Biomaterials 31 (2010) 4781–4788

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Nucleic acid transfer with hemifluorinated polycationic lipids

Emmanuel Klein [a], Christian Leborgne [b], Miahala Ciobanu [a], Jérôme Klein [a], Benoît Frisch [a], Françoise Pons [a], Guy Zuber [a], Daniel Scherman [c], Antoine Kichler [b,c], Luc Lebeau [a,*]

[a] Laboratoire de Conception et Application de Molécules Bioactives, UMR 7199 CNRS-Université de Strasbourg, Faculté de Pharmacie, 74 route du Rhin, BP 60024, 67401 Illkirch Cedex, France
[b] Généthon, 1 rue de l'Internationale, BP 60, 91002 Évry, France
[c] Laboratoire de Pharmacologie Chimique et Génétique U640 INSERM – UMR 8151 CNRS, Faculté de Sciences Pharmaceutiques et Biologiques, 75270 Paris, France

ARTICLE INFO

Article history:
Received 13 January 2010
Accepted 17 February 2010
Available online 29 March 2010

Keywords:
Fluorocarbon
Gene transfer
Lipid
Self-assembly
DNA
siRNA

ABSTRACT

In this study, the ability of synthetic fluorinated lipospermines to bind DNA and siRNA was investigated and the transfection efficiency and toxicity of the resulting lipoplexes in cell lines were evaluated. Three lipopolyamines displaying fluorous tags close to their cationic polar head ("HFP" polyamines) were synthesized. Their ability to condense pDNA and siRNA, and to form nanoparticles were characterized. Lipoplex stability was investigated in the presence of different surface active compounds and was shown to be significantly improved due to the presence of the fluorous tags. Transfection efficiencies were studied in HepG2 and 911 cell lines, and compared to that of DOGS, DOTAP, and Lipofectamine™ 2000. Also, the ability of these compounds to deliver nucleic acids into cells in the presence of high concentration of serum was quantified. By incorporating two fluorous tags in the direct vicinity of the polycationic head group of the lipospermines, we show efficient pDNA and siRNA formulation, and delivery to cultured cells. Fluorinated lipoplexes exhibit improved stability in the presence of amphiphilic compounds and retain high transfection efficiency in the presence of 50–75% serum. These results demonstrate that lipospermines displaying fluorous tags close to their cationic polar head bind to and deliver pDNA and siRNA with high cell viability in different cell lines. They are efficient non-viral vectors that exhibit remarkable serum compatibility.

1. Introduction

Gene therapy relies on devising vectors able to promote efficient and durable expression of selected genetic material inside a target cell. Among vectors, engineered viruses offer high efficiency that stay unmatched by any other vector system so far. However significant problems still are associated with viral gene therapy that are inherent in the very nature of the vehicles (limited payload of DNA, immunogenic reactions, insertional mutagenesis, and sometimes fatal toxicity) [1,2]. For these reasons non-viral vectors, especially cationic lipids, do present attractive features that did not fade over the past two decades although their efficiency still remains far beyond that of viral vectors. As a matter of fact, they do not present limitation in the payload of nucleic acid and, comparatively, display very few problems associated with their production, purification and immunogenicity. Consequently, hundreds of cationic lipids have been developed so far, most of which being active *in vitro* but practically devoided of potency *in vivo* [3–8]. Thus much progress still is required for non-viral gene therapy becoming an efficient medicine for the treatment of inherited diseases and cancer.

The key requirements for gene delivery are the transport of negatively charged genetic material through the cell membrane, and ultimately to the nucleus. Discovered during the past decade, RNA interference (RNAi) is an essential biological process that may be diverted to provide a powerful experimental tool with the potential to be used in therapeutic development. RNAi is a potent and specific gene silencing event in which a small interfering RNA (siRNA) degrades mRNA target in a temporally and spatially regulated manner [9]. As the site of action of siRNA is in the cytosol, there is no requirement to enter the nucleus for controlling the expression of target genes. On the other hand, siRNA lipoplex must efficiently afford protection from the nuclease activity which otherwise results in fast degradation of nucleic acid with no chance of a therapeutic endpoint. The technologies developed for delivery of nucleic acids such as plasmid DNA have paved the way to rapid progress for *in vivo* delivery of siRNA. However, there is no immediate correlation between the efficiency of a vector used for DNA

* Corresponding author. Tel.: +33 (0)3 68 85 43 03; fax: +33 (0)3 68 85 43 06.
E-mail address: llebeau@unistra.fr (L. Lebeau).

0142-9612/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2010.02.047

and siRNA delivery, and structure–activity studies still are required for non-viral vectors in siRNA delivery [10–13].

Optimum transfection efficacy invariably requires excess of cationic lipid for full nucleic acid condensation and protection from nucleases. The net positive charge displayed by transfection particles facilitates absorptive endocytosis but makes them especially vulnerable in serum, mostly due to their interaction with negatively charged serum components. Among the strategies developed to improve the stability of lipoplexes, one is involving the introduction of a fluorous tag in the structure of the cationic lipid. Fluorine increases the hydrophobicity of the amphiphilic compound and, consequently, the stability of its self-assembly so it prevents the lipoplex from degradation to a larger extent than conventional cationic lipids and finally improves transfection efficacy [14–20]. For synthetic convenience, the fluorous tag was introduced at the extremity of the fatty chains, *i.e.* in the region of the molecule located at the opposite of its polar head. These lipids are referred to as "FHP" lipids (a fluoroalkyl moiety-F is separated by an alkyl segment-H from to the polar head-P). More recently it was reported that amphiphilic compounds displaying a fluorous tag in the direct vicinity of the polar head show unusual self-assembly properties. Such compounds, named "HFP" lipids (a terminal alkyl chain-H being separated by a fluoroalkyl segment-F from the polar head-P), self-organize to form supramolecular structures that are remarkably resistant to hydrophobic insertion [21,22] and protein adsorption [23], both of these processes being responsible for the destruction of lipoplexes in biological media.

Our aims are to design and develop non-viral vectors taking advantage of the unique properties of amphiphilic compounds that incorporate a fluorous tag located close to their polar head (HFP lipids). In a previous report we have described HFP monocationic compounds that were shown to constitute very promising transfection systems [24]. Herein we describe a series of HFP lipopolyamines that were designed for the formulation of a circular plasmid DNA encoding for *firefly* luciferase, and of a siRNA. We investigated whether these hemifluorinated HFP polyamines are suitable for transfection of target cells by forming nanoparticles which can efficiently enter cells by endocytosis and provoke intracellular protein synthesis or RNA knock-down.

2. Materials and methods

2.1. Materials

Unless otherwise stated, all chemical reagents were purchased from Alfa Aesar (Bischeim, France) and used without purification. *N*-[1-(2,3-Dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTAP chloride), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), 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). The lipospermine DOGS (Transfectam™) was kindly given by Dr. J.-P. Behr. Lipofectamine™ 2000 was from Invitrogen. Culture media Dulbecco's Modified Eagle Medium Glutamax (DMEM) was 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) were from ATCC-LGC (Molsheim, France). The human embryonic retinoblasts (cell line 911) stably transfected with a luciferase encoding expression vector (911-Luc) were kindly given by Dr. C. Le Bec (Genethon, France). The two small double stranded RNAs: siRNA-Luc and siRNA-GFP were from Sigma–Aldrich.

When required, solvents were dried by standard procedures just before use [25]. Thin layer chromatography (TLC) was performed on precoated plates (0.25 mm Silica Gel 60, F_{254}, Merck, Darmstadt, Germany). Products were purified by chromatography over silica gel (Silica Gel 60, 40–63 μm, Merck, Darmstadt, Germany). NMR spectra were recorded on Bruker 200 MHz and 300 MHz Avance DPX instruments. ^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR chemical shifts δ are reported in ppm relative to their standard reference (^{1}H: $CHCl_3$ at 7.27 ppm, HDO at 4.63 ppm, CD_2HOD at 3.31 ppm, DMSO-D_5 at 2.50 ppm; ^{13}C: $CDCl_3$ at 77.0 ppm, CD_3OD at 49.00 ppm, DMSO-D_6 at 39.52 ppm; ^{19}F: CF_3CH_2OH external at −76.99 ppm). IR spectra were recorded on a FT-IR Nicolet 380 spectrometer in the ATR mode and absorptions values ν are in wave numbers (cm^{-1}). Mass Spectra (MS) were recorded on a Waters Micromass ZQ instrument, using electrospray ionization (ESI) mode. Mass data are reported in mass units (m/z). High-resolution mass spectra (HRMS) were recorded on a Bruker Daltonics MicrOTOF-Q spectrometer in ESI mode.

2.2. Synthesis of hemifluorinated HFP lipospermines

2.2.1. 1,2-Bis(3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorotetradecanoyloxy)-3-{2,5-bis-[(3-aminopropyl)amino]pentanamido}propane, trifluoroacetic acid salt (**1a**)

Compound **5a** (21.4 mg, 13.6 μmol) was stirred in TFA (1 mL) for 3 h at RT. Trifluoroacetic acid was removed under vacuum and the residue was solubilized in *tert*-butyl alcohol (2 mL), and lyophilized to yield **1a** (21.9 mg, 99%) as a white waxy solid. ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1, 200 MHz) δ 5.53-5.25 (m, CO_2CH); 4.53–4.19 (m, CH_2OCO); 4.10–3.63 (m, $CONHCH_2$); 3.60–2.90 (m, $5NCH_2$, $2CF_2CH_2CO_2$, $CHCONH$); 2.26–1.60 (m, $3NCH_2CH_2$, $NCHCH_2$, $2CF_2CH_2CH_2$); 1.70–1.28 (m, $2CF_2CH_2CH_2$, $2CH_3CH_2CH_2CH_2$); 0.91 (t, $J = 6.4$ Hz, $2CH_3$). ^{19}F NMR ($CDCl_3/CD_3OD$ 1:1, 188 MHz) δ −74.7 (s, $4CF_3CO_2$); −110.6 (m, $2CF_2CH_2COO$); −113.3 (m, $2CF_2CH_2CH_2$); −120.7 (m, $2C_2F_4CF_2CF_2C_2F_4$); −121.9 (m, $2CF_2CF_2CH_2COO$); −122.4 (m, $2CF_2CF_2CH_2CH_2$). IR (film) ν 3400; 2929; 2860; 1751; 1670; 1186, 1136. ESI-HRMS for $C_{42}H_{61}F_{24}N_5O_5$: calcd. 586.7217 $[M + 2H]^{2+}$, found 586.7225.

2.2.2. 1,2-Bis(3,3,4,4,5,5,6,6-octafluorohexadecanoyloxy)-3-{2,5-bis[(3-aminopropyl)amino]pentanamido}propane, trifluoroacetic acid salt (**1b**)

Compound **1b** (23.0 mg, 99%) was obtained as a white waxy solid from **5b** following the same procedure as for **1a**. ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1, 200 MHz) δ 5.45–5.28 (m, CO_2CH); 4.50–4.19 (m, CH_2OCO); 4.03–3.61 (m, $CONHCH_2$); 3.53–2.99 (m, $5NCH_2$, $2CF_2CH_2CO_2$, $CHCONH$); 2.26–1.63 (m, $3NCH_2CH_2$, $NCHCH_2$, $2CF_2CH_2CH_2$); 1.60–1.27 (m, $2CF_2CH_2CH_2$, $2CH_3C_7H_{14}$); 0.89 (t, $J = 6.4$ Hz, $2CH_3$). ^{19}F NMR ($CDCl_3/CD_3OD$ 1:1, 188 MHz) δ −74.7 (s, $4CF_3CO_2$); −110.8 (m, $2CF_2CH_2COO$); −113.3 (m, $2CF_2CH_2CH_2$); −122.2 (m, $2CF_2CF_2CF_2CF_2$). IR (film) ν 3416; 2924; 2855; 1753; 1672; 1169, 1129. ESI-HRMS for $C_{46}H_{77}F_{16}N_5O_5$: calcd. 1084.5742 $[M + H]^{+}$, found 1084.5785.

2.2.3. 1,2-Bis(3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorooctadecanoyloxy)-3-{2,5-bis[(3-aminopropyl)amino]pentanamido}propane, trifluoroacetic acid salt (**1c**)

Compound **1c** (25.3 mg, 99%) was obtained as a white waxy solid from **5c** following the same procedure as for **1a**. ^{1}H NMR ($CDCl_3/CD_3OD$ 1:1, 200 MHz) δ 5.50–5.25 (m, CO_2CH); 4.49–4.21 (m, CH_2OCO); 4.05–3.62 (m, $CONHCH_2$); 3.55–3.01 (m, $5NCH_2$, $2CF_2CH_2CO_2$, $CHCONH$); 2.25–1,73 (m, $3NCH_2CH_2$, $NCHCH_2$, $2CF_2CH_2CH_2$); 1.70–1.25 (m, $2CF_2CH_2CH_2$, $2CH_3C_7H_{14}$); 0.89 (t, $J = 6.3$ Hz, $2CH_3$). ^{19}F NMR ($CDCl_3/CD_3OD$ 1:1, 188 MHz) δ −74.7 (s, $4CF_3CO_2$); −110.6 (m, 2 CF_2CH_2COO); −113.2 (m, $2CF_2CH_2CH_2$); −120.6 (m, $2C_2F_4CF_2CF_2C_2F_4$); −121.9 (m, $2CF_2CF_2CH_2COO$); −122.4 (m, $2CF_2CF_2CH_2CH_2$). IR (film) ν 3412; 2922; 2853; 1752; 1667; 1180, 1135. ESI-HRMS for $C_{46}H_{77}F_{16}N_5O_5$: calcd. 642.7843 $[M + 2H]^{2+}$, found 642.7842.

2.2.4. 1,2-Dihydroxy-3-{2,5-bis[(3-tert-butoxycarbonylaminopropyl)tert-butoxycarbonylamino]pentanamido}propane (**3**)

DCC (22.4 mg, 108 μmol), HOBt (14.6 mg, 108 μmol), and (*S*)-2,5-bis[(*tert*-butoxycarbonyl){3-[(*tert*-butoxycarbonyl)amino]propyl}amino]pentanoic acid **2** (53.7 mg, 83 μmol) were stirred for 2 h in anhydrous DMF (1 mL) at RT. Then (±)-3-aminopropane-1,2-diol (7.6 mg, 83 μmol) in DMF (0.5 mL) was added and the reaction mixture was stirred overnight. Solvent was removed under vacuum and the crude residue was purified by flash chromatography over silica gel (AcOEt/MeOH 95:5) to yield compound **3** (56.0 mg, 94%) as a white powder (mixture of diastereomers). TLC R_f 0.4 (AcOEt/MeOH 9:1). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 4.40–4.25 (m, $NCHCONH$); 3.77–3.69 (m, CH_2CHOH CH_2); 3.56–3.01 (m, $5CH_2NBoc$, $NHCH_2CHOH$, CH_2OH); 2.25–1.84 (m, $2NHCH_2CH_2$, $NC_4H_4CH_2CHN$); 1.78–1.56 (m, $NCH_2CH_2CH_2CHN$); 1.46, 1.44, 1.43, and1.42 (4s, $4tC_4H_9$). IR (film) ν 3350; 2975; 2930; 1671. ^{13}C NMR ($CDCl_3$, 100 MHz) δ 169.03 ($CHCONH$); 156.37, 156.25, 156.15, and 156.07 ($4NHCO_2tBu$); 81.24, 80.06, 79.87, and 79.39 ($4CMe_3$); 70.82 ($CHOH$); 63.86 (CH_2OH); 58.66 ($CHCONH$); 46.72, 46.31, 44.54, 43.91, and 43.02 ($2NCH_2CH_2CH_2N$, $NCH_2C_2H_4CHN$); 44.24 ($CHCONHCH_2$); 29.70, 26.09, 25.51, and 24.81 ($2BocNHCH_2CH_2$, $NCHCH_2CH_2CH_2N$). ESI-HRMS for $C_{34}H_{65}N_5O_{11}$: calcd. 720.4753 $[M + H]^{+}$, found 720.4750.

2.2.5. 1,2-Bis(3,3,4,4,5,5,6,6,7,7,8,8-dodecafluorotetradecanoyloxy)-3-{2,5-bis[(3-tert-butoxycarbonylaminopropyl)tert-butoxycarbonylamino]pentanamido}-propane (**5a**)

3,3,4,4,5,5,6,6,7,7,8,8-Dodecafluorotetradecanoic acid **4a** [24] (120 mg, 0.27 mmol) in anhydrous CH_2Cl_2 (2 mL) was stirred at room temperature with oxalyl chloride (120 μL, 1.4 mmol) and a catalytic amount of DMF (1 μL) for 4 h. Volatile was 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 to a mixture of diol **3** (64 mg, 89 μmol), pyridine (22 μL, 270 μmol), and 4-DMAP (22 mg, 180 μmol) in anhydrous THF. The resulting suspension was stirred for 20 h at RT. Saturated aqueous NH_4Cl was added