

Rational design of novel cationic lipids for use in next-generation siRNA delivery systems

Supplementary Table 1. Linker modifications to DLinDMA

Abbreviated Name	Chemical Name	Structure	Mean Particle Size (nm) ^a	ED ₅₀ (mg/kg)
DLinDMA (Benchmark)	1,2-Dilinoleoxy-3-dimethylaminopropane		71 ± 24	~1
DLinDAP	1,2-Dilinoleoyl-3-dimethylaminopropane		65 ± 21	40-50
DLin-2-DMAP	1-Linoleoyl-2-linoleoxy-3-dimethylaminopropane		79 ± 26	5-12
DLin-C-DAP	1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane		73 ± 25	12-25
DLin-S-DMA	1,2-Dilinoleylthio-3-dimethylaminopropane		69 ± 25	12-25
DLin-K-DMA	2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane		67 ± 22	~0.4

^a Mean particle size of the PFV formulation as tested for FVII activity and containing the indicated cationic lipid at 40 mol%.

Supplementary Syntheses 1

Synthesis of 1,2-Dilinoleoxy-3-dimethylaminopropane (DLinDMA)

DLinDMA was synthesized according to the method previously published by Heyes et. al¹⁵.

Synthesis of 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP)

DLinDAP was synthesized according to the method previously published by Bailey and Cullis¹⁷.

1-Linoleoyl-2-linoleoxy-3-dimethylaminopropane (DLin-2-DMAP)

Synthesis of 1-Triphenylmethyloxy-3-(N,N-dimethylamino)-2-propanol (I)

A mixture of 3-(dimethylamino)-1,2-propanediol (3.0 g, 25 mmol) and triphenylmethyl chloride (7.75 g, 27.8 mmol) in dry pyridine (100 mL) was refluxed for 30 minutes. Upon cooling, most of the solvent was evaporated in vacuo, and the resulting residual was re-dissolved in 400 mL of dichloromethane. The organic phase was washed with water (3 x 200 mL), then brine (150 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 6.3 g of yellow oil as a crude product. The crude product was purified by column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 0-10% methanol gradient in dichloromethane. This afforded 4.0 g of **I** as yellow oil.

Synthesis of 1-Triphenylmethyloxy-2-linoleoxy-3-N,N-dimethylaminopropane (II)

NaH (60%, 2.17 g, 54 mmol) was washed with hexanes (3 x 40 mL) under nitrogen and then suspended in anhydrous benzene (60 mL). To the suspension was added **I** (4.0 g, 11 mmol) dropwise in 20 mL of anhydrous benzene. The resulting mixture was stirred at room temperature for 20 minutes, then a solution of linoleyl methanesulfonate (4.5 g, 13 mmol) in 40 mL of anhydrous benzene was added dropwise under nitrogen. The mixture was stirred at room temperature for 30 minutes and then refluxed overnight. Upon cooling to room temperature, 30 mL of 1:1 (V:V) ethanol-benzene solution were added dropwise under nitrogen followed by 100 mL of benzene and 100 mL of water. Upon shaking, the aqueous phase was separated. The organic phase was washed with brine (2 x 100 mL) and dried over anhydrous sodium sulfate. Evaporation of the solvent afforded 6.8 g of yellowish oil. The crude product was

chromatographed on a silica gel column (230-400 mesh, 400 mL) eluted with 0-3% methanol gradient in chloroform. 5.8 g (84%) of **II** were obtained as yellowish oil.

Synthesis of 2-Linoleyloxy-3-(N,N-dimethylamino)-1-propanol (III)

II (5.8 g, 9.2 mmol) was refluxed in 80% HOAc (25 mL) under nitrogen for 10 minutes. Upon cooling to room temperature, the mixture was diluted with water (100 mL). The resulting aqueous solution was neutralized to about pH 6 with 0.5% NaOH solution. The aqueous phase was then extracted with dichloromethane (4 x 100 mL). The combined organic phase was washed with 0.1% NaOH solution (100 mL), water (100 mL), then brine (100 mL), and dried over anhydrous sodium sulfate. Evaporation of the solvent gave 5.6 g of a mixture of product and starting material as yellowish oil. The mixture was chromatographed on a silica gel column (230-400 mesh, 400 mL) eluted with 0-10% methanol gradient in chloroform. 2.2 g (62%) of **III** were afforded as yellowish oil.

Synthesis of 2-Linoleyloxyl-3-linoleyloxyl-1-N,N-dimethylaminopropane (DLin-2-DMAP)

To a solution of linoleic acid (2.36 g, 8.4 mmol) in anhydrous benzene (50 mL) was added dropwise oxalyl chloride (1.45 g, 11.4 mmol) under nitrogen. The resulting mixture was stirred at room temperature for 4 hours. Solvent and excess of oxalyl chloride was removed in vacuo to give linoleoyl chloride as light yellowish oil. This was re-dissolved in anhydrous benzene (85 mL). To the resulting solution was added dropwise a solution of **III** (2.9 g, 7.5 mmol) and dry pyridine (1 mL) in 15 mL of anhydrous benzene. The mixture was then stirred at room temperature under nitrogen for 2 days, resulting in a suspension. The mixture was diluted with benzene (100 mL). The organic phase was washed with a solution of 3:5 (V:V) ethanol-water (320 mL), then brine (2 x 75 mL), and dried over anhydrous Na₂SO₄. The solvent was removed in vacuo affording 5.2 g of oil. The crude product was purified by column chromatography on silica gel (230-400 mesh, 450 mL) eluted with 0-4% methanol gradient in chloroform. This afforded 3.9 g (80%) of **DLin-2-DMAP** as yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ: 5.25 (8H, m, 4 x CH=CH), 4.17 (1H, dd, J = 11.6 and 4 Hz, OCH), 3.96 (1H, dd, J = 11.6 and 5.2 Hz, OCH), 3.53-3.64 (1H, m, OCH), 3.35-3.53 (2H, m, OCH₂), 2.68 (4H, t, =CH-CH₂-CH=), 2.41 (2H, m, CH₂), 2.25 (6H, s, 2 x NCH₃), 2.21 (2H, m, CH₂), 1.96 (8H, q, allylic 4 x CH₂), 1.4-1.6 (4H, m, 2 x CH₂), 1.21 (30H, s, 15 x CH₂), 0.80 (6H, t, 2 x CH₃) ppm.

1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP)

Preparation of Linoleyl Phthalimide

A mixture of potassium phthalimide (11.2 g, 59.5 mmol) and linoleyl methanesulfonate (9.3 g, 27 mmol) in 250 mL of anhydrous DMF was stirred at 70°C under nitrogen overnight. The resulting suspension was poured into 500 mL of cold water. The aqueous phase was extracted with EtOAc (3 x 200 mL). The combined extract was washed with water (200 mL), then brine (200 mL), and dried over anhydrous Na₂SO₄. Solvent was evaporated to give a mixture of solid and oily materials. To the mixture was added 300 mL of hexanes. The solid was filtered and washed with hexanes (2 x 25 mL). The filtrate and washes were combined, and the solvent was evaporated to result in 11 g of Linoleyl Phthalimide, which was used in the next step without further purification.

Preparation of Linoleylamine

The above crude linoleyl phthalimide (11 g, ca. 27 mmol) and hydrazine (10 mL) were refluxed in 350 mL of ethanol under nitrogen overnight. The resulting white solid was filtered upon cooling the mixture to about 40 to 50°C and the solid was washed with warm EtOH (2 x 30 mL). The filtrate and washes were combined and solvent evaporated. To the residual was added 400 mL of chloroform which resulted in precipitation of white solid. The solid was filtered again. The organic phase of the resulting filtrate was washed with water (2 x 100 mL), then brine (100 mL), and dried over anhydrous Na₂SO₄. Solvent was removed in vacuo to afford 7.3 g of yellow oil as a crude product. Pure linoleylamine was obtained by column chromatography on silica gel eluted with 0-20% methanol gradient in chloroform.

Preparation of Linoleyl Isocyanate

Anhydrous sodium carbonate (11 g) was suspended in a solution of linoleylamine (7.3 g, ca. 27 mmol) in anhydrous CH₂Cl₂ (200 mL) under good stirring and nitrogen. The suspension was cooled to 0 to 5°C with an ice bath. To the suspension was added diphosgene (8.2 g, 41 mmol) in 10 mL of anhydrous CH₂Cl₂ under vigorous stirring. Upon addition, the resulting suspension was stirred at 0 to 5°C under nitrogen for 60 minutes and then at room temperature for 2 hours. Upon completion of the reaction, 100 mL of water was added to the mixture and the mixture was stirred at room temperature for 30 minutes. The organic layer was separated, and washed with

water (100 mL) then brine (100 mL). After drying with anhydrous Na₂SO₄, the solvent was evaporated to give 7.6 g of yellow oil as a crude product, which was used in the following step without further purification.

1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP)

To a solution of crude linoleyl isocyanate (7.6 g, ca. 25 mmol) in 150 mL of anhydrous benzene under nitrogen was added dropwise a solution of 3-(dimethylamino)-1,2-propanediol (0.99 g, 8.3 mmol) in 20 mL of anhydrous benzene. The resulting mixture was stirred at room temperature for 60 minutes and then refluxed for 4 hours, followed by stirring at room temperature overnight. Upon dilution of the mixture with 150 mL benzene, the organic phase was washed with water (3 x 100 mL), then brine (100 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 8.4 g of yellow oil. Column purification of the oily material (500 mL silica gel, 230-400 mesh, eluted with 0-3% methanol gradient in chloroform) afforded 2.2 g (38%) of yellowish oil as the product **DLin-C-DAP**. ¹H NMR (400 MHz, CDCl₃) δ: 5.37 (8H, m, 4 x CH=CH), 5.06 (1H, br. CONH), 4.91 (1H, br. CONH), 4.79 (1H, m, OCH), 4.28 (1H, br. d, J = 11 Hz, OCH), 4.16 (1H, dd, J = 12 and 6 Hz, OCH), 3.16 (4H, m, 2 x NCH₂), 2.77 (4H, t, J = 6.4 Hz, =CH-CH₂-CH=), 2.4-2.7 (2H, m, NCH₂), 2.33 (6H, s, 2 x NCH₃), 2.05 (8H, m, allylic 4 x CH₂), 1.4-1.55 (4H, m, 2 x CH₂), 1.29 (40H, s, 20 x CH₂), 0.89 (6H, t, 2 x CH₃) ppm.

1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA)

Synthesis of Linoleylthio Acetate

First linoleyl mercaptane was synthesized. To a solution of triphenylphosphine (18.0g, 68.2 mmol) in 250 mL of anhydrous THF under nitrogen at 0-5°C was added dropwise diisopropyl azodicarboxylate (DIAD, 14.7 mL, 68 mmol). Upon addition, the resulting mixture was stirred at 0 to 5°C for 45 minutes. A yellow suspension was resulted. A solution of linoleyl alcohol (9.1g, 34 mmol) and thiolacetic acid (5.1 mL, 68 mmol) was then added at 0 to 5°C dropwise over 30 minutes to the yellow suspension under nitrogen. The resulting mixture was stirred at 0 to 5°C for 1 hour and then allowed to warm up to room temperature. After stirring at room temperature for 60 minutes, a brown solution was obtained. After solvent evaporation the residual was re-dissolved in 600 mL of ether. The ether phase was washed with water (2 x 250 mL), then brine (250 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated to afford 31g of brown

oil which partially solidified overnight. This crude mixture was treated with 100 mL of hexanes. The solid was filtered off and washed with hexanes (2 x 30 mL). The filtrate and washes were combined and solvent evaporated to give 13 g of brown oil as a crude product. The crude product was purified by column chromatography twice on silica gel (230-400 mesh, 600 mL) eluted with 0-3% ether gradient in hexanes. This gave 10.0 g (91%) of linoleylthio acetate as yellowish oil.

Synthesis of Linoleyl Mercaptane

To a suspension of LiAlH_4 (4.7g, 124 mmol) in 150 mL of anhydrous ether under nitrogen at 0 to 5°C was added dropwise a solution (one crystal of iodine in 200 mL of anhydrous ether) under nitrogen followed by linoleylthio acetate (10.0g, 30.8 mmol) in 100 mL of anhydrous ether. Upon addition, the suspension was allowed to warm up to room temperature and then stirred at room temperature for 4 hours. The resulting mixture was cooled to 0 to 5°C and 10 mL of NaCl saturated aqueous solution was added very slowly. After stirring at room temperature for 60 minutes, the suspension was filtered through a pad of diatomaceous earth. The solids were washed with ether (3 x 100 mL). The filtrate and washes were combined and the solvent evaporated, resulting in 7.2 g (83%) of linoleyl mercaptane as colourless oil.

Synthesis of 1-Triphenylmethyloxy-2-hydroxy-3-dimethylaminopropane (I)

A mixture of 3-(dimethylamino)-1,2-propanediol (6.3g, 53 mmol) and triphenylmethyl chloride (15.5g, 55.6 mmol) in anhydrous pyridine (200 mL) was refluxed for 40 minutes. Upon cooling to room temperature, most of the solvent was removed in vacuo. To the resulting oily residual was added 400 mL of ethyl acetate. A large amount of solid was formed. The solid was filtered off and dried in air. The filtrate phase was washed with water (2 x 150 mL), then brine (150 mL) and dried over anhydrous Na_2SO_4 . Evaporation of the solvent afforded 8.5 g of brown oil as a crude product (**I**), which was purified by column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 0-10% methanol gradient in chloroform.

Synthesis of 1-Triphenylmethyloxy-2-methylsulfonyloxy-3-dimethylaminopropane (II)

To a solution of **I** (4.2g, 11.7 mmol) and anhydrous triethylamine (2.5 mL, 17.9 mmol) in 150 mL of anhydrous dichloromethane under nitrogen was added dropwise with an ice-water cooling bath methylsulfonyl chloride (1.0 mL, 13 mmol). Upon addition, the cooling bath was removed and the mixture stirred at room temperature under nitrogen overnight (20 hours). The

resulting mixture was diluted with 100 mL of dichloromethane. The organic phase was washed with water (2 x 100 mL), then brine (100 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 4.3 g of crude product (**II**), which was used in the next step without further purification.

Synthesis of 1-Triphenylmethoxy-2-linoleylthio-3-dimethylaminopropane (III)

To a suspension of NaH (2.0 g, 95%, 79 mmol) in 100 mL of anhydrous benzene under nitrogen was added dropwise a solution of linoleyl mercaptane (3.1 g, 11 mmol) in 30 mL of anhydrous benzene. The resulting mixture was stirred at room temperature for 20 minutes. A solution of **II** (4.5g, 10 mmol) in 30 mL of anhydrous benzene was then added dropwise. After stirring at room temperature for 15 minutes, the mixture was refluxed gently under nitrogen for 3 days. Upon cooling, 30 mL of 1:1 (V:V) ethanol-benzene was added slowly to the mixture. The organic phase was washed once with 1:2 ethanol-water (360 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 7.1 g of yellowish oil as a crude product (**III**), which was purified by column chromatography on silica gel (230-400 mesh, 250 mL) eluted with 0-5% methanol gradient in chloroform to yield 5.5 g (88%) of **III**.

Synthesis of 1-Hydroxy-2-linoleylthio-3-dimethylaminopropane (IV)

III (5.5g, 8.8 mmol) was refluxed in 150 mL of 80% HOAc under nitrogen for 7 hours. Upon cooling, the solvent was removed to give a pale semi-solid. The material was re-dissolved in 200 mL of ethyl acetate. The organic phase was washed subsequently with 0.5% NaOH aqueous solution (100 mL), water (100 mL), and brine (100 mL). After drying over anhydrous Na₂SO₄, the solvent was evaporated. 5.1 g of a pale solid was resulted. Column chromatography of the crude product on silica gel (230-400 mesh, 250 mL) eluted with 0-7% methanol gradient in chloroform afforded 1.3 g (39%) of **IV**.

Synthesis of 1-Methylsulfonyloxy-2-linoleylthio-3-dimethylaminopropane (V)

Methylsulfonyl chloride (0.5g, 4.3 mmol) was added dropwise to a solution of **IV** (1.3g, 3.2 mmol) and anhydrous triethylamine (0.7 mL, 5 mmol) in 50 mL of anhydrous dichloromethane under nitrogen. The resulting mixture was stirred at room temperature overnight (19 hours). The reaction mixture was diluted with 50 mL of dichloromethane. The organic phase was washed with water (2 x 50 mL), then brine (50 mL), and dried over anhydrous

Na₂SO₄. Evaporation of the solvent resulted in 1.4 g of yellowish oil as a crude product (**V**), which was used in the following step without further purification.

Synthesis of 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA)

NaH (0.89g, 60%, 22 mmol) was washed twice with hexanes (2 x 15 mL) under nitrogen and then suspended in 70 mL of anhydrous benzene. To the suspension was added dropwise a solution of linoleyl mercaptane (1.1 g, 3.9 mmol) in 15 mL of anhydrous benzene. The resulting mixture was stirred at room temperature for 20 minutes. A solution of **V** (1.4g, 3.0 mmol) in 15 mL of anhydrous benzene was then added dropwise. After stirring at room temperature for 20 minutes, the mixture was refluxed gently under nitrogen for 2 days. Upon cooling, 200 mL of 1:1 (V:V) ethanol-benzene was added slowly to the mixture. The organic phase was washed with water (200 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 2.5 g of yellowish oil as a crude product. The crude product was purified by repeated column chromatography on silica gel (230-400 mesh, 250 mL) eluted with 0-3% methanol gradient in chloroform. This afforded 0.4 g (20%) of **DLin-S-DMA** as yellowish oil. ¹H NMR (400 MHz, CDCl₃) δ: 5.27-5.48 (8H, m, 4 x CH=CH), 2.88-3.0 (1H, m), 2.83 (2H, d, CH₂), 2.7 (4H, t, 2 x C=C-CH₂-C=C), 2.63-2.73 (1H, m), 2.58 (4H, double triplet, 2 x SCH₂), 2.39-2.49 (1H, m), 2.31 (6H, s, 2 x NCH₃), 2.06 (8H, q, 4 x allylic CH₂), 1.52-1.65 (4H, m, 2 x CH₂), 1.23-1.45 (32H, m), 0.90 (6H, t, 2 x CH₃) ppm.

2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA)

Synthesis of Linoleyl Bromide

A mixture of linoleyl methane sulfonate (6.2g, 18 mmol) and magnesium bromide etherate (17g, 55 mmol) in anhydrous ether (300 mL) was stirred under argon overnight (21 hours). The resulting suspension was poured into 300 mL of chilled water. Upon shaking, the organic phase was separated. The aqueous phase was extracted with ether (2 x 150 mL). The combined ether phase was washed with water (2 x 150 mL), then brine (150 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated to afford 6.5g of colourless oil. The crude product was purified by column chromatography on silica gel (230-400 mesh, 300 mL) eluted with hexanes. This gave 6.2 g (approximately 100%) of linoleyl bromide.

Synthesis of Dilinoleyl Methanol

To a suspension of Mg turnings (0.45g, 18.7 mmol) with one crystal of iodine in 200 mL of anhydrous ether under nitrogen was added a solution of linoleyl bromide in 50 mL of anhydrous ether at room temperature. The resulting mixture was refluxed under nitrogen overnight. The mixture was cooled to room temperature. To the cloudy mixture under nitrogen was added dropwise at room temperature a solution of ethyl formate (0.65g, 18.7 mmol) in 30 mL of anhydrous ether. Upon addition, the mixture was stirred at room temperature overnight (20 hours). The ether layer was washed with 10% H₂SO₄ aqueous solution (100 mL), then water (2 x 100 mL), then brine (150 mL), and then dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 5.0g of pale oil. Column chromatography on silica gel (230-400 mesh, 300 mL) with 0-7% ether gradient in hexanes as eluent afforded two products, dilinoleyl methanol (2.0g) and dilinoleylmethyl formate (1.4g). Dilinoleylmethyl formate (1.4g) and KOH (0.2g) were stirred in 85% EtOH at room temperature under nitrogen overnight. Upon completion of the reaction, half of the solvent was evaporated. The resulting mixture was poured into 150 mL of 5% HCL solution. The aqueous phase was extracted with ether (3 x 100 mL). The combined ether extract was washed with water (2 x 100 mL), then brine (100 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 1.0 g of dilinoleyl methanol as colourless oil. Overall, 3.0 g (60%) of dilinoleyl methanol were obtained.

Synthesis of Dilinoleyl Ketone

To a mixture of dilinoleyl methanol (2.0g, 3.8 mmol) and anhydrous sodium carbonate (0.2g) in 100 mL of CH₂Cl₂ was added pyridinium chlorochromate (PCC, 2.0g, 9.5 mmol). The resulting suspension was stirred at room temperature for 60 minutes. Ether (300 mL) was then added into the mixture, and the resulting brown suspension was filtered through a pad of silica gel (300 mL). The silica gel pad was further washed with ether (3 x 200 mL). The ether filtrate and washes were combined. Evaporation of the solvent gave 3.0 g of an oily residual as a crude product. The crude product was purified by column chromatography on silica gel (230-400 mesh, 250 mL) eluted with 0-3% ether in hexanes. This gave 1.8 g (90%) of dilinoleyl ketone.

Synthesis of 2,2-Dilinoleyl-4-bromomethyl-[1,3]-dioxolane (I)

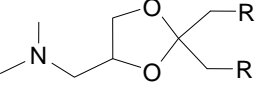
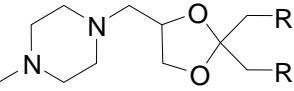
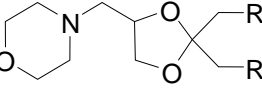
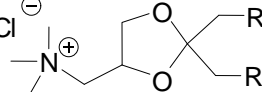
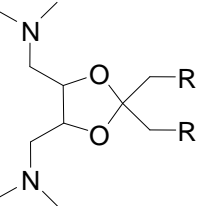
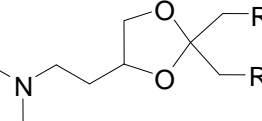
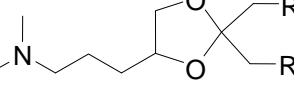
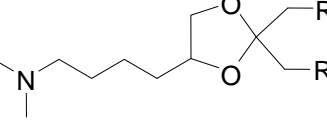
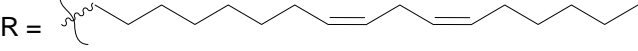
To make **I**, a mixture of dilinoleyl methanol (1.3g, 2.5 mmol), 3-bromo-1,2-propanediol (1.5g, 9.7 mmol) and p-toluene sulfonic acid hydrate (0.16g, 0.84 mmol) in 200 mL of toluene was refluxed under nitrogen for 3 days with a Dean-Stark tube to remove water. The resulting

mixture was cooled to room temperature. The organic phase was washed with water (2 x 50 mL), then brine (50 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-6% ether gradient in hexanes as eluent afforded 0.1 g of pure product and 1.3 g of a mixture of product and the starting material.

Synthesis of 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane (DLin-K-DMA)

Anhydrous dimethyl amine was bubbled into an anhydrous THF solution (100 mL) containing 1.3 g of a mixture of **I** and dilinoleyl ketone at 0°C for 10 minutes. The reaction flask was then sealed and the mixture stirred at room temperature for 6 days. Evaporation of the solvent left 1.5 g of a residual. The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) eluted with 0-5% methanol gradient in dichloromethane. This gave 0.8 g of **DLin-K-DMA**. ¹H NMR (400 MHz, CDCl₃) δ: 5.25-5.45 (8, m, 4x CH=CH), 4.28-4.4 (1H, m, OCH), 4.1 (1H, dd, OCH), 3.53 (1H, t OCH), 2.78 (4H, t, 2 x C=C-CH₂-C=C), 2.5-2.65 (2H, m, NCH₂), 2.41 (6H, s, 2 x NCH₃), 2.06 (8H, q, 4 x allylic CH₂), 1.56-1.68 (4H, m, 2 x CH₂), 1.22-1.45 (32H, m), 0.90 (6H, t, 2 x CH₃) ppm.

Supplementary Table 2. Headgroup modifications to DLin-K-DMA

Abbreviated Name	Chemical Name	Modification	Mean Particle Size (nm) ^a	ED ₅₀ (mg/kg)
DLin-K-DMA (Benchmark)	2,2-Dilinoleyl-4-dimethylamino methyl-[1,3]-dioxolane		67 ± 22	~0.4
DLin-K-MPZ	2,2-Dilinoleyl-4-N-methyl piperazino-[1,3]-dioxolane		71 ± 21	~1.5
DLin-K-MA	2,2-Dilinoleyl-4-N-morpholino-[1,3]-dioxolane		58 ± 21	>15
DLin-K-TMA.Cl	2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane Chloride		150 ± 69	>5 ^b
DLin-K ² -DMA	2,2-Dilinoleyl-4,5-bis (dimethylamino methyl)-[1,3]-dioxolane		68 ± 32	~0.4
DLin-KC2-DMA	2,2-Dilinoleyl -4-(2-dimethyl aminoethyl)-[1,3]-dioxolane		72 ± 23	~0.1
DLin-KC3-DMA	2,2-Dilinoleyl -4-(3-dimethyl aminopropyl)-[1,3]-dioxolane		80 ± 22	~0.6
DLin-KC4-DMA	2,2-Dilinoleyl -4-(4-dimethyl aminobutyl)-[1,3]-dioxolane		64 ± 15	>3
R = 				

^a Mean particle size of the PFV formulation as tested for FVII activity and containing the indicated cationic lipid at 40 mol%.

^b No activity observed at 5 mg/kg and lethal at next dose of 15 mg/kg.

Supplementary Syntheses 2

2,2-Dilinoleyl-4-N-methylpiperazino-[1,3]-dioxolane (DLin-K-MPZ)

A mixture of dilinoleyl ketone (1.3 gm, 2.5mmol), 3-bromo1,2-propane diol (1.5 gm, 9.7 mmol) and PPTS (pyridinium p-toluene sulfonate) (100 mg) in 25 mL of toluene was refluxed under nitrogen overnight with a Dean-stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2 x 50 mL) and saturated NaHCO₃ solution, then dried over anhydrous Na₂SO₄. Evaporation of solvent resulted in a yellowish oily residue. Column Chromatography on silica (230-400 mesh) with 0-5% ether as eluent in hexanes afforded 750 mg of the corresponding ketal. To a mixture of the ketal (250mg, 0.37 mmol) and K₂CO₃ (138 mg, 1 mmol) in 5 mL of acetonitrile was added N-methylpiperazine (50 mg, 0.50 mmol). The resulting solution was refluxed under argon overnight. The resulting mixture was cooled to room temperature, the solvent was evaporated, and the organic phase was washed with water (2 x 50 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 25-50% hexanes and ethyl acetate and then eluted with 0-5% methanol gradient in dichloromethane gave 225 mg of the desired product **DLin-K-MPZ**.

2,2-Dilinoleyl-4-N-morpholino-[1,3]-dioxolane (DLin-K-MA)

To a mixture of the above ketal (250mg, 0.37 mmol) and K₂CO₃(138 mg, 1 mmol) in 5 mL of acetonitrile was added morpholine (50 mg, 0.57 mmol). The resulting solution was refluxed under argon overnight. The resulting mixture was cooled to room temperature, the solvent was evaporated, and the organic phase was washed with water (2 x 50 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 25-50% hexanes and ethyl acetate and then eluted with 0-5% methanol as gradient in dichloromethane gave 225 mg of the desired product **DLin-K-MA**: ¹H NMR (300 MHz, CDCl₃) δ: 5.27-5.46 (8H, m), 4.21-4.31 (1H, m), 4.06-4.09 (1H, t), 3.71-3.73 (4H, t) 3.49-3.55 (1H, t), 2.78 (4H, t) 2.42-2.62 (6H, m), 2.02-2.09 (8H, m) 1.55-1.65 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane Chloride (DLin-K-TMA.Cl)

Synthesis of 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane Iodide (I)

A mixture of DLin-K-DMA (1.5g, 2.4 mmol) and CH₃I (4.0 mL, 64 mmol) in 10 mL of anhydrous CH₂Cl₂ was stirred under nitrogen at room temperature for 9 days. Evaporation of the solvent and excess of iodomethane afforded 20 g of yellow syrup as crude **I**, which was used in the following step without further purification.

Preparation of 2,2-Dilinoleyl-4-trimethylamino-[1,3]-dioxolane Chloride (DLin-K-TMA.Cl)

I (2.0 g) was dissolved in 100 mL of CH₂Cl₂ in a separatory funnel. 30 mL of 1N HCl methanol solution was added, and the resulting solution was shaken well. To the solution was added 50 mL of brine and the mixture was shaken well. The organic phase was separated. The aqueous phase was extracted with 10 mL of CH₂Cl₂. The organic phase and extract were then combined. This completed the first step of ion exchange. The ion exchange step was repeated four more times. The final organic phase was washed with brine (2 x 75 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave 2.0 g of yellowish viscous oil. The product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) eluted with 0-15% methanol gradient in chloroform. This afforded 1.2 g of **DLin-K-TMA.Cl** as a pale waxy material. ¹H NMR (300 MHz, CDCl₃) δ: 5.25-5.45 (8H, m, 4 x CH=CH), 4.55-4.75 (2H, m, 2 x OCH), 4.26-4.38 (1H, dd, OCH), 3.48-3.57 (1H, dd, NCH), 3.51 (9H, s, 3 x NCH₃), 3.11-3.22 (1H, dd, NCH), 2.77 (4H, t, 2 x C=C-CH₂-C=C), 2.05 (8H, q, 4 x allylic CH₂), 1.49-1.7 (4H, m, 2 x CH₂), 1.2-1.45 (30H, m), 0.89 (6H, t, 2 x CH₃) ppm.

2,2-Dilinoleyl-4,5-bis(dimethylaminomethyl)-[1,3]-dioxolane (DLin-K²-DMA)

Synthesis of DLin-K-diethyltartarate

A mixture of dilinoleyl ketone (1 g, 1.9 mmol), diethyl-D-tartarate (412 mg, 2 mmol) and pyridinium p-tolene sulfonate (250 mg, 1 mmol) in 25 mL of toluene was refluxed under nitrogen for 2 days with a Dean-stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water NaHCO₃ and brine (2 X 50 mL) and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residue. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 0-10% ether gradients in hexanes as eluent afforded 400 mg of pure DLin-K-diethyltartarate.

Synthesis of DLin-K-diethyldiol

To a solution of lithium aluminum hydride (32 mg, 1 mmol) in dry THF was added a solution of DLin-K-diethyltartarate (600mg, 0.85 m mol) in dry THF at 0°C under argon atmosphere; the reaction was stirred for 4 hours at room temperature. The reaction mixture was quenched with ice-cold water and then filtered through celite. The evaporation of solvent gave crude reduced alcohol. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 10-40% ethyl acetate gradients in hexanes as eluent afforded 350 mg of pure DLin-K-diethylidol.

Synthesis of DLin-K-diethyldimesylate

To a mixture of DLin-K-diethylidol (570 mg, 0.95 mmol) in dry dichloromethane was added pyridine (275 mg, 3.85 mmol) and 4-(dimethylamino)pyridine (122 mg, 1 mmol) under argon, followed by a solution of methane sulfonyl chloride (500 mg, 2.5 mmol). The resulting mixture was stirred overnight. The organic phase was washed with water and brine (2 x 50 mL), then the solvent was evaporated to give a yellowish oil residue. Column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 10-40% ethyl acetate gradients in hexanes as eluent afforded 300 mg of pure DLin-K-diethyldimesylate.

2,2-Dilinoleyl-4,5-bis(dimethylaminomethyl)-[1,3]-dioxolane (DLin-K²-DMA)

Anhydrous dimethyl amine solution in THF was added to the reaction vessel containing 300 mg of DLin-K-diethyldimesylate at room temperature for 5 minutes. The reaction flask was then sealed and the mixture stirred at room temperature for 6 days. Evaporation of the solvent left 300 mg of residual. The crude product was purified by column chromatography on silica gel (230-400 mesh, 500 mL) eluted with 0-10% methanol gradients in chloroform as eluent and afforded 50 mg of pure **DLin-K²-DMA**. ¹H NMR (300 MHz, CDCl₃) δ: 5.27-5.46 (8H, m), 3.72-3.80 (2H, t), 2.75 (4H, t), 2.49 (4H, d), 2.30 (12H, s), 2.02-2.09 (8H, m) 1.62-1.72 (4H, m), 1.2-1.47 (32 H, m), 0.87-0.90 (6H, t) ppm.

2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA)

Synthesis of 2,2-Dilinoleyl-4-(2-hydroxyethyl)-[1,3]-dioxolane (I)

A mixture of dilinoleyl ketone (527 mg, 1.0 mmol); 1,2,4-butanetriol (technical grade, ca. 90%, 236 mg, 2 mmol); and pyridinium p-toluenesulfonate (50 mg, 0.2 mmol) in 50 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2 x 30 mL),

then brine (50 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residual (0.6 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with dichloromethane as eluent. This afforded 0.5 g of pure **I**.

Synthesis of 2,2-Dilinoleyl-4-(2-methanesulfonylethyl)-[1,3]-dioxolane (II)

To a solution of **I** (500 mg, 0.81 mmol) and dry triethylamine (218 mg, 2.8 mmol) in 50 mL of anhydrous CH₂Cl₂ was added methanesulfonyl anhydride (290 mg, 1.6 mmol) under nitrogen. The resulting mixture was stirred at room temperature overnight. The mixture was diluted with 25 mL of CH₂Cl₂. The organic phase was washed with water (2 x 30 mL), then brine (50 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated to afford 510 mg of yellowish oil. The crude product **II** was used in the following step without further purification.

Synthesis of 2,2-Dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA)

To the above crude material under nitrogen was added 20 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 6 days. An oily residual was obtained upon evaporation of the solvent. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-5% methanol gradient in dichloromethane as eluent resulted in 380 mg of the product **DLin-KC2-DMA** as pale oil. ¹H NMR (400 MHz, CDCl₃) δ: 5.27-5.49 (8, m, 4x CH=CH), 4.01-4.15 (2H, m, 2 x OCH), 3.49 (1H, t OCH), 2.78 (4H, t, 2 x C=C-CH₂-C=C), 2.34-2.54 (2H, m, NCH₂), 2.30 (6H, s, 2 x NCH₃), 2.06 (8H, q, 4 x allylic CH₂), 1.67-1.95 (2H, m, CH₂), 1.54-1.65 (4H, m, 2 x CH₂), 1.22-1.45 (32H, m), 0.90 (6H, t, 2 x CH₃) ppm.

2,2-Dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-KC3-DMA)

Synthesis of 1,2,5-Pentanetriol

To a suspension of LiAlH₄ (1.75g) in 80 mL of anhydrous THF was added dropwise under nitrogen a solution of (R)-γ-hydroxymethyl-γ-butarolactone (0.50 g, 4 mmol) in 20 mL of anhydrous THF. The resulting suspension was stirred at room temperature under nitrogen overnight. To this mixture was added 5.5 mL of NaCl-saturated aqueous solution very slowly using an ice-water bath. The mixture was further stirred under nitrogen overnight. The white solid was filtered and washed with THF (2 x 20 mL). The filtrate and washes were combined. Evaporation of the solvent gave 0.25 g of colourless oil, which was used in the next step without further purification.

Synthesis of 2,2-Dilinoleyl-4-(3-hydroxypropyl)-[1,3]-dioxolane (I)

A mixture of dilinoleyl ketone (1.0 g, 2 mmol); 1,2,5-pentanetriol (crude, 0.25 g, 2 mmol); and pyridinium p-toluenesulfonate (100 mg, 0.4 mmol) in 150 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (3 x 40 mL), then brine (50 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent gave a yellowish oily residual (1.1 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with 0-1% methanol in dichloromethane as eluent. This afforded 0.90 g of pure **I** as colourless oil.

Synthesis of 2,2-Dilinoleyl-4-(3-methanesulfonylpropyl)-[1,3]-dioxolane (II)

To a solution **I** (0.90 g, 1.4 mmol) and dry triethylamine (0.51 g, 5 mmol) in 100 mL of anhydrous CH₂Cl₂ was added methanesulfonyl anhydride (0.70 g, 4 mmol) under nitrogen. The resulting mixture was stirred at room temperature overnight. The organic phase was washed with water (2 x 40 mL), then brine (50 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated to afford 1.0 g of brownish oil as a crude product. The crude product was used in the following step without further purification.

Synthesis of 2,2-Dilinoleyl-4-(3-dimethylaminopropyl)-[1,3]-dioxolane (DLin-KC3-DMA)

To the above crude material (**II**, 1.0 g) under nitrogen was added 40 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 8 days. The solid was filtered. Upon evaporation of the solvent, an orange residual was obtained. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-40% ethyl acetate gradient in hexanes as eluent resulted in 510 g of the product **DLin-KC3-DMA** as pale oil. ¹H NMR (400 MHz, CDCl₃) δ: 5.22-5.50 (8, m, 4x CH=CH), 3.95-4.15 (2H, m, 2 x OCH), 3.35-3.55 (1H, m OCH), 2.78 (4H, t, 2 x C=C-CH₂-C=C), 2.45-2.55 (2H, m, NCH₂), 2.35 (6H, s, 2 x NCH₃), 2.05 (8H, q, 4 x allylic CH₂), 1.45-1.75 (6H, m, CH₂), 1.2-1.45 (32H, m), 0.90 (6H, t, 2 x CH₃) ppm.

2,2-Dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-KC4-DMA)

Synthesis of 2,2-Dilinoleyl-4-(4-hydroxybutyl)-[1,3]-dioxolane (I)

A mixture of dilinoleyl ketone (1.05 g, 2.0 mmol); 1,2,6-hexanetriol (0.54 g, 4 mmol); and pyridinium p-toluenesulfonate (100 mg, 0.4 mmol) in 150 mL of toluene was refluxed under nitrogen overnight with a Dean-Stark tube to remove water. The resulting mixture was cooled to room temperature. The organic phase was washed with water (2 x 60 mL), then brine (60 mL), and dried over anhydrous Na₂SO₄. Evaporation of the solvent resulted in a yellowish oily residual (1.5 g). The crude product was purified by column chromatography on silica gel (230-400 mesh, 100 mL) with 0-0.5% methanol in dichloromethane as eluent. This afforded 1.4 g of **I**.

Synthesis of 2,2-Dilinoleyl-4-(4-methanesulfonylbutyl)-[1,3]-dioxolane (II)

To a solution of **I** (1.4 g, 2 mmol) and dry triethylamine (0.73 g, 7.2 mmol) in 150 mL of anhydrous CH₂Cl₂ was added methanesulfonyl anhydride (1.0 g, 5.7 mmol) under nitrogen. The resulting mixture was stirred at room temperature overnight. The organic phase was washed with water (2 x 75 mL), then brine (75 mL), and dried over anhydrous Na₂SO₄. The solvent was evaporated to afford 1.45 g of **II**.

Synthesis of 2,2-Dilinoleyl-4-(4-dimethylaminobutyl)-[1,3]-dioxolane (DLin-KC4-DMA)

To **II** (1.45 g) under nitrogen was added 60 mL of dimethylamine in THF (2.0 M). The resulting mixture was stirred at room temperature for 6 days. The solid was filtered. An oily residual (1.2 g) was obtained upon evaporation of the solvent. Column chromatography on silica gel (230-400 mesh, 100 mL) with 0-5% methanol gradient in dichloromethane as eluent resulted in 0.95 g of the product **DLin-KC4-DMA** as pale oil. ¹H NMR (400 MHz, CDCl₃) δ: 5.26-5.49 (8, m, 4x CH=CH), 3.97-4.15 (2H, m, 2 x OCH), 3.45 (1H, t OCH), 2.78 (4H, t, 2 x C=C-CH₂-C=C), 2.45-2.55 (2H, m, NCH₂), 2.40 (6H, s, 2 x NCH₃), 2.05 (8H, q, 4 x allylic CH₂), 1.45-1.75 (8H, m, CH₂), 1.2-1.45 (32H, m), 0.90 (6H, t, 2 x CH₃) ppm.

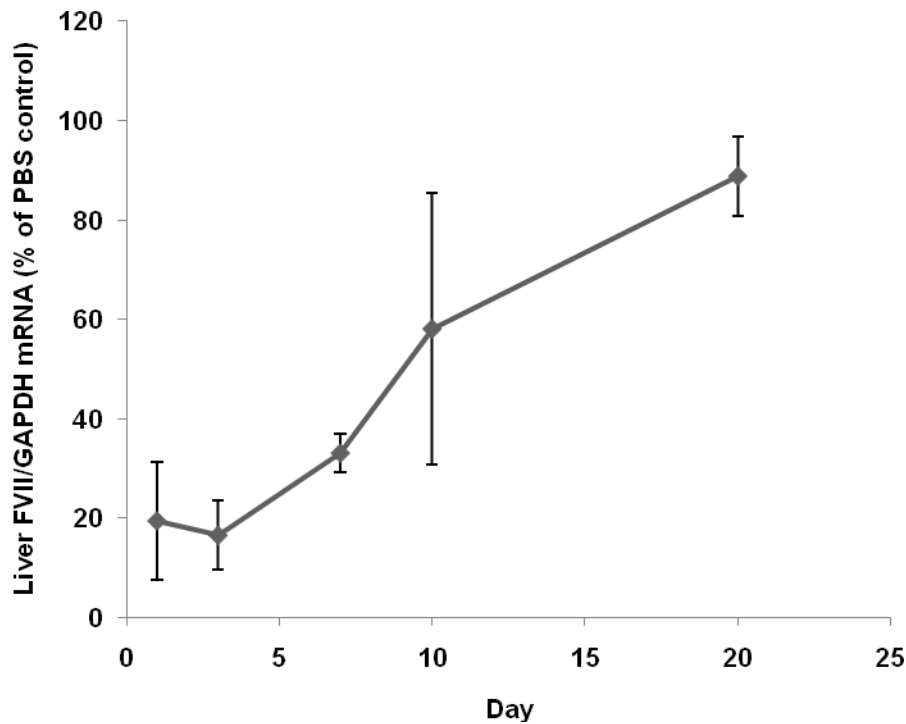
Supplementary Table 3. Pharmacokinetics and biodistribution of LNP (PFV) containing the cationic lipids indicated. All formulations were administered at an siRNA dose of 5 mg/kg and had an siRNA-to-lipid ratio of 0.04 to 0.06, wt/wt. Results are expressed as the percentage of the injected dose administered (mean value; n=3-5 mice).

Lipid	0.5 h			3.0 h			pKa	ED ₅₀
	Plasma	Liver	Spleen	Plasma	Liver	Spleen		
DLin-K-DMA	1.1	32.0	<0.1	0.4	4.0	<0.1	5.9	~0.3
DLinDMA	15.3	50.0	0.8	0.7	17.0	0.2	6.8	~1
DLinMPZ	20.3	52.0	1.5	0.4	37.5	0.1	6.2	12-25
DLinDAC	27.1	29.0	0.2	0.3	6.5	0.1	-	12-25
DLin-2-DMAP	17.5	20.5	0.3	8.8	2.5	0.1	-	12-25
DLinAP	86.2	11.5	0.4	23.1	5.0	0.2	-	12-25
DLin-C-DAP	69.4	28.5	0.8	19.0	13.5	0.1	-	>25
DLinMA	20.2	10.5	0.1	0.4	4.5	0.3	5.6	>25
DLin-S-DMA	10.7	2.5	<0.1	5.4	<0.1	<0.1	-	>25
DLinDAP	46.6	20.5	0.7	3.3	16.5	0.2	6.2	40-50

Supplementary Table 4. Clinical chemistry and hematology parameters in NHPs

Treatment	siRNA dose (mg/kg)	ALT (U/L)	AST (U/L)	Total Bilirubin (mg/dL)	BUN (mg/dL)	RBC (x 10 ⁶ /μL)	Hemoglobin (g/dL)	WBC (x 10 ³ /μL)	PLT (x 10 ³ /μL)
PBS	-	54 ± 25	51 ± 27	0.3 ± 0.1	27 ± 4	4.6 ± 0.5	13.8 ± 0.6	17.6 ± 3.0	515 ± 70
siApoB	1	42 ± 11	49 ± 12	0.3 ± 0.1	23 ± 3	6.0 ± 0.2	14.2 ± 0.9	13.6 ± 3.7	508 ± 49
siTTR	0.03	57 ± 11	47 ± 12	0.1 ± 0	15 ± 4	4.8 ± 0.4	11.5 ± 0.9	10.9 ± 2.2	495 ± 105
siTTR	0.1	50 ± 22	63 ± 47	0.13 ± 0.1	20 ± 3	5.0 ± 0.0	11.1 ± 0.4	12.9 ± 3.3	528 ± 22
siTTR	0.3	67 ± 38	66 ± 18	0.1 ± 0	21 ± 6	5.1 ± 0.2	11.0 ± 0.5	11.1 ± 5.5	529 ± 72
siTTR	1	47 ± 5	43 ± 7	0.13 ± 0.1	19 ± 1	4.9 ± 0.1	10.9 ± 0.4	10.7 ± 1.6	477 ± 34

ALT, alanine aminotransferase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; RBC, red blood cells; WBC, white blood cells; PLT, platelets.



Supplementary Figure 1. Durability of gene silencing for FVII siRNA formulated in KC2-SNALP. Rats (n = 5) received a single iv dose of 0.25 mg/kg FVII siRNA formulated in KC2-SNALP. At various time points after administration, animals were sacrificed and liver mRNA levels were determined. Liver mRNA levels were reported relative to PBS treated control animals. Earlier studies have established that, at the dose levels employed, treatment with formulated control siRNAs do not alter liver FVII levels relative to PBS treated animals in this model (data not shown). Data are expressed as group mean \pm s.d.