post-administration and snap-frozen in liquid nitrogen. TTR mRNA levels, relative to GAPDH mRNA levels, were determined in ground liver samples using a branched DNA assay (QuantiGene Assay, Affymetrix, CA) (14).

Nucleic Acids. Luciferase GL3 siRNA (Dharmacon) was used for in vitro silencing experiments. For in vivo studies, siRNAs were produced by Alnylam. All siRNAs were tested negative for activation of the immune system (specifically, IFN-α and TNF-α) in primary human blood monocytes. All siRNAs were characterized by electrospray mass spectrometry and anion exchange HPLC. The sequences for the sense and antisense strands are as follows:

- siLuc sense: 5'-CUUACCGCUGAGUACUUCGATT-3', antisense: 5'-UCGAAGUACUCAGCGUAAGTT-3'
- siFVII sense: 5'-GGAAUCuuAuAuuuGAUCcAsA-3', antisense: 5'-AAGUCGUGCUGCUUCAUGUdTdT-3'
- siRSK9 sense: 5'-GccuGGAGuuuAuuuGAAGAdTdT-3', antisense: 5'-UucccGAAuuAAACCUCcAGGcTsTdT-3'
- siBIP1 sense: 5'-cAccucGAuuuAuuuGuucudTdT-3', antisense: 5'-AGAcAAUGAAUucAGGGUGdTsdT-3'
- siApOB sense: 5'-GGAAUcCuAuAuuuGAUcCaAsA-3', antisense: 5'-uucAGAcAAUcCuAuAAGGauuUCcscsU-3'
- siSort1 sense: 5'-AuGuGaAuuuAAGuucucAcdTdT-3', antisense: 5'-uGuUGAGACCuAAuAucAcAdTdTsT-3'
- siTTR sense: 5'-GuAaccAGAGAuAuccuAuAdTdTsT-3', antisense: 5'-AUGGAAuACUUCUUGGuACdTdT-3'

GFP-Alexa647 sense: 5'-AcuAAGAcGAcAGCCuUdTsdT-3', antisense: 5'-AGUCGGUCGCUCACAUCuAdTdTsT-3'

Alexa647 sense: 5'-UGGAGAACCuAAuAcAdTdTsT-3'

2'-OMe modified nucleotides are in lower case.
Fig. S1. In vitro cytotoxicity assay of top 10 performing lipidoids. Viability of HeLa cells after transfection with lipidoid:siRNA complexes at varying w:w ratios as measured by MTS assay. Relative to untreated cells, minimal toxicity is observed 24 h after transfection.

Fig. S2. Room temperature 600 MHz $^1$H NMR spectrum of C12-200 in CDCl$_3$. 

Current Data Parameters
NAME C12-200
CHIRP 1
PROG 1

F2 - Acquisition Parameters
Date 20090907
Time 14:14
DPPM 5 mm GPRM 1H-
PULPROG zg90
TD 6536
SOLVENT CDCl$_3$
NB 16
DS 3
SNR 6009.615 Hz
PD1 0.09669 Hz
AQ 5.4529453 msec
BG 14.3
DM 81.200 usec
DS 6.00 usec
TE 9.100 K
DI 1.00000000 msec
T0 1

--------- CHANNEL F2 ---------
HNC 1H
P1 11.00 usec
P2 4.00 usec
SPD1 600.3339998 MHz

F2 - Processing parameters
S1 65536
SF 600.156119 MHz
NDW RM
SSB 0
LB 0.30 Hz
DR 0
PC 1.00

2-(2-(4-(cyanomethyl)piperazin-1-yl)ethylamino)acetonitrile. Anhydrous ethanol (150 mL) was added to an oven dried 250 mL round bottom flask containing a magnetic stir bar. 1-(2-aminoethyl)piperazine (205, 4.00 mL, 30.4 mmol) was added to the ethanol using a syringe. Chloroacetonitrile (4.25 mL, 67.2 mmol, 2.2 equiv) was added to the stirred ethanolic solution slowly. Anhydrous sodium carbonate (12.9 g, 122 mmol, 4 equiv) was added to the mixture in one portion. The round bottom flask was fitted with a reflux condenser with drying tube and submersed in an oil bath; the solution was heated to reflux with vigorous stirring. After 7 h, the reflux condenser was removed from the flask, and the flask was removed from the oil bath. A portion of decolorizing charcoal was added cautiously to the reaction mixture. The hot suspension was stirred briefly and then gravity filtered through paper. The yellow filtrate was concentrated by rotary evaporation affording an orange oil that crystallized. The crude product was purified by chromatography on silica gel (gradient elution from CH2Cl2 to 9:1 CH2Cl2/MeOH). Fractions containing the desired product were pooled and concentrated by rotary evaporation; residual solvent was removed under reduced pressure affording the nitrile (3.7 g, 58%) as a pale yellow solid. 1H NMR (600 MHz, CDCl3): δ 3.57 (s, 2H), 3.47 (s, 2H), 2.74 (t, J = 5.4 Hz, 2H), 2.56 (t, J = 9.3 Hz, 4H), 2.48 (t, J = 5.4 Hz, br s, overlapped, 6H), 1.81 (br s, 1H); 13C NMR (151 MHz, CDCl3): δ 118.2, 114.9, 57.0, 52.6, 51.9, 46.1, 45.1, and 37.6.

N1-(2-(4-(2-aminoethyl)piperazin-1-yl)ethyl)ethane-1,2-diamine (200). 2-(2-(4-(cyanomethyl)piperazin-1-yl)ethylamino)acetonitrile (500 mg, 2.41 mmol) was added to a 100 mL oven dried round bottom flask containing a magnetic stir bar. MeOH (25 mL) and concentrated aqueous NH4OH (3 mL) were added to the flask. Raney®-Nickel-2400 catalyst (approximately 250 mg, in water) was transferred into a small pipette plugged with cotton. The catalyst was washed sequentially with water and MeOH (as the dried catalyst is flammable in air, pressurized Ar gas was used to force solvent through the pipette). The catalyst was transferred into the reaction solution, and the flask was placed into a stainless steel high pressure vessel (Parr apparatus); the pressure vessel was assembled. The vessel was charged with H2 gas to 500 psi, then vented. This was repeated once. Stirring was activated, and the vessel was filled with H2 to 1,000 psi; this pressure was maintained for 6 h by occasional addition of H2. The pressure in the vessel was released slowly and the reaction mixture was filtered through paper. The filtrate was concentrated by rotary evaporation affording a bluish-green oil. This oil was dissolved in 50 mL EtOH; HCl(g) was bubbled into the solution causing the formation of an off-white precipitate. The precipitate (the HCl salt of amine 200) was filtered and dried under reduced pressure. This solid was dispersed in CHCl3 by sonication. NH3 (g) was bubbled into the CHCl3 solution; the amine salt dissolved; this was accompanied by formation of solid NH4Cl. The CHCl3 solution was filtered; the filtrate was concentrated by rotary evaporation affording a pale yellow oil (300 mg). This material was used in the following reaction without further purification. 1H NMR (600 MHz, CDCl3): δ 2.82–2.77 (m, 4H), 2.74–2.67 (m, 4H), 2.50 (t, J = 9.3 Hz, 8H), 2.42 (t, J = 9.3 Hz, 4H), 1.32 (s, 5H).

Scheme 2. Synthesis of C12-200 from amine 200. C12-200. 1,2-epoxydodecane (2.10 g, 11.4 mmol, 7 equiv) was added to a 4 mL vial containing crude amine 200 (300 mg) and a magnetic stir bar. The vial was sealed and warmed to 80 °C. The reaction mixture was stirred for 2 days, whereupon analysis of the reaction mixture by MALDI-TOF spectroscopy suggested that the crude reaction mixture contained a significant amount of the desired product (MALDI-TOF-MS m/z calcld for C70H146N5O5 [M + H]+ 1,137, found 1,137). The crude mixture was purified by chromatography on silica gel (gradient elution from CH2Cl2 to 87.5:11.0:1.5 CH2Cl2/MeOH/NH4OH (aq.) affording C12-200 (668 mg, 42% over two steps) as a pale yellow viscous oil. 1H NMR (600 MHz, CDCl3): δ 5.00–4.00 (br s, 5H), 3.62 and 3.55 (apparent br s, 5H), 3.00–2.00 (m, 30H), 1.52–1.25 (m, 90H), 0.87 (t, J = 7.2 Hz, 15H).
Table S1. Summary of particle sizing data for formulated lipidoid particles used for in vivo screening as measured by dynamic light scattering

<table>
<thead>
<tr>
<th>Lipidoid</th>
<th>Mean Particle Diameter</th>
<th>Polydispersity</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14-100</td>
<td>251.2–8.4</td>
<td>0.224–0.047</td>
</tr>
<tr>
<td>C14-96</td>
<td>126.1–0.5</td>
<td>0.243–0.017</td>
</tr>
<tr>
<td>C12-120</td>
<td>65.1–0.2</td>
<td>0.128–0.023</td>
</tr>
<tr>
<td>C12-113</td>
<td>67.7–0.4</td>
<td>0.147–0.021</td>
</tr>
<tr>
<td>C18-62</td>
<td>167.4–5.9</td>
<td>0.142–0.070</td>
</tr>
<tr>
<td>C14-98</td>
<td>250.6–16.1</td>
<td>0.227–0.045</td>
</tr>
<tr>
<td>C18-96</td>
<td>252.2–5.9</td>
<td>0.178–0.024</td>
</tr>
<tr>
<td>C14-113</td>
<td>135.7–1.0</td>
<td>0.184–0.016</td>
</tr>
<tr>
<td>C14-120</td>
<td>135.5–5.6</td>
<td>0.227–0.015</td>
</tr>
<tr>
<td>C14-110</td>
<td>236.2–1.1</td>
<td>0.162–0.080</td>
</tr>
<tr>
<td>C16-96</td>
<td>181–2.1</td>
<td>0.117–0.027</td>
</tr>
<tr>
<td>C14-120</td>
<td>135.5–5.6</td>
<td>0.227–0.015</td>
</tr>
<tr>
<td>C14-110</td>
<td>236.2–1.1</td>
<td>0.162–0.080</td>
</tr>
</tbody>
</table>

Table S2. A summary of the calculated and observed m/z ratios in the MALDI-TOF spectra of a selection of crude reaction mixtures

<table>
<thead>
<tr>
<th>Amine</th>
<th>Epox.</th>
<th>N°</th>
<th>calcd.</th>
<th>observed</th>
<th>calcd.</th>
<th>observed</th>
<th>calcd.</th>
<th>observed</th>
<th>other</th>
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<tbody>
<tr>
<td>114</td>
<td>C14</td>
<td>5</td>
<td>1165.2</td>
<td>1165.6</td>
<td>953.0</td>
<td>953.4, 975.4(M + Na⁺)</td>
<td>740.8</td>
<td>741.0</td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>C12</td>
<td>4</td>
<td>854.9</td>
<td>855.2, 877.2</td>
<td>670.7</td>
<td>670.9, 692.8(M + Na⁺)</td>
<td>486.5</td>
<td>486.5, 508.5</td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>C10</td>
<td>4</td>
<td>885.9</td>
<td>866.2, 908.1(M + Na⁺)</td>
<td>701.7</td>
<td>701.9, 723.8(M + Na⁺)</td>
<td>517.5</td>
<td>517.5</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>C15</td>
<td>6</td>
<td>1588.6</td>
<td>1590.3(trace)</td>
<td>1348.4</td>
<td>1349.0, 1371.0(M + Na⁺)</td>
<td>1108.1</td>
<td>1108.6, 868.3(N – 3)</td>
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<tr>
<td>103</td>
<td>C18</td>
<td>2</td>
<td>349.3</td>
<td>349.1, 371.1(M + Na⁺)</td>
<td>249.2</td>
<td>248.8, 270.8(M + Na⁺)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>C10</td>
<td>4</td>
<td>773.7</td>
<td>774.1, 796.0(M + Na⁺)</td>
<td>617.6</td>
<td>617.8, 639.7(M + Na⁺)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>C15</td>
<td>2</td>
<td>517.5</td>
<td>517.6, 539.5(M + Na⁺)</td>
<td>333.3</td>
<td>333.1, 355.1(M + Na⁺)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>C13</td>
<td>3</td>
<td>657.8</td>
<td>657.8, 679.8(M + Na⁺)</td>
<td>473.5</td>
<td>473.5, 495.4(M + Na⁺)</td>
<td>289.3</td>
<td>289.0</td>
<td></td>
</tr>
</tbody>
</table>

The m/z ratios are reported as (M + H⁺) unless otherwise noted. Notes: (A) N equals maximum number of epoxide tails for the given amine ([number of 2° amines] plus [2 times number of 1° amines] in the polyamine starting material).
Table S3. A summary of the calculated and observed m/z ratios in the MALDI-TOF spectra of a selection of the epoxide lipids following column purification

<table>
<thead>
<tr>
<th>Amine</th>
<th>Epox.</th>
<th>A/B</th>
<th>N</th>
<th>m/z: [N] tails</th>
<th>m/z: [N − 1] tails</th>
<th>m/z: [N − 2] tails</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>C₁₄</td>
<td>B</td>
<td>3</td>
<td>725.8</td>
<td>513.5</td>
<td>301.3</td>
</tr>
<tr>
<td>120</td>
<td>C₁₂</td>
<td>A</td>
<td>4</td>
<td>885.9</td>
<td>886.14, 908.11</td>
<td>701.7</td>
</tr>
<tr>
<td>120</td>
<td>C₁₂</td>
<td>B</td>
<td>4</td>
<td>885.9</td>
<td>701.68</td>
<td>517.5</td>
</tr>
<tr>
<td>96</td>
<td>C₁₈</td>
<td>B</td>
<td>3</td>
<td>893.9</td>
<td>894.26, 916.3</td>
<td>625.66</td>
</tr>
<tr>
<td>100</td>
<td>C₁₄</td>
<td>A</td>
<td>4</td>
<td>924.0</td>
<td>924.4, 946.3</td>
<td>711.7</td>
</tr>
<tr>
<td>100</td>
<td>C₁₄</td>
<td>B</td>
<td>4</td>
<td>924.0</td>
<td>711.7</td>
<td>499.5</td>
</tr>
<tr>
<td>114</td>
<td>C₁₆</td>
<td>B</td>
<td>5</td>
<td>1305.3</td>
<td>1065.1</td>
<td>824.9</td>
</tr>
<tr>
<td>114</td>
<td>C₁₄</td>
<td>B</td>
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<td>1165.2</td>
<td>953.0</td>
<td>740.8</td>
</tr>
<tr>
<td>98</td>
<td>C₁₄</td>
<td>A</td>
<td>6</td>
<td>1420.4</td>
<td>1208.2</td>
<td>996.0</td>
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<tr>
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<td>C₁₄</td>
<td>B</td>
<td>6</td>
<td>1420.4, 1444.0</td>
<td>1208.2, 1230.7</td>
<td>996.0</td>
</tr>
<tr>
<td>63</td>
<td>C₁₈</td>
<td>A</td>
<td>2</td>
<td>639.7</td>
<td>639.8, 661.7</td>
<td>371.4</td>
</tr>
<tr>
<td>63</td>
<td>C₁₈</td>
<td>B</td>
<td>2</td>
<td>639.7</td>
<td>967.3</td>
<td>371.4</td>
</tr>
<tr>
<td>113</td>
<td>C₁₄</td>
<td>B</td>
<td>4</td>
<td>967.0</td>
<td>967.3</td>
<td>542.6</td>
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<tr>
<td>113</td>
<td>C₁₂</td>
<td>B</td>
<td>4</td>
<td>854.8</td>
<td>855.3, 877.3</td>
<td>486.5</td>
</tr>
<tr>
<td>120</td>
<td>C₁₄</td>
<td>A</td>
<td>4</td>
<td>998.0</td>
<td>998.4, 1020.4</td>
<td>573.6</td>
</tr>
<tr>
<td>120</td>
<td>C₁₄</td>
<td>A</td>
<td>4</td>
<td>998.0</td>
<td>785.8</td>
<td>573.6(trace)</td>
</tr>
<tr>
<td>110</td>
<td>C₁₄</td>
<td>B</td>
<td>6</td>
<td>1420.4</td>
<td>1208.2</td>
<td>996.0</td>
</tr>
<tr>
<td>110</td>
<td>C₁₄</td>
<td>B</td>
<td>6</td>
<td>1420.4, 1420.7</td>
<td>1208.2</td>
<td>996.0</td>
</tr>
<tr>
<td>120</td>
<td>C₁₆</td>
<td>B</td>
<td>4</td>
<td>1110.1</td>
<td>869.9</td>
<td>629.6</td>
</tr>
</tbody>
</table>

m/z ratios are reported as (M + H⁺) unless otherwise noted. “A”: fractions enriched in those less polar compounds expected to have [N] tails. “B”: fractions enriched in more-polar compounds expected to have [N − 1] tails.