"HFP" Fluorinated Cationic Lipids for Enhanced Lipoplex Stability and Gene Delivery

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Although a great number of cationic lipids have been designed and evaluated as gene delivery systems, there is still a need for improvement of nonviral vectors. Recently, cationic lipids incorporating terminal fluoroalkyl segments ("FHP" lipids) have been described to display remarkable transfection potency. Here, we describe the synthesis of a new family of fluorinated triblock cationic lipids in which a fluorous segment lays between the cationic and the lipophilic parts of the molecule ("HFP" lipids). The compounds were designed so their self-assembly would offer enhanced resistance toward the host's degradation mechanisms mediated by lipophilic insertion. Self-assembly properties of these cationic lipids were evaluated at the air−water interface where they collapse in a highly ordered liquid phase. The HFP lipids efficiently condense DNA, and the resulting lipoplexes display enhanced resistance to amphiphilic agents when compared to nonfluorinated or FHP cationic lipids. Transfection properties of the fluorinated vectors, alone or as mixtures with different helper lipids (DOPE and a fluorinated analogue of DOPE), were then investigated on different cell lines (BHK-21, HepG2, and HeLa) and compared to those of the reference cationic lipid DOTAP. Data show that impermeabilization of the lipidic phase by fluorous segments alter significantly the gene transfection activities. Remarkably, incorporation of DOPE within the lipoplexes provides the particles with high gene transfection activity without reducing their resistance to amphiphilic agents.

INTRODUCTION

The delivery of a "medicine" gene in somatic cells involves the use of vectors, the function of which is to help the therapeutic gene cross the biological barriers and reach the nucleus of the target cells where it will be processed into mRNA and, later on, translated into proteins. Due to their high transfection efficiency, viral vectors such as adenoviruses, adeno-associated viruses (AAVs), or retroviruses are the most widespread vectors used in the laboratory or in clinical trials (*1*). However, their performance cannot overcome several major side effects, which include adverse immunogenic reactions, insertional mutagenesis, and sometimes fatal toxicity (*2, 3*). An additional limitation of these vectors lies in the restricted size of the DNA fragments they can introduce into a cell (less than 5 Kbp). The nonviral vectors, including cationic lipids, present interesting activity in vitro, but always appeared rather disappointing when evaluated in vivo. However, these vectors are devoid of some of the limitations of viruses (size restriction of the nucleic acid to be transfected, difficulty of production and purification, immunogenicity, etc.) and allow for specialized delivery options such as time-enhanced circulation, time-dependent release, and targeted delivery (*4*).

Since the first studies were conducted, hundreds of cationic lipids have been synthesized as candidates for nonviral gene delivery (*5*−*7*). Cationic lipids interact with negatively charged DNA through electrostatic interactions leading to condensed nucleic acid particles (lipoplexes). Optimum transfection efficacy invariably requires excess of cationic lipid for full DNA condensation and protection from serum nuclease. On the other hand, the cationic residual charge induces a poor circulation time of lipoplexes in blood, mostly due to their interaction with serum components. Many strategies have been developed to improve gene transfer mediated by cationic lipids. Those aiming at improving lipoplex circulation time are essentially based on the use of a poly(ethylene glycol) (PEG) coating of the cationic particles. However, introduction of PEG at the lipoplex surface unexpectedly does not highly increase the residential time of particles in the blood, which may be related to the drastic increase of the critical micelle concentration (cmc) of a lipid provoked by PEGylation (by a ca. 10^6-fold factor) (*8*). In addition, it may have rather adverse effects by inhibiting gene transfer in some cases (*9, 10*). A related strategy proposed by Mignet and Scherman results from the combination of cationic lipoplexes and PEGylated anionic liposomes (*11*). These pH-sensitive PEGylated anionic particles display interesting DNA delivery properties and are supposed to reverse to non-PEGylated cationic ones in the acidic endosome compartment. Behr and Vierling developed an alternative strategy to improve lipoplex circulation time by introducing a highly hydrophobic fluoroalkyl short segment at the extremity of the fatty acid chains of the cationic lipids (*12*−*17*). Owing to their increased hydrophobic character, these lipids (further named "FHP" fluorinated lipids to indicate that the fluoroalkyl moiety, F, is separated by an alkyl segment, H, from to the polar head, P) condense DNA into lipoplexes with higher stability than conventional cationic lipids. FHP lipid assemblies, however, are not expected to efficiently prevent anchoring/insertion of amphiphilic or lipophilic compounds to/within the bilayer structure, with such an event most often being a prelude to lipoplex destruction (i.e., lipid solubilization, recruitment of amphiphilic proteins triggering particle elimination by the reticuloendothelial system, activation of the complement system and opsonization, etc.). Importantly, FHP helper lipids have been

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described to enhance in vitro and in vivo gene delivery by cationic lipids (*18, 19*).

Recently, some of us showed that phospholipids that are fluorinated close to their polar head spread at the air−water interface into monolayers which are stable for several hours in the presence of high concentrations of detergents (*20, 21*). In the same conditions, conventional phospholipid monolayers hardly resist a few seconds. This remarkable property only partly results from the high hydrophobicity of fluorinated species as is the case with FHP lipids. Indeed, displaying the fluoroalkyl segment close to the lipid polar head likely induces a stabilization of higher magnitude, which may be explained by the interactions between adjacent fluorine atoms in the same perfluorinated chain that cause the chain to twist into a rigid, helical conformation (*22*). Consequently, close packing of the rigid fluoroalkyl segments does substantiate a kind of fluorous repelling shield that is highly impermeable to nonfluorinated hydrophilic, lipophilic, as well as amphiphilic compounds (*23*). Thus, by preventing or reducing insertion of surface active compounds within the lipid layer it is possible to efficiently prevent the destruction of the lipid assembly. We made the hypothesis that cationic lipids displaying such characteristics would condense DNA into fluorinated lipoplexes with original capabilities in gene transfer experiments. By resisting serum component adsorption (and subsequent destruction triggered by insertion of hydrophobic anchors of recruited proteins or other destruction mechanisms) (*24*), such lipoplexes are expected to present an extended half-life time, and possibly a higher transfection efficiency, however keeping in mind that intracellular trafficking of the delivery system requires at some points disassembly of the lipid−DNA complexes. Therefore, the immediate goal of this study was to prepare cationic lipids fluorinated close to their polar head (further named "HFP" fluorinated lipids, a terminal alkyl chain, H, being separated by a fluoroalkyl segment, F, from the polar head, P) and to evaluate (i) their capability to form complexes with DNA, (ii) the stability of the resulting lipoplexes, and (iii) the gene delivery efficacy of the latter in vitro. In the following, we describe the design, synthesis, characterization, and evaluation of a series of unprecedented HFP fluorinated triblock cationic lipids.

EXPERIMENTAL PROCEDURES

Chemicals and Materials. Unless otherwise stated, all chemical reagents were purchased from Alfa Aesar (Bischeim, France) and used without purification. α,ω-Diiodoperfluoroalkanes were from Apollo Scientific Ltd. (Bredbury, Cheshire, UK). When required, solvents were dried by standard procedures just before use (*25*). Products were purified by chromatography over silica gel (Silica Gel 60, 40−63 μm, Merck, Darmstadt, Germany). Reverse-phase filtration was carried out with Sep-Pack classic short-body C-18 cartridges (Waters, Division of Millipore, Milford, MA, USA).

N-[1-(2,3-Dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTAP chloride), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), sodium *n*-dodecyl sulfate (SDS), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), sodium cholate (NaCh), cetyltrimethylammonium bromide (CTAB), Tween 80, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), penicillin, streptomycin, L-glutamine, D-glucose, and tryptose phosphate broth were from Sigma-Aldrich (Saint-Quentin Fallavier, France). Culture media Dulbecco's Modified Eagle Medium Glutamax (DMEM) and Minimum Essential Eagle Medium (MEM) were from GIBCO-BRL (Cergy-Pontoise, France). Fetal calf serum (FCS) was from Perbio (Brebières, France). Lysis and luciferin solutions for monitoring luciferase activity were purchased from Promega (Charbonnières, France). Human hepatocarcinoma cells (HepG2), Syrian hamster kidney cells (BHK-21), and immortalized human epithelial cells (HeLa) were from ATCC-LGC (Molsheim, France). pSMD2-LucΔITR (7.6 kpb) or pEGFP-Luc (6.4 kpb) expression plasmids (BD Biosciences Clontech, Franklin Lakes, NJ, USA) were used as reporter genes to monitor transfection activity. Both plasmids encoded the firefly luciferase gene under the control of a strong promoter. HepG2 cells were transfected with the pSMD2-Luc and the other cell lines with the pEGFP-Luc.

Preparation of Cationic Lipids. *2,3-Bis(3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorotetradecanoyloxy)-propyl-1-trimethylammonium Chloride (**1a**).* The acid **7a** (209 mg, 0.47 mmol) in anhydrous CH_2Cl_2 (2 mL) was stirred at room temperature with oxalyl chloride (0.2 mL, 2.29 mmol) and a catalytic amount of DMF (5 μL) for 4 h. Volatiles were removed under reduced pressure, and the residue was coevaporated twice with anhydrous toluene. The crude acid chloride was dissolved in anhydrous CH_2Cl_2 (2 mL), and the resulting solution was added dropwise to a mixture of anhydrous diol **8** (32 mg, 0.19 mmol) and pyridine (38 μL, 0.47 mmol) in THF/CH_2Cl_2 (2:1 v/v, 3 mL). The resulting suspension was sonicated for 2 h and stirred overnight at room temperature. Solvent was removed under reduced pressure at room temperature, and the residue was purified by flash chromatography over silica gel ($CHCl_3$/MeOH 100:0 to 60:40) to yield compound **1a** (51 mg, 26%; containing 37% of elimination byproduct) as a white wax.

*2,3-Bis(3,3,4,4,5,5,6,6-octafluorohexadecanoyloxy)-propyl-1-trimethylammonium Chloride (**1b**).* Compound **1b** (79 mg, 24%; containing 18% of elimination byproduct) was obtained as a white wax starting from compound **7b** following the same procedure as described for the preparation of **1a**.

*2,3-Bis(3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorooctadecanoyloxy)-propyl-1-trimethylammonium Chloride (**1c**).* Compound **1c** (54 mg, 28%; containing 11% of elimination byproduct) was obtained as a white wax starting from compound **7c** following the same procedure as described for the preparation of **1a**.

*2,3-Bis(3,4,4,5,5,6,6,7,7,8,8-undecafluorotetradecen-2-oyloxy)-propyl-1-trimethylammonium Chloride (**2a**).* Compound **1a** (25 mg, 24.5 μmol) in CH_3CN/CH_2Cl_2 (2:1 v/v, 3 mL) was stirred for 2 h with 2 M triethylamine in THF (36 μL, 62.0 μmol) at room temperature. The reaction mixture was filtered over a pad of finely powdered $NaHCO_3$, and the filtrate was reduced under vacuum to yield **2a** (20 mg, 84%) as a white wax.

*2,3-Bis(3,4,4,5,5,6,6-heptafluorohexadecen-2-oyloxy)-propyl-1-trimethylammonium Chloride (**2b**).* Compound **2b** (6 mg, 88%) was prepared from **1b** following the same procedure as described for **1a**.

*2,3-Bis(3,4,4,5,5,6,6,7,7,8,8-undecafluorooctadecen-2-oyloxy)-propyl-1-trimethylammonium Chloride (**2c**).* Compound **2c** (10 mg, 86%) was prepared from **1c** following the same procedure as described for **1a**.

*1,2-Di[(Z)-3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorohexadec-9-enoyl]-sn-glycero-3-phosphatidylethanolamine (**3**).* Triphenyl phosphine (4.1 mg, 15.7 μmol) and azido compound **15** (6.0 mg, 5.2 μmol) in THF/CH_2Cl_2/CF_3CH_2OH/HCl 2% (3:1:1:0.1 v/v, 3 mL) were stirred for 48 h at room temperature. Volatiles were removed under reduced pressure, and the residue was purified by silica gel chromatography ($CHCl_3$/MeOH/H_2O 10:0:0 to 10:6:1). The fluorinated analogue of DOPE was obtained as a colorless oil (3.0 mg, 51%; mixture of isomers, Z/E = 85:15).

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluoro-1,10-diiodotetradecyl Benzoate (**4a**).* Sodium dithionite (3.14 g, 17.8 mmol) was slowly added to a mixture of 1,6-diiodoperfluorohexane (5.00 g, 9.0 mmol), vinyl benzoate (1.25 mL, 9.0 mmol), 1-hexene (1.12 mL, 9.0 mmol), and $NaHCO_3$ (1.91 g, 18.0 mmol) in CH_3CN/H_2O (8:7 v/v, 18.4 mL) at 0 °C. The resulting solution was stirred for 50 min at 0 °C, brought to pH 5−7 with HCl 3N,

and stirred for an additional 20 min period at room temperature. Water was added, and the solution was extracted with CH_2Cl_2. The organic layer was dried over $MgSO_4$, filtered, and reduced under vacuum. The residue was purified by silica gel chromatography (C_6H_{14}/CH_2Cl_2 95:5) to yield benzoate **4a** (2.03 g, 28%) as a slightly yellow waxy solid.

*3,3,4,4,5,5,6,6-Octafluoro-1,8-diiodohexadecyl Benzoate (**4b**).* Compound **4b** (2.46 g, 29%) was prepared from 1,4-diiodoperfluorobutane, vinyl benzoate, and 1-decene following the same procedure as described for **4a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluoro-1,10-diiodooctadecyl Benzoate (**4c**).* Compound **4c** (1.53 g, 33%) was prepared from 1,6-diiodoperfluorohexane, vinyl benzoate, and 1-decene following the same procedure as described for **4a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorotetradecyl Benzoate (**5a**).* Tributyltin hydride (173 μL, 0.64 mmol) was added to compound **4a** (100 mg, 0.13 mmol) and AIBN (2 mg, 0.01 mmol) in Et_2O (3 mL). The reaction mixture was refluxed under the light of a domestic halogen lamp (I.CEM Züblin AG, type 0201, 500 W) for 15 h. Solvent was removed under reduced pressure and the residue was purified by flash chromatography over silica gel (n-C_6H_{14}/CH_2Cl_2 9:1) to yield benzoate **5a** (61 mg, 90%) as a white waxy solid.

*3,3,4,4,5,5,6,6-Octafluorohexadecyl Benzoate (**5b**).* Compound **5b** (1.38 g, 96%) was prepared from **4b** following the same procedure as described for **5a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorooctadecyl Benzoate (**5c**).* Compound **5c** (1.49 g, 90%) was prepared from **4c** following the same procedure as described for **5a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorotetradecanol (**6a**).* Compound **5a** (3.0 g, 5.61 mmol) was stirred in methanolic 1 M LiOH (20 mL) for 3 h at room temperature. Solvent was removed under vacuum, and the residue was dissolved in CH_2Cl_2, then washed with water and brine. The organic layer was dried over $MgSO_4$ and evaporated, and compound **6a** (2.21 g, 91%) was obtained as a white waxy solid after purification over silica gel (n-C_6H_{13}/CH_2Cl_2 1:1).

*3,3,4,4,5,5,6,6-Octafluorohexadecanol (**6b**).* Compound **6b** (0.60 g, 86%) was prepared from **5b** following the same procedure as described for **6a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorooctadecanol (**6c**).* Compound **6c** (0.47 g, 77%) was prepared from **5c** following the same procedure as described for **6a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorotetradecanoic Acid (**7a**).* Alcohol **6a** (1.0 g, 2.32 mmol) was dissolved in acetone (40 mL) and water (0.5 mL). Jones' reagent was added dropwise at room temperature until a persistent red−brown solution was obtained. The reaction mixture was stirred for 1 h, added with *i*-PrOH (2 mL), and stirred for an additional 15 min period. Solvent was then removed under reduced pressure, water (5 mL) was added, and the resulting mixture was extracted with Et_2O. The organic layer was washed with H_2O, brine, dried over $MgSO_4$, filtered, and reduced under vacuum to yield acid **7a** (0.93 g, 90%) as a white waxy solid.

*3,3,4,4,5,5,6,6-Octafluorohexadecanoic Acid (**7b**).* Compound **7b** (0.37 g, 92%) was prepared from **6b** following the same procedure as described for **7a**.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorooctadecanoic Acid (**7c**).* Compound **7c** (1.64 g, 89%) was prepared from **6c** following the same procedure as described for **7a**.

*N-[1-(2,3-Dihydroxy)propyl]-N,N,N-trimethylammonium Chloride (**8**).* (±)-Glycidol (2 mL, 30.1 mmol), trimethylamine hydrochloride (2.88 g, 30.1 mmol), and *N,N*-diisopropylethylamine (5.25 mL, 30.1 mmol) in methanol (20 mL) were stirred for 12 h at room temperature. Solvent was removed under reduced pressure, and compound **8** (3.65 g, 71%) was purified by recrystallization in EtOH/acetone (1:1 v/v).

*Benzyl 2-Bromoethyl Phosphite (**9**).* *N,N*-Diisopropylethylamine (1.0 mL, 5.73 mmol) was added dropwise to phosphorus trichloride (0.5 mL, 5.73 mmol) in anhydrous THF (10 mL). The reaction mixture was cooled down to −78 °C, and benzyl alcohol (0.6 mL, 5.73 mmol) in THF (20 mL) was added with a syringe driver under vigorous stirring over a 3 h period. The resulting suspension was stirred for 30 min, and a second portion of *N,N*-diisopropylethylamine (1.0 mL, 5.73 mmol) was added. Then, freshly distilled 2-bromoethanol (0.4 mL, 5.73 mmol) in THF (20 mL) was added dropwise over a 3 h period. Stirring was prolonged for 90 min at −78 °C before water (7 mL) was added. The reaction mixture was warmed to room temperature, THF was removed under vacuum, and the residue was washed twice with AcOEt. The organic layer was dried over MgSO4, reduced under vacuum, and purified over silica gel (n-C_6H_{14}/AcOEt 5:5) to yield compounds **9** (1.08 g, 67%) as a slightly yellow oil.

*Benzyl 2-Bromoethyl (2,2-Dimethyl-1,3-dioxolan-4-yl)methyl Phosphate (**10**).* Sulfuryl chloride (4.3 mL, 53.5 mmol) was added dropwise at 0 °C to phosphite **9** (5.0 g, 17.9 mmol) in dry CCl_4 (50 mL). Stirring was prolonged 30 min at 0 °C and 10 min at room temperature. Volatile was removed under vacuum, and the residue was coevaporated twice with anhydrous toluene. Benzyl 2-bromoethyl chlorophosphate was obtained as a colorless oil and was used without purification. (*R*)-(-)-2,2-Dimethyl-1,3-dioxolane-4-methanol (2.37 g, 17.9 mmol) in anhydrous THF (200 mL) was treated with sodium hydride (60% oil suspension, 0.86 g, 21.5 mmol) at 0 °C for 30 min. Then, previously prepared chlorophosphate was added, and the reaction mixture was stirred for 3 h at room temperature. THF was removed under vacuum, saturated aqueous NH_4Cl (100 mL) was added, and the resulting solution was extracted with AcOEt. The organic layer was washed with H_2O, brine, dried over $MgSO_4$, and reduced under vacuum. The residue was purified over silica gel (n-C_6H_{14}/Et_2O 5:5 to 8:2) to yield **10** (4.6 g, 63%, mixture of diastereomers) as a yellow oil.

*2-Azidoethyl Benzyl (2,2-Dimethyl-1,3-dioxolan-4-yl)methyl Phosphate (**11**).* Compound **10** (0.50 g, 1.22 mmol) and sodium azide (0.10 g, 1.54 mmol) were stirred in anhydrous DMF (6 mL) for 2 h at 50 °C. Water (15 mL) was added, and the resulting solution was extracted with Et_2O. The organic layer was dried over $MgSO_4$, reduced under vacuum, and purified by flash chromatography (n-C_6H_{14}/AcOEt 5:5 to 8:2) to yield **11** (0.34 g, 75%, mixture of diastereomers) as a yellow oil.

*2-Azidoethyl Benzyl 2,3-dihydroxypropyl Phosphate (**12**).* Compound **11** (118 mg, 0.32 mmol) was stirred in EtOH (8 mL) with strongly acidic cation exchange resin Dowex 50 × 8 (H^+ form) for 12 h at room temperature. The resin was filtered off, solvent was removed under vacuum, and the residue was purified by flash chromatography (AcOEt/EtOH 10:0 to 8:2) to yield **12** (69 mg, 64%) as a yellow oil.

*(Z)-3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorohexadec-9-enoic Acid (**13**).* Unsaturated acid **13** (0.14 g, 99%, Z/E = 85:15) was obtained as a colorless oil starting from alcohol **17** and following the same procedure as described for **7a**.

*2-Azidoethyl Benzyl 3-{1,2-di[(Z)-3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorohexadec-9-enoyl]-sn-glycero} Phosphate (**14**).* Unsaturated acid **13** (250 mg, 0.53 mmol) in anhydrous CH_2Cl_2 (2 mL) was stirred at room temperature for 4 h with oxalyl chloride (0.25 mL, 2.86 mmol) and DMF in catalytic amount. Volatiles were removed under reduced pressure, and the residue was coevaporated twice with anhydrous toluene and dissolved in CH_2Cl_2 (2 mL). Then, diol **12** (68 mg, 0.21 mmol) and pyridine (45 μL, 0.56 mmol) in anhydrous THF (3 mL) were added, and the reaction mixture was stirred at room temperature for 8 h. Saturated aqueous NH_4Cl was added, and the mixture was extracted with Et_2O. The organic layer was dried over $MgSO_4$,

filtered, and reduced under vacuum. The residue was purified by flash chromatography (n-C_6H_{14}/CH_2Cl_2/AcOEt 6:2:2 to 0:0:10) to yield **15** (172 mg, 68%; mixture of isomers, Z/E = 85:15) as a colorless oil.

*2-Azidoethyl 3-{1,2-di[(Z)-3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorohexadec-9-enoyl]-sn-glycero} Hydrogen Phosphate (**15**).* Bromotrimethylsilane (35 μL, 265 μmol) was added to compound **14** (84 mg, 68 μmol) in anhydrous CH_2Cl_2 (5 mL). The reaction mixture was stirred for 3 h at room temperature and moist THF is added. Solvent was removed under vacuum, and the residue was purified over silica gel ($CHCl_3$/MeOH 10:0 to 6:4) to yield compound **15** (74 mg, 95%; mixture of isomers, Z/E = 85:15) as a colorless oil.

*3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluoro-1,10-diiodotetradec-9-enyl Benzoate (**16**).* A degassed mixture of 1,6-diiodoperfluorohexane (200 mg, 0.36 mmol), 1-octyne (80 μL, 0.54 mmol), vinyl benzoate (75 μL, 0.54 mmol), and AIBN (12 mg, 0.07 mmol) was stirred for 15 h at 80 °C in a sealed tube. The resulting mixture was cooled down to room temperature, volatiles were removed under reduced pressure, and the residue was purified by silica gel chromatography (n-C_6H_{14}/CH_2Cl_2 9:1 to 0:10) to yield compound **16** (105 mg, 36%, Z/E = 15:85) as a yellow waxy solid.

*(Z)-3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorohexadec-9-enyl Alcohol (**17**).* Diiodoester **16** (354 mg, 0.44 mmol) in anhydrous THF (4 mL) was added dropwise to a suspension of $LiAlH_4$ (100 mg, 2.63 mmol) in refluxing THF (8 mL). The resulting mixture was stirred at 66 °C for 30 min and cooled down at room temperature, and excess reagent was slowly decomposed by addition of solid NH_4Cl. Ether (15 mL) was then added, and the organic layer was washed with water and brine, dried over $MgSO_4$, filtered, and reduced under vacuum. The crude residue was purified over silica gel (n-C_6H_{14}/CH_2Cl_2 5:5 to 0:10) to yield alcohol **17** (135 mg, 68%, Z/E = 88:12) as a colorless oil.

Film Formation and Surface Pressure Measurements. Monolayer experiments were performed on a computer-controlled Langmuir film balance KSV 3000 (KSV, Helsinki, Finland). Surface pressure isotherms were recorded on a Teflon trough (570 × 150 mm) with a Wilhelmy plate coupled to a linear transducer. Monolayers were spread from 0.5 mM lipid solutions in hexane/chloroform (1/1: v/v). Volumes ranging from 100 to 300 μL were delivered at several locations across the water surface with a Hamilton microsyringe. At least 15 min were allowed for the spreading solvent to evaporate before monolayer compression. The subphase consisted of purified water (pH 5.35, Millipore filtration system, Barnstead, NANO pure 11) with a resistivity of $18.2 \cdot 10^6$ Ω cm. Compressions were realized using a hydrophilic Delrin barrier (polyacetal). Surface pressure vs molecular area curves were recorded at 21.0 ± 0.5 °C with a compression speed of 2−5 Å^2 per lipid molecule per minute. All measurements were carried out in triplicate. Specific areas A_s, lift-off areas A_{lo}, and collapse pressures π_c were determined as previously described (*26*).

Cell Culture. Human hepatocarcinoma cells (HepG2) were cultured in DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 292 mg/L L-glutamine, 4.5 g/L D-glucose, and 10% heat-inactivated FCS. The syrian hamster kidney fibroblast cell line (BHK-21) was cultured in MEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 10 mg/mL L-glutamine, 50 mg/mL tryptose, and 10% FCS. The HeLa cell line was cultured in MEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, 292 mg/L L-glutamine, and 10% FCS.

Lipoplex Preparation. For transfection experiments, cationic lipid (30 μL of a 1.8 mM solution in ethanol) and DOPE (6 μL of a 18 mM solution in ethanol) were mixed and added to 10 mM Hepes pH 7.5, 150 mM NaCl (150 μL). After stirring by vortex, the solution was added to the plasmid DNA (6 μg) in 10 mM Hepes pH 7.5, 150 mM NaCl (150 μL). The solution was vortexed again and incubated for 20 min at room temperature before addition to the cells. For gel retardation analysis, the same concentration and procedure were used except that the amount of DNA per sample was 1 μg.

Dynamic Light Scattering Measurements. The average particle size and the polydispersity of the lipoplexes were measured by using photon correlation spectroscopy (Malvern Zetasizer nanoZS, Malvern Instruments Inc., UK) using the multimodal analysis and the automatic mode. Lipoplex solutions (100 μL, vide supra) were diluted 10 times, in DMEM medium unless stated otherwise.

DNA Retardation Assays. After 15 min incubation, complexes were analyzed by electrophoresis through a 1% agarose gel. The gel was run in a 40 mM Tris-acetate-EDTA buffer pH 8.0 (TAE). DNA was then visualized with ethidium bromide solution at 0.5 μg/mL.

Transfection Procedure. Cells were plated in 24-well plates one day before transfection in order to obtain 60−80% confluent cultures at the time of the experiment. Transfection mixture (110 μL containing 2 μg of DNA plasmid, vide supra) was deposited in each well of the triplicate. After a 24 h incubation period, cells were harvested in lysis buffer (250 μL: 8 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA, 1% Triton X-100, 15% glycerol, in 25 mM Tris-phosphate buffer pH 7.8). The cell lysate was centrifuged for 5 min at 10 000 g to pellet cell debris. Luciferase light units were measured in a 96-well plate format with a PhL luminometer (Mediators Diagnostika) from an aliquot of the supernatant (50 μL) with 10 s integration after automatic injection of assay buffer (100 μL: lysis buffer without Triton X-100 but supplemented with 2 mM ATP) and 167 μM luciferin (100 μL). The protein content of each cell lysate was measured using the BioRad protein assay. The transfection efficiency was expressed as relative light units/10 s/mg protein (RLU/mg protein) after subtraction of background luciferase value from each sample value. Values of each sample are the means of triplicate determinations.

Lipoplex Cytotoxicity Assessment. Lipoplex cytotoxicity was assessed using a tetrazolium-based assay. Cells were seeded in 24-well culture plates at a density of 5.0×10^4 cells per well and cultured at 37 °C for 24 h. On the next day, culture medium was changed to fresh DMEM medium supplemented with 10% FCS before addition of lipoplexes (100 μL) prepared as previously described. After a 24 h incubation period at 37 °C, culture supernatant was removed, cells were carefully washed with PBS, and 0.5 mg/mL MTT (1 mL) in complete culture medium was added. After a 2 h incubation period at 37 °C, MTT solution was removed and DMSO (1 mL) was added to lyse cells and dissolve reduced MTT. Intensity of MTT reduction was then evaluated by measuring absorbance at 570 nm. Viability of cells treated with lipoplexes was expressed as a percentage of the absorbance measured in untreated cells. Each treatment was assessed in triplicate, and data are presented as means ± sem of n = 3 values.

RESULTS AND DISCUSSION

Molecular Design and Chemistry. To investigate the influence of fluoroalkyl moieties located in the vicinity of the headgroup of a cationic lipid on its transfection efficiency, we first need a reference compound and selected the widely used DOTAP (Figure 1). The introduction of fluoroalkyl segments in its structure must fulfill some preliminary requirements in terms of chemical stability. Considering the high reactivity of α,α′-difluoroesters toward water with half-life time for hydrolysis in the range 0.1−5 min (*27*), we decided on structures in which one methylene group lies in between the fluoroalkyl chain

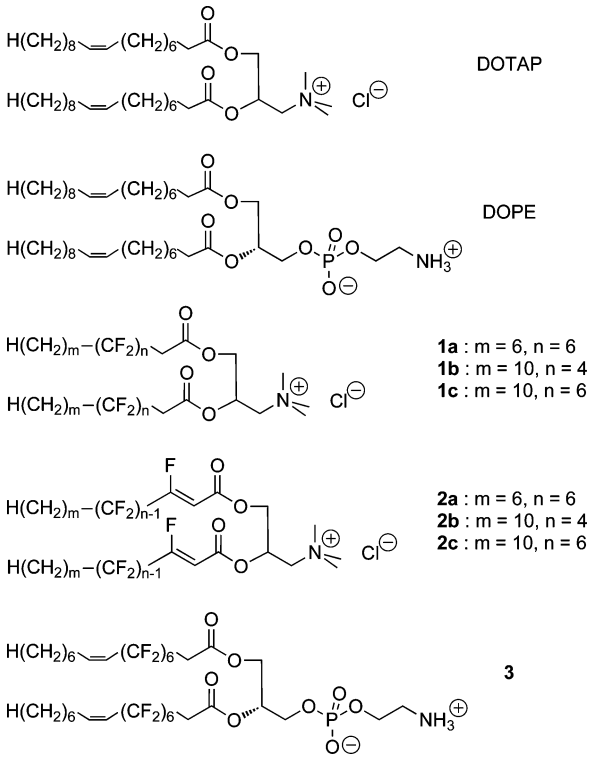
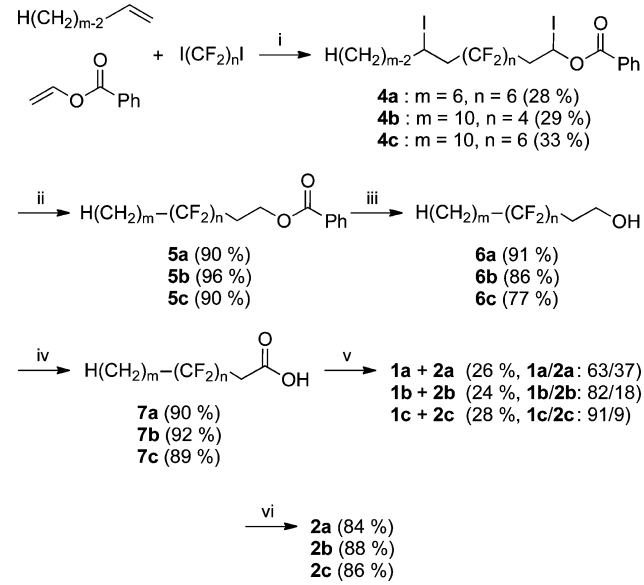


Figure 1. Structures of DOTAP, DOPE, and fluorinated cationic lipids developed.

and the carboxyl group. We thus designed compounds **1a**−**c** with modulated lengths for fluoroalkyl and terminal alkyl moieties. In order to target compounds with hydrophobicity tentatively comparable with that of DOTAP, we varied the number of methylene (*m*) and difluoromethylene (*n*) groups in the chains, considering the "1 CF_2 = 1.5 CH_2" rule (*28, 29*) and its variations (1.5 $CH_2 \leq 1$ $CF_2 \leq 4$ CH_2) (*30*). Under mild basic conditions, β,β'-difluoroesters easily eliminate HF, and compounds **2a**−**c** result from a double elimination in **1a**−**c** (vide infra).

A number of transfection protocols with cationic lipids use dioleoylphosphatidylethanolamine (DOPE) as a helper lipid (Figure 1). The role of DOPE in transfection assays has been extensively studied (*31, 32*). The mechanism of action of that lipid has been attributed to its capacity to induce transition of a lipid bilayer from the liquid-crystalline lamellar phase into the inverted hexagonal H_{II} phase. Vierling et al. examined the use of terminal fluorinated glycerophosphoethanolamines analogues of DOPE as helper lipids in nonfluorinated cationic lipid-mediated gene transfer (*18, 19*). A fluorinated segment was introduced at the extremity of the ester at C^1 (or C^1 and C^2) on the glycerol backbone, and the resulting increase in transfection efficiency by cationic lipids was attributed to the pronounced cone-shaped geometry of the molecule, displaying a greater tendency to promote a transition from the lamellar phase into the inverted hexagonal H_{II} phase. Considering the results obtained with lipoplexes prepared from mixtures of **1a**−**c** and DOPE (vide infra), we were especially interested in evaluating a fluorinated analogue of DOPE displaying two fluorous segments close to the lipid polar head. Consequently, we designed HFP compound **3** that displays all structural features of DOPE and displays a fluorous segment in between the double bond and the carboxyl group of the two fatty acid chains. Chain length is less than C_{18} to compensate for enhanced hydrophobicity due to fluorine. Stereochemistry of the double bond is *Z* in order to tentatively preserve the original chain conformation of oleoyl chains. Last, C^2 stereochemistry on glycerol backbone was imposed to fit with that in natural phospholipids.

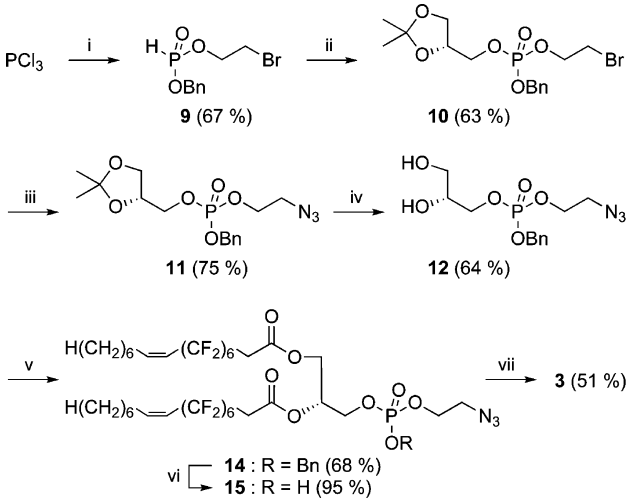
Scheme 1. Synthesis of Compounds 1a−c and 2a−c[a]



[a] Conditions: (i) $NaHCO_3$, $Na_2S_2O_4$; (ii) Bu_3SnH, AIBN; (iii) LiOH; (iv) CrO_3, H_2SO_4; (v) $(COCl)_2$, *N*-[1-(2,3-dihydroxy)propyl]-*N,N,N*-trimethylammonium chloride (**8**), py; (vi) Et_3N.

The synthesis of compounds **1a**−**c** was realized starting from α,ω-diiodoperfluoroalkanes according to Scheme 1. Hemifluorinated fatty acids **7a**−**c** were key intermediates. Their preparation involved the well-described radical addition of a iodoperfluoroalkane to an alkenyl compound (*33*). However, the homobifunctional fluorinated starting material introduced some difficulty, as heterofunctionalization was required. The reaction of a large excess of diiodoperfluoroalkane with 1 equiv of alkene allows high yield but low conversion and is not acceptable due to high cost of the fluorinated reagents. When the reaction was carried out with diiodo and alkenyl compounds in stoichiometric amounts, the proportion of monoalkylated product obtained was close to 50%, which is in agreement with statistics. Nevertheless, the process of the radical reaction remained difficult to monitor and reduction compounds R_F-H rapidly appeared as alkene concentration decreased in the reaction mixture. Consequently, the heterobifunctionalization of the diiodo starting material was carried out using a one-step procedure involving stoichiometric amounts of both alkenyl reagents. Perfluoroalkyl iodide addition to alkene can be promoted in many ways (by photolysis, pyrolysis, electrolysis, free radical initiator, enzymes, metals, and transition metal complexes). In that work, radicals were generated under the conditions described by Rong and Keese, and using sodium dithionite and sodium carbonate in CH_3CN/H_2O (*34*). The yield of the double transformation was close to 30% and was higher than that by any multistep procedure we have experienced. Efforts to run the reaction under anhydrous conditions with triethylborane (*35*) did not give better results either. Radical reduction of compounds **4a**−**c** using Bu_3SnH and AIBN (*36*) proved efficient and **5a**−**c** were obtained in high yield. Benzoate hydrolysis and Jones oxidation of the resulting alcohols **6a**−**c** afforded hemifluorinated fatty acids **7a**−**c** (*37, 38*). Further conversion into acyl chlorides allowed direct coupling with *N*-[1-(2,3-dihydroxy)propyl]-*N,N,N*-trimethylammonium chloride **8** straightforwardly prepared from glycidol. Fluorinated cationic lipids **1a**−**c** were obtained in 26−28% yield. Important to note is the high sensitivity of compounds **1a**−**c** that very easily eliminate HF even at room temperature. As a consequence, they were obtained as a mixture with eliminated byproducts that were very difficult to separate (**1a**, 37% elimination byproduct; **1b**, 18% elimination byproduct; **1c**, 11% elimination byproduct).

Scheme 2. Synthesis of the Fluorinated Analogue of DOPE 3[a]



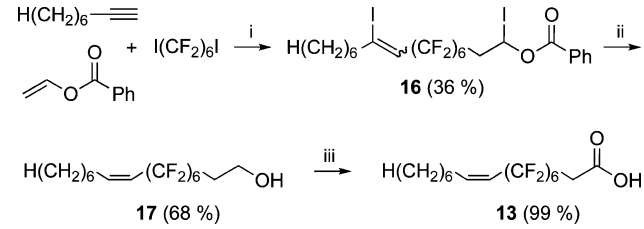
[a] Conditions: (i) Et_3N, BrC_2H_4OH, H_2O; (ii) SO_2Cl_2, (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol, NaH; (iii) NaN_3; (iv) Dowex 50 × 8 (H^+ form); (v) **13**, $(COCl)_2$, py; (vi) TMSBr, H_2O; (vii) PPh_3, H_2O.

In order to tentatively assume better control of the composition and properties of later lipoplexes, compounds **1a**−**c** were quantitatively transformed into the corresponding unsaturated cationic lipids **2a**−**c** upon treatment with Et_3N. The unsaturated double bonds are in the *Z* configuration as was determined from the coupling constant $^3J_{H-F} \approx 29$ Hz, a value which is characteristic of the trans position between fluorine and hydrogen on ethylenic double bonds (*39, 40*). Lipid **1c**, however, was considered for further biophysical and biological investigations (vide infra), as it was obtained at the lower contamination rate.

Synthesis of the fluorinated analogue of DOPE **3** was achieved according to Scheme 2. Benzyl 2-bromoethyl phosphite **9** was prepared in one step from phosphorus trichloride. Transformation of **9** into corresponding chlorophosphate allowed condensation with (*S*)-2,2-dimethyl-1,3-dioxolane-4-methanol. Resulting phosphotriester **10** was obtained in 53% yield. Bromide nucleophilic displacement with sodium azide provided compound **11**, and the isopropylidene protecting group was removed upon treatment with a strongly acidic cation exchange resin. Resulting diol **12** was then acylated with unsaturated hemifluorinated fatty acid **13** to give **14** in 68% yield. Interestingly, elimination byproducts were not obtained or could not be detected. In the preparation of compounds **2a**−**c**, due to the amphiphilicity of the compounds, the crude reaction mixture was not submitted for aqueous work up but reduced in vacuo and directly purified by silica gel chromatography. This could possibly indicate that HF elimination occurs during concentration of the crude reaction mixture and may be avoided by aqueous workup prior to solvent evaporation. Debenzylation of **14** was achieved with bromotrimethylsilane (*41, 42*). The final step was reduction of the azide group in **15** via a Staudinger reaction affording **3** in 51% yield.

Unsaturated fluorinated fatty acid **13** was a key compound for the preparation of the fluorinated DOPE analogue. It was prepared in three steps (Scheme 3). The first one involved the reaction of R_FI with an alkynyl compound (*43*−*45*). It was similar to the preparation of **4a**−**c** except that 1-alkene was replaced with 1-octyne. The double radical oxidative addition of 1,6-diiodoperfluorohexane on a stoichiometric mixture of vinylbenzoate and alkyne furnished vinyl iodide **16** as *E*/*Z* mixture (*E*:*Z* = 85:15). Reduction of compound **16** under the conditions employed by Takagi (*n*BuLi, Et_2O, −78 °C) for the synthesis of fluorinated analogues of oleic acid displaying

Scheme 3. Synthesis of Unsaturated Hemifluorinated Fatty Acid 13[a]



[a] Conditions: (i) Na_2CO_3, $Na_2S_2O_4$, AIBN. (ii) $LiAlH_4$. (iii) CrO_3, H_2SO_4.

the fluorous part opposite the carboxyl group proved inadequate (*44, 45*). Reduction using *n*Bu_3SnH-AIBN (*46*) gave a mixture of cis and trans alkenes (*Z*:*E* = 60:40) revealing extended isomerization of the intermediate vinyl radical. More satisfactorily, treatment with lithium aluminum hydride allowed extensive reduction of compound **16** into **17** in better favor of the cis isomer (*47*). Under these conditions, reduction proceeded with full retention of the configuration (*Z*:*E* = 88:12), and concomitant reduction of the benzoate moiety occurred with release of the free primary hydroxyl group. The last step consisted of oxidation of alcohol **17** that was quantitatively transformed into unsaturated hemifluorinated fatty acid **13** upon treatment with CrO_3−H_2SO_4.

Self-Assembly Properties of the Fluorinated Cationic Lipids. Self-assembly properties of fluorinated cationic lipids were investigated at the air/water interface (due to their high content in elimination byproduct, samples **1a** and **1b** were not subjected to monolayer study). Compounds were spread from a hexane/chloroform solution, and the surface pressure π vs area A was recorded. All the compounds analyzed exhibited similar behavior (see Supporting Information). The pressure regularly increased up to collapse pressure at 28−34 mN/m. No surface pressure decrease was recorded at high lateral pressure ($\pi < 30$ mN/m) when the compression barrier was stopped, indicating that the monolayers formed at the air/water interface were stable. The values for specific area A_s, lift-off area A_{lo}, and collapse pressure π_c for compounds **1c** and **2a**−**c** are presented in Supporting Information.

Monolayer spread from saturated lipid **1c** (containing 11% of elimination products) collapsed in a more condensed state than those from unsaturated compounds **2a**−**c**. This indicates that unsaturation to some extent opposes close packing of the compounds. That is further supported by the lower value for the lift-off area recorded for **1c** when compared to those of **2a**−**c**. However, the *Z* configuration of the double bonds in **2a**−**c** seems to be contradictory to these results. Indeed, it is expected that **1c** and the *Z* conformer of **2c** display comparable monolayer properties and more particularly similar specific areas (mean molecular area in the more condensed state of the monolayer). The large difference in specific area between the two compounds could then reflect some unusual conformation of the glycerol backbone in **2c**, and computational modeling would be necessary to get a deeper insight in the packing properties of the compounds. The measured value for specific area of lipid **1c** at the air/water interface was close to twice that of a section of crystalline fluoroalkyl chain (2 × 24.9 Å^2) (*48*). That indicates that compound **1c** collapsed in a highly ordered liquid phase, as no phase transition could be detected through isothermal monolayer compression. The same held for compounds **2a**−**c** though slightly higher A_s values were recorded. For comparison, the A_s value for DOTAP is 50 Å^2, which is larger than twice the section of alkyl chain (16 Å^2) and accounts for a higher free volume within the lipidic phase. That may be correlated to susceptibility to detergents (vide infra).

Table 1. Semiquantitative Evaluation of Lipoplex Stability in the Presence of Surface Active Compounds

	detergent resistance (mM)[b]				
cationic lipid[a]	SDS	NaCh	Tween 80	CHAPS	CTAB
DOTAP	(1)[c]	10	<1	50	1
1c	1 (10)[c]	100	150	150	150
2a	1	10	<1	10 (50)[c]	150
2b	1	10	<1	10	1 (150)[c]
2c	1	50 (150)[c]	1	50	10 (150)[c]
DOTAP/DOPE[d]	1	10	<1	10	1
1c/DOPE[d]	(1)[c]	100	<1	150	1 (150)[c]
2a/DOPE[d]	1	10	<1	50	1 (150)[c]
2b/DOPE[d]	1	10	<1	10	1
2c/DOPE[d]	1	150	<1	50	150

[a] Lipoplexes were prepared as indicated in the experimental section, with N/P = 3. [b] Detergent was added to reach 1, 10, 50, 100, and 150 mM final concentration (concentration of cationic lipid: 0.18 mM). Concentration reported corresponds to the higher one tested at which lipoplexes were 100% preserved. [c] At that detergent concentration, only partial release of plasmid DNA (±10%) is observed. [d] Cationic lipid/DOPE was in the ratio 1/2.

Characterization of Lipid−DNA Complexes. The ability of the fluorinated cationic lipids to interact electrostatically with DNA was studied by conventional electrophoretic DNA retardation assays in which a full retard of plasmid DNA is observed at a lipid/DNA phosphate ratio (N/P) corresponding to electroneutrality. As expected, all the fluorinated lipids led to a full complexation of the plasmid at N/P 1, indicating that the fluorous block does not impact on the ability of the cationic headgroup to interact with the DNA phosphate groups (data not shown).

Cationic lipoplexes destruction may occur essentially through ionic competition or detergence of the lipidic phase (*49*−*52*). Both cause DNA to dissociate from the complex and release nucleic acids then being vulnerable to nuclease attack. We therefore evaluated the resistance of fluorinated lipoplexes in the presence of various surfactants: anionic, cationic, or neutral ones (Table 1). Plasmid DNA was first condensed with fluorinated lipids **1c** and **2a**−**c** at N/P 3 to form overall cationic complexes with putative transfection activity. Complexes were then treated with increasing amounts of surfactant. The resulting mixtures were homogenized and analyzed by gel electrophoresis to allow semiquantitative evaluation of lipoplex destruction by monitoring DNA release. Results indicate that, regardless of the cationic lipid, lipoplexes hardly resisted to anionic surfactant SDS. This is consistent with an effect mediated by electrostatic competition between sulfate and phosphate for the cationic lipid headgroup, coupled to high lipid detergence by the alkyl chain. Sodium cholate (NaCh), the other anionic detergent tested, is a poorer competitor of phosphate for ammonium complexation (due to a higher pk_a value) and lipoplexes resisted better. Integrity of DOTAP, **2a**, and **2b** lipoplexes was respected to up to 10 mM cholate. Remarkably, the higher fluorinated homologues **1c** and **2c** were revealed to form highly stable complexes with DNA that resisted 150 mM sodium cholate, suggesting that these lipids may be impermeable to the hydrophobic cholyl moiety. A similar but less pronounced effect was reported by Vierling's group with FHP lipospermine−DNA complexes that resist a taurocholate concentration twice as high as the corresponding nonfluorinated lipoplex (17.5 mM vs. 10 mM) (*13, 16*). Next, and to minimize destabilization by anionic contribution, we evaluated the stability of DNA lipoplexes in the presence of neutral (Tween 80), zwitterionic (CHAPS), and cationic (CTAB) detergents. A little surprisingly, neutral surfactant Tween 80 showed drastic effects on lipoplex stability. DOTAP-DNA complexes did not resist much and were fully destroyed even at the lowest detergent concentration investigated (1 mM). The same held for **2a**−DNA and **2b**−DNA complexes. As already observed with SDS and sodium cholate, cationic lipids **1c** and **2c** were revealed to be more potent, likely due to their greater hydrophobic character (longer fatty acid chains, higher fluorine content). The zwitterionic detergent CHAPS, though neutral, exhibited very different behavior and destabilized lipoplexes poorly. Most of them survived to 10−50 mM, and once again, **1c**−DNA and **2c**−DNA were characterized by higher stability. Lastly, cationic CTAB proved highly detrimental to DOTAP-DNA complexes but affected fluorinated lipoplexes only weakly. Thus, even if a superficial positive charge of the lipoplexes may shield the particles from other cations, 1 mM CTAB revealed enough to fully destabilize DOTAP-DNA complexes, whereas fluorinated lipoplexes mostly resisted up to 150 mM. It is interesting to note that the stability of **1c**−DNA complexes was invariably higher than for **2c**−DNA, especially in the presence of Tween 80. The difference in lipoplex stability between **1c**−DNA and **2c**−DNA probably cannot be explained just considering fluorine content (24 vs 22 F atoms). More likely, chain conformation imposed by unsaturation in **2c** thwarts close packing of helical fluorinated chains (vide supra), resulting in higher free volume in the external lipid layer of the lipoplex and consequently higher permeability. Such a permeability increase facilitates access of detergent molecules to the condensed DNA core and favors lipoplex destruction.

As DOPE was used as the lipid helper (vide infra), we characterized the mixed lipoplexes (lipid/DOPE/DNA) and looked at their stability. Considering DOTAP and DOTAP/DOPE lipoplexes, no significant difference was observed in detergent resistance. As DOTAP and DOPE are more likely similarly solubilized by detergents, behavior of a mixed lipoplex was indeed expected to be the same as for the pure DOTAP lipoplex. For mixed fluorinated lipid/DOPE lipoplexes, we could possibly anticipate some differences. Actually, no such deviation was observed, and DNA condensed particles resisted detergents in the same concentration range as with pure fluorinated cationic lipids. Whether DOPE remained associated to the lipoplexes or was depleted via micelle formation was not checked.

From that series of experiments, we conclude that the fluorous segments within the lipid structure indeed increase the stability of the lipoplexes in the presence of surfactants, and the longer the fatty acid chains and the higher the fluorine content, the more stable the lipoplexes are.

It is well-known that the size of transfecting complexes is an important parameter that impacts the cell entry mechanism of the particles. Most cationic particles establish electrostatic interaction with proteoglycans and enter adherent cells entrapped into endosomal vesicles. Escape from these recycling compartments occurs only for complexes incorporating molecules with endosomolytic activity and is the result of the specific activity of such endosomolytic element(s) (*53, 54*). Efficiency of current and multipurpose lipid or polymeric nucleic acid delivery agents seems to rely on the preparation of "large" complexes with diameter in the 100−1000 nm range for optimal cell anchorage and subsequent intracellular routing activity. Such complexes offer excellent transfection efficiency for adherent cell lines for which multitissular organization does not impede accessibility of large particles to cell surface receptors (*55*−*60*). Even if particles smaller than 100 nm might be more suited for in vivo administration, their efficiency seems to rely on the use of endosomolytic agents with high activity (*61*), and large delivery systems have shown some effectiveness upon regional administration (*62, 63*).

Classically, optimal gene transfection efficacies are obtained when DNA lipoplexes are prepared in 150 mM NaCl at N/P charge ratio of 3 and above. In these conditions, DNA plasmids assemble with cationic lipids to form cationic aggregates of optimal size. We therefore carried out dynamic light scattering

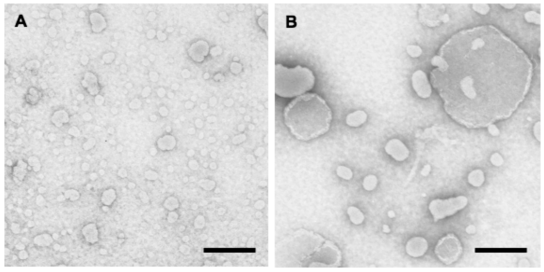


Figure 2. TEM images of **1c**/DOPE (A) and **1c**/DOPE-DNA (B). Figure shows negatively stained samples (uranyle acetate) obtained with fluorinated lipid/DOPE molar ratio 1/2 and total lipid concentration of 1.16 mM (A) and 0.42 mM (B, see Experimental Procedures). Charge ratio N/P is 3. Scale bars correspond to 200 nm.

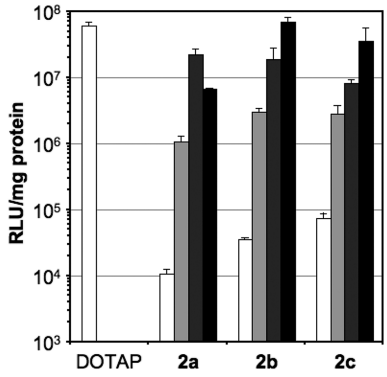


Figure 3. Expression of luciferase in HepG2 cells treated with DNA complexes with DOTAP and fluorinated **2a**−**c** with various compositions. Lipids were mixed with DOPE in different molar ratios (lipid/DOPE molar ratio: white, 1/0; light gray, 1/1; deep gray, 1/2; black, 1/3). Lipoplexes were prepared in 150 mM NaCl, 10 mM Hepes, pH 7.5, at charge ratio N/P 4.

measurements for evaluating the size of lipoplexes DOTAP/DOPE-DNA, **1c**/DOPE-DNA, and **2c**/DOPE-DNA, assembled in 150 mM NaCl (cationic lipid/DOPE 1/2, N/P 3). In these conditions, DOTAP-based lipoplexes show a mean particle size of 140 nm. Lipoplexes prepared from **1c** and **2c** appear as larger particles with mean diameter of 250 and 400 nm, respectively. Lipoplexes were analyzed as well by electron microscopy. No significant difference was observed between DOTAP and fluorinated particles, which presented similar globular morphology, except that negative staining of the latter was more difficult and provided lower contrast (Figure 2). That last point is consistent with some exacerbation of the peripheral hydrophobicity of the particles due to the presence of fluorous segments very close to the surface exposed to the hydrophilic staining material (UO_2^{2+}).

Transfection Biology. The transfection activity of fluorinated lipoplexes was first evaluated on HepG2 cells. The activity of different formulations of lipids **2a**−**c** is shown in Figure 3. Experiments were carried out at a charge ratio commonly used with cationic lipids (N/P 4), and DOTAP was used as a control. Lipoplexes were classically incubated with cells in serum-free conditions for a 4 h period before addition of serum-complemented culture medium. The expression level of luciferase was low for **2a**−**c**/DNA complexes. Transfection efficiency was, however, drastically increased upon addition of the helper lipid DOPE. With **2**/DOPE molar ratio between 1/2 and 1/3, transfection efficacy of most fluorinated lipoplexes was comparable to that of DOTAP. Further optimization of the charge ratio (in the N/P range 1−6) indicated that the transfection efficacy reached a plateau for N/P 3−4, a behavior that was already noticed for classical cationic lipids (*64*).

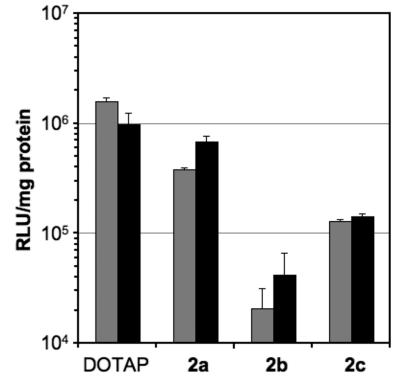


Figure 4. Expression of luciferase in HepG2 cells treated with DNA complexes with cationic lipids DOTAP (N/P 4) and **2a**−**c**/DOPE (1/1 molar ratio, N/P 3) in serum-free conditions (gray) or in the presence of 25% FCS (black).

One serious shortcoming of gene delivery mediated by cationic lipids is that the transfection mostly requires serum-free conditions to be effective. Serum proteins are negatively charged and interact with cationic liposomes, thereby competing with DNA for cationic lipids (*50*). Untimely DNA decomplexation even to a low extent usually results in transfection inhibition. To investigate the effect of serum, we then tested transfection activity of our fluorinated lipoplexes by direct addition of DNA complexes to cells in serum-free conditions and in the presence of 25% fetal calf serum (Figure 4). Lipoplexes were incubated with cells for a 4 h period before the cell culture medium, which contained either too much or too little FCS for optimal cell growth, was replaced with DMEM containing 10% serum. Luciferase expression was monitored 24 h later. Whereas DOTAP transfection efficiency was invariably reduced by 30−40% in the presence of serum, that of fluorinated lipoplexes was preserved or even increased. This might be tentatively interpreted considering that lower adsorption of plasma elements onto fluorinated particles would not significantly provoke their degradation (as is the case with DOTAP particles) but rather would induce or amplify sedimentation, which would result in improved cellular internalization.

Having shown that the transfection activity of fluorinated lipoplexes is not impaired by a high concentration of FCS, we simplified the gene transfection procedure. The following experiments were conducted on the BHK-21 cell line, which is more convenient to culture and to handle than HepG2. DNA lipoplexes were directly added to cells in culture medium containing 10% serum, and transfection/toxicity activities were measured 24 h later without any medium change. The effect of chain unsaturation in fluorinated cationic lipids is presented in Figure 5. At optimal charge ratio N/P 3, DOTAP/DOPE, **1c**/DOPE, and **2c**/DOPE (in a 1/2 molar ratio) roughly displayed similar transfection efficiency in BHK-21 cells. An increase of charge ratio provoked a rapid loss of transfection activity with saturated lipid **1c**, whereas activity remained much better preserved with unsaturated compound **2c** or DOTAP.

The efficiency of DOPE as a helper lipid in fluorinated lipoplexes was somehow uncertain, as DOPE and fluorinated cationic lipids are supposed to segregate. In fact, DOPE was revealed to be even more potent when mixed with compound **1c** than when mixed with DOTAP (Figure 6). DOPE increased DOTAP transfection efficiency by a 40-fold factor, whereas that of **1c** gained up to 4 orders of magnitude. From there, the evaluation of a HFP fluorinated analogue of DOPE appeared inevitable to us. We assumed that the mixture of **1c** and fluorinated DOPE analogue **3** would exhibit different aggregation properties than **1c** and DOPE. Properties of the resulting lipoplexes should thus differ from and, possibly, outdo those

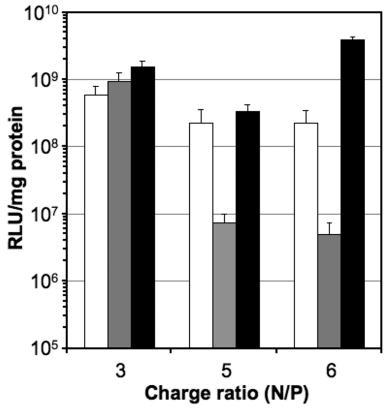


Figure 5. Expression of luciferase in BHK-21 cells treated with DNA complexes with DOTAP (white) and saturated and unsaturated fluorinated lipids **1c** and **2c** (gray and black, respectively). Cationic lipids were mixed in a 1/2 molar ratio with DOPE. Lipoplexes were prepared in 150 mM NaCl, 10 mM Hepes, pH 7.5. Transfection was performed in cell culture medium containing 10% FCS.

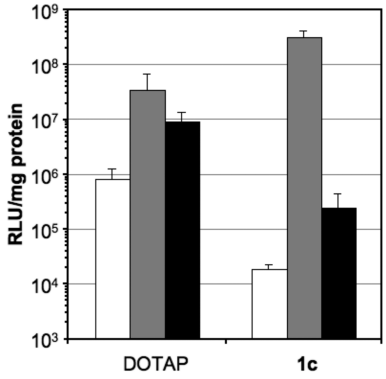


Figure 6. Transfection efficiency of DOTAP and fluorinated cationic lipid **1c**, pure (white) and mixed with DOPE (gray) and its fluorinated analogue **3** (black) in a 1/2 molar ratio. Lipoplexes were prepared at charge ratio N/P 3, in 150 mM NaCl, 10 mM Hepes, pH 7.5. BHK-21 cells were transfected in the presence of 10% FCS.

of **1c**/DOPE-DNA particles. Results did not completely meet our expectations, as **3** only improved transfection efficiency of **1c** by 1 order of magnitude. However, the same quantitative effect was observed with DOTAP and a similar effect was observed with FHP DOPE analogues (*18, 19*). Although they are surprising at first sight, these results may be rationalized and attributed to the introduction of some lipid heterogeneity in membranes upon fusion/internalization of liposomes/lipoplexes and possibly modifying the internalization pathway and intracellular trafficking. On one hand, mixed lipoplexes, i.e., those containing both fluorinated and nonfluorinated lipids (DOTAP/**3**-DNA and **1c**/DOPE-DNA), most likely exhibit a heterogeneous lipid distribution resulting from partial segregation of the fluorinated and nonfluorinated components. Lipoplexes may thus display discrete nonfluorinated lipid domains at their periphery. These domains are brought into close contact with the negatively charged membrane of cells through electrostatic interactions and may trigger the fusion/internalization process. Incorporation of some fluorinated lipid components into the cell membrane is supposed to introduce additional heterogeneity in the lipid bilayer, possibly resulting in endosome destabilization. On the other hand, fusion of homogeneous fully fluorinated lipoplexes (**1c/3**−DNA) with cell membrane is made difficult due to the low miscibility of fluorocarbon−hydrocarbon mixture. Though contact between the fluorinated cationic surface of lipoplexes and the negatively charged cell membrane may be tight, most probably it does not suffice to provoke efficient

Table 2. Mean Size of Lipoplexes in Different Media as Determined by Light Scattering Experiments[a]

	DOTAP/DOPE-DNA (nm)[b]		**1c**/DOPE-DNA (nm)[b]	
incubation medium	− FCS	+ FCS	− FCS	+ FCS
water[c]	100 (0.1)	180 (0.22)	731 (0.25)	1635 (1.00)
150 mM NaCl[c]	140 (0.08)	225 (0.24)	255 (0.38)	400 (0.68)
5% glucose[c]	95 (0.11)	175 (0.22)	1200 (0.55)	1015 (0.47)
MEM	1400 (0.2)	2080 (0.93)	675 (0.35)	680 (0.93)

[a] Complexes were prepared as described for gene transfection assays (cationic lipid/DOPE 1/2, charge ratio N/P 3) and incubated in the absence or presence of 10% FCS. [b] Polydispersity index is indicated into brackets. [c] Solution contains 10 mM Hepes, pH 7.5.

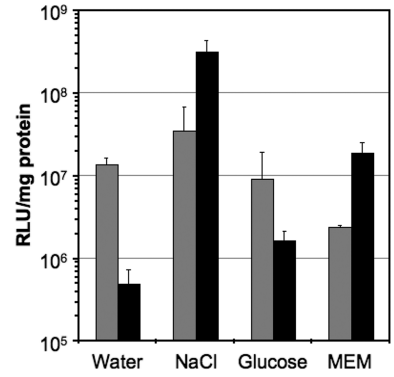


Figure 7. Transfection efficiency of DOTAP/DOPE (gray) and **1c**/DOPE (black) in a 1/2 molar ratio as a function of medium used for lipoplex preparation (for composition, see text). Lipoplexes were prepared at charge ratio N/P 3, and BHK-21 cells were transfected in the presence of 10% FCS.

lipid exchange from lipoplexes to lipid bilayer of the endosomes. Consequent rupture of the endosome and DNA escape are therefore reduced.

Transfection with cationic lipids is known to be significantly affected by the procedure and medium used for the preparation of lipoplexes. Differences probably may be interpreted by considering the size of the resulting complexes, but not exclusively. Therefore, we investigated the influence of the lipoplex preparation medium on the size of the particles (Table 2) and on the level of luciferase expression in BHK-21 cells (Figure 7). Dried lipid mixtures (DOTAP/DOPE and **1c**/DOPE in 1/2 molar ratio) were hydrated in different solutions ((i) 10 mM Hepes buffer, pH 7.5; (ii) 150 mM NaCl, 10 mM Hepes, pH 7.5; (iii) 5% glucose, 10 mM Hepes, pH 7.5; (iv) MEM) before DNA condensation at N/P 3. The size and gene delivery efficacy of these complexes were then evaluated. With the DOTAP-based lipoplex, the mean particle size was in the 100−255 nm range except in MEM (>1 μm). In the presence of 10% FCS, size of the lipoplexes was increased by ca. 50−80%, and the larger particles (obtained in MEM) showed poor transfection efficacy, likely because micrometric size impairs particle endocytosis. The same was observed with fluorinated lipoplexes unless smaller particles were produced in 150 mM NaCl and larger in water or glucose 5%. The fluorinated segment, however, has a significant effect on the assembly process, and **1c**/DOPE-DNA complexes formed in 150 mM NaCl were roughly twice as large as DOTAP/DOPE-DNA complexes. Obtainment of these larger complexes resulted in enhanced transfection activity for reasons discussed earlier. It is important to note, in identical conditions, that the size of **2c**/DOPE-DNA complexes was 690 and 400 nm with and without FCS, respectively, indicating that unsaturation does not significantly alter the assembly process. The larger size of lipoplexes and associated transfection efficacies measured when they were prepared in the medium with higher ionic strength (150 mM NaCl, 10 mM Hepes, pH 7.5) may be explained by considering

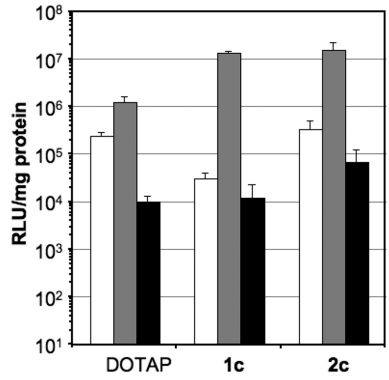


Figure 8. Expression of luciferase in cells treated with pEGFP-Luc complexed by DOTAP/DOPE, **1c**/DOPE, and **2c**/DOPE (1/2 molar ratio) at N/P 3. Three cell lines were investigated: HepG2 (white), BHK-21 (gray), and HeLa (black). Transfection experiments were carried out in the presence of 10% FCS.

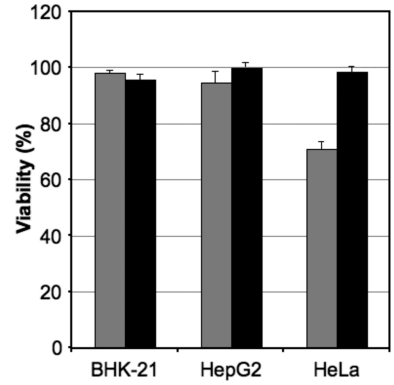


Figure 9. Cytotoxicity of DOTAP/DOPE-DNA (gray) and **1c**/DOPE-DNA (black) on different cell lines. Experiments were conducted at N/P 3 in culture medium supplemented with 10% FCS.

that increasing ionic strength exacerbates hydrophobic interactions and shields electrostatic repulsions. Such conditions allowed kinetically formed and thermodynamically unstable cationic lipoplexes to equilibrate toward lower-energy systems (aggregation). As two fluoroalkyl segments increase hydrophobicity in **1c**, exacerbation of cohesive interactions by ionic strength is stronger than with DOTAP-based particles, provoking a stabilizing effect on the lipid phase and micellar aggregation of higher magnitude. The behavior of **1c**/DOPE-DNA in low ionic strength media is interesting. The absence of salts is supposed to stabilize the colloidal solution to small sizes by favoring electrostatic repulsions (*56*) and large sizes were observed. One possible explanation is that hydration of the fluorinated lipid is much more sensitive to the medium composition than that of classical lipids. When NaCl was substituted for glucose, transfection efficiency was close to that observed when lipoplexes were prepared in 10 mM Hepes buffer pH 7.5, which is consistent with the previous results. The negative influence of MEM on transfection efficiency might be interpreted in terms of both equilibration between phosphate (DNA) and carboxylate (MEM amino acids, 5−6 mM) for ammonium complexation and possible insertion of the side chain of the more hydrophobic amino acids into the lipid phase of the particles. That could account for both destabilization of the DOTAP-based particles and stabilization of **1c**-based ones, the latter being more resistant to both phenomena (vide supra) and taking advantage of ionic strength increase to a greater extent.

Transfection efficiency mediated by nonviral vectors is strongly affected by the cell type. We investigated three different cell lines: HepG2, BHK-21, and HeLa (respectively hepatoma, kidney, and epithelial cells). These cells were treated with mixed **1c**/DOPE, **2c**/DOPE, or DOTAP/DOPE lipoplexes in the presence of FCS. Results are shown in Figure 8. In the BHK-21 cell line, fluorinated lipoplexes appeared more potent than DOTAP-based lipoplex by 1 to 2 orders of magnitude. In HeLa cells, **2c**/DOPE proved superior by almost an order of magnitude to the other formulations. Formulations **2c**/DOPE and DOTAP/DOPE displayed same transfection efficiency in hepatoma cells HepG2, whereas **1c**/DOPE appeared less effective.

To complete our evaluation of fluorinated lipoplexes, we assessed their cytotoxicity as a function of charge ratio and cell type. In a first set of experiments, BHK-21 cells were treated with **1c**/DOPE-DNA or **2c**/DOPE-DNA complexes at various charge ratios (cationic lipid/DOPE 1/2, molar ratio) and using the same amount of DNA plasmid (2 μg) when compared to gene transfection assays (see Supporting Information). Cell viability generally tends to decrease with increasing N/P ratio. That may be explained by considering that positively charged lipoplexes strongly interact with negatively charged cell membranes through electrostatic forces, with potent negative impact on cell membrane integrity and therefore cell homeostasis. In the case of DOTAP lipoplexes, 36% cytotoxicity was observed at N/P 6, and a similar tendency was observed with fluorinated lipoplexes, although these complexes exhibited a limited effect, with cytotoxicity not exceeding 10−20% at N/P 6. In another set of experiments, cytotoxicity of **1c**/DOPE-DNA was evaluated at N/P 3 on three different cell lines, BHK-21, HepG2, and HeLa (Figure 9). No real difference was observed between DOTAP and fluorinated lipoplexes, except for the HeLa cell line. In this cell line, whereas DOTAP lipoplexes provoked 30% cytotoxicity, viability was fully maintained in the presence of **1c**/DOPE-DNA complexes.

CONCLUSIONS

Although a great number of cationic lipids have been designed and evaluated as gene delivery systems, there is still a need for improvement in the design of synthetic lipids for gene transfer. Novel cationic lipids with triblock structure were designed with the purpose of achieving optimal condensation of DNA and enhancing transfection performances. Cationic lipids displaying a fluorous moiety close to their ammonium headgroup were prepared. Self-assembly properties of these cationic lipids were evaluated. They revealed efficient DNA condensing agents, and the resulting lipoplexes displayed enhanced resistance to amphiphilic agents. Transfection data obtained from the fluorinated vectors, alone or as a mixture with different helper lipids, on different cell lines and at various charge ratio revealed in vitro activities that are comparable to that of parent DOTAP, with similar or lower associated toxicity. Due to the fluorous segments within the lipid structure, lipoplexes displayed improved stability, and gene delivery appeared to be regardless of the presence of serum. On the other hand, in vitro transfection efficiency appeared to be very sensitive to the preparation protocol when compared to DOTAP-based lipoplexes. This work highlights the very atypical properties of these fluorinated triblock cationic lipids. Although their in vitro use did not lead to a significant improvement of transfection efficiency, this result does not prejudge their behavior in vivo, as many additional variables, and especially resilience of delivery systems, may induce changes in the behavior of lipoplexes in vivo. Effective impermeabilization of the lipid phase, combined with a preserved ability to deliver genes across endosomal membrane, make these molecules potent building blocks for the preparation of novel gene delivery systems with improved stability and resilience in an in vivo context. In vivo evaluation is currently underway to establish the utility of these new vectors in gene therapy.

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Supporting Information Available: Characterization of compounds; isothermal pressure−area curves, values for specific area A_s, lift-off area A_{lo}, and collapse pressure π_c for compounds **1c** and **2a**−**c**; additional cytotoxicity data for compounds **1c** and **2c**. This material is available free of charge via the Internet at http://pubs.acs.org.

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