Supplementary Material (ESI) for Chemical Communications

New Helper Phospholipids for Gene Delivery

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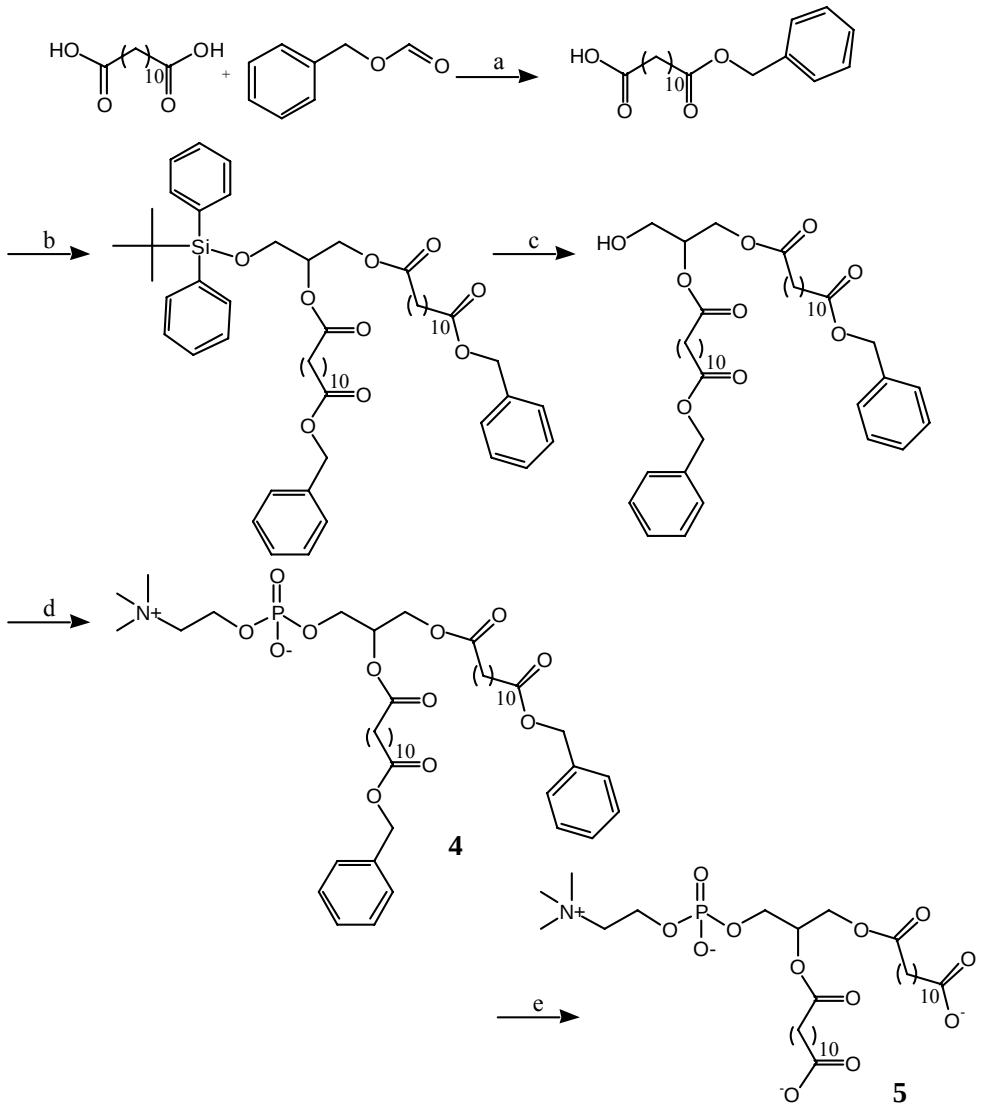
Supporting Information:

Instrumentation:

All solvents were dried and freshly distilled prior to use. All chemicals were purchased from Aldrich and used without further purification. All reactions were performed under nitrogen atmosphere. NMR spectra were recorded on a Varian INOVA spectrometer operating at 400 MHz (for ^{1}H and ^{13}C at 300 and 80 Mhz, respectively). Elemental analysis was obtained from Atlantic Microlab, Inc. A TA Instruments DSC 2920 Modulated DSC was used to collect thermal data. Calf thymus DNA and EtBr were purchased from Sigma. Fluorescence studies were carried out with a PTI emission instrument. Dynamic light scattering was performed with a 90 Plus particle size analyzer from Brookhaven Instruments. The HPLC experiments were run on a LC-MS waters apparatus.



Reagents and conditions : a) octane, Dowex 50W-X2, 80 ºC, 12 h, yield 79 %; b) sn-glycerol -3-t-butyl-diphenyl silane, DCC, DCM, rt, 18 h, yield 70 %. c) TBAF, THF, 3 h, yield 70 %. d) chloro-oxo-dioxaphospholane, TEA, THF, 0 ºC, 18 h ; trimethylamine, CH_3CN, THF, 60 ºC, 24 h, yield 70 %. e) THF, Pd/C, H_2, 90%.

Dodecanedioic acid monobenzyl ester[1]: Dodecanoic diacid (1 mmol) and Dowex 50W-X2 (50-100 mesh) (1.0 g) were stirred in benzyl formate/ octane (2:8, 10 mL) at 80 ºC. The reaction was stirred for 12 h. The solution was then filtrated and the filtrate evaporated. The crude was purified by column chromatography (hexanes/ethyl acetate 8:2) to afford the compound as a white powder (79 % yield). ^{1}H NMR ($CDCl_3$) δ ppm 1.34 (m, 10 H,

$(CH_2)_n$), 1.61 (m, 4H, CH_2), 2.32 (m, 4H, CH_2), 5.08 (s, 2H, CH_2), 7.34 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ ppm 25.16 ($\underline{C}H_2CH_2CO_2Ph$), 25.25 ($\underline{C}H_2CH_2CO_2H$), 29.40 $(CH_2)_n$, 29.57 $(CH_2)_n$, 29.71 $(CH_2)_n$, 34.40 ($\underline{C}H_2CO_2Ph$), 34.62 ($\underline{C}H_2CO_2H$), 66.55 ($CO_2\underline{C}H_2Ph$), 127.22 (Ph), 127.86 (Ph), 128.38 (Ph), 128.76 (Ph), 136.29 (Ph), 174.04 ($\underline{C}O_2Ph$), 179.73 ($\underline{C}O_2H$). MH^+ (GC-CIMS) = 321.

3-tert-butyldiphenyl silyl-sn-glycerol: Glycerol (1 mmol), tert-butyldiphenyl silane chloride (1 mmol) and imidazole (1 mmol) were dissolved in DMF. The reaction mixture was stirring for 2 days. The solution was filtered and the solvent removed under reduced pressure. The crude was purified by column chromatography (hexane/ethyl acetate 8:2) to afford the compounds as a white powder (8 g). ^1H NMR ($CDCl_3$) δ ppm 1.07 (m, 9 H, $(CH_3)_3$), 2.33 (s, 1H, OH), 3.64-3.80 (m, 5H, glycerol), 7.41-7.66 (m, 5H, Ph); ^{13}C NMR ($CDCl_3$) δ ppm 26.76 ($(CH_3)_3$), 63.74 ($\underline{C}H_2$), 65.03 ($\underline{C}H$), 71.98 ($\underline{C}H_2$), 127.75 (Ph), 129.82 (Ph), 132.82 (Ph), 135.43 (Ph). MH^+ (Fab+) = 331.2. HRMS calcd. for $[C_{19}H_{26}O_3Si+H]^+$ 331.1729, found 331.1729.

1,2-Di-dodecanedioyl benzyl ester-3-tert-butyl diphenyl silyl-rac-glycerol : DCC (2.2 mmol) was added to a solution of dodecanoic acid benzyl ester (2.2 mmol), sn-glycero-3-tert-butyl diphenyl silane (1, mmol) and DMAP (catalytic amount) in DCM (20 mL). The solution was stirred for 18 h and then the reaction solution was filtered to remove the DCU precipitate. Concentration of the filtrate followed by chromatography (hexane/ethyl acetate 9:1) afforded the product as a white powder (70 %). ^1H NMR ($CDCl_3$) δ ppm 1.07 (m, 9 H, $(CH_3)_3$), 1.26 (m, 24H, $(CH_2)_n$), 1.62 (m, 8H, CH_2), 2.27 (m, 4H, CH_2), 2.36 (m, 4H, CH_2), 3.78 (m, 2H, CH_2), 4.23 (dd, 1H, CH_2), 4.39 (dd, 1H, CH_2), 5.12 (s, 4H, CH_2Ph), 5.13 (m, 1H, CH), 7.41-7.66 (m, 15H, Ph); ^{13}C NMR ($CDCl_3$) δ ppm 25.05 ($\underline{C}H_2$), 25.13 ($\underline{C}H_2$), 26.91 ($(CH_3)_3$), 29.31 ($\underline{C}H_2$), 29.40 ($\underline{C}H_2$), 29.42 ($\underline{C}H_2$), 29.54 ($\underline{C}H_2$), 29.58 ($\underline{C}H_2$), 34.30 ($\underline{C}H_2$), 34.50 ($\underline{C}H_2$), 62.49 ($\underline{C}H_2$), 66.25($\underline{C}H$, $CO_2\underline{C}H_2Ph$), 71.67 ($\underline{C}H_2$), 127.94 (Ph), 128.36 (Ph), 128.72 (Ph), 130.02 (Ph), 133.18 (Ph), 135.71 (Ph), 135.77 (Ph), 136.30 (Ph), 173.20 ($\underline{C}O_2$), 173.59 ($\underline{C}O_2$), 173.85 ($\underline{C}O_2$). MH^+ (Fab+) = 935.0. HRMS calcd. for $[C_{57}H_{78}O_3Si+H]^+$ 935.5493, found 935.5489.

1,2-Di-dodecanedioyl benzyl ester-rac-glycerol: The compound prepared above,1,2-di-dodecanedioyl benzyl ester-3-tert-butyl diphenyl silyl-rac-glycerol (1 mmol), was dissolved in 50 mL of THF. Tetrabutylammonium fluoride trihydrate (4 mmol) was then added to the reaction and the mixture was stirred for 1 hour. After one hour the reaction was complete as indicated by TLC. The solution was diluted with 10 mL of H_2O and acidified with 1N HCl to a pH of 3. The product was extracted into DCM, dried over Na_2SO_4, and rotoevaporated to dryness. The residue was purified by chromatography (hexane/ethyl acetate 8:2) to afford the product as colorless oil (70%). ^{1}H NMR ($CDCl_3$) δ ppm 1.26 (m, 24H, $(CH_2)_n$), 1.68 (m, 8H, CH_2), 2.33 (m, 8H, CH_2), 4.06-4.19 (m, 5H, glycerol), 5.10 (s, 4H, CH_2Ph), 7.32 (m, 10H, Ph); ^{13}C NMR ($CDCl_3$) δ ppm 25.09 ($\underline{C}H_2$), 25.15 ($\underline{C}H_2$), 29.31 ($\underline{C}H_2$), 29.42 ($\underline{C}H_2$), 29.56 ($\underline{C}H_2$), 34.31 ($\underline{C}H_2$), 34.55 ($\underline{C}H_2$), 65.26 ($\underline{C}H_2$), 66.31 ($\underline{C}H$, $CO_2\underline{C}H_2Ph$), 68.51.67 ($\underline{C}H_2$), 128.40 (Ph), 128.77 (Ph), 136.33 (Ph), 173.99 ($\underline{C}O_2$), 174.16 ($\underline{C}O_2$). MH^+ (Fab+) = 697.4. HRMS calcd. for $[C_{19}H_{26}O_3Si+H]^+$ 697.4316, found 697.4297.

1,2-Di-dodecanedioyl benzyl ester-3-phosphocholine-rac-glycerol (4): A solution of 1,2-di-dodecanedioyl benzyl ester-rac-glycerol (0.97 mmol) and TEA (19 mmol) in THF was cooled to 0º C and chloro-2-oxo-1,2,3-dioxaphosphonate (1.55 mmol) was added drop wise. The reaction mixtures was stirred at room temperature for 18 h followed by the filtration of the TEA salts at 0º C. The solvent was evaporated and the residue was used in the next step without purification. Anhydrous trimethylamine was condensated at 0º C under nitrogen in a pressure tube. Next, the solution of oxo-dioxaphospholane product was added. The reaction mixture was stirred at 60º C for 24 h. Evaporation of the solvent and purification by reverse phase chromatography (acetonitrile/water) afforded the product (yield 70%). ^{1}H NMR ($CDCl_3$) δ ppm 1.25 (s, 24H), 1.60 (m, 8H), 2.31 (m, 8H), 3.33 (s, 9H), 3.79 (m, 2H),), 3.96 (m, 2H), 4.13 (m, 1H), 4.36 (m, 2H), 5.10 (s, 4H), 5.20 (m, 1H), 7.26 – 7.37 (m, 10H); ^{13}C NMR ($CDCl_3$) δ ppm 25.03, 28.80, 29.24, 29.28, 29.38, 29.42, 29.57, 34.20, 34.41, 54.52, 59.57, 63.08, 63.74, 66.19, 66.24, 70.59, 128.38, 128.77, 136.36, 173.53, 173.94; MH^+ (Fab+) = 862.7. HRMS calcd. for $[C_{46}H_{72}NO_{12}P+ H]^+$ 862.4871, found 862.4882.

1,2-Di-dodecanedioic acid-3-phosphocholine-rac-glycerol (5): To a solution of 1,2-Di-dodecanedioyl benzyl ester-3-phosphocholine-rac-glycerol in methanol was added a catalytic amount of Pd/C. The solution was then placed in a Parr tube on a hydrogenator and shaken under 60 psi H_2 for 12 hours. The solution was then filtered over wet celite, concentrated, and placed on a high vacuum line to yield the product (90%). ^{1}H NMR (CD_3OD) δ ppm 1.25 (s, 24H), 1.60 (m, 8H), 2.31 (m, 8H), 3.33 (s, 9H), 3.79 (m, 2H),), 3.96 (m, 2H), 4.13 (m, 1H), 4.36 (m, 2H); ^{13}C NMR (CD_3OD) δ ppm, 28.80, 29.24, 29.28, 29.38, 29.42, 29.57, 34.20, 34.41, 54.52, 59.57, 63.08, 63.74, 70.59, 178.83, 179.04; MH^+ (Fab+) = 682.5. HRMS calcd. for $[C_{46}H_{72}NO_{12}P+ H]^+$ 682.3932, found 682.3936.

Liposome preparation

A chloroformic solution 4 or 4/DOTAP or DPPC or DOPE or DOTAP/DOPE was introduced into a pear shaped flask and the solution was evaporated under vacuum leaving a thin film deposited onto the flask wall. A total of 1 mL Tris buffer (100mM Tris, 100 mM NaCl, pH 7.4) was then added and the film was peeled off by vortexing. The concentration of each lipid was 1.15 mM. The milky aqueous suspension was extruded through a polycarbonate membrane (100 nm) using a Aventi polar lipids mini-extruder until a homogeneous liposomes solution was obtained (typically 20 extrusions). The lipoplexes were prepared by addition of the DNA to the liposomes solution previously prepared, in a ratio lipid/DNA 2:1. The diameter of the liposomes and lipid/DNA assemblies was measured at a 90° angle using 90 Plus particle size analyzer (Brookhaven Instruments Model 90 Plus). All solutions used in the study were filtered through a 0.02 μm membrane prior to use. The results are resumed in the table.

Compound	Diameter (nm)	Polydispersity
4	106	0.2
4/DOTAP	127	0.5
4/DOTAP+DNA	884.4	0.2
DOPC	117.5	0.5

DOPC + DOTAP	139.5	1.8
DOPC + DOTAP + DNA	1060.3	0.2

Modulated differential scanning calorimetry.

The thermal properties of the lipid were measured on a DSC (TA Q100). 0.5 mg of lipid in 5 µL of water was hermetically sealed in an aluminum pan. The modulation was set to ±1.00 ºC every 40 s, and the pan was equilibrated at –15 ºC. The temperature was increased at 0.5 ºC/min to 70 ºC where it has held for 2 min. The temperature was then reduced to –10 ºC and held at this temperature for 2 min. This heating-cooling cycle was repeated two more times before the sample was held isothermal at –10 ºC for 20 min. The data colleted on the third cycle were analyzed.

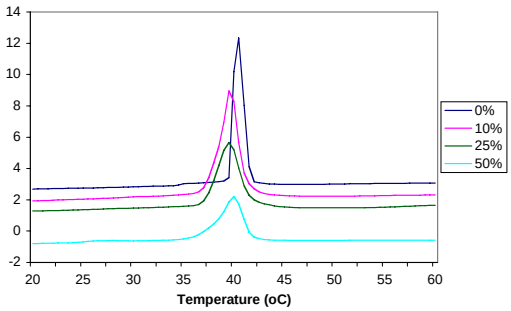


Figure S2. Reduction of the Tm peak with increasing added amounts of **5**.

In order to determine if **5** destabilize a membrane bilayer a few studies were performed. The DPPC undergoes a melting transition at Tm = 41 ^{o}C.(Aventi polar lipids technical report) According to our calorimetric data, DPPC exhibited a melting transition at 40.58 ^{o}C. When DPPC was mixed with different amounts of **5**, the calorimetric data showed a decrease of the transition and the peak became broader indicating a disruption in the bilayer organization (Figure S2).

DNA binding affinities [3]

A competitive displacement fluorometric assay using ethidium bromide was used for the DNA binding studies. This assay involves the addition of aliquots of the compound to a 3 mL solution of EthBr (1.3 µM) and calfus thymus DNA (3 µM) in buffer (100 mM NaCl, 100 mM Tris, pH 7.4) with the decrease of fluorescence (λ_{exc}=546 nm, λ_{em}=600 nm; 1 cm path length glass cuvette, slit width 3 nm) recorded after 5 minutes of equilibrium time following each addition. Figure S3 shows the fluorescence intensity as a function of

vector/DNA charge ratio. The fluorescence intensity decreases upon addition of DOTAP, **4**/DOTAP, DOTAP/DOPE, and DOPC/DOTAP. The results obtained with DOTAP, DOTAP/DOPE, and DOPC/DOTAP are consistent with previous reports. A 1:1 assembly is formed between the lipids and DNA. The zwitterionic lipids **4,** DOPC, and DOPE as well as the anionic lipid, **5** do not displace EtBr consistent with the unfavorable electrostatic interactions with the anionic DNA.

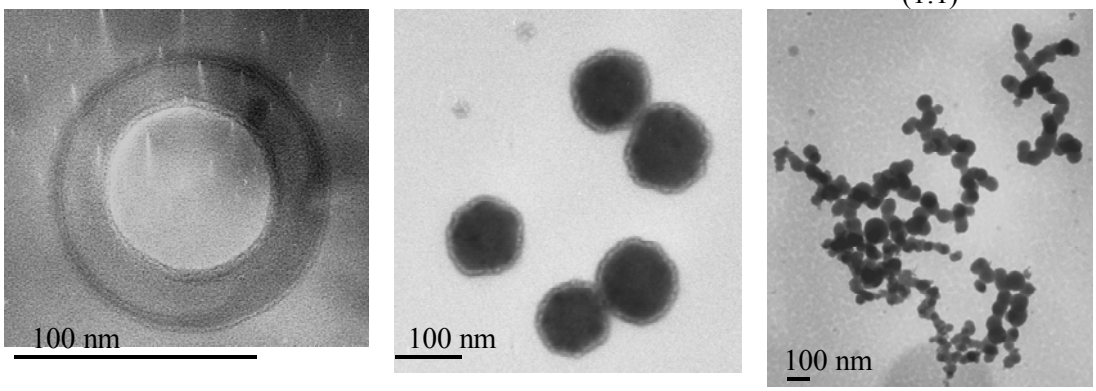
X-ray diffraction

For **4**, X-ray diffraction was performed on both fully hydrated liposomes and oriented multilayers by methods detailed in previous publications (references 4 and 5). In the case of the fully hydrated preparations, the liposomes were pelleted by centrifugation, the pellet was then transferred to a sealed quartz-glass X-ray capillary and analyzed with a point collimated X-ray beam. For the oriented multilayers, liposomes were partially dried onto a curved glass surface under a controlled relative humidity atmosphere. These oriented multilayers were transferred to a 98% humidity chamber and X-rayed with a line-focussed beam oriented at a grazing angle to the glass surface. Typically, for both unoriented and oriented preparations, the specimen to film distances were 10 cm and the exposure times were 2-6 hours. In the case of **4**, the fully hydrated liposomes gave a lamellar repeat period of 6.2 nm and the partially hydrated multilayers gave nearly the same lamellar repeat period (6.1 nm), indicating that the liposomal bilayers imbibed relatively little water (Ref 4). At 98% relative humidity the oriented multilayers of **4**/DOTAP/DNA gave a d spacing of 7.6 nm. A stationary anode Jerrel-Ash generator (Jerrel-Ash Div., Fisher Scientific Co., Waltham, MA) was used to produce Cu Kα X-radiation.[4] Diffraction patterns were obtained using a flat plate film cassette loaded with Kodak DEF X-ray film. The specimen to film distance was 10 cm with exposure times of 2-6 hours. The low angle reflections were determined in accordance with Bragg's law $2d \sin\theta = h\lambda$, where λ is the wavelength (1.54Å), d is the repeat period, h is the number of the diffraction order, and θ is the Bragg angle.

TEM

TEM microscopy experiments were performed on a JEOL 200 CX (Cu/Pd carbon coated grids).

4 DOTAP+4 (1:1) DOTAP+4 (1:1)+ DNA (1:1)



Zeta potential measurements

A zeta potential analyzer (Nano ZS, Malvern Instrument) was used to measure the zeta potential of the different solutions. The liposomes solutions were prepared as described previously. Each sample was diluted with distilled water until the appropriate concentration of particles was achieved. Each sample was measured 3 times to calculate the average zeta potential.

Compounds	Zeta potential (mV)			
	Without DNA	DNA (1:20)	DNA (1:5)	DNA (1:1)
DOTAP	60.1 ± 0.4	63.5 ± 0.7	61.4 ± 2.2	48.8 ± 0.9
DOTAP + DOPE (1:1)	63.6 ± 1.6	60.4 ± 1.1	61.5 ± 0.6	57.3 ± 0.9
DOTAP + 4 (1:1)	62.6 ± 2.2	61.5 ± 0.1	62.1 ± 0.6	54.7 ± 0.1
DOTAP + 4 (1:2)	68.9 ± 0.2	72.2 ± 1.1	70.1 ± 4.1	62.1 ± 2.0
DOTAP + 4 (1:5)	82.1 ± 1.4	78.7 ± 3.3	74.1 ± 1.8	65.9 ± 1.0
4	-22.0 ± 0.4			

DOPE	-29.4 ± 0.6			

The Zeta potential increases with the addition of the zwiterionic lipid.

Cell culture and Transfection experiment

Chinese hamster ovarian cells (CHO, ATCC, Manassas, VA) were cultured in complete F12K media (ATCC) containing 10% fetal calf serum (Sigma) and 1% penicillin and streptomycin (500 IU/mL and 5000 μg/ml, respectively, Mediatech, Herndon, VA) at 37 ^{o}C in 5% CO_2 with humidity. When the CHO cells reached about 90% confluency, they were split into 48-well plates with a 1:4 ratio using a standard trypsin-based technique. Transfections were performed 24 hours later by modification of previously published methods.[2] Briefly, plasmid DNA coding for a reporter gene, β-galactosidase (β-gal, pVax-LacZ1, Invitrogen) was first mixed with lipids in potassium phosphate buffer (PBS) at room temperature. Depending on the experimental design, the ratio of DNA and amphiphile, the pH of the buffer used, and incubation time was varied. The mixture was incubated for 15 minutes at room temperature before adding to the cells. The amount of DNA used was the same as used in naked DNA control and positive control (commercially available transfection reagents). After incubation at 37 °C and 5% CO_2 for 2 hours, medium containing the mixtures was gently removed and fresh growth medium was added. Transfection efficiencies were assessed at 48 h post transfection depending on the experimental design. Negative controls were constructed with 1.0 mL of serum-free F12 K medium and naked DNA controls were using 1.0 mL of serum-free F12 K medium with 10.0 μL (1 μg) of reporter gene. All experiments were performed in triplicate.

Reporter gene transfection efficiency assay

Reporter gene (β-gal) assay was performed with a β-galactosidase enzyme assay system (Promega, Madison, WI) following the manufacturer protocol. Briefly cells were first lysed using M-PER buffer (Pierce, Rockford, Illinois) and enzyme activities were determined. A standard curve was constructed for each experiment using dilutions of purified β-gal protein. The β-gal activities from experimental samples were determined by

comparison to the standard curve (enzyme activity vs. enzyme concentration). Efficiency of each transfection was calculated as β-gal activity normalized to total protein.

Cytotoxity

Cytotoxity was assessed using both a formazan-based proliferation assay (CellTiter 96 AQueous One Solution Cell Proliferation Assay kit, Promega) and a total protein-based assay (Pierce). Briefly CHO cells were seeded onto a 96-well microliter plate with an appropriate density of 1 x 10^4 cells per well. After 48h the MTS substrate was added to each well and the plate was incubated for 4 h at 37 ^oC in a humidified, 5% CO_2 incubator. The amount of soluble formazan produced by cellular reduction of the substrates MTS was recorded at 490 nm using a multi-well plate reader. For the total protein-based proliferation assay, cells were lysed at the same time when transfection efficiency was assayed. A 5 µL of lysates were transferred to a separate multi-well plate. Total protein contents were assessed using the Coomassie Blue protein kit (Pierce, Rockford, IL) following the manufacturer protocol. Negative and positive controls were non-treated cells and commercial lipids treated cells, respectively. The proliferation results were expressed as percentages of non-treated cells. All experiments were performed in triplicate.

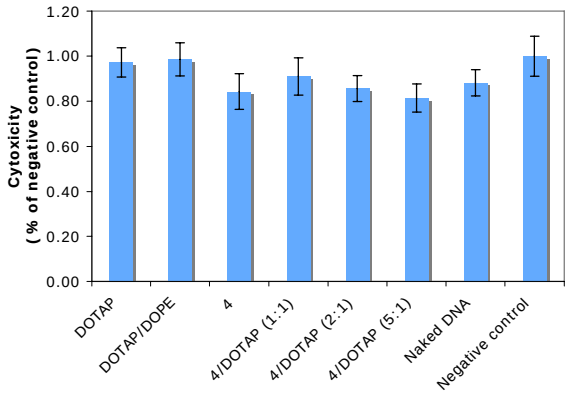


Figure S3. Results of the cytotoxicity study.

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