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A single thiourea group is not enough to get stable thiourea lipoplexes

Eihab Kabha, Claire Jacquement, Gaëlle Pembouong, Nathalie Mignet, Daniel Scherman and Jean Herscovici*

Inserm, U640, Paris F-75006, France
CNRS, UMR8151, Paris F-75006, France
Ecole Nationale Supérieure de Chimie de Paris, Unité de Pharmacologie Chimique et Génétique, Paris F-75005, France
Université Paris Descartes, Faculté de Pharmacie Paris, F-75270, France
Institut de Chimie Moléculaire de Paris Centre, CNRS FR 2769, F-75005, France

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Abstract—The preparation of a new family of lipothiourea is reported using an automatic synthetic workstation. In these compounds the headgroups were made from single thiourea derivatives. The physicochemical properties and the transfection efficiency of several members of the family were studied. It was found that in the presence of DMPC small lipoplexes could be prepared. In opposite to the previously described di- and tri-lipothioureas most of these liposomes are unstable overtime. In addition, even the stable ones show no transfecting efficiency. All these data demonstrate that at least two thiourea groups are necessary to produce stable lipoplexes, to condense DNA and to give efficient transfection.

1. Introduction

Anion receptors have been extensively studied using charged species or neutral hydrogen bond donor.[1,2] Among neutral receptors thioureas have attracted a special interest because of the acidic nature of the NH protons that led to strong hydrogen bond.[3] On the other hand phosphate anions[4] show the strongest affinity among carboxylate and inorganic anions for thioureas.[5]

Chemical vectors[6–8] are with viruses[9,10] and physical methods,[11,12] the three main processes for gene transfer. During the past year a great effort was made toward the preparation of synthetic vector of nucleic acids. Due to the polyanionic nature of nucleic acid and of plasmic membrane most of them are derived from cationic lipid or cationic polymer. The cationic nature of the DNA lipid or DNA polymer complexes is one of the main problems of their poor in vivo efficiency. These complexes aggregate with blood protein, which in turn, leads to their elimination.[13] One of the solutions proposed to resolve this problem is the use of neutral derivatives.[14] However, with the exception of glycocluster, neutral liposomes has no or very low-transfecting efficiency.[15]

With these data in mind we proposed to replace the cationic head of the synthetic vector by a thiourea one (Fig. 1). Our previous studies indicated that compounds bearing two to four thiourea groups were able to condense DNA[16,17] and to deliver it to the cell.[18] Recently we showed that T-shape lipodithioureas transfect cells with the same efficiency as cationic derivatives used as reference when the experiments were performed in the presence of serum.

These results raised the question of the number of thiourea group necessary for a stable interaction between non cationic lipids and nucleic acids. In this report we describe the synthesis of a small library of lipomonothiourea and the evaluation of their transfecting properties.

2. Results and discussion

2.1. Synthesis

The synthesis was carried out in four steps (Scheme 1). First, didecylamine was reacted with Boc-protected

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* Corresponding author. Tel.: +33 1 44 27 67 48; fax: +33 1 53 10 12 95; e-mail: jean-herscovici@enscp.fr

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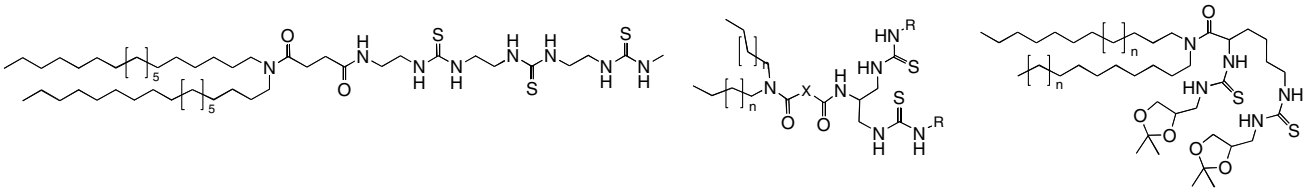
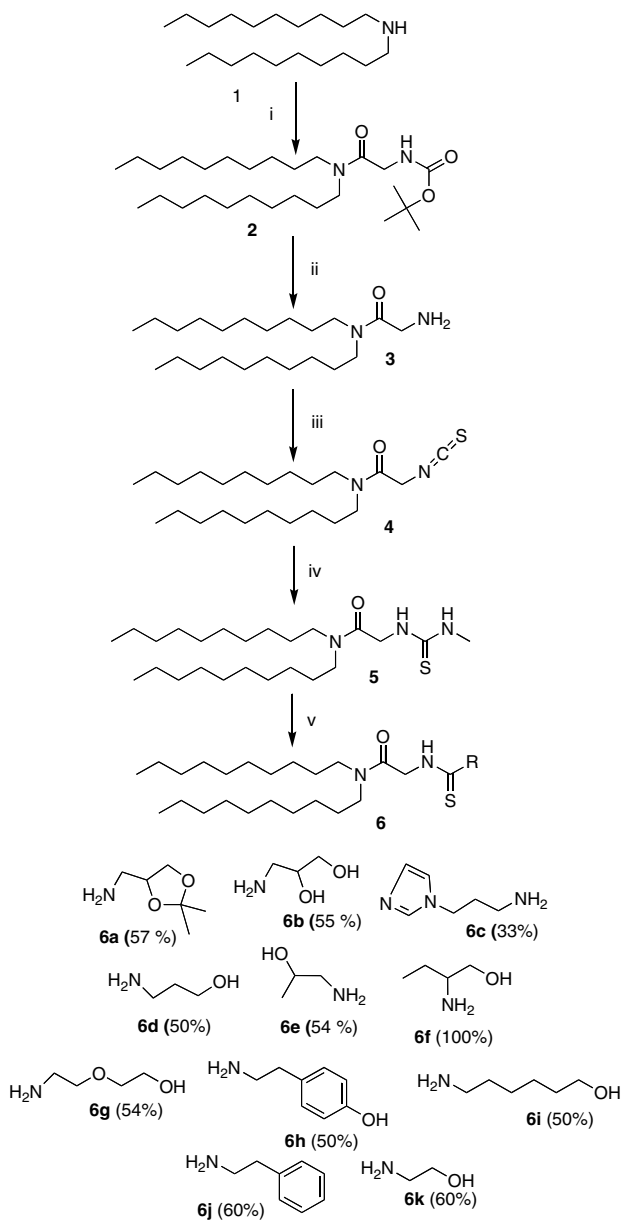


Figure 1. Lipopolythioureas.

glycine in the presence of DCC and *N*-hydroxysuccinimide to give **2** in 50–60% yield. After removal of the *tert*-butoxy carbamate, using trifluoroacetic acid, the resulting amine **3** was treated with carbon disulfide in the presence of *p*-TSCl, DIPEA to give the lipoisothiocyanate **4** in 89% yield. Finally, The scaffold **4** was treated with different amines onto a Chemspeed ASW 2000 synthetic workstation and the lipomonothiourea library was obtained in 50–60% yield.



Scheme 1. Preparation of the Lipomonothiourea. Reagents and conditions: (i) *N*-hydroxysuccinimide, DCC, THF, 60%; (ii) trifluoroacetic acid, rt, 70% (iii) carbon disulfide, *p*-TSCl, DIPEA, CH_2Cl_2, 89%; (iv) amine, DMF, 50–60%.

2.2. Physico-chemical studies

Different attempts were performed to suspend the lipids in aqueous media. They were formulated via an ethanolic injection method[19] in the presence of DPPC as a ratio 1/1 or as a lipidic film with DMPC as a ratio DMPC/LMTU 0.65:0.45. Both ways allowed the formation of nanometer range particles with the characteristics indicated below (Table 1). Compounds **5** and **6a** could also be formulated but the formulations were poorly stable and we did not pursue our efforts on these two compounds. The size of the particles formed was in the same range 100–130 nm and zeta potential close to zero. Aggregation of these formulations after few days of storage at 4 °C could be expected considering the log *P* of these compounds (**5** 6.26, **6a** 6.83).[18]

Compound **6b** did not show a very low electrophoretic mobility as expected, giving a zeta potential of 6.5 ± 1.5 mV. Zeta potential of compound **6c** was 2.5 ± 2 at pH 7 and 35 ± 0.5 mV at pH 5.5 relating to the protonation of the imidazole ring.

LMTU/DNA interaction was evaluated by dynamic light scattering experiments. A fixed amount of DNA was added to an increasing amount of lipid to form the complexes (Table 2). The lipid/DNA ratio is given as the amount of thiourea function per phosphate. A size increase occurred below a ratio of 1 TU/PO leading to an aggregation, confirming the high interaction efficiency that we previously observed between the thiourea moiety and the phosphates.[16] We cannot exclude that the thiourea function not only reacts on DNA phosphate but also on the phosphate functions of phospholipids. However, we have proven on the trithiourea lipid that removing the colipid did not change the lipop-

Table 1. Characteristics of the particles LMTU/DMPC obtained via the film hydration technique measured by dynamic light scattering experiments

	Size (nm)	PDI	Zeta potential (mV)
6b	99.3	0.02	6.5 ± 1.5
6c	130 (pH 5.5)	0.07	35 ± 0.5
			2.5 ± 2 (pH 7)

PDI is the polydispersity index; zeta potential (mV) was measured in 20 M NaCl, pH 5.5.

Table 2. Size of the lipomonothiourea/DNA complexes in PBS as measured by dynamic light-scattering

TU/PO	*Z*-average nm (PDI)	
	6b/DMPC/DNA	**6c**/DMPC/DNA
1	461.9 (0.38)	331.9 (0.312)
5	8032 (0.11)	641.8 (0.7)
10	761.6 (0.77)	/
20	113.2 (0.27)	344.5 (0.4)
40	135.7 (0.09)	158.4 (0.4)

The polydispersity index is indicated in brackets.

olythiourea/DNA interaction.[16] The system undergoes a precipitation and then stabilizes at a ratio TU/PO = 20. A similar pattern was evidenced for the two lipids.

We could evidence a lipid/DNA interaction by dynamic light scattering while DNA was not retained on an agarose gel when the previous complexes **6b**/DMPC/DNA and **6c**/DMPC/DNA were loaded on a gel. DNA migrated into the wells even when using a low electrophoretic field. This suggests that even though one thiourea function appears to be enough for the lipid to interact with DNA, this association is probably weaker than the one obtained with the lipids bearing two thiourea functions for which DNA was partially retained in the wells or for the previously reported lipopolythiourea, which fully blocked DNA migration on the gel.[18] Finally, we evaluated the transfection capacity of the complexes on B16 cells, using a luciferase encoding gene. No transfection could be evidenced, probably due to the lack of stability of the complexes in the biological medium.

3. Conclusion

In conclusion we have developed a new family of lipothiourea using an automation strategy. Among these compounds thioureas **5**, **6a**, **6b**, and **6c** were studied for their physicochemical and transfecting properties. As it could be expected from the properties of thiourea/phosphate complexes the described lipopolythioureas were able to interact with DNA in the presence of DMPC leading to small particles using a thiourea/phosphate ratio of the same magnitude that was previously described.[17,18] However, lipoplexes **5** and **6a** were poorly stable. In addition, the more stable lipothiourea **6b** did not possess any transfecting properties. All these data demonstrated that at least two thiourea groups are necessary to produce stable lipoplexes, to condense DNA and to give efficient transfection.

4. Experimental

4.1. Material

All solvents were purchased from Carlo Erba-SDS (Peypin France). Dichloromethane was distilled from P_2O_5, and THF was distilled from Na in the presence of benzophenone. DMF was dried over 3 Å molecular sieves. All chemicals were purchased from Sigma–Aldrich–Fluka. 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 silica gel according to the method of Still, Khan, and Mitra or by using small column flash chromatography (SFC) according to the following procedure. A plastic syringe was filled with silica gel (product/silica gel 1:5) and connected to a vacuum pump. The column was equilibrated with heptane then the sample dissolved in 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 silica gel volume. For each fraction, the amount of ethyl acetate was increased 10% (v/v) to 100% ethylacetate in heptane. The ^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. The NMR spectra were processed using Xwinnmr (Bruker) or SwaN-MR. Mass Spectrometry was carried out on a Shimazu 2010A LC–MS on ESI mode. Dimyristoyl phosphatidylcholine (DMPC) and Dipalmytoylphosphatidylcholine (DPPC) were purchased from Aventi Polar Lipids (Alabaster Alabama). log *P* calculation was performed using MarvinSketch (Chemaxon Ltd Budapest Hungary). Particle diameter was determined by dynamic light scattering on a Zeta Sizer NanoSeries Malvern (Malvern Instruments, France).

4.2. Synthetic procedures

4.2.1. *tert*-Butyl *N*-{[bis(decyl)carbamoyl]methyl} carbamate (2). Boc-glycine (5.2 g, 29.7 mmol), DCC (15.25 g, 74 mmol) and *N*-hydroxysuccinimide (3.42 g, 30 mmol) were dissolved in dry THF (200 mL). The reaction mixture was stirred for 3 h at room temperature, didecylamine (7.43 g, 25 mmol) was then added and the resulting mixture was stirred overnight at the same temperature. The precipitated DCU was removed by filtration, and the filtrate was evaporated under reduced pressure. The residual oil was dissolved in Et_2O (600 mL) and the solution was stirred overnight. An additional amount of DCU was removed by filtration, and the filtrate was washed with $NaHCO_3$ (3× 200 mL), water, and brine. The organic layer was dried ($MgSO_4$) and evaporated under reduced pressure. Purification by chromatography (diethyl ether) yielded 5.85 g (51%) of *tert*-butyl *N*-{[bis(decyl)carbamoyl]methyl}carbamate (**2**) as yellow oil.

^1H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.29 (m, 28H, H-2′–H-9′), 1.3 (m, 4H, H-8′), 1.48 (s, 9H, Boc), 3.15 (m, 2H, H-1′), 3.34 (m, 2H, H-1′), 3.975 (d, 2H,J = 7 Hz, H-2), 5.06 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 23.1 (C-9′), 27.4 (C-3′), 28.5 (C-Boc), 42.4 (C-2′), 32.3 (C-8′), 46.4 (C-1′), 47.4 (C-1′). MS (ESI) MH^+ = 355 (MH^+-Boc).

4.2.2. 2-Amino-*N*,*N*-bis(decyl)acetamide (3). Carbamate **2** (5.84 g, 12.86 mmol) was dissolved in trifluoroacetic acid (55 mL) in a round bottomed flask equipped with

a $CaCl_2$ tube and the mixture was stirred for 1 h. TFA was evaporated and the residue was dissolved in CH_2Cl_2, washed successively with $NaHCO_3$ (×3), water and brine. The organic layer was dried ($MgSO_4$), evaporated under a reduced pressure afford 3 g (65%) of 2-amino-*N*,*N*-bis(decyl)acetamide **3** as yellow oil.

^{1}H NMR ($CDCl_3$) δ (ppm) 0.87 (t, 6H, J = 7 Hz, H-10′), 1.26 (m, 4H, H-9′), 1.27 (m, 28H, H-3′–H-8′), 1.49 (m, 2H, H-2′), 1.5 (m, 2H, H-2′), 3.14 (m, 2H, H-1′), 3.32 (m, 2H, H-1′), 4.09 (s, 2H, H-2).^{13}C NMR ($CDCl_3$) δ (ppm) 14.1 (C-10′), 23.1 (C-9′), 27–30.3 (C-2′–C-7′), 32.3 (C-8′), 41.7 (C-2), 46.7 (C-1′), 47.4 (C-1′), 171.7 (C-1), MS (ESI) MH^+ = 355.

4.2.3. *N*,*N*-Bis(decyl)-2-isothiocyanatoacetamide (4)

4.2.3.1. Procedure 1. A solution of DCC (0,757 g, 3,67 mmol) in dry CH_2Cl_2 (25 mL) was added dropwise to a cold (−7 °C/NH_4Cl/ice) mixture of 2-amino-*N*,*N*-bis(decyl)acetamide **3** (1.301 g, 3.67 mmol), and carbon disulfide (1.50 mL, 24.96 mmol) in dry CH_2Cl_2 (25 mL). The solution was stirred for 30 min at 0 °C then for 25 h at room temperature. The solvent was evaporated under reduced pressure. Purification by SFC chromatography (Heptane/EtOAc) yielded 1,13 g (89%) of *N*,*N*-bis(decyl)-2-isothiocyanatoacetamide (**4**) as yellow oil.

4.2.3.2. Procedure 2. *p*-Toluenesulfonyl chloride (0,7 g, 3.67 mmol) in dry CH_2Cl_2 (25 mL) was added dropwise to a cold (−7 °C/NH_4Cl/ice) solution of 2-amino-*N*,*N*-bis(decyl)acetamide **3** (1.301 g, 3.67 mmol) and carbon disulfide (1.50 ml, 24.96 mmol) in dry CH_2Cl_2 (25 mL). The solution was stirred for 30 min at 0 °C then for 25 h at room temperature. Purification by SFC chromatography (Heptane/EtOAc) yield 1.13 g (89%) of *N*,*N*-bis(decyl)-2-isothiocyanatoacetamide (**4**) as yellow oil.

^{1}H NMR ($CDCl_3$) δ (ppm) 0.86 (t, 6H, J = 7 Hz, H-10′), 1.25 (m, 32H, H-3′–H-9′), 1.54 (m, 4H, H-2′), 3.06 (m, 2H, H-1′), 3.33 (m, 2H, H-1′), 4.25 (s, 2H, H-2).^{13}C NMR ($CDCl_3$) δ (ppm) 14.18 (C-10′), 22.75 (C-9′), 26.92–29.61 (C-2′–C-7′), 31.95 (C-8′), 46.99 (C-2), 46.47 (C-1′), 47.47 (C-1′), 139.55 (C-3), 164.01 (C-1). MS (ESI) MH^+ = 397, HPLC purity 98% by ELSD.

4.2.4. *N*,*N*-Bis(decyl)-2-[(methylcarbamothioyl)amino]-acetamide (5).

Isothiocyanate (0.082 g, 1.12 mmol) was added to a solution of 2-amino-*N*,*N*-bis(decyl)acetamide **3** (0.199 g, 0.56 mmol), triethylamine (0.227 g, 2.24 mmol) in dry CH_2Cl_2 (5.6 mL). The reaction solution was stirred 6 h at room temperature. Purification by chromatography (Heptane/EtOAc) to yield 0.15 g (62%) of *N*,*N*-bis(decyl)-2-[(methylcarbamothioyl)amino]acetamide **5** as yellow oil.

^{1}H NMR ($CDCl_3$) δ (ppm) 0.87 (t, 6H, J = 7 Hz, H-10′), 1.26 (m, 4H, H9′), 1.32 (m, 4H, H-8′), 1.38–1.17 (m,H, 20H, H-3′–H-7′), 1.5 (m, 2H, H-2′), 1.59 (m, 2H, H-2′), 2.99 (s, 3H, H-4), 3.23 (m, 2H, H-1′), 3.23 (m, 2H, H-1′), 7.18 (br s, 1H, NH), 7.53 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 22.8 (C-9′), 27–30 (C-2′–C-7′), 32 (C-8′), 46.9 (C-1′), 46.9 (C-2), 47.7 (C-1′). MS (ESI) MH^+ = 429, MNa^+ = 451, HPLC purity by ELSD 98%.

4.2.5. Automated thioureas synthesis. The reactions were performed in an ASW2000 automated synthesis workstation (Chemspeed Augst Switzerland). Amines (0.08 mmol) were dissolved in dry DMF (1 mL), added to solutions of *N*,*N*-bis(decyl)-2-isothiocyanatoacetamide **4** (0.08 g, 0.2 mmol) in dry DMF (1 mL). The solutions were stirred for 16 h at 70 °C. All the products were purified by SFC chromatography (Heptane → EtOAc → MeOH) to yield the thioureas in 50–60% yields as yellow oil.

4.2.6. *N*,*N*-Bis(decyl)-2-({[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]carbamothioyl}amino)acetamide (6a).

2,2-Dimethyl-1,2-dioxalane-4-methanamine was added to a solution of **4** to yield 0.060 g of *N*,*N*-bis(decyl)-2-({[(2,2-dimethyl-1,3-dioxolan-4-yl)methyl]carbamothioyl} amino)-acetamide **6a** (57%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.89 (t, 6H, J = 7 Hz, H-10′), 1.28 (m, 32H, H-3′–H-9′), 1.35 (s, 3H, H-8), 1.41 (s, 3H, H-9), 3.25 (m, 2H, H-1′), 3.35 (m, 2H, H-1′), 3.67 (br s, 2H, H-4), 3.67 (dd, 1H, J = 9 Hz, H-6), 4.04 (dd, 1H, J = 9 Hz, H-6), 4.32 (m, 1H, H-5), 4.43 (s, 2H, H-2), 7.05 (br s, 1H, NH), 7.55 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 23.1 (C-9′), 25.6 (C-9), 27.3 (C-8), 27.4 (C-3′) 28 (C-7′), 29.1 (C-6′), 29.8 (C-4′ and C-5′), 30.0 (C-2′), 32.3 1(C-8′), 46.7 (C-1′), 47.4 (C-1′), 67 (C-6), 75 (C-5), 109 (C-7), 168 (C-1), 182.7 (C-3). MS (ESI) MH^+ = 529, M+Na = 551; HPLC purity by ELSD 98%.

4.2.7. *N*,*N*-Bis(decyl)-2-{[(2,3-dihydroxypropyl)carbamothioyl]amino}acetamide (6b).

3-Amino-1,2-propanediol was added to a solution of **4** to yield 0.054 g of *N*,*N*-bis(decyl)-2-{[(2,3-dihydroxypropyl) carbamothioyl] amino}acetamide **6b** (55%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.89 (t, 6H, J = 7 Hz, H-10′), 1.3 (m, 4H, H9′), 1.18–1.46 (m, 20H, H-3′–H-7′), 1.324 (m, 4, H-8′), 1.52 (m, 2H, H-2′), 1.62 (m, 2H, H-2′), 3.27 (m, 2H, H-1′), 3.31 (m, 2H, H-1′), 3.60 (m, 4H, H-4 and H-6), 3.9 (m, 1H, H-5), 4.47 (s, 2H, H-2), 7.56 (br s, 1H, NH), 7.78 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 23.1 (C-9′), 27–30 (C-2′–C-7′), 32.3 (C-8′), 46.9 (C-4), 46.9 (C-1′), 47.9 (C-1′), 64 (C-6), 71.5 (C-5), 169.4 (C-1), 182.4 (C-3). MS (ESI) MH^+ = 488, HPLC purity by ELSD 98%.

4.2.8. *N*,*N*-Bis(decyl)-2-({[3-(1H-imidazol-1-yl)propyl]-carbamothioyl}amino)acetamide (6c).

1-(3-Aminopropyl)imidazole was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-({[3-(1H-imidazol-1-yl)propyl]carbamothioyl}amino)acetamide **6c** 0.033 g (33%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.90 (t, 6H, J = 7 Hz, H-10′), 1.30 (m, 4H, H9′), 1.21–145 (m, 20H, H-3′–H-7′), 1.324 (m, 4H, H-8′), 1.52 (m, 2H, H-2′), 1.63 (m, 2H, H-2′), 2.14 (m, 2H, H-5), 3.26 (m, 2H, H-1′), 3.33 (m, 2H, H-1′), 3.51 (m, 2H, H-4), 4.09 (m, 2H, H-6), 4.44 (s,

2H, H-2), 7.02 (s, 1H, H-8), 7.11 (s, 1H, H-7), 7.53 (br s, 2H, NH), 7.85 (br s, 1H, H-9). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 23.1 (C-9′), 27–30 (C-2′–C-7′), 32.3 (C-8′), 30.8 (C-5), 41.4 (C-4′), 45.2 (C-6′), 46.7 (C-1′), 46.7 (C-2), 47.7 (C-1′), 120 (C-8), 129 (C-7), 138 (C-9). MS (ESI) MH^+ = 522, HPLC purity by ELSD 98%.

4.2.9. *N*,*N*-Bis(decyl)-2-{[(3-hydroxypropyl)carbamothioyl]-amino}acetamide (6d). 3-Amino-L-propanol was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-{[(3-hydroxypropyl)carbamothioyl]amino}acetamide **6d** 0.048 g (50%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.305 (m, 4H, H9′), 1.24–145 (m, 20H, H-3′–H-7′), 1.364 (m, 4H, H-8′), 1.55 (m, 2H, H-2′), 1.64 (m, 2H, H-2′), 1.75 (m, 2H, H-5), 3.26 (m, 2H, H-1′), 3.33 (m, 2H, H-1′), 3.36(m, 2H, H-4), 3.64 (m, 2H, H-4), 3.72 (m, 2H, H-6), 4.5 (s, 2H, H-2), 6.90 (s, 1H, NH), 7.45 (s, 1H, NH).^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 22.8 (C-9′), 26.8–30 (C-2′–C-7′), 32.3 (C-8′), 32.3 (C-5), 46.9 (C-1′), 46.9 (C-4), 46.9 (C-2), 47.7 (C-1′), 59.3 (C-6), 169 (C-1), 182 (C-3). MS (ESI) MH^+ = 473, M+Na = 495, HPLC purity by ELSD 98%.

4.2.10. *N*,*N*-Bis(decyl)-2-{[3-(2-hydroxypropyl)carbamothioyl]}acetamide (6e). 1-Amino-2-propanol was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-[3-(2-hydroxypropyl)carbamothioyl]acetamide **6e** 0.051 g (54%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.2 (d, 3H, H-6), 1.305 (m, 4H, H9′), 1.26–145 (m, 20H, H-3′–H-7′), 1.283 (m, 4H, H-8′), 1.53 (m, 2H, H-2′), 1.65 (m, 2H, H-2′), 3.25 (m, 2H, H-1′), 3.23 (m, 2H, H-4), 3.35 (m, 2H, H-1′), 3.76 (br s, 1H, OH), 3.96 (m, 1H, H-5), 4.38 (dd, 1H, H-2), 4.65 (dd, 1H, H-2), 7.132 (br s, 1H, NH), 7.904 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 21.3 (C-6), 23.1 (C-9′), 27–30 (C-2′–C-7′), 32.3 (C-8′), 46.9 (C-1′), 46.9 (C-4), 47.9 (C-1′), 67 (C-5). MS (ESI) MH^+ = 473, M+Na = 495. HPLC purity by ELSD 98%.

4.2.11. *N*,*N*-Bis(decyl)-2-{[(1-hydroxybutan-2-yl)carbamothioyl]amino}acetamide (6f). 2-Amino-1-butanol was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-{[(1-hydroxybutan-2-yl)carbamothioyl]amino}acetamide **6f** 0.067 g (100%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.89 (t, 6H, J = 7 Hz, H-10′), 1.263 (m, 4H, H-8′), 1.305 (m, 4H, H-9′), 0.97 (t, 3H, H-7), 1.57 (m, 2H, H-6), 1.21–144 (m, 20H, H-3′–H-7′), 1.57 (m, 2H, H-2′), 1.65 (m, 2H, H-2′), 3.25 (m, 2H, H-1′), 3.173 (m, 1H, H-4), 3.356 (m, 2H, H-1′), 3.57 (dd, 1H, H-5), 3.76 (dd, 1H, H-5), 4.12 (s, 2H, H-2), 7.74 (br s, 1H, NH), 7.78 (br s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 10.9 (C-7), 14.4 (C-10′), 22.8 (C-9′), 24.8 (C-6), 27–30 (C-2′–C-7′), 32.3 (C-8′), 46.7 (C-4), 47 (C-1′), 47.8 (C-1′), 64.7 (C-5). MS (ESI) MH^+ = 487, M+Na = 509, HPLC purity by ELSD 98%.

4.2.12. *N*,*N*-Bis(decyl)-2-({[2-(2-hydroxyethoxy)ethyl]-carbamothioyl}amino)acetamide (6g). 2-(2-Aminoethoxy)ethanol was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-({[2-(2-hydroxyethoxy)ethyl]carbamothioyl}amino)acetamide **6g** 0.054 g (54%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.30 (m, 4H, H9′), 1.21–145 (m, 20 H, H-3′–H-7′), 1.34 (m, 4H, H-8′), 1.53 (m, 2H, H-2′), 1.60 (m, 2H, H-2′), 3.23 (m, 2H, H-1′), 3.356 (m, 2H, H-1′), 3.36 (m, 2H, H-4), 3.61 (m, 4H, H-5 and H-6), 3.73 (m, 2H, H-7), 4.14 (s, 1H, OH), 4.43 (s, 2H, H-2), 6.82 (s, 1H, NH), 7.72 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 22.9 (C-9′), 26.8–30 (C-2′–C-7′), 32.3 (C-8′), 46.7 (C-4 and C-1′), 47.5 (C-1′), 62.1 (C-7), 74.2 (C-5, C-6). MS (ESI) MH^+ = 503, M+Na = 525, HPLC purity by ELSD 95%.

4.2.13. *N*,*N*-Bis(decyl)-2-({[2-(4-hydroxyphenyl)ethyl]-carbamothioyl}amino)acetamide (6h). Tyramin was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-({[2-(4-hydroxyphenyl) ethyl]carbamothioyl}amino)acetamide **6h** 0.054 g (50%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.92 (t, 6H, J = 7 Hz, H-10′), 1.32 (m, 4H, H-9′), 1.20–1.45 (m, 20H, H-3′–H-7′), 1.32 (m, 4H, H-8′), 1.48 (m, 2H, H-2′), 1.59 (m, 2H, H-2′), 2.85 (t, 2H, J = 6 Hz, H-5), 3.23 (m, 4H, H-1′), 3.7 (br s, 2H, H-4), 4.41 (s, 2H, H-2), 6.31 (br s, 1H, OH), 6.67 (s, 1H, NH), 6.79 and 7.02 (AA'XX' system), 7.22 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.4 (C-10′), 23.1 (C-9′), 27–30 (C-2′–C-7′), 32 (C-8′), 34.2 (C-5), 45.7 (C-4), 46.7 (C-2 and C-1′), 116 and 130 (AA'XX'). MS (ESI) MH^+ = 535, MNa^+ = 557. HPLC purity by ELSD 98%.

4.2.14. *N*,*N*-Bis(decyl)-2-{[(6-hydroxyhexyl)carbamothioyl]-amino}acetamide (6i). Amino-1-hexanol was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-{[(6-hydroxyhexyl)-carbamothioyl]amino}acetamide **6i** 0.0563 g (50%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.30 (m, 4H, H-9′), 1.21–1.37 (m, 26 H, H-3′–H-7′ and H-5–H-7), 1.32 (m, 4H, H-8′), 1.42 (m, 4H, H-2′), 1.62 (m, 2H, H-8), 3.23 (m, 2H, H-4), 3.355 (m, 4H, H-1′), 3.68 (t, 2H, H-9), 4.41 (s, 2H, H-2), 6.34 (s, 1H, NH), 7.06 (s, 1H, NH). ^{13}C NMR ($CDCl_3$) δ (ppm) 14.5 (C-10′), 22.9 (C-9′), 26.8–30 (C-2′–C-7′ and C-5–C-7), 32.3 (C-8′), 32.9 (C-8), 46.6 (C-4), 47.2 (C-1′ and C-2), 63 (C-9). MS (ESI) MH^+ = 515, MNa^+ = 537, HPLC purity by ELSD 98%.

4.2.15. *N*,*N*-Bis(decyl)-2-{[(2-phenylethyl)carbamothioyl]amino}acetamide (6j). 2-Phenyl-ethylamine was added to a solution of **4** to yield *N*,*N*-bis(decyl)-2-{[(2-phenylethyl)carbamothioyl]amino}acetamide **6j** 0.062 g (60%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.921 (t, 6H, J = 7 Hz, H-10′), 1.32 (m, 4H, H-9′), 1.20–145 (m, 20H, H-3′–H-7′), 1.324 (m, 4H, H-8′), 1.48 (m, 2H, H-2′), 1.59 (m, 2H, H-2′), 2.93 (t, 2H, J = 6 Hz, H-5), 3.30 (m, 4H, H-1′), 3.70 (br s, 2H, H-4), 4.42 (s, 2H, H-2), 6.52 (s, 1H, NH), 7.23–7.33 (m, 5H, Ph).MS(ESI) MH^+ = 518, HPLC purity by ELSD 90%.

4.2.16. *N*,*N*-Bis(decyl)-2-{[(2-hydroxyethyl)carbamothioyl]-amino}acetamide (6k). Ethanalamine was added to a solution of **4** to yield 0.055 g *N*,*N*-bis(decyl)-2-{[(2-hydroxyethyl)carbamothioyl]-amino}acetamide **6k** (60%).

^{1}H NMR ($CDCl_3$) δ (ppm) 0.91 (t, 6H, J = 7 Hz, H-10′), 1.20–145 (m, 28H, H-3′–H-9′), 1.56 (m, 2H, H-2′), 1.66 (m, 2H, H-2′), 3.35 (m, 4H, H-1′), 3.73 (br s, 2H, H-4), 4.57 (s, 2H, H-2), 7.34 (s, 1H, NH), 7.95 (s, 1H, NH). MS (ESI) MH^+ = 459, M+Na = 481, HPLC purity by ELSD 95%.

4.3. Physico-chemical procedures

4.3.1. Preparation of the liposomes by the film/hydration method. All compounds were formulated the same way. Example is given for compound **6b**. Compound **6b** (0.85 mg, 1.7 μmol) and DMPC (1.9 mg, 2.8 μmol) were dissolved in 600 μL of $CHCl_3$. After solvent removal under vacuum, the film obtained was hydrated with 400 μL of H_2O at room temperature to give a multilamellar liposome. Liposomes were successively heated at 37 °C and extruded to form a homogeneous population of liposomes.

4.3.2. Preparation of the complexes DMPC/LMTU/DNA. Plasmid DNA (pVax2Luc[18]), 50 μL, 0.01 g/L in sucrose 15%, was added to various amounts of DMPC/LMTU liposomes at room temperature. The mixture was vortexed during 10 s and left at room temperature for 1 h before size measurements using water viscosity and refractive index. TU/PO indicates the ratio in nmol of thiourea function versus nmol of DNA phosphates

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

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