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Design, Synthesis, and Evaluation of Enhanced DNA Binding New Lipopolythioureas

Jeanne Leblond, Nathalie Mignet, Lucie Leseurre, Céline Largeau, Michel Bessodes, Daniel Scherman, and Jean Herscovici*

Inserm, U640, Paris, F-75006 France, CNRS, UMR8151, Paris, F-75006 France, Ecole Nationale Supérieure de Chimie de Paris, Paris, F-75005, Unité de Pharmacologie Chimique et Génétique, France, Université Paris Descartes, Faculté de Pharmacie Paris, F-75270 France, and Institut de Chimie Moléculaire de Paris Centre, CNRS FR 2769, F-75005. Received April 27, 2006; Revised Manuscript Received July 6, 2006

Nonviral gene delivery is limited to a large extent by the cationic nature of most of the chemical vector. We have shown that lipopolythioureas interact with DNA. However, lipopolythioureas were not very efficient at transfecting cells, probably due to reduced interaction between the noncationic synthetic lipid and the cell membrane. Here, we report that liposomes made from a new thiourea lipid, DPPC, and a lipid bearing an RGD ligand allowed very efficient entry of the lipopolythioureas into integrin $\alpha_v\beta_3$ expressing cells. In addition, we show that a stable interaction between DNA and lipopolythiourea could be obtained with two thiourea groups. Moreover, the addition of a hydrophilic terminus improves the formulation of these new DNA binding agents.

INTRODUCTION

Gene therapy considers the use of nucleic acid as a drug to treat or cure acquired or innate diseases. In most cases, the genetic material must be packed in a delivery system to be protected from nucleases and to cross physiological barriers and/or cell membranes (1). During the past years, efforts have been undertaken to develop physical (2), viral (3, 4), or synthetic DNA delivery processes (5, 6). Because of the polyanionic nature of DNA and the overall negative charge of cell membranes, nonviral vectors are mostly cationic. They have been proven to efficiently transfect cells in culture. However, most of them are ineffective for in vivo DNA delivery, due to interaction with serum, opsonins, or other physiological components (7). To improve synthetic vector efficiency, several attempts have been made to mask (8) or suppress cationic charges (9, 10).

Thiourea derivatives have been intensively investigated in the area of molecular recognition due to their strong hydrogen-bonding activity (11). Moreover, bis-thiourea moieties selectively bind to dihydrogenphosphate via multitopic hydrogen bonding, giving stronger complexes with H_2PO_4^- than any synthetic neutral receptor known so far (12).

On the basis of these data, which suggested that stable lipoplexes could be made from the interaction between thiourea and nucleic acid, we reported that *N*-(2-{3-[2-(2-{3-[2-(3-methylthioureido)ethyl]thioureido}ethylamino)ethyl]thioureido}ethyl)-*N,N'*-ditetradecylsuccinamide (DTTU, Figure 1) formulated with DPPC were able to interact with DNA and formed nanometric particles (13). This new lipopolythiourea lipid (LPT) was composed of a hydrophobic anchor connected to a linear tri-thiourea. Lipopolythiourea lipoplexes made from DTTU exhibited a longer circulation time in the blood than cationic lipid RPR209120 (14) taken as a reference compound. However, this particular lipopolythiourea was difficult to formulate as liposome because of its highly hydrophobic nature and presented very poor transfecting properties. These primary results clearly indicated that the LPT physicochemical properties and perhaps their interaction with cell membranes have to be improved.

A recent study using density functional theory (DFT) calculation (15) predicts that DTTU binds to DNA by six hydrogen bonds with a major contribution of thioureas **II** and **III** that form four of the six bonds (Figure 1). This interaction induces a conformational change with the three thiourea groups, normally in trans from one another in the bare molecule, which moves into the cis position. From these predictions, it could be expected that a headgroup containing two thioureas will lead to a stable complex with nucleic acid. On the other hand, it has been reported that a T-shaped branched structure improved the transfecting properties of cationic polyamine (16). Moreover, such a structure will probably avoid the constraints due to the cis conformation of DTTU.

So, we designed new noncationic lipids with a T-shaped bis-thiourea headgroup. We introduced also hydroxyl and diol termini, which have been proven to have a positive effect on transfection efficiency (17). Finally, we chose two different linkers composed of a benzyl group or a 3-motives-PEG to connect the hydrophobic anchor to the headgroup. These new lipopolythioureas (LPT) have been synthesized and evaluated in terms of DNA compaction and transfection efficiency. As a final point, we investigated RGD-mediated internalization of endothelial cells expressing integrin $\alpha_v\beta_3$.

MATERIALS AND METHODS

All solvents were purchased from Carlo Erba-SDS (Peypin France). Dichloromethane was distilled from P_2O_5 . DMF was dried over 3 Å molecular sieves and pyridine over KOH. All chemicals were purchased from Sigma-Aldrich-Fluka or Lancaster. Tosyl chloride (petroleum ether) and cholesteryl chloroformate (acetone) were recrystallized, and diglycolic anhydride was dehydrated (P_2O_5) prior to use. Other solvents and products were used without further purification. Reactions were monitored by thin-layer chromatography using Merck precoated 60 F254 silica gel plates. Column chromatography was performed over SDS (Peypin France) 35–70 m silicagel according to the method of Still, Khan, and Mitra (18) or using small-column flash chromatography (SFC) according to the following procedure. A plastic syringe was filled with silicagel (product/silicagel 1/5) and connected to vacuum (12 mmHg). The column was

* Email: jean-herscovici@enscp.fr.

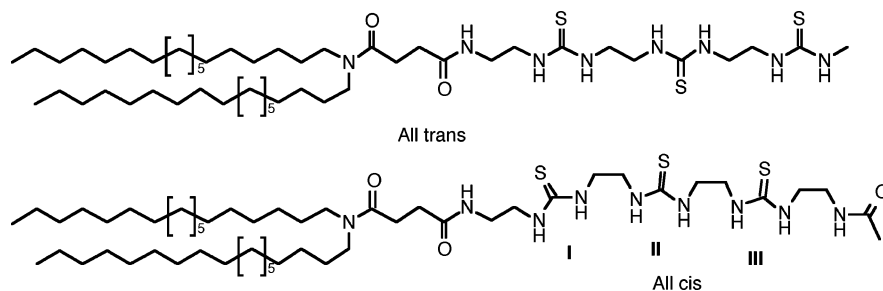


Figure 1. Structures of the linear trithiourea DTTU.

equilibrated with heptane, then the sample dissolved in a minimum of dichloromethane was added to the top. The column was eluted by ten fractions of a heptane/ethyl acetate mixture. The volume was equal to the silicagel volume. For each fraction, the amount of ethyl acetate was increased (10% (v/v) to 100% ethyl acetate in heptane). Inverse-phase solid-phase extraction (SPE) was performed on a Supelco Superclean C8 cartridge using water/acetonitrile/methanol mixture. ^1H and ^{13}C NMR spectra were recorded on a Bruker Advance DRX-300 spectrometer at 300.13 MHz for ^1H and 75.47 MHz for ^{13}C . NMR spectra were processed using XwinNmr (Bruker) or SwaN-MR (19). Mass spectra were carried out on a Shimadzu 2010A LC-MS on ESI mode. High-resolution mass spectra and elemental analysis were carried out by the "Service Central de Micro-analyse du CNRS" Vernaison France. Dipalmytoylphosphatidylcholine (DPPC) and phosphatidylethanolamine lissamine rhodamine were purchased from Aventi Polar Lipids (Alabaster, Alabama).

Synthesis of Lipopolythioureas. *2,3-Dihydroxy-1,4-di-para-toluenesulfonyloxy butane (2)*. A dichloromethane solution (60 mL) of tosyl chloride (15.6 g, 82 mmol) was added dropwise for 5 h to a cooled (-20°C) pyridine solution (16.6 mL, 0.21 mole) of D,L-threitol (5.0 g, 41 mmol). The reaction mixture was stirred overnight at 0°C , then the solvent was evaporated. 1 N HCl (100 mL) was added to the residual oil that was extracted six times with dichloromethane. The combined organic layers were washed with brine, dried (MgSO_4), and then evaporated under reduced pressure. The residual oil was purified by chromatography (heptane/ethyl acetate 6:4) to yield 9.1 g of **2** as a white solid (52%). mp 83°C . ^1H NMR (CDCl_3) δ (ppm) 2.44 (s, 6H, CH_3), 2.95 (m, 2H, OH), 3.88 (m, 2H, CHOH), 4.05 (d, 4H, $J = 5$ Hz, CH_2O), 7.35 (d, 4H, $J = 8.1$ Hz, H_{arom}), 7.88 (d, 4H, $J = 8.1$ Hz, H_{arom}). ^{13}C NMR (CDCl_3) δ (ppm) 21.80 (s, CH_3), 68.62 (s, CHOH), 71.05 (s, CH_2O), 128.96 (s, CH_{arom}), 130.21 (CH_{arom}), 132.68 (s, C_{arom}), 145.81 (s, C_{arom}). MS (ESI, m/z) 431 ($\text{M} + \text{H}^+$), HPLC purity 96% by ELSD. Anal. Calcd for $\text{C}_{18}\text{H}_{22}\text{O}_8\text{S}_2$: C, 50.22; H, 5.15; N, 14.90. Found: C, 50.21; H, 5.35; N, 14.64.

4-[4,5-Bis-(toluene-4-sulfonyloxymethyl)-1,3-dioxolan-2-yl]-benzoic Acid Methyl Ester (4). A solution of diol **2** (1.07 g, 2.5 mmol), *para*-toluene sulfonic acid monohydrate (48 mg, 0.25 mmol), and 4-dimethoxymethylbenzoic acid methyl ester **3** (0.58 g, 2.75 mmol) in dry benzene (30 mL) was heated under reflux with a Dean–Stark trap during 5 h. Then, pyridine was added (0.5 mL), and the reaction mixture was concentrated under reduced pressure. The resulting solid was purified by flash chromatography (heptane/ethyl acetate/ CH_2Cl_2 4:1:1, then 4:4:1) to afford 1.1 g of dioxolane **4** as a white solid (75%). mp 130°C . ^1H NMR (CDCl_3) δ (ppm) 2.45 (s, 6H, CH_3), 3.96 (s, 3H, OCH_3), 4.14 (m, 2H, CHO), 4.22 (m, 4H, CH_2O), 5.82 (s, 1H, CHO_2), 7.28–7.37 (m, 6H, H_{arom}), 7.71–7.77 (m, 4H, H_{arom}), 7.97 (d, 2H, $J = 7.5$ Hz, H_{arom}). ^{13}C NMR (CDCl_3) δ (ppm) 21.80 (s, CH_3), 52.36 (s, OCH_3), 68.51 (d, CH_2O), 75.68 (d, CHO), 103.98 (s, CHO_2), 126.59 (s, CH_{arom}), 128.10 (s, CH_{arom}), 129.74 (s, CH_{arom}), 130.17 (s, CH_{arom}), 131.23 (s,

C_{arom}), 132.14 (s, C_{arom}), 141.12 (s, C_{arom}), 145.61 (s, C_{arom}), 166.71 (s, $\text{C}=\text{O}$). MS (ESI) m/z 577 ($\text{M} + \text{H}^+$), HPLC purity 100% by ELSD. Anal. Calcd for $\text{C}_{27}\text{H}_{28}\text{O}_{10}\text{S}_2$: C, 56.24; H, 4.89; S, 11.12. Found: C, 55.65; H, 5.01; S, 11.44.

4-(4,5-Bis-azidomethyl-1,3-dioxolan-2-yl)benzoic Acid Methyl Ester (5). A solution of ditosyl **4** (1.48 g, 2.5 mmol) and sodium azide (0.97 g, 15.0 mmol) in DMF (25 mL) was heated overnight under reflux. The reaction mixture was evaporated under reduced pressure, then the residual solution was coevaporated several times with toluene. The resulting oil was treated with brine (30 mL) and extracted with ethyl acetate (3×20 mL). The combined organic extracts were dried (MgSO_4) and evaporated under reduced pressure to yield a yellow oil (0.75 g, 94%) that was used without further purification. ^1H NMR (CDCl_3) δ (ppm) 3.49 and 3.60 (m, 4H, CH_2), 3.90 (s, 3H, OCH_3), 4.20 (m, 2H, CHO), 6.03 (s, 1H, CHO_2), 7.57 (d, 2H, $J = 8.1$ Hz, H_{arom}), 8.06 (d, 2H, $J = 8.1$ Hz, H_{arom}). ^{13}C NMR (CDCl_3) δ (ppm) 51.88 (s, CH_2), 52.24 (s, CH_3), 77.54 and 78.36 (2s, CHO), 103.58 (s, CHO_2), 126.56 (s, CH_{arom}), 129.83 (s, CH_{arom}), 131.32 (s, C_{arom}), 141.57 (s, C_{arom}), 166.72 (s, $\text{C}=\text{O}$). MS (ESI, m/z) 378 ($\text{M} + \text{H}^+$), HPLC purity 82% by ELSD.

4-(4,5-Bis-azidomethyl-1,3-dioxolan-2-yl)benzoic Acid (6). Ester **5** was treated with a solution of sodium hydroxide (0.64 g, 16.0 mmol) in a mixture of $\text{H}_2\text{O}/\text{CH}_3\text{OH}/\text{THF}$ (1:1:1 v/v/v, 45 mL). When all the starting material had disappeared, iced 2 N HCl was added to reach pH 3. Then, the reaction mixture was extracted with ethyl acetate (3×20 mL). The combined organic extracts were washed with brine, dried (MgSO_4), and evaporated under reduced pressure to give a yellow oil (0.75 g, 94%) that was used without further purification. ^1H NMR (CDCl_3) δ (ppm) 3.44–3.67 (m, 4H, CH_2), 4.23 (m, 2H, CHO), 6.07 (s, 1H, CHO_2), 7.61 (d, 2H, $J = 8.1$ Hz, H_{arom}), 8.15 (d, 2H, $J = 8.1$ Hz, H_{arom}). ^{13}C NMR (CDCl_3) δ (ppm) 51.80 (s, CH_2), 77.54 and 78.36 (s, CHO), 103.48 (s, CHO_2), 126.71 (s, CH_{arom}), 130.50 (s, CH_{arom}), 131.31 (s, C_{arom}), 142.39 (s, C_{arom}), 171.85 (s, $\text{C}=\text{O}$). MS (ESI, m/z) 302 ($\text{M} + \text{HCOO}^-$). HPLC purity 98% by ELSD.

4-(4,5-Bis-azidomethyl-1,3-dioxolan-2-yl)-N,N-ditetradecylbenzamide (7). *N*-methylmorpholin (0.20 mL, 1.8 mmol) was added to a THF solution (30 mL) of **6** (0.54 g, 1.8 mmol) cooled to -40°C . Then, a THF solution (3 mL) of isobutyl chloroformate (0.23 mL, 1.8 mmol) was added slowly. The reaction mixture was stirred for 1 h at -40°C , then a THF solution (5 mL) of ditetradecylamine (0.68 g, 1.8 mmol) and *N*-methylmorpholin (0.20 mL, 1.8 mmol) was added slowly. The resulting mixture was stirred vigorously for 20 h at RT. Then, ethyl acetate (150 mL) and water (150 mL) were added. The water layer was extracted twice with ethyl acetate; then, the combined organic layers were successively washed twice with 1 N HCl, twice with a saturated solution of NaHCO_3 , twice with brine, and then dried (MgSO_4). The solvent was removed under reduced pressure, then the resulting oil was purified by chromatography (heptane/ethyl acetate 1:4, then 3:7) to yield **7** as a clear oil (0.55 g, 46%). ^1H NMR (CDCl_3) δ (ppm) 0.87 (t,

6H, $J = 6.9$ Hz, CH₃), 1.21–1.46 (m, 44H, –CH₂–), 1.63 (m, 4H, CH₂), 3.14 (t, 2H, $J = 7.2$ Hz, CH₂N), 3.38–3.63 (m, 6H, CH₂N, CH₂N₃), 4.20 (m, 2H, CHO), 6.01 (s, 1H, CHO₂), 7.37 (d, 2H, $J = 7.1$ Hz, CH_{arom}), 7.52 (d, 2H, $J = 7.1$ Hz, CH_{arom}). ¹³C NMR (CDCl₃) δ (ppm) 14.20 (s, CH₃), 22.78 (s, –CH₂–), 27.17 (s, –CH₂–), 28.77 (s, –CH₂–), 29.73 (m, –CH₂–), 32.01 (s, –CH₂–), 44.76 and 49.04 (2s, CH₂N), 51.85 (s, CH₂N₃), 77.80 (d, CHO), 103.82 (s, CHO₂), 126.69 (s, CH_{arom}), 137.57 (s, C_{arom}), 138.76 (s, C_{arom}), 171.09 (s, C=O). MS (ESI, m/z) 697 (M + H)⁺, HPLC purity 99% by ELSD.

4-(4,5-Bis-aminomethyl-1,3-dioxolan-2-yl)-*N,N*-ditetradecylbenzamide (**8**). A 25 mL round-bottom flask equipped with a magnetic stirring bar was filled successively with diazide **7** (0.35 g, 0.5 mmol), ethanol (10 mL), Pd/C (50 mg), and hydrogen. The suspension was stirred for 19 h at RT, then the mixture was filtered through celite to yield after evaporation the diamine **8** (0.31 g, 96%), which was used without further purification. ¹H NMR (CDCl₃) δ (ppm) 0.84 (t, 6H, $J = 6.9$ Hz, CH₃), 1.04–1.46 (m, 44H, –CH₂–), 1.56 (m, 4H, –CH₂–), 2.99–3.40 (m, 8H, CH₂N, CH₂NH₂), 4.24 (m, 2H, CHO), 5.97 (s, 1H, CHO₂), 6.40 (m, 4H, NH₂), 7.28 (d, 2H, $J = 7.1$ Hz, CH_{arom}), 7.55 (d, 2H, $J = 7.1$ Hz, CH_{arom}). ¹³C NMR (CDCl₃) δ (ppm) 14.12 (s, CH₃), 22.71 (s, –CH₂–), 27.25 (s, –CH₂–), 28.82 (s, –CH₂–), 29.73 (m, –CH₂–), 31.97 (s, –CH₂–), 41.73 (d, CH₂NH₂), 45.02 and 49.24 (2s, CH₂–N), 78.15 (d, CHO), 102.87 (s, CHO₂), 126.51 (s, CH_{arom}), 127.31 (s, CH_{arom}), 138.16 (s, C_{arom}), 171.29 (s, C=O). MS (ESI, m/z) 645 (M + H)⁺. HPLC purity 92% by ELSD.

4-(4,5-Bis-isothiocyanatomethyl-1,3-dioxolan-2-yl)-*N,N*-ditetradecylbenzamide (**9**). Diisopropylethylamine (0.26 mL, 1.5 mmol) and carbon disulfide (0.12 mL, 2 mmol) were successively added to a solution of diamine **8** (0.32 g, 0.5 mmol) in dichloromethane (5 mL). The solution was stirred for 90 min, then a solution of tosyl chloride (0.19 g, 1 mmol) in dichloromethane was added dropwise. The mixture was stirred for 20 h at room temperature, diluted with dichloromethane, and washed three times with brine. The organic solution was dried (MgSO₄) and evaporated under vacuum. The crude was purified by chromatography (heptane/ethyl acetate 8:2, 7:3, and 6:4) to yield **9** as a colorless oil (0.24 g, 66%). ¹H NMR (CDCl₃) δ (ppm) 0.84 (t, 6H, $J = 6.9$ Hz, CH₃), 1.04–1.30 (m, 44H, –CH₂–), 1.46 (m, 4H, –CH₂–), 3.15 and 3.46 (m, 4H, CH₂N), 3.80 and 3.90 (m, 4H, CH₂NCS), 4.28 (m, 2H, CHO), 6.06 (s, 1H, CHO₂), 7.39 (d, 2H, $J = 6.5$ Hz, CH_{arom}), 7.53 (d, 2H, $J = 6.5$ Hz, CH_{arom}). ¹³C NMR (CDCl₃) δ (ppm) 14.07 (s, CH₃), 22.71 (s, –CH₂–), 27.17 (s, –CH₂–), 29.04 (s, –CH₂–), 29.73 (m, –CH₂–), 31.93 (s, –CH₂–), 44.97 and 49.19 (s, –CH₂N), 46.59 (CH₂NCS), 77.1 (d, CHO), 104.40 (s, CHO₂), 126.74 (s, CH_{arom}), 132.02 (s, C=S), 138.58 (s, C_{arom}), 138.83 (s, C_{arom}), 171.17 (s, C=O). IR (NaCl): σ (cm⁻¹) 1086 (ν_{C-O-C}), 1465 (δ_{CH_2}), 1629 ($\nu_{C=O}$), 2048 ($\nu_{N=C=S}$), 2842 (ν_{C-H_2}), 2934 (ν_{CH_2}), 3196 ($\nu_{C-H_{arom}}$).

4-{4,5-Bis-[3-(2-hydroxyethyl)thioureidomethyl]-1,3-dioxolan-2-yl}-*N,N*-ditetradecylbenzamide (**10**). Ethanolamine (0.026 mL, 0.4 mmol) was added to a solution of diisothiocyanate **9** (62 mg, 0.09 mmol) in THF (1 mL), then the mixture was stirred for 22 h at room temperature. The solution was concentrated under reduced pressure. The resulting oil was purified by SPE (C-8 100% acetonitrile to 100% methanol) to afford the dithiourea **10** (29.8 mg, 39%) as a colorless oil. ¹H NMR (CDCl₃) δ (ppm) 0.87 (m, 6H, CH₃), 1.25 (m, 44H, –CH₂–), 1.62 (m, 4H, –CH₂–), 3.14 (m, 2H, –CH₂NCO), 3.44–3.78 (m, 14H, –CH₂NCO, CH₂NCS, CH₂OH), 4.16 (m, 2H, CHO), 5.94 (s, 1H, CHO₂), 7.25 (m, 4H, NH), 7.33 (m, 2H, CH_{arom}), 7.52 (m, 2H, CH_{arom}). ¹³C NMR (CDCl₃) δ (ppm) 14.09 (s, CH₃), 22.71 (s, –CH₂–), 27.22 (s, –CH₂–), 28.83 (s, –CH₂–), 29.73 (m, –CH₂–), 31.97 (s, –CH₂–), 45.30 (s, CH₂NCS),

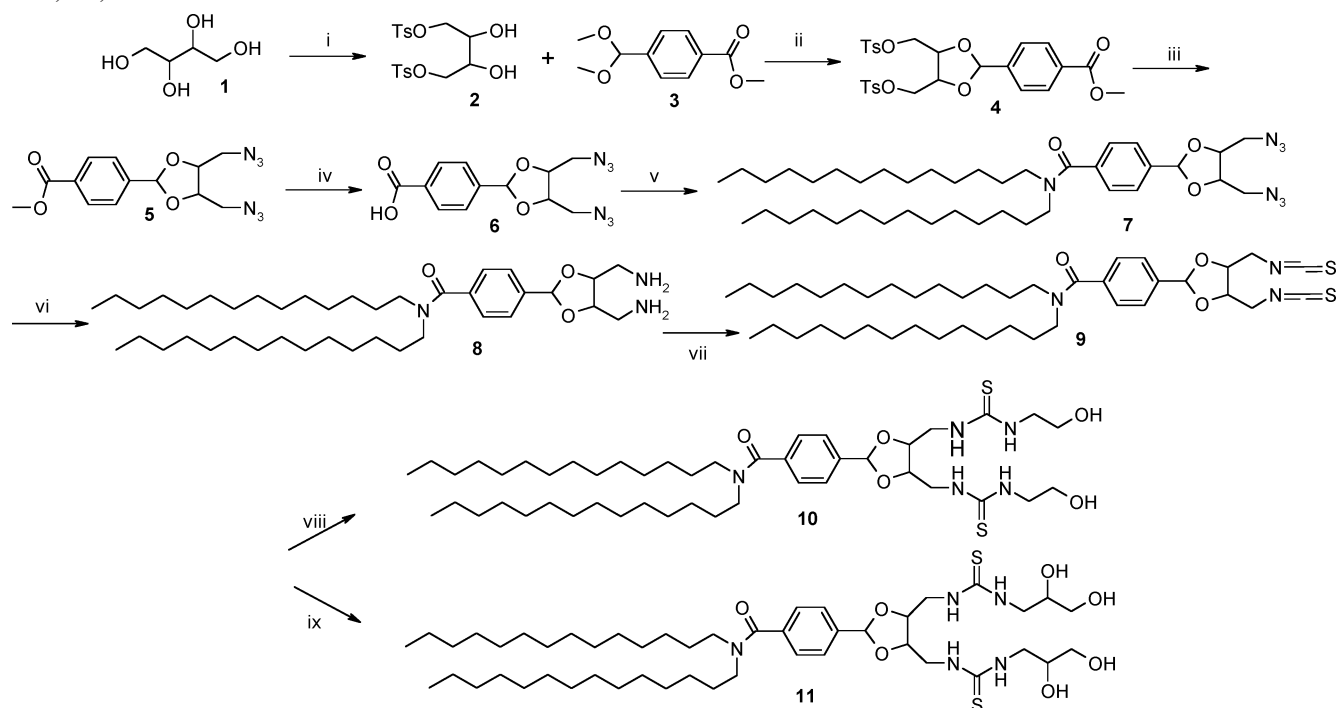
45.80 (s, CH₂NCS), 46.97 and 49.45 (s, CH₂NCO), 61.42 (s, CH₂OH), 78.23 (d, CHO), 102.83 (s, CHO₂), 126.48 (s, CH_{arom}), 127.05 (s, CH_{arom}) 138.14 (s, C_{arom}), 138.80 (s, C_{arom}), 171.71 (s, C=O), 183.35 (s, C=S).

N,N-ditetradecyl-4-(4,5-bis-azidomethyl-1,3-dioxolan-2-yl)-benzamide (**11**). 3-Amino-1,2-propanediol (22 mg, 0.24 mmol) was added to a solution of **9** (41 mg, 0.056 mmol) in dry DMF. The solution was stirred at RT for 20 h then concentrated to dryness. The crude was dissolved in ethyl acetate and washed three times with brine. The organic layer was dried (MgSO₄) and evaporated to dryness. Purification as for **10** gave **11** as a colorless oil (20 mg, 38%). ¹H NMR (CDCl₃) δ (ppm) 0.88 (m, 6H, CH₃), 1.27 (m, 44H, –CH₂–), 1.65 (m, 4H, –CH₂–), 3.16 (m, 2H, –CH₂NCO), 3.35–3.48 (m, 6H, –CH₂NCO, CH₂NCS), 3.47–3.68 (m, 8H, CH₂NCS, CH₂OH), 3.97 (m, 2H, CHOH), 4.16 (m, 2H, CHO), 5.97 (s, 1H, CHO₂), 7.35 (m, 2H, CH_{arom}), 7.55 (m, 2H, CH_{arom}). ¹³C NMR (CDCl₃) δ (ppm) 13.96 (s, CH₃), 22.64 (s, –CH₂–), 27.11 (s, –CH₂–), 28.78 (s, –CH₂–), 29.73 (m, –CH₂–), 31.91 (s, –CH₂–), 45.27 (s, –CH₂NCS), 45.83 (s, –CH₂NCS), 46.91 and 49.44 (s, –CH₂NCO), 63.65 (s, CH₂OH), 70.99 (s, CHOH), 78.33 (d, CHO), 102.88 (s, CHO₂), 126.42 (s, CH_{arom}), 126.86 (s, CH_{arom}), 138.02 (s, C_{arom}), 138.72 (s, C_{arom}), 171.87 (s, C=O), 183.49 (s, C=S). ES-HRMS Calcd. for C₄₈H₈₇N₅O₇NaS₂: 932.5945. Found: 932.5965.

2-(2-{2-[2-(2,2-Diethoxyethoxy)ethoxy]ethoxy}ethoxy)-tetrahydropyran (**13**). Sodium hydride (1.7 g, 43 mmol) was added portionwise to an iced cooled solution of **12** (previously dried by codistillation with THF) in THF (50 mL). The suspension was stirred for 30 min, then bromoacetaldehyde diethylacetal (2.3 mL, 17 mmol) was added dropwise. The mixture was stirred for 3 h at RT, then heated overnight at 60 °C. The reaction mixture was cooled with an ice bath, then the reaction was stopped by the addition of water (2 mL). Most of the solvent was removed by evaporation under reduced pressure, then water and dichloromethane were added. The water layer was extracted three times with dichloromethane, then the combined organic layer was washed with brine, dried (MgSO₄), and concentrated under reduced pressure. The residual oil was purified by chromatography (heptane/ethyl acetate 1:1) to afford **13** (3 g, 100%), which was used without further purification. ¹H NMR (CDCl₃) δ (ppm) 1.11 (t, 6H, $J = 6.9$ Hz, CH₃), 1.41–1.72 (m, 6H, –CH₂–ring), 3.43 (m, 4H, OCH₂–CH₃), 3.48 (m, 2H, CH₂O–ring), 3.53–3.59 (m, 12H, –CH₂O–), 3.75 (m, 2H, CH₂–CH), 4.53 (m, 2H, CHO₂). ¹³C NMR (CDCl₃) δ (ppm) 15.32 (s, CH₃), 19.45 (s, CH₂–ring), 25.46 (s, CH₂–ring), 30.58 (s, CH₂–ring), 62.21 (–CH₂O–), 66.64 (CH₂O–ring), 70.62 (s, –CH₂O–), 70.91 (s, –CH₂O–), 71.95 (s, –CH₂O–), 98.86 (s, CHO₂), 101.22 (s, CHO₂). MS (ESI, m/z) 373 (M + H)⁺. Anal. Calcd for C₁₇H₃₄O₇: C, 58.26; H, 97.8; Found: C, 57.79; H, 9.90.

2-{2-[2-(4,5-Bis-(toluene-4-sulfonyloxymethyl)-[1,3]-dioxolan-2-ylmethoxy)ethoxy]ethoxy}ethanol (**14**). Ditosyl **2** (0.28 g, 0.65 mmol) and *para*-toluenesulfonic acid (0.022 g, 0.13 mmol) was added to a solution of **13** (0.23 g, 0.65 mmol) in benzene (10 mL), then the solution was heated to reflux for 22 h. Pyridine (0.1 mL) was added at RT, then the solvent was removed under reduced pressure, and the residual oil was purified to yield a mixture of the acetals protected by THP (0.14 g, 31%) or with free hydroxyl terminus **14** (0.096 g, 24%). After dissolution of the THP-protected compound in methanol (2 mL), *para*-toluenesulfonic acid was added to the solution, and the reaction mixture was stirred for 6 h, then poured in an Erlenmeyer flask filled with solid potassium carbonate. The solution was filtered, and the filtrate was concentrated under reduced pressure. The residual oil was dissolved in dichloromethane and washed three times with a saturated NaHCO₃ solution and brine successively.

Scheme 1. (i) TsCl, Pyridine, CH₂Cl₂, -20 °C, 52%; (ii) APTS, 3, Benzene, Reflux, 75%; (iii) NaN₃, DMF, Reflux, 94%; (iv) NaOH, MeOH/H₂O/THF, RT, 97%; (v) Isobutyl Chloroformate, *N*-Methylmorpholin, Ditetradecylamine, THF, -40 °C, 46%; (vi) Pd/C, H₂, Ethanol, RT, 96%; (vii) CS₂, TsCl, DIPEA, CH₂Cl₂, RT, 66%; (viii) Ethanolamine, THF, RT, 39%; (ix) 3-Aminopropane-1,2-diol, DMF, RT, 38%



acetamide (**20**). 3-Amino-1,2-propanediol (0.008 g, 0.092 mmol) in DMF (0.1 mL) was added to a solution of **19** (41 mg, 0.056 mmol) in dry DMF (0.2 mL). The solution was stirred at RT for 20 h, then concentrated to dryness. The crude was dissolved in ethyl acetate and washed three times with brine. The organic layer was dried (MgSO₄) and evaporated to dryness. SFC purification yield **20** as a colorless oil (0.016 g, 72%). ¹H NMR (CDCl₃) δ (ppm) 0.88 (t, 6H, *J* = 6.6 Hz, CH₃), 1.22–1.29 (m, 44H, -CH₂-), 1.52 (m, 4H, -CH₂-), 3.11 and 3.28 (t, 4H, *J* = 7.5 Hz, -CH₂N), 3.50–3.75 (m, 22H, -CH₂O-, -CH₂NCS, CH₂OH), 4.13–4.34 (m, 6H, -CH₂CO, CHO, CHOH), 5.25 (t, 1H, *J* = 3.6 Hz, CHO₂). ¹³C NMR (CDCl₃) δ (ppm) 14.13 (s, CH₃), 22.72 (s, -CH₂-), 27.16 (s, -CH₂-), 28.94 (s, -CH₂-), 29.67 (m, -CH₂-), 31.98 (s, -CH₂-), 45.44 (s, -CH₂NCS), 46.48 and 47.14 (s, -CH₂NCO), 63.74 (s, CH₂OH), 68.98 (s, CHOH), 69.84 (s, -CH₂O-), 70.28 (m, -CH₂O-), 70.59 (s, -CH₂CO), 71.39 (s, -CH₂O-), 78.35 (d, CHO), 101.93 (s, CHO₂), 169.06 (s, C=O), 183.55 (s, C=S). ES-HRMS Calcd for C₄₈H₉₅N₅O₁₀NaS₂: 988.6418. Found: 988.6389.

Lipopolythiourea Formulation. ARTUtetraol (2.27 mg, 2.5 μmol) and DPPC (3.66 mg, 5.0 μmol) were dissolved in a chloroform/acetone/ethanol mixture (1/5/4 v/v/v, 500 μL). This mixture was then added dropwise to 5 mL of stirred H₂O and vigorously stirred overnight. Most of the solvents were removed under vacuum to obtain a fairly concentrated suspension of liposomes (concentration of ARTUdiol = 8.5 mM). Similar procedures were used for the other LPT.

Complex Size. The complex diameter was determined by dynamic light scattering on a Zetasizer Nano Series Malvern. 10 μL of ARTUdiol/DPPC 1/2 (*C* = 8.5 mM) was added to 800 μL of H₂O; mean value of three runs is given (1 min equilibration, 3 min run).

Preparation of the Complexes LPT/DPPC/DNA. Plasmid DNA (pVax2luc) (100 μL, 0.02 g/L in H₂O) was added dropwise with constant vortexing to various amounts of ARTUdiol/DPPC 1/2 particles (for a ratio value of thiourea function over DNA phosphate of 20: 100 μL, 1.2 mM) at RT.

The samples were left to incubate at RT overnight before size measurements, which were performed on a Zetasizer Nano Series Malvern.

Agarose Gel Electrophoresis. Samples were prepared as described above (20 μL), and 5 μL of bromophenol blue was added. The mixtures were loaded into 0.8% agarose gel in TBE buffer (1 M Tris, 0.9 M boric acid, 0.01 M EDTA) at 80 V/cm. DNA was revealed with ethidium bromide and visualized under UV light.

Luciferase Measurement. The NIH3T3, mice fibroblasts cells, were grown in DMEM medium, supplemented with 10% fetal bovine serum, 1% antibiotics (penicillin and streptomycin), and 1% glutamine. HUVEC cells, primocultures of human umbilical vein endothelial cells, were grown into endothelial cell growth medium kit (Promo Cell) medium. One day before transfection, the cells were treated with trypsin, deposited into 24-well plates (3T3, 50 000 cells/well; HUVEC, 70 000 cells/well) and incubated 24 h at 37 °C. A volume of 200 μL of LPT/DNA (corresponding to 0.5 μg DNA) complexes was loaded on each well, and the plates are incubated at 37 °C for 48 h. Then, the cells were washed twice with PBS and lysed with 200 μL of a passive lysis buffer (Promega) containing SDS (2.5%) and TRIS (50 mM) at pH 7.5. After 15 min, the cells were centrifuged for 5 min at 12 000 rpm. 10 μL of supernatant and 10 μL iodoacetamide were deposited onto a 96-well plate, which was incubated at 37 °C for 1 h. Protein quantification was performed with the BCA protein assay kit (Pierce) and reported to the BSA taken as a reference curve. Luciferase activity was quantified using a commercial kit Luciferase assay system (Promega). On 10 μL of the lysed cells, 50 μL of the luciferin substrate was injected via an injector, and the absorbance was read immediately at 563 nm on a Wallac Victor2 1420 Multilabel Counter (Perkin-Elmer).

RGD-Mediated Lipopolythiourea Liposomes Delivery to HUVEC and NIH3T3 Fibroblast Cells. One day before transfection, HUVEC or NIH3T3 cells were deposited into 12-well plates with glass lamellae (3T3, 70 000 cells/well; HUVEC, 150 000 cells/well) and incubated at 37 °C for 24 h in MEM

Scheme 2. (i) NaH, Bromoacetaldehyde Diethyl Acetal, THF, 60 °C, 100%; (ii) APTS, 2, Benzene, Reflux; (iii) APTS, Methanol, RT, 52% Two Steps; (iv) TEMPO, Trichloroisocyanuric Acid, NaBr, Acetone, RT, 71%; (v) Isobutyl Chloroformate, *N*-Methylmorpholin, Ditretracyclamine, THF, -40 °C, 42%; (vi) NaN₃, DMF, Reflux, 100%; (vii) H₂, Pd/C, Ethanol, RT, 100%; (viii) CS₂, TsCl, DIPEA, CH₂Cl₂, RT, 35%; (ix) 3-Amino-1,2-propanediol, DMF, RT, 72%

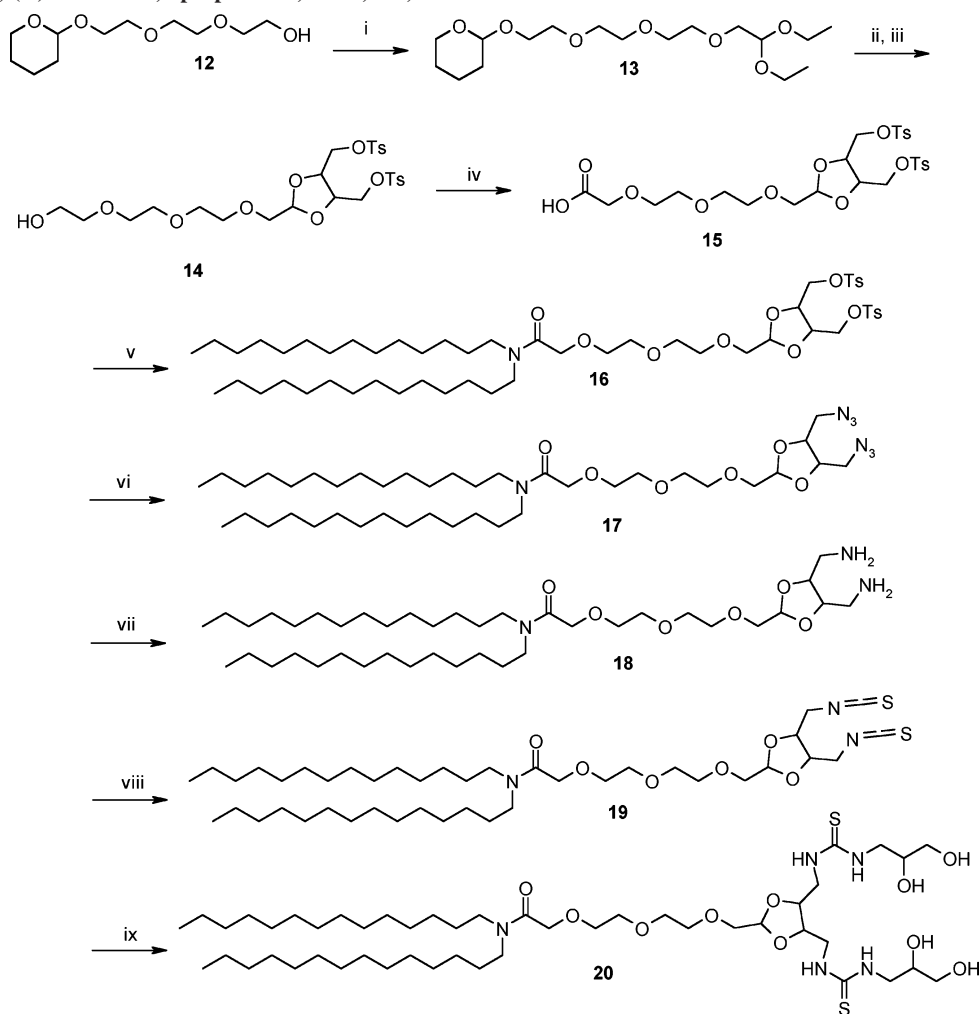


Table 1. Size (nm) of LPT/Colipid Particles Measured in H₂O on a Malvern Instrument Zetasizer NanoSeries

formulation	ARTUdiol	ARTUtetraol	PEG ₃ TUtetraol
LPT/DPPC (1:1)	no particles	79 ± 20	no particles
LPT/DPPC (1:2)	107 ± 26	122 ± 28	142 ± 49
LPT/Chol-PEG ₄₅ (10:1)	264	130	ND

medium. Lipopolythiourea/DNA complexes (200 μ L, 0.5 μ g DNA) were loaded in each well. After 7 h at 37 °C, the wells were washed twice with PBS, and 500 μ L of PFA (3% in PBS) was added. The plates were left 30 min in the dark at RT before being washed twice with PBS. DAPI (0.05 μ g in 500 μ L PBS) was then added. The plates were left 30 min in the dark, washed with PBS, and treated with mowiol. Fluorescence was observed on a ZEISS Axiophot noninverted microscope.

RESULTS

Synthesis. Three myristyl compounds were chosen as models to evaluate the impact of LPT structural modifications to DNA compaction and to transfection efficacy. First, the synthesis of compounds **10** and **11**, bearing an aromatic linker, is described in Scheme 1.

Considering the structure of **10** and **11**, we chose to investigate a strategy starting from the readily available *D,L*-threitol (**1**). Tosylation of **1** with two tosyl chloride equivalents proceeded selectively (6:1) to give **2** in 52% yield with the tritosyl

derivative as a minor product (8%) (**20**). Attempts to increase the selectivity using the more bulky trisyl chloride provided no improvement (**21**). Transacetalation with methyl 4-(dihydroxymethyl)cyclohexa-1,3-diene-1-carboxylate **3** (**22**) in the presence of a catalytic amount of APTS and using a Dean–Stark trap afforded the five-membered-ring acetal **4** in 75% yield (**23**). Azidation of **4** afforded the diazide **5** (94%), then saponification of the methyl ester gave the carboxylic acid **6** (97%).

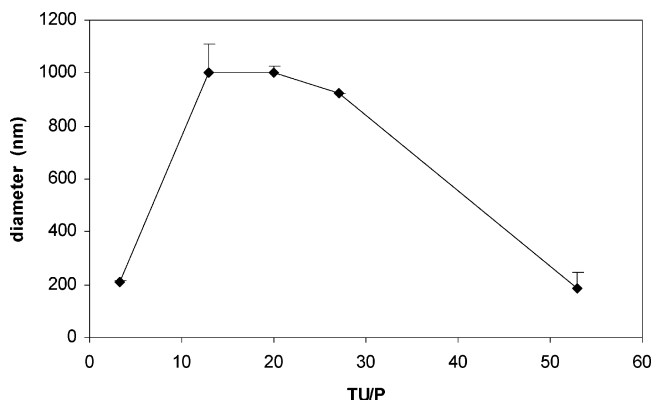


Figure 2. Diameter of particles PEG₃TUtetraol/DPPC (1:2) with increasing amounts of DNA measured in H₂O on a Malvern Instrument Zetasizer NanoSeries (ratio TU/P = mol thiourea functions/mol DNA phosphate groups).

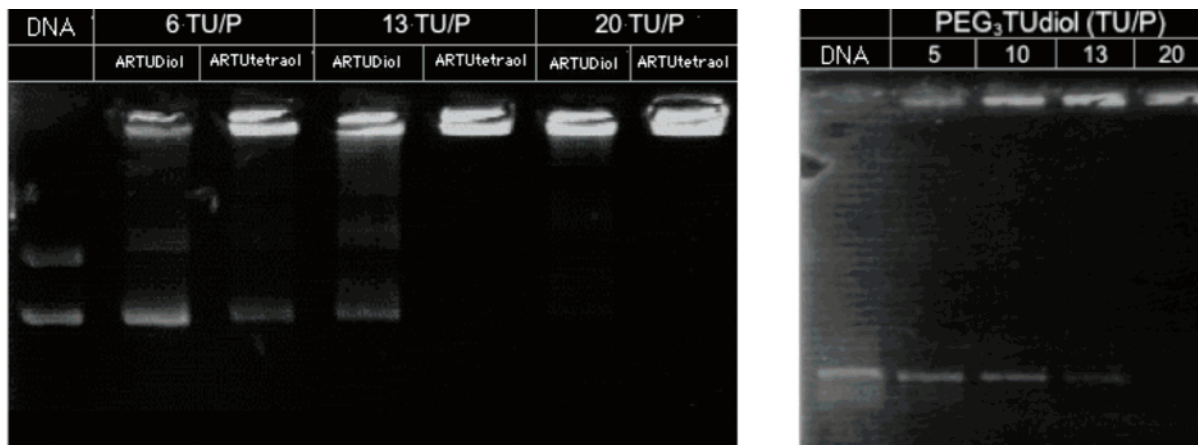


Figure 3. Migration of the ARTUdiol/DPPC(1:2)/DNA and ARTUtetraol/DPPC(1:2)/DNA complexes (left) and PEG₃TUtetraol/DPPC(1:2)/DNA complexes (right) on a 0.8% agarose gel in TBE (1×). DNA was revealed with ethidium bromide and visualized under UV light.

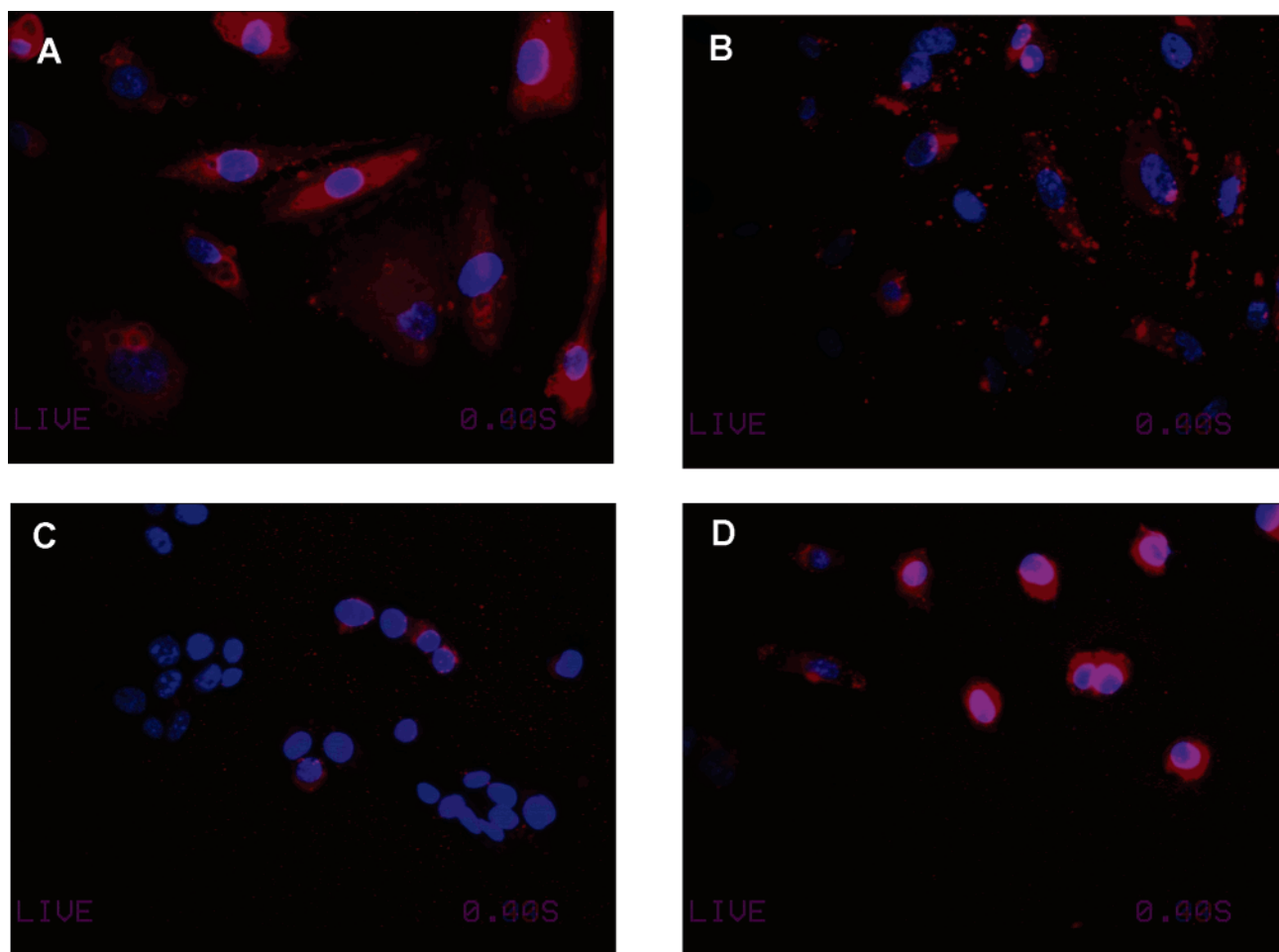


Figure 4. Fluorescence microscopy of ARTUtetraol particles. ARTUtetraol/DPPC/ CholPEG₄₅/DOPE–Rhodamine (10:10:0.20:0.1) ± Chol–PEG₇₅–RGD–4C (0.2) complexes were incubated for 7 h on HUVEC cells (A and B) in the absence (A) or presence (B) of RGD ligand, and on NIH3T3 cells (C and D) in the absence (C) or presence (D) of RGD ligand. Nuclei are stained with DAPI.

Subjecting **6** to amidation with ditetradecylamine using a mixed-anhydride procedure (24) provided good access to compound **7**. Catalytic reduction of the diazide **7** effected the conversion to the diamine **8** in high yield. The bis-isothiocyanate **9** was obtained in good yield by the reaction of **8** with CS₂ in the presence of tosyl chloride (25). The desired lipopolythioureas **10** and **11** were ultimately prepared by condensation of **9** with ethanolamine and 3-aminopropane-1,2-diol, respectively.

The synthesis of the lipopolythiourea bearing a short PEG linker is described in Scheme 2. It started with the synthesis of

2-{2-[2-(tetrahydropyranyl-2-yloxy)ethoxy]ethoxy}ethanol (**12**) as described in the literature (26).

Then, substitution of the hydroxyl terminus with bromoacetaldehyde diethyl acetal (27) led to the diethoxy acetal **13** that was converted to the 1,3-dioxolane **14** through treatment with diol **2** in the presence of APTS. Oxidation of **14** was performed using TEMPO and trichloroisocyanuric acid (28). The resulting acid **15** was treated with isobutyl chloroformate and ditradecylamine to afford the amide **16**. Substitution of the tosyl group of **16** with sodium azide followed by palladium

reduction of the azido group gave the diamine **18** quantitatively. Elaboration of the diisothiocyanate **19** from the diamine **18** proved to be difficult and led to a modest 35% yield. Addition of 3-amino-1,2-propanediol to **19** completed the synthesis of the lipopolythiourea **20**.

Formulation Study. Compounds **10** (ARTUdiol) and **11** (ARTUtetraol) bear the same aromatic linker and differ only by the number of hydroxyl groups attached to the thiourea functionalities. Compound **20** is similar to compound **11** (PEG₃TUtetraol) considering the thiourea part, but differs by its linker. In terms of the hydrophilic/hydrophobic balance, we expected ARTUdiol < ARTUtetraol < PEG₃tetraol.

The ability of these 3 LPTs to form micellar or liposomal particles was evaluated. Similarly to the previously studied linear LPT (*13*), we chose DPPC or PEG₄₅ lipid as co-lipids because of the hydrophobic behavior of the LPT. Several formulations were evaluated (Table 1). ARTUdiol/DPPC (1:2), ARTUtetraol/DPPC (1:1 and 1:2), ARTUdiol/Chol-PEG₄₅ (10:1), ARTUtetraol/Chol-PEG₄₅ (10:1), and PEG₃TUtetraol/DPPC (1:2) led to small particles whose diameters ranged from 100 to 250 nm, depending on the co-lipid. It is noteworthy that only ARTUtetraol could be formulated with 1 equiv of DPPC. Comparison among the aromatic lipids indicated that a tetraol terminus eased the formulation, since ARTUtetraol required a lower amount of DPPC than ARTUdiol, with the tetraol moiety probably increasing the hydrophilicity. Comparison of the two tetraol lipids showed that the lipid bearing an aromatic linker had a better capacity to form particles, possibly due to aromatic π - π stacking within the lipid bilayer (*29*).

Interaction of LPT/DPPC with DNA. The capacity of LPT/DPPC (1:2) particles to associate to plasmid DNA was then studied. Setting a fixed DNA amount, we added on the DNA solution an increasing amount of lipids. After one night of incubation, we measured the size of the complexes by dynamic light scattering. The results are reported for PEG₃TUtetraol (Figure 2), but similar data have been obtained for ARTUdiol and ARTUtetraol. In the presence of DNA, we observed a size increase from 200 nm to the micrometer range, proving an interaction between lipopolythiourea particles and plasmid DNA. Adding an excess of lipids (30 equiv thiourea functions/DNA phosphate groups) let the system return to a colloidal stability.

Gel retardation experiments of LPT/DPPC/DNA complexes were performed to evaluate DNA association to particles ARTUdiol/DPPC (1:2), ARTUtetraol/DPPC (1:2), and PEGTUtetraol/DPPC (1:2). Partial retardation was observed above 6 thiourea functions/DNA phosphate groups (TU/P), and complete retardation was observed for ratios of 13 TU/P for ARTUtetraol and 20 TU/P for ARTUdiol and PEG₃TUtetraol (Figure 3). In our previous study (*13*), complete retardation was obtained for a ratio equal to 20 thiourea functions/DNA phosphate groups (TU/P). Thus, we observed that, using branched lipids bearing only two thiourea functions, we could obtain an interaction with DNA at lower or identical ratio TU/P, as compared to the DTTU lipid bearing three linear functions. These data indicated that a stable interaction between LPT and DNA could be established with only two thiourea groups.

Transfection Activity and RGD-Mediated Delivery into Endothelial Cells. DNA transfection mediated by ARTUdiol, ARTUtetraol, and PEGTUtetraol led to disappointing results, since no significant luciferase activity could be measured on the treated B16 cell. This lack of transfection activity may be explained by the noncationic nature of the LPT, causing poor contact with cell membranes. To improve these interactions, RGD-mediated internalization of endothelial cells expressing integrin $\alpha_v\beta_3$ was investigated. Thus, we added a PEG₇₅-lipid bearing a RGD peptide (*14*) to the formulation, and a rhodamine-labeled lipid/DNA complex were prepared. Their intracellular

fate was investigated using fluorescence microscopy. These experiments were carried out on two cell types: human umbilical vein endothelial cells (HUVEC), which express integrin $\alpha_v\beta_3$, and NIH3T3 fibroblasts, taken as a negative control. Only the results with ARTUtetraol are shown in Figure 4. In the absence of Chol-PEG-RGD, we only observed extracellular binding of lipoplexes to the cell membrane. Such a result has also been reported for PEGylated lipoplexes (*30*). However, in its presence, we clearly saw intracellular punctuations, which indicated particle internalization. In opposition, no internalization was observed in NIH3T3 cell fibroblasts, although the extracellular binding to cell membranes seemed to be stronger with the RGD group. Thus, addition of Chol-PEG-RGD allowed a specific uptake of the LPT/DNA complexes into the cells. These results are in agreement with the specific capture previously reported on RGD-functionalized spherulites (*31*). However, this has been found to lead to neither HUVEC nor NIH3T3 transfection. This might result from a lack of DNA release from the LPT/DNA complex, possibly due to the PEG-lipid, that was shown to stabilize lipoplexes and reduce transfection efficiency (*32*).

CONCLUSION

These results present new lipopolythioureas able to associate DNA. Adding a diol or a tetraol terminus to the thiourea part improved the hydrophilicity and eased the DNA formulation. Use of Chol-PEG-RGD allowed observation of a specific internalization of the complexes in HUVEC cells as shown earlier with other particle types, such as spherulite. On the other hand, these particles were unable to transfect DNA, an effect which might be related to the presence of a PEG moiety in the formulation, thus inducing a shielding effect, which has been shown to affect transfection. Work is in progress to obtain more hydrophilic thiourea compounds and to study the internalization using an RGD derivative without any PEG moiety.

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