

Electronic Supplementary Information

Safe and Efficient *In Vitro* and *In Vivo* Gene Delivery: Tripodal Cationic Lipids with Programmed Biodegradability

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1. Characterization of the novel compounds

1.1. General Remarks

TLC was performed on silica plates using varying systems as stated. Plates were visualised under an UV lamp at 254 nm or by a ninhydrin test.

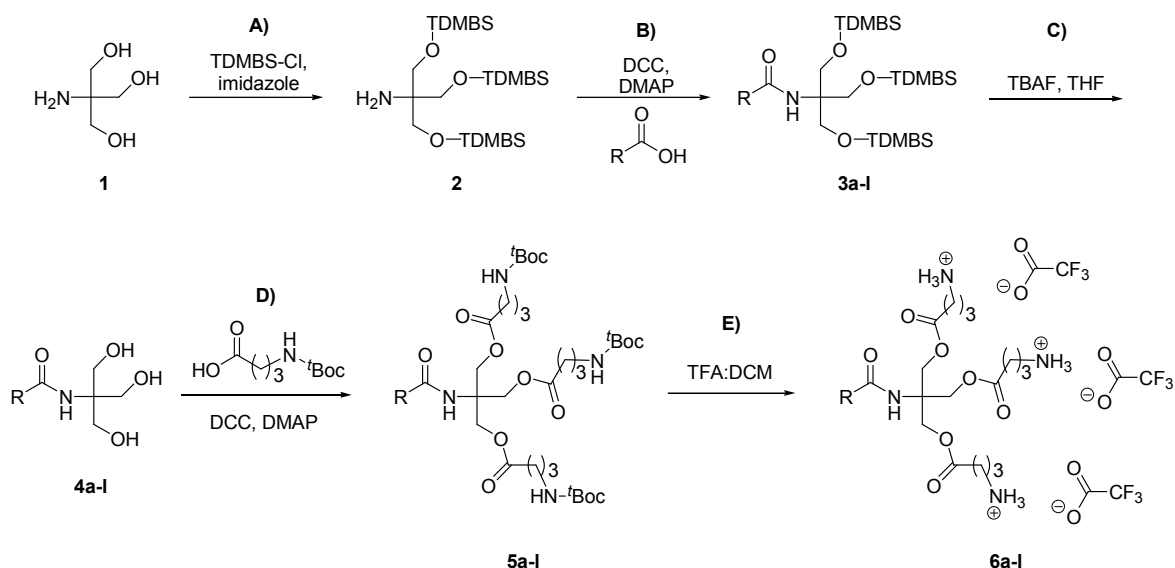
^1H , ^{13}C and DEPT NMR spectra were recorded on a Bruker AC300 spectrometer operating at 250MHz for ^1H and 62.5MHz for ^{13}C and DEPT at 298K. Samples were dissolved in CDCl_3 or DMSO-d_6 or methanol- d as stated. Chemical shifts are quoted in parts per million relative to the solvent peak. Abbreviations for multiplicity are s, singlet, d, doublet, t, triplet, q, quadruplet and m, multiplet. All coupling constants were measured in Hz.

Electrospray mass spectroscopy spectra were recorded using an Agilent 1100 series VG platform Quadruple Electrospray Ionisation mass spectrometer model G1946B. Sonification was done using a Hilsonic water bath and flow cytometry using a BD FACS Aria flow cytometer.

In vivo imaging of luminescence was carried out using an IVIS SPECTRUM Imaging System (Caliper).

Organic solvents and laboratory reagents were supplied by Fisher Scientific and Sigma-Aldrich.

1.2. Synthesis and characterization of compounds 6a-l



Scheme 1: Synthetic Route.

A) Synthesis of tris-(*tert*-butyldimethylsilyloxymethyl)aminomethane^{1, 2}

Butyldimethylsilyl chloride (6.7g, 44.6mmol) and imidazole (6.3g, 92.9mmol) were dissolved in DMF (5mL). Tris(hydroxymethyl)methyl amine, **1** (1.5g, 12.4mmol) was added and the mixture stirred at room temperature for 1 h. The product was washed with H₂O, extracted with DCM, dried over anhydrous MgSO₄ and filtered. Solvent was removed under reduced pressure to give a white powder (5.6g, 12.1mmol, 98%): ¹H NMR (CDCl₃) δ: 3.4 (s, 6H), 0.85 (s, 27H), 0.0 (s, 18H). ¹³C NMR (CDCl₃) δ: 64.5, 57.1, 26.3, 17.9. LRMS (ES+) *m/z* 464.5 [100, (M+H)⁺].

B) Synthesis of fatty derivatives 3a-l

Method A. Either decanoyl chloride or cholesteryl chloroformate was added dropwise into a solution of compound **2** (1 equiv.), pyridine (1.1 equiv.) and dimethylaminopyridine (DMAP, 0.1 equiv.) in DCM and stirred for 2 h. Subsequently, the solution was filtered and the solvent removed under reduced pressure and the crude product redissolved in DCM (three times). The product was washed with H₂O, extracted with DCM, dried over anhydrous MgSO₄ and filtered. Solvent was removed under reduced pressure to give the product.

Method B. The corresponding fatty acid (1.1 equiv.) and N,N'-dicyclohexylcarbodiimide (DCC)(1.1 equiv.) were dissolved in DCM and stirred for 30 min. Subsequently DMAP (0.1 equiv.) and compound **2** (1 equiv.) were successively added and the resulting mixture stirred for 2 h. The solution was filtered and the solvent removed under reduced pressure and the crude product redissolved in DCM (three times). The product was washed with H₂O, extracted with DCM, dried over anhydrous MgSO₄ and filtered. Solvent was removed under reduced pressure to give the product.

N-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]decamide, **3a** (85%): ¹H NMR (CDCl₃) δ: 5.5 (s, 1H), 3.8 (s, 6H), 2.1-0.9 (m, 19H), 0.85 (s, 27H), 0.0 (s, 18H). ¹³C NMR (CDCl₃) δ: 171.9, 61.0, 60.0, 37.0, 29.7, 26.2, 23.1, 18.6, 14.5. LRMS (ES+) 618.2 [100, (M+H)⁺].

N-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]undecamide, **3b** (88%): ¹H NMR (CDCl₃) δ: 5.5 (s, 1H), 3.8 (s, 6H), 2.0-1.0 (m, 21H), 0.85 (s, 27H), 0.0 (s, 18H). ¹³C NMR (CDCl₃) δ: 171.9, 62.0, 61.2, 29.6, 29.7, 26.2, 23.1, 18.6, 14.5. LRMS (ES+) 632.2 [100, (M+H)⁺].

N-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]dodecamide, **3c** (83%): ¹H NMR (CDCl₃) δ: 5.5 (s, 1H), 3.8 (s, 6H), 2.1-0.9 (m, 23H), 0.85 (s, 27H), 0.0 (s, 18H). ¹³C NMR (CDCl₃) δ: 171.9, 61.0, 38.2, 29.6, 29.7, 26.2, 23.1, 18.6, 14.5. LRMS (ES+) 646.7 [100, (M+H)⁺].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]tridecamide, 3d** (83%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.0-0.9 (m, 25H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.9, 61.0, 60.0, 38.2, 32.6, 31.8, 30.2, 26.2, 23.1, 18.6, 14.5. LRMS (ES+) 660.3 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]tetradecamide, 3e** (79%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.1-0.9 (m, 27H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.9, 59.6, 30.9, 28.4, 28.2, 24.8, 23.7, 21.7, 17.2, 13.1. LRMS (ES+) 674.8 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]pentadecamide, 3f** (93%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.1-1.0 (m, 29H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.9, 59.6, 30.9, 28.4, 28.2, 24.8, 23.7, 21.7, 17.2, 13.1. LRMS (ES+) 688.8 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]hexadecamide, 3g** (90%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.1-0.9 (m, 31H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.9, 60.2, 36.7, 33.3, 30.9, 28.7, 28.5, 25.4, 24.8, 23.7, 17.2, 13.1. LRMS (ES+) 702.8 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]octadecamide, 3h** (92%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.1-0.9 (m, 35H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.7, 59.6, 47.7, 32.9, 28.7, 28.4, 28.1, 25.4, 24.8, 24.7, 23.9, 21.5, 17.2, 13.1. LRMS (ES+) 730.8 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]-9-octadecenamide, 3i** (85%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 5.3 (t, $J=5.5$, 2H), 3.8 (s, 6H), 2.1-0.9 (m, 31H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 162.0, 60.2, 36.4, 35.8, 32.6, 31.8, 29.6, 29.4, 29.3, 26.3, 25.8, 24.7, 22.6, 18.2, 14.1. LRMS (ES+) 728.8 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]eicosanamide, 3j** (78%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.3-0.9 (m, 39H), 0.87 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.7, 59.6, 47.7, 32.9, 28.7, 28.4, 28.1, 25.4, 24.8, 24.7, 23.9, 21.5, 17.2, 13.1. LRMS (ES+) 758.5 [100, (M+H) $^+$].

***N*-[Tris(*tert*-butyldimethylsilyloxymethyl)methyl]tetraeicosanamide, 3k** (72%): ^1H NMR (CDCl_3) δ : 5.5 (s, 1H), 3.8 (s, 6H), 2.2-0.8 (m, 47H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 171.7, 61.7, 60.7, 37.7, 31.9, 29.6, 29.3, 25.8, 25.8, 22.6, 18.1, 14.1. LRMS (ES+) 814.6 [100, (M+H) $^+$].

***O*-(Cholest-5-en-3-yl)-*N*-[tris(*tert*-butyldimethylsilyloxymethyl)methyl]carbamate, 3l** (70%): ^1H NMR (CDCl_3) δ : 5.47 (s, 1H), 3.8 (s, 6H), 2.3-0.5 (m, 45H), 0.85 (s, 27H), 0.0 (s, 18H). ^{13}C NMR (CDCl_3) δ : 150.7, 140.0, 123.6, 64.0, 60.0, 56.7, 56.2, 50.1, 39.5, 38.6, 37.1, 36.5, 35.8, 31.9, 30.0, 28.0, 25.8, 23.8, 22.8, 21.0, 19.3, 18.2. LRMS (ES+) 876.7 [100, (M+H) $^+$].

C) Synthesis of deprotected derivatives 4a-l

General TDMBS deprotection procedure: Tetrabutylammonium fluoride (1.5equiv.) in THF was added to the compound and the mixture left stirring at room temperature for 3 h. The solvent was removed under reduced pressure and water added. The suspension was sonicated for 1 h and left to precipitate overnight. The product was recovered by vacuum filtration washing with diethyl ether to obtain the pure compound as a white solid (yield=55-90%).

N-[Tris(hydroxymethyl)methyl]decamide, 4a (88%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.6 (s, 6H), 2.2-1.4 (m, 16H), 1.1 (t, 3H, $J=6.5$). ^{13}C NMR (DMSO-*d*) δ : 173.7, 62.1, 62.2, 35.8, 31.2, 28.8, 28.7, 28.4, 25.2, 22.0, 13.9.

N-[Tris(hydroxymethyl)methyl]undecamide, 4b (85%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.5 (s, 6H), 2.1-1.2 (m, 18H), 0.8 (t, 3H, $J=6.5$). ^{13}C NMR (DMSO-*d*) δ : 173.8, 62.1, 61.2, 35.7, 31.2, 28.9, 25.3, 22.1, 13.9.

N-[Tris(hydroxymethyl)methyl]dodecamide, 4c (69%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.5 (s, 6H), 2.2-1.5 (m, 20H), 1.1 (t, 3H, $J=6.5$). ^{13}C NMR (DMSO-*d*) δ : 174.2, 62.5, 61.2, 36.2, 31.7, 31.1, 29.4, 29.0, 25.7, 22.4, 14.3.

N-[Tris(hydroxymethyl)methyl]tridecamide, 4d (78%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.5 (s, 6H), 2.1-1.4 (m, 22H), 1.2 (t, 3H, $J=6.5$). ^{13}C NMR (DMSO-*d*) δ : 173.0, 62.2, 60.8, 35.8, 31.3, 29.0, 28.9, 25.7, 22.1, 13.9.

N-[Tris(hydroxymethyl)methyl]tetradecamide, 4e (61%): ^1H NMR (DMSO-*d*) δ : 7.5 (s, 1H), 5.3 (s, 3H), 3.5 (s, 6H), 1.2-2.2 (m, 24H), 1.1 (t, 3H, $J=6.6$). ^{13}C NMR (DMSO-*d*) δ : 174.2, 62.6, 61.2, 36.2, 31.7, 29.4, 29.1, 25.7, 22.4, 14.3.

N-[Tris(hydroxymethyl)methyl]pentadecamide, 4f (81%): ^1H NMR (DMSO-*d*) δ : 7.5 (s, 1H), 5.3 (s, 3H), 3.5 (s, 6H), 2.2-1.3 (m, 26H), 1.1 (t, 3H, $J=6.0$). ^{13}C NMR (DMSO-*d*) δ : 174.2, 62.6, 61.2, 36.2, 31.7, 29.4, 29.0, 25.7, 22.4, 14.3.

N-[Tris(hydroxymethyl)methyl]hexadecamide, 4g (66%): ^1H NMR (DMSO-*d*) δ : 7.5 (s, 1H), 5.2 (s, 3H), 3.45 (s, 6H), 1.2-2.1 (m, 28H), 0.95 (t, 3H, $J=6.6$). ^{13}C NMR (DMSO-*d*) δ : 174.2, 62.7, 61.2, 36.2, 31.7, 29.4, 29.0, 25.7, 22.4, 14.3.

N-[Tris(hydroxymethyl)methyl]octadecamide, 4h (86%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.5 (s, 6H), 1.5-2.2 (m, 32H), 1.1 (t, 3H, $J=6.6$). ^{13}C NMR (DMSO-*d*) δ : 174.2, 62.5, 61.2, 36.2, 31.7, 31.1, 29.4, 29.0, 25.7, 22.4, 14.3.

***N*-[Tris(hydroxymethyl)methyl]-9-octadecenamide, 4i** (55%): ^1H NMR (DMSO-*d*) δ : 7.3 (s, 1H), 5.3 (t, 2H), 5.0 (s, 3H), 3.5 (s, 6H), 0.9-2.1 (m, 28H), 1.1 (t, 3H, $J=6.6$). ^{13}C NMR δ : (DMSO-*d*) 174.2, 57.8, 47.9, 33.7, 25.7, 24.8, 23.4, 19.5, 13.8.

***N*-[Tris(hydroxymethyl)methyl]eicosamide, 4j** (75%): ^1H NMR (CDCl_3) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.6 (s, 6H), 2.3-1.2 (m, 36H), 1.0 (t, 3H, $J=6.5$). ^{13}C NMR (CDCl_3) δ : 61.2, 33.9, 33.2, 31.9, 30.9, 29.7, 22.6, 14.1.

***N*-[Tris(hydroxymethyl)methyl]tetraeicosamide, 4k** (60%): ^1H NMR (CDCl_3) δ : 7.3 (s, 1H), 5.0 (s, 3H, OH), 3.5 (s, 6H), 2.3-1.0 (m, 44H), 1.1 (t, 3H, $J=6.5$). ^{13}C NMR (CDCl_3) δ : 174.2, 62.4, 61.6, 34.2, 31.6, 29.4, 29.0, 25.5, 24.8, 22.4, 13.8.

***O*-(Cholest-5-en-3-yl)-*N*-[tris(hydroxymethyl)methyl]carbamate, 4l** (80%): ^1H NMR (CDCl_3) δ : 7.3 (s, 1H), 5.0 (s, 3H), 3.5 (s, 6H), 2.1-0.6 (m, 45H). ^{13}C NMR (CDCl_3) δ : 156.7, 139.4, 122.2, 62.7, 59.9, 56.3, 55.8, 49.7, 42.0, 40.0, 39.7, 39.4, 36.2, 31.5, 27.6, 23.4, 22.5, 22.2, 18.4.

D) Synthesis of protected compounds 5a-l

General coupling with t Boc-protected amino acids: The coupling compound (3.43mmol, 3.3eq) was dissolved in DCM (40mL) and DMF (5mL) and DCC (702mg, 3.43mmol, 3.3eq) added and the mixture was stirred at room temperature for 30 min. A solution of one of **4a-l** (400mg, 1.04mmol) was added slowly to the mixture with DMF (5mL) and left to stir for 10 min. DMAP (13.0mg, 0.1mmol, 0.1eq) was then added and the mixture left to stir at room temperature overnight. The solution was filtered and the crude product purified by column chromatograph using ethyl acetate/hexane mixtures. The pure product fractions were combined and the solvent evaporated under reduced pressure giving colourless oils, which crystallised under vacuum (yield 40-60%).

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)decamide, 5a** (42%) ^1H NMR (CDCl_3) δ : 6.6 (s, 1H), 5.0 (br t, 3H), 4.4 (s, 6H), 3.0 (m, 6H), 2.3 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.3 (s, 27H), 1.1-1.6 (m, 14H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 173.8, 172.3, 156.0, 79.0, 62.1, 57.8, 39.4, 36.7, 31.6, 31.0, 29.2, 29.2, 29.0, 29.0, 28.2, 25.3, 25.0, 22.4, 13.9. LRMS (ES+) m/z 853.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)undecamide, 5b** (45%): ^1H NMR (CDCl_3) δ : 6.5 (s, 1H), 4.9 (br t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.3 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.3 (s, 27H), 1.1-1.6 (m, 16H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 173.8, 172.3, 156.0, 79.1, 62.1, 57.9, 39.5, 36.8, 31.7, 31.0, 29.4, 29.4, 29.1, 29.0, 28.2, 25.4, 25.1, 22.5, 14.0. LRMS (ES+) m/z 867.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)dodecamide, 5c** (63%): ^1H NMR (CDCl_3) δ : 6.7 (s, 1H), 5.0 (br t, 3H), 4.3 (s, 6H), 3.0 (m, 6H), 2.3 (t, 6H, $J=7.5$), 2.0 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.6-1.1 (m, 45H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 173.8, 172.2, 155.9, 78.8, 62.0, 57.7, 39.4, 36.6, 36.2, 31.6, 31.1, 30.9, 29.4, 29.3, 29.1, 29.0, 28.9, 28.1, 25.3, 25.0, 22.4, 13.8. LRMS (ES+) m/z 881.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)tridecamide, 5d** (58%): ^1H NMR (CDCl_3) δ : 6.5 (s, 1H), 4.9 (br t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.3 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.3 (s, 27H), 1.2-1.6 (m, 20H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 174.0, 172.4, 156.0, 79.2, 62.2, 58.0, 39.5, 36.8, 31.8, 31.0, 29.5, 29.4, 29.2, 29.2, 29.0, 28.2, 25.4, 25.1, 22.5, 14.0. LRMS (ES+) m/z 895.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)tetradecamide, 5e** (50%): ^1H NMR (CDCl_3) δ : 6.5 (s, 1H), 4.8 (t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.3 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.8 (m, 6H), 1.4 (s, 27H), 1.2-1.6 (m, 22H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 173.9, 172.5, 156.0, 79.3, 62.3, 58.0, 39.6, 37.0, 31.9, 31.0, 29.6, 29.5, 29.3, 29.3, 29.2, 28.3, 25.5, 25.2, 22.6, 14.0. LRMS (ES+) m/z 909.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)pentadecamide, 5f** (55%): ^1H NMR (CDCl_3) δ : 6.7 (s, 1H), 5.0 (br t, 3H), 4.3 (s, 6H), 3.0 (m, 6H), 2.2 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.3 (s, 27H), 1.1-1.5 (m, 24H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 173.8, 172.3, 156.0, 78.9, 62.0, 57.7, 39.4, 36.6, 36.2, 31.6, 31.1, 30.9, 29.4, 29.3, 29.1, 29.1, 28.0, 28.1, 25.3, 25.0, 22.4, 13.9. LRMS (ES+) m/z 923.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)hexadecamide, 5g** (52%): ^1H NMR (CDCl_3) δ : 6.5 (s, 1H), 4.8 (br t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.4 (t, 6H, $J=7.1$), 2.1 (t, 2H, $J=7.5$), 1.8 (m, 6H), 1.4 (s, 27H), 1.2-1.6 (m, 26H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 174.0, 172.5, 156.0, 78.3, 62.3, 58.0, 39.7, 36.9, 31.9, 31.1, 29.7, 29.5, 29.4, 29.3, 28.4, 25.5, 25.3, 22.7, 14.1. LRMS (ES+) m/z 937.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)octadecamide, 5h** (43%): ^1H NMR (CDCl_3) δ : 6.4 (s, 1H), 4.7 (t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.3 (t, 6H, $J=7.5$), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.3 (s, 27H), 1.6-1.1 (m, 30H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 172.9, 171.5, 155.0, 78.3, 61.3, 59.3, 57.0, 38.7, 35.9, 30.1, 29.8, 29.0, 28.7, 27.4, 24.5, 24.3, 21.3, 20.0, 13.1. LRMS (ES+) m/z 965.8 [100, (M+Na) $^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)-9-octadecenamide, **5i** (49%):** ^1H NMR (CDCl_3) δ : 6.4 (s, 1H), 4.7 (br t, 3H), 4.4 (s, 6H), 3.1 (m, 6H), 2.3 (t, $J=7.5$, 6H), 2.1 (t, 2H, $J=7.5$), 1.7 (m, 6H), 1.6-1.1 (m, 57H), 0.8 (t, 3H, $J=6.6$). ^{13}C NMR (CDCl_3) δ : 172.9, 171.5, 155.0, 78.3, 61.3, 59.3, 57.0, 38.7, 35.9, 30.1, 29.8, 29.0, 28.7, 27.4, 24.5, 24.3, 21.3, 20.0, 13.1. LRMS (ES+) m/z 963.8 [100, $(\text{M}+\text{Na})^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)eicosanamide, **5j** (43%):** ^1H NMR (CDCl_3) δ : 6.5 (s, 1H, NH), 4.8 (t, 3H, NHBoc), 4.4 (s, 6H, $\text{CH}_2\text{-O}$), 3.1 (m, 6H, CH_2NHBoc), 2.3 (t, $J=7.5$, 6H, $\text{-CO}_2\text{CH}_2$), 2.1 (t, 2H, $J=7.5$, $\text{CH}_2\alpha$), 1.7 (m, 6H, CH_2), 1.4 (s, 27H, $\text{O-C-(CH}_3)_3$), 1.6-1.2 (m, 34H, $(\text{CH}_2)_{17}$), 0.8 (t, 3H, $J=6.6$, CH_3). ^{13}C NMR (CDCl_3) δ : 173.8, 172.4, 156.0, 79.2, 62.2, 58.0, 39.7, 39.6, 36.9, 31.8, 31.1, 29.6, 28.3, 25.4, 25.2, 22.6, 14.0. LRMS (ES+) m/z 1021.8 [100, $(\text{M}+\text{Na})^+$].

***N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)tetraeicosanamide, **5k** (39%):** ^1H NMR (CDCl_3) δ : 6.5 (s, 1H, NH), 4.8 (t, 3H, NHBoc), 4.4 (s, 6H, $\text{CH}_2\text{-O}$), 3.1 (m, 6H, CH_2NHBoc), 2.3 (t, $J=7.5$, 6H, $\text{-CO}_2\text{CH}_2$), 2.1 (t, 2H, $J=7.5$, $\text{CH}_2\alpha$), 1.8 (m, 6H, CH_2), 1.4 (s, 27H, $\text{O-C-(CH}_3)_3$), 1.6-1.2 (m, 42H, $(\text{CH}_2)_{21}$), 0.8 (t, 3H, $J=6.6$, CH_3). ^{13}C NMR (CDCl_3) δ : 173.8, 172.4, 156.0, 79.2, 62.3, 58.0, 39.6, 39.6, 36.9, 31.1, 29.7, 28.4, 25.3, 22.7, 14.1. LRMS (ES+) m/z 1049.8 [100, $(\text{M}+\text{Na})^+$].

***O*-(Cholest-5-en-3-yl)-*N*-Tris([4-(*N*-*tert*-butoxycarbonylamino)butanoyl]oxymethyl)carbamate, **5l** (48%):** ^1H NMR (CDCl_3) δ : 4.7 (t, 3H, NHBoc), 4.4 (s, 6H, $\text{CH}_2\text{-O}$), 3.1 (m, 6H, CH_2NHBoc), 2.3 (t, $J=7.5$, 6H, $\text{-CO}_2\text{CH}_2$), 1.8 (m, 6H, CH_2), 1.4 (s, 27H, $\text{O-C-(CH}_3)_3$), 1.9-0.6 (m, 45H). ^{13}C NMR (CDCl_3) δ : 172.5, 156.0, 139.5, 122.6, 79.2, 62.4, 57.1, 56.6, 56.2, 50.0, 42.3, 39.7, 39.0, 36.5, 36.1, 35.7, 31.1, 28.4, 25.2, 23.8, 22.8, 22.5, 20.9, 18.6, 11.8. LRMS (ES+) m/z 1111.8 [100, $(\text{M}+\text{Na})^+$].

E) Synthesis of final compounds **6a-l**

General deprotection protocol: A 1:1 (v/v) mixture of trifluoroacetic acid (99%) and DCM (10mL) was added to **5a-l** (0.1mmol) and left to stir at room temperature for 30 min. The solvent was evaporated under reduced pressure and the product re-dissolved in DCM and the solvent evaporated under reduced pressure (three times) to give final compounds **6a-l** either as sticky white solids or colourless oils (quantitative yield).

***N*-[tris-(4-aminobutirylloximethyl)methyl]decamide, trifluoroacetic salt, **6a**.** ^1H NMR (250 MHz, CD_3OD) δ : 4.44 (s, 6 H), 2.99 (t, $J=7.5$ Hz, 6 H), 2.52 (t, $J=7.5$ Hz, 6 H), 2.19 (t, $J=7.5$ Hz, 2 H), 1.95 (q, $J=7.5$ Hz, 6 H), 1.65-1.12 (m, 14 H), 0.90 (t, $J=6.0$ Hz, 3 H). ^{13}C NMR (62.5 MHz, CD_3OD)

δ : 177.0, 173.4, 63.1, 59.1, 40.0, 37.5, 33.1, 31.4, 30.7, 30.5, 30.5, 30.3, 27.0, 23.8, 23.7, 14.5. LRMS (ES+) m/z 531.5 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]undecamide, trifluoroacetic salt, 6b.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.67-1.09 (m, 16 H), 0.90 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.2, 40.0, 37.5, 33.1, 31.4, 30.7, 30.7, 30.5, 30.3, 27.0, 23.7, 23.7, 14.5. LRMS (ES+) m/z 545.6 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]dodecamide, trifluoroacetic salt, 6c.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.68-1.06 (m, 18 H), 0.90 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.2, 40.0, 37.5, 33.1, 31.4, 30.7, 30.7, 30.5, 30.3, 27.0, 23.7, 23.7, 14.5. LRMS (ES+) m/z 559.6 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]tridecamide, trifluoroacetic salt, 6d.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.66-1.00 (m, 20 H), 0.89 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.2, 40.0, 37.5, 33.1, 31.5, 30.8, 30.7, 30.5, 30.3, 27.0, 23.7, 23.7, 14.5. LRMS (ES+) m/z 545.6 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]tetradecamide, trifluoroacetic salt, 6e.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.67-1.02 (m, 22 H), 0.90 (t, J = 6.5 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.1, 40.0, 37.5, 33.1, 31.4, 30.8, 30.7, 30.5, 30.3, 27.0, 23.8, 23.7, 14.5. LRMS (ES+) m/z 587.7 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]pentadecamide, trifluoroacetic salt, 6f.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.69-1.06 (m, 24 H), 0.90 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.2, 40.0, 37.5, 33.1, 31.4, 30.9, 30.7, 30.5, 30.3, 27.0, 23.7, 23.7, 14.5. LRMS (ES+) m/z 601.7 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]hexadecamide, trifluoroacetic salt, 6g.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.70-1.03 (m, 26 H), 0.90 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz,

CD₃OD) δ : 177.0, 173.4, 63.1, 59.1, 40.0, 37.5, 33.1, 31.4, 30.8, 30.7, 30.5, 30.3, 27.0, 23.8, 23.7, 14.5.
LRMS (ES+) m/z 615.7 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]octadecamide, trifluoroacetic salt, 6h.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.67-1.01 (m, 30 H), 0.90 (t, J = 6.0 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.1, 40.0, 37.4, 33.1, 31.4, 30.8, 30.7, 30.5, 30.3, 27.0, 23.7, 23.7, 14.5.
LRMS (ES+) m/z 643.7 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]-9-octadecenamide, trifluoroacetic salt, 6i.** ¹H NMR (250 MHz, CD₃OD) δ : 5.41-5.28 (m, 2H) 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.0 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.71-0.92 (m, 26 H), 0.90 (t, J = 6.5 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 130.9, 130.8, 63.1, 59.2, 40.0, 37.4, 33.1, 31.4, 30.9, 30.6, 30.4, 30.4, 30.4, 30.3, 28.2, 27.0, 23.7, 23.7, 14.5. LRMS (ES+) m/z 641.7 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]eicosanamide, trifluoroacetic salt, 6j.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.0 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.65-0.99 (m, 34 H), 0.90 (t, J = 6.5 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.4, 63.1, 59.2, 40.0, 37.5, 33.1, 31.4, 30.8, 30.5, 30.3, 27.0, 23.8, 23.7, 14.5.
LRMS (ES+) m/z 671.8 [100, (M+H)⁺].

***N*-[tris-(4-aminobutirylloximethyl)methyl]tetraeicosanamide, trifluoroacetic salt, 6k.** ¹H NMR (250 MHz, CD₃OD) δ : 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 2.19 (t, J = 7.5 Hz, 2 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.69-1.06 (m, 42 H), 0.90 (t, J = 6.5 Hz, 3 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 177.0, 173.8, 63.5, 59.5, 40.4, 37.9, 33.5, 31.8, 31.2, 30.9, 30.7, 27.4, 24.2, 24.1, 14.9. LRMS (ES+) m/z 727.7 [100, (M+H)⁺].

***O*-(Cholest-5-en-3-yl)-*N*-[tris-(4-aminobutirylloxymethyl)methyl]carbamate, trifluoroacetic salt, 6l.** ¹H NMR (250 MHz, CD₃OD) δ : 5.47 (s, 1H), 4.44 (s, 6 H), 2.99 (t, J = 7.5 Hz, 6 H), 2.51 (t, J = 7.5 Hz, 6 H), 1.95 (q, J = 7.5 Hz, 6 H), 1.70-0.69 (m, 45 H). ¹³C NMR (62.5 MHz, CD₃OD) δ : 173.4, 157.0, 141.1, 123.6, 63.3, 58.4, 58.2, 57.6, 54.8, 51.6, 43.5, 41.1, 40.8, 40.7, 40.2, 40.1, 40.0, 40.0, 38.3, 37.8, 37.4, 37.1, 33.2, 33.1, 31.4, 29.3, 29.2, 25.3, 25.0, 23.6, 23.3, 23.0, 22.2, 19.8, 19.3, 12.4.
LRMS (ES+) m/z 789.8 [100, (M+H)⁺].

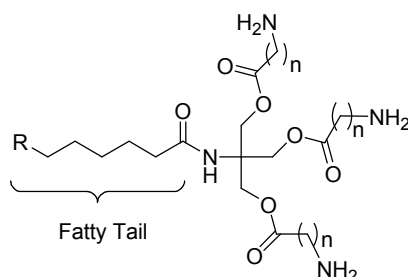
1.3. Elemental analysis data of the final compounds

Compound	Formula		C%	H%	N%
6a	$C_{32}H_{53}F_9N_4O_{13}$	Calcd	44.04	6.12	6.42
		Found	43.93	6.24	6.39
6b	$C_{33}H_{55}F_9N_4O_{13}$	Calcd	44.70	6.25	6.32
		Found	44.31	6.53	6.29
6c	$C_{34}H_{57}F_9N_4O_{13}$	Calcd	45.33	6.38	6.22
		Found	44.98	6.42	5.84
6d	$C_{35}H_{59}F_9N_4O_{13}$	Calcd	45.95	6.50	6.12
		Found	45.67	6.77	6.18
6e	$C_{36}H_{61}F_9N_4O_{13}$	Calcd	46.55	6.62	6.03
		Found	46.23	6.59	6.12
6f	$C_{37}H_{63}F_9N_4O_{13}$	Calcd	47.13	6.73	5.94
		Found	47.00	6.88	5.76
6g	$C_{38}H_{65}F_9N_4O_{13}$	Calcd	47.70	6.85	5.85
		Found	47.62	6.99	5.50
6h	$C_{40}H_{69}F_9N_4O_{13}$	Calcd	48.78	7.06	5.69
		Found	48.89	7.03	5.58
6i	$C_{40}H_{67}F_9N_4O_{13}$	Calcd	48.88	6.87	5.70
		Found	48.57	6.99	5.93
6j	$C_{42}H_{73}F_9N_4O_{13}$	Calcd	49.80	7.26	5.53
		Found	49.58	7.39	5.08
6k	$C_{46}H_{81}F_9N_4O_{13}$	Calcd	51.68	7.64	5.24
		Found	51.99	7.52	5.42
6l	$C_{59}H_{100}F_9N_4O_{13}$	Calcd	65.05	9.25	5.14
		Found	64.68	9.67	4.79

2. Preliminary Transfection Studies

To study the influence of aminoacid type in the transfection abilities of the novel compounds, a library of compounds was synthesized combining two fatty tails (stearyl and oleyl) and three aminoacids (glycine, beta-alanine and GABA) (see Table below).

Table S1: List of compounds synthesized.



Compound	Aminoacid (n)	Fatty Tail
8a	Glycine (1)	Stearyl (18:0)
8b	Glycine (1)	Oleyl (18:1)
9a	β -Alanine (2)	Stearyl (18:0)
9b	β -Alanine (2)	Oleyl (18:1)
6h	γ -Aminobutyric acid (3)	Stearyl (18:0)
6i	γ -Aminobutyric acid (3)	Oleyl (18:1)

Transfection assays were carried out with glycine- (**8a** and **8b**), beta-alanine- (**9a** and **9b**), and GABA-containing tripodal biodegradable cationic lipids (**6h** and **6i**), plus two positive controls: Lipofectamine2000 (Invitrogen/Life Technologies) and Effectene (Qiagen). Liposomes were formulated with and without DOPE to study the importance of the presence/absence of this co-lipid on the transfection abilities of the compounds. The resulting formulations were then complexed at various N/P ratios with pEGFP-C1 (enhanced green fluorescent protein (EGFP)-reporter plasmid). As our goal was to find the candidates for both *in vitro* and *in vivo* studies, experiments were performed in serum-containing media. EGFP expression was determined by measuring fluorescence emission using a BioTek microplate reader FLx800 (485/20 excitation, 530/25 emission). Results are shown in Figure S1.

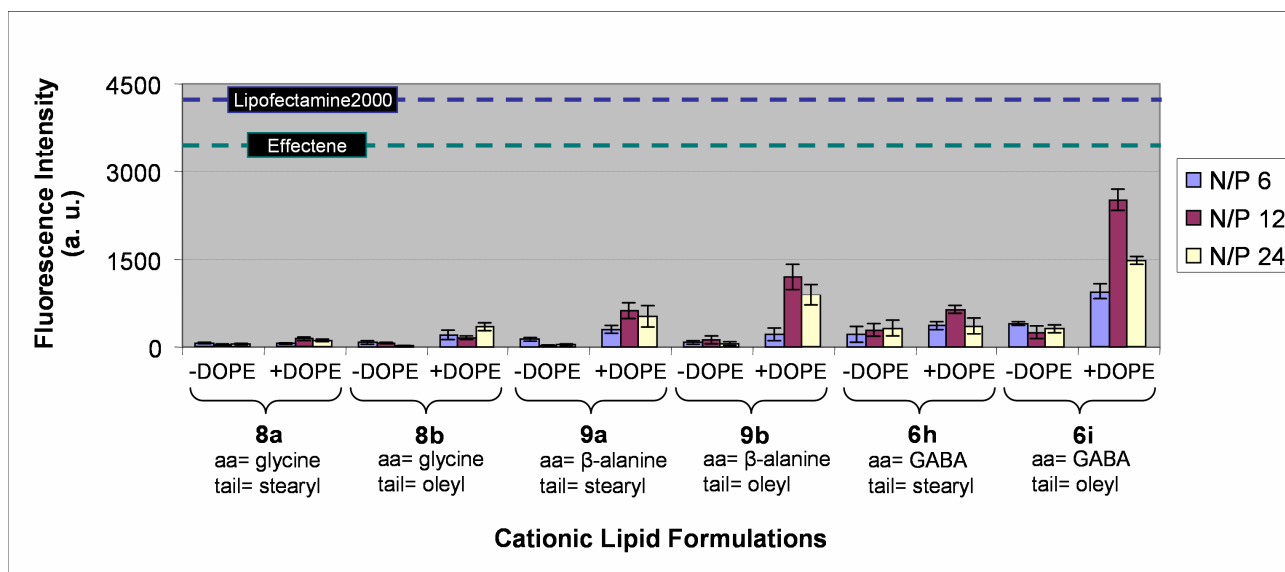


Figure S1. Fluorescence intensity obtained from the screening of HeLa cells two days after adding tripodal biodegradable cationic lipids complexed with pEGFP-C1 with/without DOPE at different charge ratios. $n=6$ wells/group. Lipofectamine2000 (purple) and Effectene (green) were used according to supplier protocol.

As observed in Figure S1, the inclusion of DOPE in the formulations produced an increment in the transfection abilities of most compounds, supporting the positive role of DOPE for enhancing the gene delivery properties of the library members and thus DOPE was used for subsequent assays.

Interestingly, transfection properties were highly dependent on the aminoacid used, with the transfection abilities following the order: GABA > beta-alanine > glycine. This might be a consequence of the higher pK_a of GABA ammonium group (pK_a (GABA) = 10.46 > pK_a (beta-alanine) = 10.19 > pK_a (glycine) = 9.78) and/or the longer space between the linker and the cationic headgroup. In accordance with these results, we used GABA for the synthesis of a library of tripodal biodegradable cationic lipids (TBCL) by modifying the lipid moiety.

These preliminary experiments also confirmed the ability of the novel compounds (in particular compound **9b** and **6i**) to transfect in the presence of serum.

3. *In vitro* assays

3.1. Transfection assay protocol

Lipoplex formulations (0.2 μ g of plasmid/experiment mixed with the corresponding amounts of compounds **6a-l** and DOPE) were added in triplicate. Media were not changed during the transfection experiments. After incubation for two days, the GFP expression was observed by microscopy (Leica) and measured using a BioTek microplate reader FLx800 (485/20 excitation, 530/25 emission). Hits were analyzed by flow cytometry: cells were washed twice with PBS, detached with trypsin/EDTA, harvested with 2% FCS in PBS, centrifuged and resuspended with 2% FCS in PBS and analyzed using a BD FACSaria flow cytometer.

Effectene[®] (Qiagen) and Lipofectamine[™] 2000 (Invitrogen) were used as positive controls and untreated cells as a negative control. Due to the high cytotoxicity levels observed with the positive controls using the protocol described above, Lipo2000 and Effectene transfection experiments were performed with the media being changed 6h after transfection as suggested by the manufacturer's protocol. It is noteworthy that the change of media was not required when using the biodegradable compounds **6a-l**. When Lipo2000 and Effectene were incubated for 12h or 24h, the transfection levels observed were higher, but the cytotoxicity increased significantly (see Table S2).

Table S2: Transfection efficiency vs cytotoxicity in HeLa cells at different incubation times.

Incubation Time	Lipofectamine 2000		Effectene	
	Transfection (%)	Cell Viability (%)	Transfection (%)	Cell Viability (%)
6 h	54 %	82 %	40 %	64 %
12 h	58 %	69 %	51 %	49 %
24 h	63 %	51 %	55 %	36 %

Ester-free tripod-like cationic lipids **7a** and **7b** (see Fig S2 [7]) were also used as positive controls. According to the optimal conditions found in our lab, compounds **7a** and **7b** were used in the presence of DOPE (molar ratio 1:2).

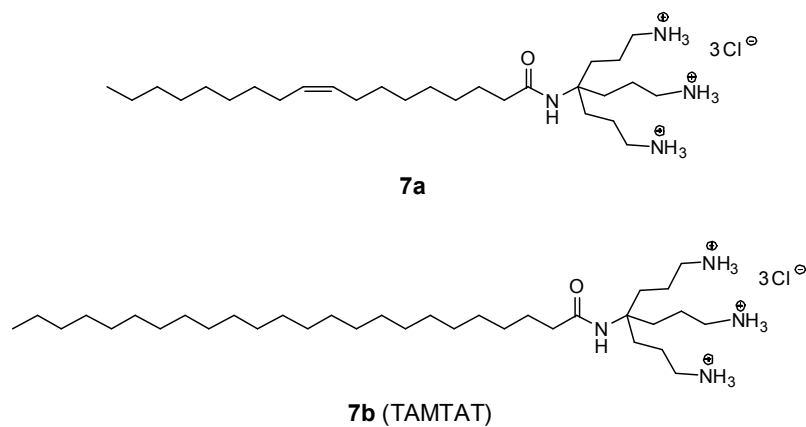


Figure S2. Chemical structure of ester-free tripod-like cationic lipids **7a** and **7b**.

3.2. Transfection Analysis of HeLa cells with compounds 6a-l

Transfection complexes were formulated following the procedure described in the section 3 and assays performed with the library members to study the influence of DOPE and N/P ratio on the transfection properties of the novel compounds to deliver pEGFP-C1.

As observed in Fig S3, none or little EGFP expression was detected with lipids **6a-c**, in accordance with their poor ability to complex DNA even at high N/P ratios. In general the lipid:DOPE ratio was critical for high transfection efficiency, with a 1:2 molar ratio being optimal for most of the library members. At this ratio, EGFP expression was very high with lipids **6d**, **6e**, **6f**, **6i** (N/P 12) and **6k** (N/P 6) (see bottom chart of Fig S3), giving greater fluorescent intensity than both Effectene and L:ipofectamine 2000, but also than the optimized formulation of ester-free compound **7b** (TAMTAT).

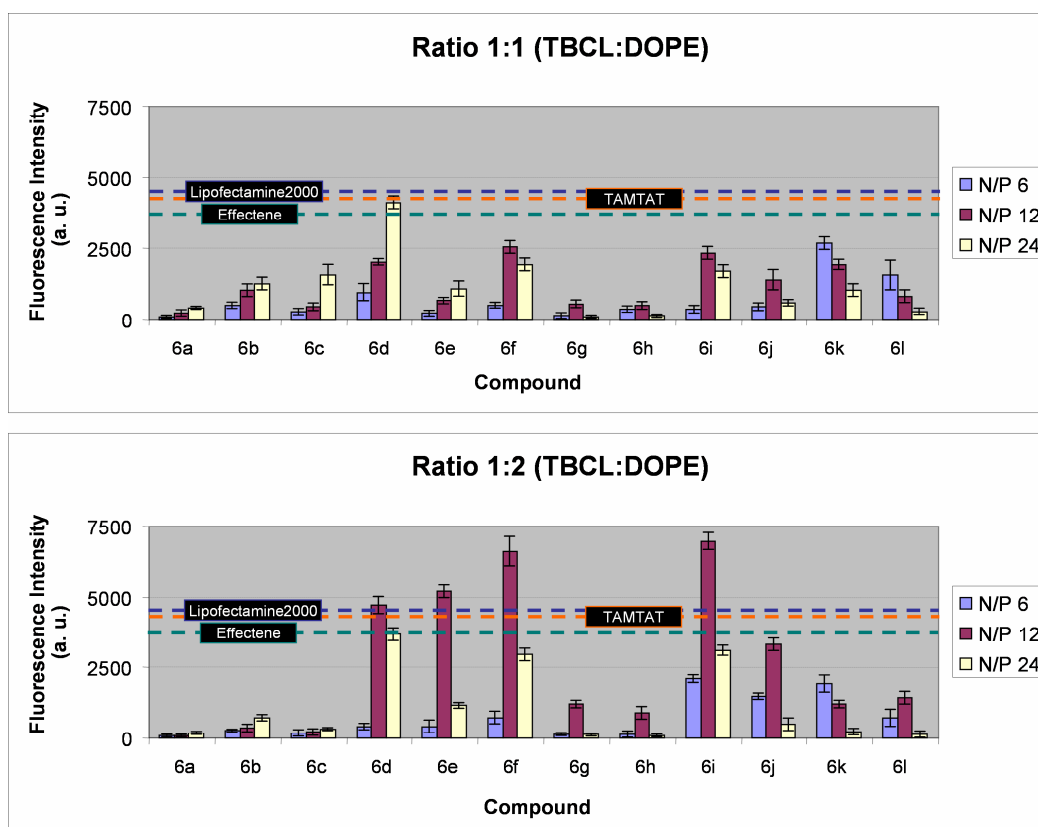


Figure S3. Fluorescence intensity obtained from the screening of HeLa cells two days after adding Tripodal Biodegradable Cationic Lipids (TBCLs) complexed with pEGFP-C1 and DOPE at different charge ratios. Top chart: liposomes formulated as TBCL:DOPE 1:1 ratio. Bottom chart: liposomes formulated as TBCL:DOPE 1:2 ratio. n=3 wells/group. Compound **7b** (TAMTAT, orange) was mixed with DOPE at 1:2 molar ratio and mixed with the plasmid at N/P 9 (formulation optimized in our lab). Lipofectamine2000 (purple) and Effectene (green) were used according to the supplier's protocol.

3.3. Cell viability assay

Toxicity measurements: Twenty-four hours after the addition of the lipoplexes, cell death was measured using an MTT cell proliferation assay. Absorbance was measured at 570nm using a spectrophotometer. In general, the absorbance readouts indicated that none of the TBCLs were toxic at the concentrations used for transfection (Figure S6).

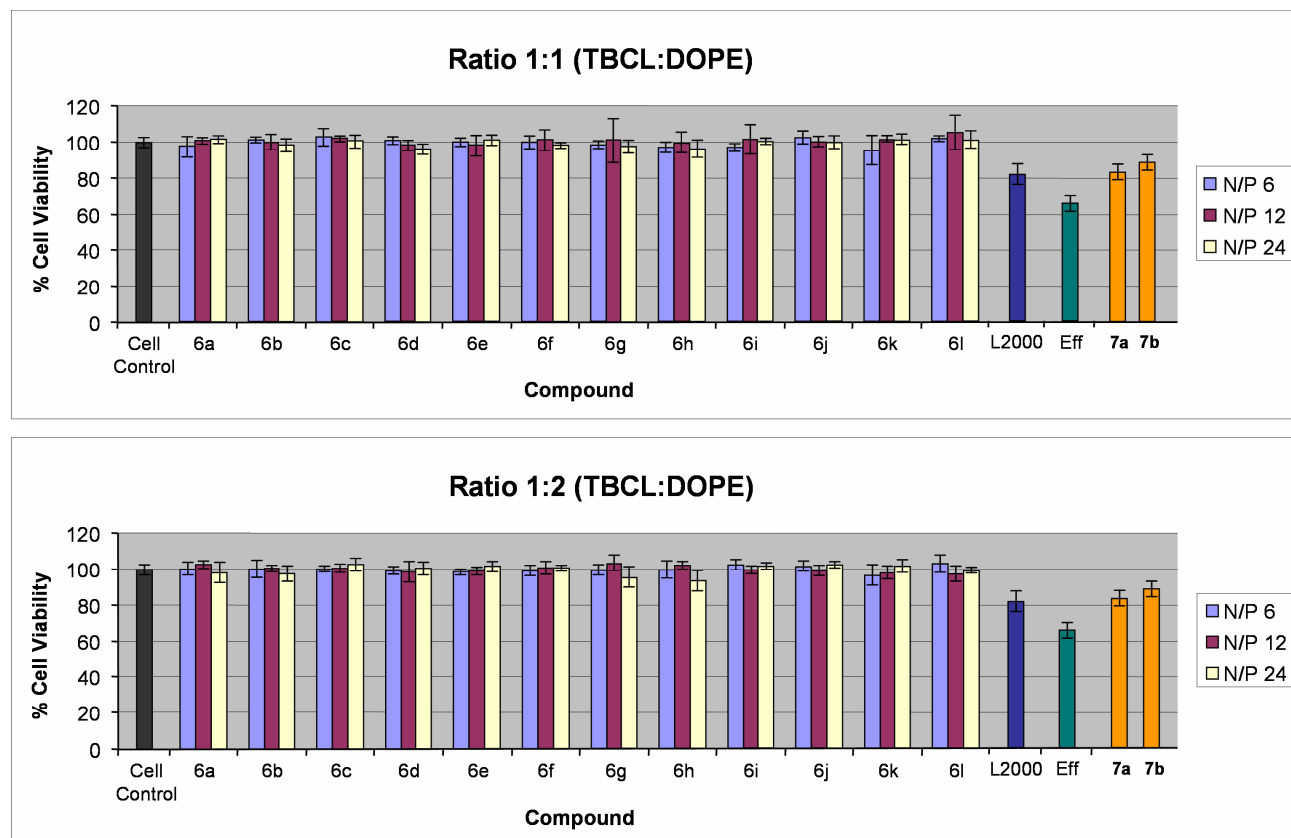


Figure S6. HeLa cell viability after transfection with TBCLs, positive controls Lipofectamine2000 and Effectene, and non-biodegradable compounds **7a** and **7b**. Top chart: liposomes formulated as TBCL:DOPE 1:1 ratio. Bottom chart: liposomes formulated as TBCL:DOPE 1:2 ratio. Data represents mean per group with standard deviation (n=3 wells/group).

3.4. Dose/response study in HeLa cells

A dose/response study (1x, 2x, 4x, 8x and 10x the standard concentration) was carried out with the optimal formulation of compound **6i** and the positive controls. Both transfection efficiency and cell viability levels were plotted in Fig. S7. In general, the absorbance readouts indicated that the **6i**:DOPE formulation became toxic at 8-10x the concentrations typically used for transfection (1x = 0.2µg/100µL of pEGFP-C1 mixed with the corresponding amounts of compound), while the positive controls showed very high toxicity levels from 2-3x the concentrations recommended by the supplier.

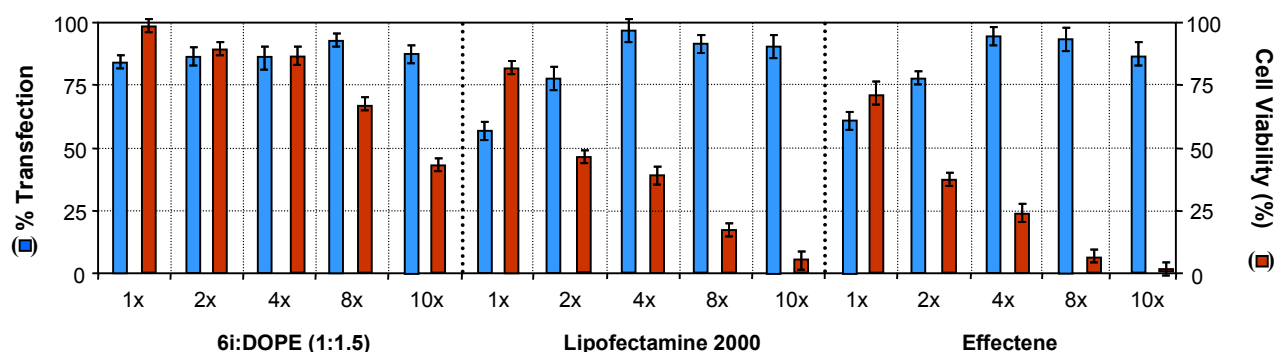


Figure S7. HeLa cell viability after pEGFP-C1 transfection with **6i** (left), positive controls Lipofectamine2000 (centre) and Effectene (right). Data represents mean per group with standard deviation (n=3 wells/group).

3.5. Transfection of E14 mouse embryonic stem (mES) cells with **6i**

Transfection of E14 (mES) cells with the optimal formulation, **6i**:DOPE 1:1.5, resulted in 18% cell population expressing EGFP after 48h. As observed by microscopy (Fig S8), the E14 cells showed no indication of cellular toxicity or altered cellular morphology after transfection.

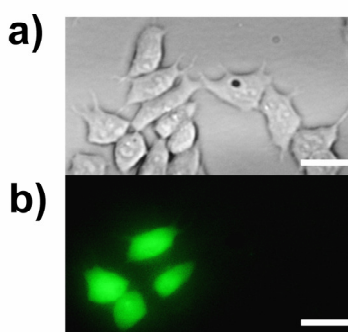


Figure S8. Brightfield (a) and fluorescent (b) images of E14 mES cells after transfection of pEGFP-C1 with compound **6i** (scale bar= 30 µm).

4. Particle size and zeta potential analysis

6i:DOPE aqueous dispersions were analyzed by dynamic light scattering and laser doppler electrophoresis using a Zetasizer ZS (Malvern Ltd).

Procedure: Lipoplexes (2 μ g of pDNA and the corresponding amount of compound **6i** and DOPE, in a total volume of 100 μ L of PBS) were prepared as described before and then diluted with 0.9 mL of either PBS or RPMI + 10% FCS. Diluted formulations were incubated at room temperature for 30 min prior to analysis. Folded capillary cells (Malvern Ltd) were loaded with each formulation (1 mL) following the procedure described by the supplier and, subsequently, both particle size distribution and zeta potential were determined for each sample using a Zetasizer ZS. All experiments were analyzed in triplicate.

Particle size distribution of serum-containing media (RPMI + 10% FCS) was also determined, giving two peaks of 8-12 and 30-50 nm. These peaks were subtracted from all lipoplex analyses performed in RPMI + 10% FCS (see Figure S9).

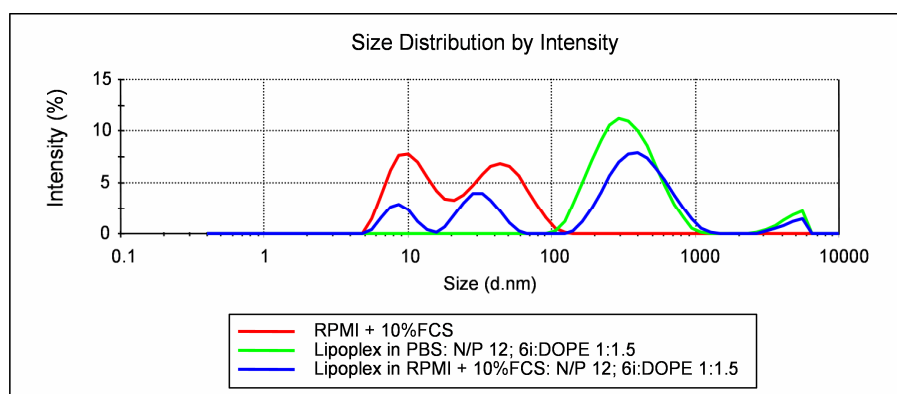


Figure S9. Representative particle size distributions of **6i**:DOPE 1:1.5 complexed with pEGFP-C1 at N/P 12 in serum-free (PBS) and serum-containing media (RPMI + 10%FCS). Control: serum-containing media without lipoplexes (red line).

The zeta potential of the **6i**:DOPE aqueous dispersions were also analyzed by laser doppler electrophoresis using the same instrument (Zetasizer ZS, Malvern Ltd).

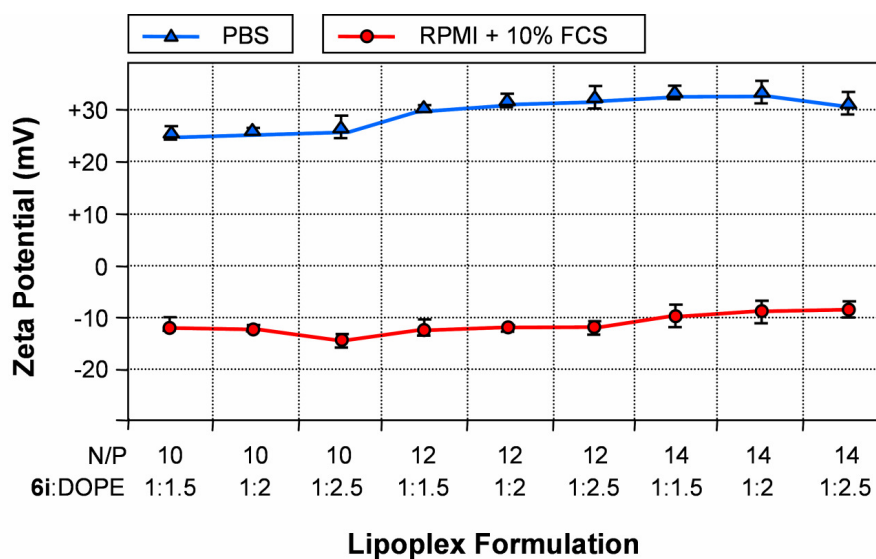


Figure S10. Zeta potential analysis of 6i:DOPE + pEGFP-C1 formulations in the presence and absence of serum.

Prior to the addition of DNA, liposome formulations gave particles with a zeta potential of 31-34 mV. Upon the addition of DNA, lipoplex potentials were slightly reduced for most formulations (25-30mV) in PBS and dramatically reduced (< -9 mV) in serum-containing media, which is the medium used for the transfection experiments (see Fig S10).