SUPPORTING INFORMATION

Cationic Nucleoside Lipids possessing a 3-Nitropyrrole Universal Base for siRNA Delivery

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Experimental Section

Material. Unless noted otherwise, all starting materiel were purchased from Sigma-Aldrich, Acros or Alfa Aesar and used as received. Some solvents were distilled under argon over appropriate drying agent: tetrahydrofuran and diethylether over sodium/benzophenone, dichloromethane over $CaCl_2$. Unless otherwise stated, the other solvents were used as commercially available. All compounds were characterized by ^{1}H NMR, ^{13}C NMR and mass spectroscopy. 250 MHz and 62.5 MHz 13C Nuclear Magnetic Resonance (NMR) spectra were recorded either on a Bruker AC 250. The spectra were taken at 20 °C and referenced against residual proton-solvent signals (^{1}H: $CDCl_3$: 7.26 ppm, and ^{13}C: 77 ppm). The ^{1}H NMR coupling constants (J) are reported in Hz. ESI-MS and elemental analyses were performed at the spectropole of the *Faculte des Sciences et Techniques de Saint Jerôme*, Marseille, France. Electrospray Ionisation Mass Spectra (ESI-MS) were recorded with an API///Plus sciex triple quadripole spectrophotometer in the positive mode. Elemental analyses were recorded with a Thermo Finnigan EA 1112 elemental analysis apparatus driven by the Eager 300 software. TEM

microscopy experiments were performed on a Philipps CM10 (negative staining with ammonium molybdate 1% in water, Cu/Pd carbon coated grids). Analytical thin layer chromatography (tlc) was performed on pre-coated glass plates with kieselgel 60F254 neutral with aluminium support plate (0.25mm layer thickness), SDS. The developed plates were air-dried and exposed to UV light and (or) sprayed with a solution of cerium sulphate (1%) and molybdic acid (1.5%) in 10% aqueous sulphuric acid and heated at 150°C. Preparative thin layer chromatography was performed on glass plates with Merck silica gel 60F-254 as the adsorbent (layer thickness 2mm or 1mm). Column chromatography was performed on Merck silica gel 60 (0.063-0.200mm). Melting point (mp) were measured with an Electrothermal 9100.

2,3-O-isopropylidene-D-ribose, 1 To a stirring solution of D-ribose (10g, 0.06 mol) in dry acetone (200 mL) was added a catalytic amount of H_2SO_4 at room temperature. Then $CuSO_4$ was added. After being stirred for 40h at 37 °C, the solution was filtered and washed with small quantities of acetone. The filtrate was neutralized with $Ca(OH)_2$ and stirred for 15 minutes. The mixture was filtered and the filtrate was evaporated under reduced pressure (T<40 °C) to give **1** (11.76g, 94%) as a yellow syrup.^{1}H NMR ($CDCl_3$): δ 5.39 (1H,m); 4.82 (1H, d, J=6.00Hz); 4.57 (1H, d, J=6.00Hz); 4.40 (1H,m); 4.22 (2H, m); 2.17 (2H,s); 1.48 (3H,s); 1.31 (3H,s). ^{13}C NMR (CDCl3): δ 102.31, 95.56, 82.36, 73.12, 72.01, 63.45, 29.03. FAB, MS m/z : 191(M+1)

5-O-*tert*-butyldimethylsilyl-2,3-O-isopropylidene-D-ribofuranose, 2

2,3-O-isopropylidene-D-ribose (6.27 g; 0.03 mol) was dissolved in dry dichloromethane (10ml) containing triethylamine (5.11 mL; 36.72 mmol) and 4-(dimethylamino)pyridine (DMAP) (450 mg; 3.69 mmol). The solution was cooled to 0°C and *tert*-butyldimethylsilylchloride (TBDMSCl) (5.40 g; 35.76 mmol) dissolved in dry dichloromethane (20 mL) was added

dropwise. The mixture was stirred at ambient temperature overnight. The mixture was then concentrated under reduced pressure, and the residue was triturated with THF. The organic phase was filtered andevaporated before the crude product was purified by flash column chromatography (90/10: pentane/Et_2O) to give **2** (3.46 g, 35%) as a yellow oil.

^1H NMR ($CDCl_3$): δ 5.33 (1H,d , J=11.37Hz); 4.70 (1H, d, J=6.00Hz); 4.57 (1H, d, J=6.00Hz); 4.36 (1H,m); 3.76 (2H, d, J=2.50Hz); 1.58 (1H,sl); 1.48 (3H,s); 1.32 (3H,s); 0.92(9H,s); 0.09 (3H,s) 0.06 (3H,s)^{13}C NMR ($CDCl_3$): δ 111.03, 102.44, 100.01, 88.64, 85.98, 80.73, 65.79, 27.36, 26.01, 24.93, 19.11, -6.01, -6.03. FAB, MS m/z : 305 (M+1)

1'-(5'-O-*tert* -butyldimethylsilyl-2',3'-O-isopropylidene-D-ribofuranosyl)-3-nitropyrrole, 3

First step : Carbon tetrachloride (433 µL, 4.49 mmol) was added to a stirring solution of **2** (1.22 g; 2.87 mmol) in anhydrous THF (10 mL). The solution was cooled to -70°C and hexamethylphosphorous triamide (700 µL, 3.82 mmol) was added dropwise for 5 minutes, and the mixture was allowed to stir at the same temperature for 5 minutes. The resulting compound was allowed to warm to room temperature and further stirred for 2 hours. The solvent was evaporated and the residue dissolved in diethyl ether. The resulting insoluble materiel was removed by decantation and the organic layer was evaporated to give the crude compound **3** as an orange oil.

Second step: To a cooled solution (0 °C) of 3-nitropyrrole (629 mg, 5.61 mmol) dissolved in anhydrous acetonitrile (9 mL) was added NaH (60%, 223 mg, 5.6 1mmol); the solution was stirred at room temperature for 30 minutes. To the resulting bright yellow solution, the chloro sugar obtained from the first step was dissolved in anhydrous acetonitrile and transferred via canula. The reaction mixture was then warmed to room temperature and stirred for 40 hours. The mixture was poured into H_2O (50 mL) and extracted with CH_2Cl_2 (3*50 mL). The organic layers

were combined and successively washed with saturated aqueous NH_4Cl and saturated aqueous NaCl. The organic layer was dried over $MgSO_4$ and concentrated in vacuo. The residue was purified by flash column chromatography (70/30: cyclohexane/EtOAc) to give **3** (579mg, 40%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.71 (1H, m); 6.72 (2H, m); 5.93 (1H, d, J=4.26Hz); 4.87 (1H, dd, J=0.63Hz, J=6.00Hz); 4.42 (1H, dd, J=2.21Hz, J=2.00Hz);); 3.89 (1H, dd, J=2.21Hz, J=11.21Hz); 3.76 (1H, dd, J=2.00Hz, J=11.05Hz); 1.42 (3H,s); 1.32 (3H,s); 0.93 (9H, s); 0.10 (6H,s). ^{13}C NMR ($CDCl_3$): δ 137.40, 119.24, 118.76, 114.14, 105.88, 95.22, 86.21, 86.14, 81.04, 63.44, 27.20, 25.77, 25.19, 18.26, -5.61, -5.71. FAB, MS m/z : 398 (M+1)

1'-(2',3'-O-isopropylidene-D-ribofuranosyl)-3-nitropyrrole, 4a and 4b

To a solution of **3** (596 mg, 1.49 mmol) in dry THF (5 mL) was added TBAF (1M in THF) (3 mL, 2.98 mmol) at room temperature and the mixture was stirred for 30 minutes. The solution was evaporated under reduced pressure, diluted with CH_2Cl_2 and washed with brine. The organic layer was dried on $MgSO_4$ and evaporated under reduced pressure. The crude product was purified by flash column chromatography (cyclohexane/EtOAc : 70/30 to 50/50) to give the anomer α **4a** (227 mg, 53%) as a white solid and the anomer β **4b** (75 mg, 18%) as a yellow oil.

1'-(2',3'-O-isopropylidene-α-D-ribofuranosyl)-3-nitropyrrole, 4a

mp=107°C. ^{1}H NMR ($CDCl_3$): δ 7.73 (1H, t, J=2.05Hz); 6.73 (2H, d, J=2.05Hz); 5.94 (1H, d, J=4.42Hz); 4.92 (1H, dd, J=1.11Hz, J=6.00Hz); 4.85 (1H, dd, J=4.42Hz, J=6.16Hz);); 4.46 (1H, t, J=2.84Hz); 3.94 (1H, td, J=2.84Hz, J=11.34Hz); 3.81 (1H, td, J=3.16Hz, J=11.21Hz); 1.40 (3H,s); 1.32 (3H,s)

^{13}C NMR ($CDCl_3$): δ 140.23, 121.12, 120.20, 113.80, 104.99, 90.73, 83.30, 81.94, 80.60, 64.30, 22.51, 24.20. FAB-MS m/z : 285 (M+1), 302 (M+16), 307 (M+23), 323 (M+29)

1'-(2',3'-O-isopropylidene-β-D-ribofuranosyl)-3-nitropyrrole, 4b

^{1}H NMR ($CDCl_3$): δ 7.80 (1H, dd, J=2.21Hz, J=4.11Hz); 6.80 (1H, dd, J=2.37Hz, J=3.32Hz); 6.74 (1H, dd, J=1.90Hz, J=3.32Hz); 5.62 (1H, d, J=3.32Hz); 4.89 (1H, dd, J=2.84Hz, J=6.32Hz);); 4.76 (1H, dd, J=3.32Hz, J=6.48Hz); 4.35 (1H, q, J=3.16Hz); 3.96 (1H, dd, J=3.47Hz, J=11.85Hz); 1.59 (3H,s); 1.36 (3H,s). ^{13}C NMR ($CDCl_3$): δ 141.68, 119.69, 119.33, 114.73, 106.22, 94.79, 85.90, 82.27, 80.74, 62.56, 27.19, 25.22. FAB-MS m/z : 285 (M+1), 302 (M+16), 307 (M+23), 323 (M+29)

1'-(2',3'-O-isopropylidene-5-O-tosyl-α-D-ribofuranosyl)-3-nitropyrrole, 5a

Compound **4a** (100mg, 0.35mmol) was dissolved in dry dichloromethane (4 mL) and the solution was cooled to 0°C. NEt_3 (196 μL, 1.41 mmol) was added, as well as tosyl chloride (200 mg, 1.05 mmol), in small portions. The solution was then allowed to warm to room temperature and stirred overnight. Dichloromethane was added and the solution was successively washed with brine and aqueous $NaHCO_3$ 5% w/v. The organic layer was dried on $MgSO_4$, filtered and concentrated under reduced pressure. The residue was purified by preparative layer chromatography (cyclohexane/EtOAc:60/40) to give **5a** (145 mg, 94%) as a white solid. mp = 111-113°C. ^{1} H NMR ($CDCl_3$): δ 7.78 (2H, d, J=8.22Hz); 7.64 (1H,m); 7.37 (2H, d, J=8.10Hz); 6.69 (2H, m); xx (1H, d, J=3.63Hz); 4.82 (2H,m); 4.51 (1H, t, J=2.35Hz); 4.19 (2H, d, J=2.60Hz); 2.44 (3H,s); 1.37 (3H,s); 1.27 (3H,s). ^{13}C NMR ($CDCl_3$): δ 145.82, 137.01, 131.83, 130.26, 127.86, 120.93, 120.11, 114.29, 105.55, 90.72, 81.55, 80.53, 80.20, 71.06, 25.47, 24.17, 21.69. FAB-MS m/z : 439 (M+1)

1'-(2',3'-O-isopropylidene-5-O-tosyl-β-D-ribofuranosyl)-3-nitropyrrole, 5b

Compound **5b** was obtained in 56% yield from compound **4b** by the procedure described above. Purification by preparative layer chromatography (cyclohexane/ EtOAc : 70/30) gave a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.75 (2H, d, J=8.37Hz); 7.60 (1H,t, J=2.05Hz); 7.34 (2H, d, J=8.50Hz); 6.72(2H, t, J=1.9Hz); 5.56(1H, d, J=3.32Hz); 4.84 (1H,dd, J=3.32Hz, J=6.30Hz); 4.45 (1H, q, J=2.84Hz); 4.28 (1H, dd, J=3.00Hz, J=11.06Hz); 4.13 (1H, dd, J=3.00Hz, J=11.06Hz); 2.44 (3H,s); 1.57 (3H,s); 1.35 (3H,s). ^{13}C NMR ($CDCl_3$): δ 145.69, 137.81, 131.10, 127.88, 119.31, 118.93, 115.08, 106.35, 94.71, 85.13, 82.78, 80.55, 68.56, 27.06, 25.13, 26.67. FAB-MS m/z : 439 (M+1)

1'-(2',3'-dioleyl-5-O-tosyl-α-D-ribofuranosyl)-3-nitropyrrole, 6a

At 0°C, compound **5a** (145 mg, 0.33 mmol) was dissolved in 3.5 mL of an aqueous trifluoroacetic acid / H_2O (6/1) solution and stirred for 1 hour at 0°C. The solvents were co-evaporated with methanol under reduced pressure, and the residue was dried in vacuo. The crude product was dissolved in dry CH_2Cl_2 (7 mL) and the solution was cooled to 0°C. Dicyclohexylcarbodiimide (271 mg, 1.32 mmol), (dimethylamino)pyridine (161 mg, 1.32 mmol) and oleic acid (372 mg, 1.32 mmol) were added, and the mixture was stirred at room temperature overnight. DCU was then filtered, and the filtrate was successively washed with aqueous 2N HCl and aqueous saturated $NaHCO_3$ solutions. The organic layer was dried on $MgSO_4$, filtered and evaporated under reduced pressure. The crude product purified by flash column chromatography (cyclohexane/EtOAc : 95/5) gave **6a** (236mg, 77%) as a yellow oil. ^{1}H NMR ($CDCl_3$): δ 7.80 (1H, d, J=8.22Hz); 7.66 (1H,t, J=2.05Hz); 7.37 (2H, d, J=7.90Hz); 6.67 (1H, dd, J=1.90Hz, J=3.32Hz); 6.59(1H, dd, J=2.53Hz, J=3.32Hz); 5.88 (1H, d, J=5.53Hz); 5.44(2H,m); 5.33 (4H,m); 4.54 (1H, q, J=2.84Hz); 4.31 (1H, dd, J=3.00Hz, J=11.37 Hz), 4.18 (1H, dd, J=2.68Hz , J= 11.21Hz); 2.45 (3H,s);); 2.33 (2H, t, J=3.00Hz); 2.31 (4H, t, J=3.00Hz); 1.99 (8H,m); 1.25

(44H,m); 0.86 (6H, t, J=6.32Hz). ^{13}C NMR ($CDCl_3$): δ 172.15, 145.59, 137.44, 132.21, 130.10, 130.05, 130.82, 130.01, 129.63, 129.60, 120.82, 105.20, 88.21, 75.86, 73.26, 70.90, 70.54, 60.34, 31.88, 29.74, 29.67, 29.51, 29.10, 29.02, 27.20, 27.14, 22.67, 14.19. FAB-MS m/z : 927 (M+1), 944 (M+18), 949 (M+23), 965 (M+29)

1'-(2',3'-dioleyl-5-O-tosyl-β-D-ribofuranosyl)-3-nitropyrrole, 6b

Compound **6b** was obtained from **5b** with the same procedure described above. Purification by flash column chromatography (cyclohexane/EtOAc : 95/5) gave **6b** as a yellow oil in 87% yield. ^{1}H NMR ($CDCl_3$): δ 7.84 (2H, d, J=8.21Hz); 7.62 (1H,t, J=2.05Hz); 7.37 (2H, d, J=8.53Hz); 6.77 (2H,m); 5.61 (1H, d, J=5.05Hz); 5.34 (6H,m); 5.34 (6H,m); 5.23 (1H,m); 4.34 (2H, m); 2.45 (3H,s), 2.33 (4H,m); 2.00 (8H,m); 1.67 (4H, m); 1.26 (44H, m); 0.87 (6H,m). ^{13}C NMR ($CDCl_3$): δ 172.05, 143.64, 136.44, 131.94, 131.72, 130.75, 129.43, 129.22, 123.58, 122.75, 121.02, 105.85, 82.12, 76.93, 70.92, 69.54, 60.29, 33.94, 33.30, 32.52, 30.32, 30.10, 29.76, 25.42, 23.14, 14.12. FAB-MS m/z : 927 (M+1), 944 (M+18), 949 (M+23), 965 (M+29)

Tosylate salt of 1'-(2',3'-dioleyl-5'-trimethylammonium-α-D-ribofuranosyl)-3-nitropyrrole, 7a

Anhydrous triethylamine (1 mL) was transferred to a pressure tube cooled at -50°C via canula. Next, anhydrous acetonitrile (2 mL) and a solution of **6a** (118 mg, 0.13 mmol) in dry THF (2 mL) were added. The tube was sealed and heated in an oil bath at 50°C during 48h then cooled to -20°C and opened. The solvents were evaporated under reduced pressure to give **7a** (125mg, quantitative) as a brown oil. ^{1}H NMR ($CDCl_3$): δ 7.68 (2H, d, J=8.06Hz); 7.64 (1H,m); 7.12 (2H, d, J=8.06Hz); 6.62 (2H,m); 6.26 (1H, d, J=4.42Hz); 5.67 (1H, t, J=4.74Hz); 5.33 (44H,m); 4.79 (1H, m); 4.46 (1H, m); 4.20 (1H,m), 3.37 (9H,s); 2.32 (3H,s); 1.99 (8H, m); 1.60 (4H, m); 1.26 (44H,m); 0.87 (6H, t, J=6.48Hz). ^{13}C NMR ($CDCl_3$): δ 173.21, 142.25, 140.51, 131.94, 137.52,

132.53, 130.73, 125.25, 123.14, 121.56, 106.83, 82.48, 78.41, 73.46, 67.88, 64.48, 51.91, 33.92, 33.32, 32.56, 30.42, 30.32, 30.12, 29.78, 26.48, 24.12, 21.19, 14.12. FAB-MS m/z : 814 (M cation). Anal Calc C,66.97 ; H, 9.30 ; N ,4.26 ; Found calc C,66.98 ; H, 9.41; N, 4.30.

Tosylate salt of 1'-(2',3'-dioleyl-5'-trimethylammonium-β-D-ribofuranosyl)-3-nitropyrrole, 7b

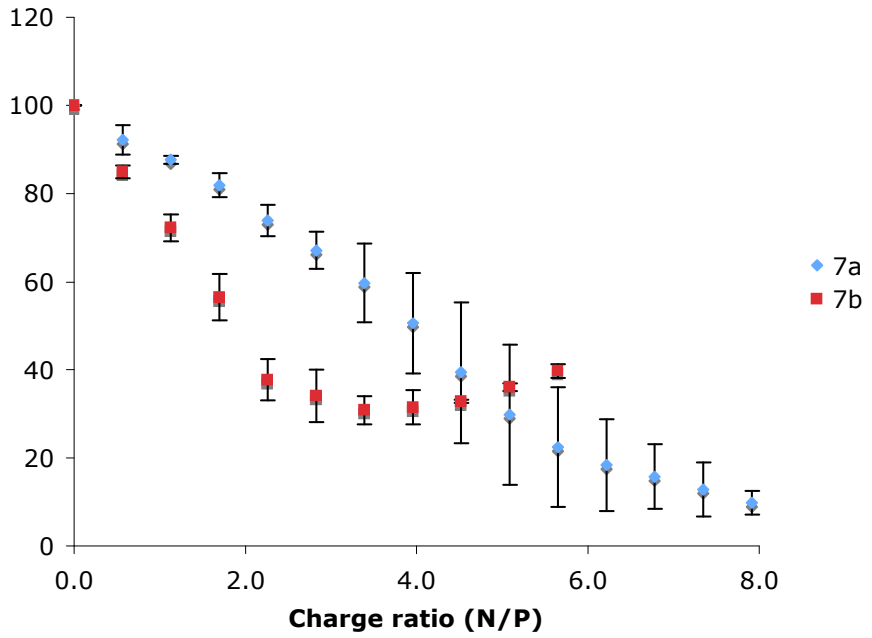
This compound was prepared from **6b** with the same procedure described above to yield **7b** as a brown oil with quantitative yield. ^{1}H NMR ($CDCl_3$): δ 7.80 (1H,m); 7.70 (2H, d, J=8.06Hz); 7.12 (2H, d, J=8.22Hz); 7.03 (1H,m); 6.60 (1H, m); 5.68 (1H, d, J=6.00Hz); 5.32 (4H,m); 4.66 (1H, dd, J=4.11Hz, J=10.27Hz); 4.42 (1H, t, J=11.05Hz); 4.06 (1H,m), 3.33 (9H,s); 2.84 (2H, d, J=7.42Hz); 2.32 (3H, m); 1.98 (8H, m); 1.56 (4H,m); 1.25 (44H,m); 0.86 (6H, t, J=6.00Hz). ^{13}C NMR ($CDCl_3$): δ 173.21, 142.25, 140.51, 131.94, 137.52, 132.53, 130.73, 125.25, 123.14, 121.56, 106.83, 82.48, 78.41, 73.46, 67.88, 64.48, 51.91, 33.92, 33.32, 32.56, 30.42, 30.32, 30.12, 29.78, 26.48, 24.12, 21.19, 14.12. FAB-MS m/z : 814 (M cation). Anal Calc C,66.97 ; H, 9.30 ; N ,4.26 ; Found calc C,66.30 ; H, 9.46; N, 4.00.

Vesicles Preparation

1 mg/mL of compounds **7a** or **7b** was dissolved in chloroform or dichloromethane. The solution was evaporated and dried on vacuum for 2 hrs. 1mL of DI water was added, and the solution was hydrated overnight at 4°C. The resulting solution was sonicated for 20 min. prior to any measurements.

DNA – Cationic Amphiphile Displacement Assay

A competitive displacement fluorescence assay using ethidium bromide (EthBr) 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 calf 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. Calf thymus DNA was used instead of siRNA due to the high costs associated with required amount of siRNA for this experiment.



Dynamic light scattering

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 vesicles of lipid and

DNA were prepared as described above in the vesicle preparation section. The diameter reported corresponds to the average of three experiments in aqueous solution. The 90 Plus particle sizing software was used for data acquisition and analysis. Calf thymus DNA was used instead of siRNA due to the high costs associated with required amount of siRNA for this experiment

siRNA transfection

Trypsine adherent cells were diluted in normal growth medium to 1 x 10^5 cells per mL. The transfection reagent was diluted in serum free medium and incubated at room temperature for 10 min before use. Next siRNA (GAPDH siRNA or negative control siRNA, Ambion) was diluted in serum free medium. The siRNA and the tranfection reagent were mixed, incubated for 10 min at room temperature and dispensed into a culture plate with cells and serum containing medium. Depending on the experimental design, the ration of lipid, siRNA, and incubation time was varied. The cell suspension was then overlayed onto the transfection mixture. The sample was then incubated at 37 °C and 5% CO_2. Gene knockdown activity was assessed after 48 h.

The gene knockdown assay performed was KDalert™ GAPDH Assay (Ambion) following the manufacturer protocol. Briefly, 48 hr after siRNA transfection, the serum-containing culture medium was aspirated from the transfected cells. Next, 200 μL KDalert Lysis Buffer was added to each sample well. The cells were incubated at 4 °C for 20 min to lyse them. The cell lysate was pipeted up and down 4–5 times to homogenize the lysate. 10 μL of each lysate or GAPDH enzyme dilution (including the GAPDH Working Stock) was transfered to the wells of a clean 96 well plate. Finally, 90 μL of KDalert Master Mix was added to each sample using a multi-channel pipettor. The increase in fluorescence of the samples was measured at

room temp.

Cytotoxicity

Cytotoxicity 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). 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, the cells were split into 24-well plates with a 1:4 ratio using a standard trypsin-based technique. For these experiments, CHO cells were seeded onto a 24-well plate with an appropriate density of 1 x 10^6 cells per well. After 48h the MTS substrate was added to each well and the plate was incubated for 4 h at 37 ^{o}C 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 10 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.