Gene Therapy (2003) 10, 1206–1215

www.nature.com/gt

RESEARCH ARTICLE

Single histidine residue in head-group region is sufficient to impart remarkable gene transfection properties to cationic lipids: evidence for histidine-mediated membrane fusion at acidic pH

VV Kumar[1], C Pichon[2], M Refregiers[2], B Guerin[2], P Midoux[2] and A Chaudhuri[1]
[1]*Division of Lipid Science and Technology, Indian Institute of Chemical Technology, India; and* [2]*Centre de Biophysique Moléculaire, CNRS UPR 4301, Orleans Cedex, France*

Presence of endosome-disrupting multiple histidine functionalities in the molecular architecture of cationic polymers, such as polylysine, has previously been demonstrated to significantly enhance their in vitro *gene delivery efficiencies. Towards harnessing improved transfection property through covalent grafting of endosome-disrupting single histidine functionality in the molecular structure of cationic lipids, herein, we report on the design, the synthesis and the transfection efficiency of two novel nonglycerol-based histidylated cationic amphiphiles. We found that* L*-histidine-(N,N-di-n-hexadecylamine)ethylamide (lipid* ***1****) and* L*-histidine-(N,N-di-n-hexadecylamine,-N-methyl)ethylamide (lipid* ***2****) in combination with cholesterol gave efficient transfections into various cell lines. The transfection efficiency of Chol/lipid* ***1*** *lipoplexes into HepG2 cells was two order of magnitude higher than that of FuGENE™6 and DC-Chol lipoplexes, whereas it was similar into A549, 293T7 and HeLa cells. A better efficiency was obtained with Chol/lipid* ***2*** *lipoplexes when using the cytosolic luciferase expression vector (pT7Luc) under the control of the bacterial T7 promoter. Membrane fusion activity measurements using fluorescence resonance energy transfer (FRET) technique showed that the histidine head-groups of Chol/lipid* ***1*** *liposomes mediated membrane fusion in the pH range 5–7. In addition, the transgene expression results using the T7Luc expression vector convincingly support the endosome-disrupting role of the presently described mono-histidylated cationic transfection lipids and the release of DNA into the cytosol. We conclude that covalent grafting of a single histidine amino acid residue to suitable twin-chain hydrophobic compounds is able to impart remarkable transfection properties on the resulting mono-histidylated cationic amphiphile, presumably* via *the endosome-disrupting characteristics of the histidine functionalities.*
Gene Therapy (2003) **10,** 1206–1215. doi:10.1038/sj.gt.3301979

Keywords: *gene transfer; cationic lipids; liposomes*

Introduction

Gene therapy, because of its aim to eradicate causes rather than symptoms of diseases, is believed by many to be the therapy of the future. Recent completion of the working draft of human genome has strengthened such belief. However, spontaneous delivery and expression of the supplemented correct copy of the malfunctioning genes into cells is unlikely to be an efficient process due to the unfavorable electrostatic interactions between macromolecular DNA and biological cell surface (both being negatively charged) and due to the nuclease sensitiveness of nonprotected DNA. To develop an efficient gene therapeutic approach, one must design safe and efficient gene delivery reagents. These goals are inseparable in a sense that any shortcomings in one will adversely affect the success of the other. In other words, realization of the full potential of gene therapy will depend, in a major way, on the future development of safe and efficient gene delivery reagents.

Transfection vectors commonly used in gene therapy are mainly of two types: viral and nonviral. The efficiencies of viral transfection vectors[1] are, in general, superior to their nonviral counterparts. However, serious immunogenicity concerns associated with the use of viral vectors[2] combined with their limited insert-size and high product cost are increasingly making the nonviral gene delivery reagents the vectors of choice. Among the existing arsenal of nonviral gene delivery reagents, the distinct advantages associated with the use of cationic liposomes include their low immunogenic response, robust manufacture, ease in handling and preparation techniques, and the ability to inject large lipid:DNA complexes. Ever since Felgner *et al*[3] reported their pioneering development of cationic lipid-mediated gene delivery protocol in 1987, many reports aimed at developing more efficient cationic transfection lipids have appeared in the literature.[4–10] Recently, we have

Correspondence: Dr A Chaudhuri, Division of Lipid Science and Technology, Indian Institute of Chemical Technology, Hyderabad 500 007, India or Dr P Midoux, Centre de Biophysique Moléculaire, CNRS UPR 4301, Orleans Cedex, France
Received 5 September 2002; accepted 4 December 2002

also developed efficient nonglycerol-based cationic transfection lipids.[11–15] In sharp contrast, details of cationic lipid-mediated transfection pathways still remain elusive. According to our current understanding, the intracellular pathways involved in lipoplex (lipid–DNA complex)-mediated transfection include: (a) endocytotic cellular uptake of lipoplex; (b) the endosomal release of DNA into the cytosol and (c) the transport of DNA into the cell nucleus followed by its transcription and expression.[16–19]

The weak efficiency of plasmid transfer into the cytosol remains one of the major limiting factors to achieve efficient transfection. For lipofection, the neutral fusogenic colipid 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE), in combination with a cationic lipid, is usually used to favor the cytosolic delivery of plasmid. In the area of cationic polymer-mediated gene delivery, remarkable transfection efficiency was previously achieved by one of our groups through covalent grafting of endosome-disrupting multiple histidine functionalities in the molecular architecture of cationic polymer.[20] Optimal conditions for transfection have been obtained when polylysine was substituted with 72 ± 9 histidyl residues corresponding to a polylysine substitution level of $38 \pm 5\%$.[21] Based on these data, *N*-Ac-poly(L-histidine)-graft-poly(L-lysine) comb-shaped polymer has also been designed.[22] More recently, Chen *et al*[23,24] have succeeded in demonstrating the enhanced transfection efficiencies of cationic liposomes used in combination with copolymers of histidine and lysine.

Herein, we report on the design, synthesis and transfection efficiency of two novel histidylated cationic amphiphiles (**1** and **2**, **Schemes I and II**) containing a single endosome-disrupting histidine head-group. As delineated below, membrane fusion activity measurements using resonance energy transfer technique in the pH range 5–7 support the view that the release of DNA from the endosomally trapped lipoplexes to the cytosol is likely to be mediated by the histidine head-groups of the cationic amphiphiles. Cytosolic transgene expression in 293T7 cells using the pT7Luc plasmid encoding luciferase gene under the control of the bacterial T7 promotor further supports this cytosolic release of DNA from lipoplexes made of the present mono-histidylated cationic lipids.

Results and discussion

Chemistry

We designed and synthesized two simple mono-histidylated cationic lipids **1** and **2** using the precursor mixed primary–tertiary amine **II** (Scheme 1) readily available in one of our laboratories. The endosome-disrupting histidine functionality in the head-group region of lipid **1** was covalently grafted by conventional dicyclohexyl-carbodiimide (DCC) coupling of di-tetr-butyl-pyrocarbonate (di-Boc)-protected histidine (**IV**) and the hydrophobic mixed tertiary–primary amine (**III**) followed by acid deprotection and chloride ion-exchange chromatography of the resulting intermediate **V** as outlined in Scheme 1. The mixed tertiary–primary amine **III** was prepared by reacting *N*,*N*-di-hexadecylamine (**I**) with *N*-tert-butyloxycarbonyl protected 2-bromoethylamine in ethyl acetate (EtoAc) in the presence of anhydrous potassium carbonate followed by deprotection and neutralization of the resulting intermediate **II** (Scheme 1).

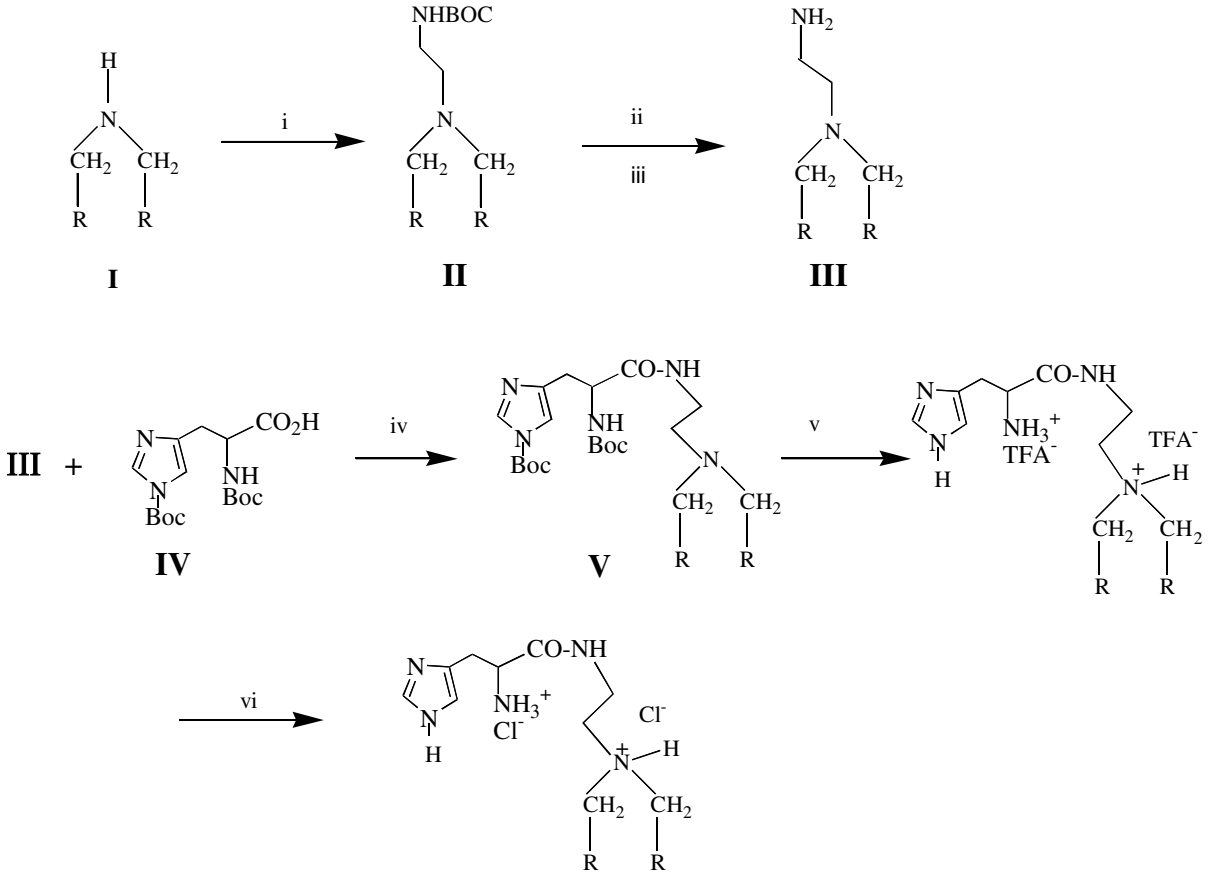
Lipid **2** was synthesized by quaternizing the common intermediate **V** with excess methyl iodide followed by acid deprotection and chloride ion-exchange chromatography of the resulting quaternized intermediate **VII** (Scheme 2).

Transfection efficiency

Toward addressing the comparative transfection properties of lipids **1** and **2** in nuclear and cytoplasmic expression systems, both a conventionally used nuclear expression plasmid pCMVLuc and a cytosolic expression plasmid pT7Luc have been used in human embryonic kidney 293-T7 cells. These cells stably express low levels of T7 RNA polymerase, an enzyme necessary for the cytoplasmic transcription of genes controlled by the bacteriophage T7 RNA polymerase promoter. The use of cytoplasmic expression systems with T7 RNA polymerase capable of ensuing cytoplasmic expression of the transfected genes has been demonstrated to be an elegant way to avoid nuclear barrier of transfection pathways.[25–29]

Experiments conducted with lipid **1** using both pCMVLuc and pT7Luc plasmids in 293T7 cell lines revealed that the transfection efficiency was not sensitive to either cholesterol-to-lipid **1** molar ratios (in the range 1:2–3:1) or to the lipid **1**-to-DNA charge ratios (in the range 3.5–1.3) (Figure 1). The observed lower transfection efficiencies for pT7Luc compared to that for pCMVLuc could result from the low level of the T7 RNA polymerase present in 293T7 cells and/or the weak strength of the promoter. Increasing the lipid-to-DNA charge ratios significantly enhanced the transfection efficiency with pCMVLuc at a constant cholesterol-to-lipid **1** molar ratio of 2:1 (Figure 2a). The luciferase expression was 10-fold higher at N/P=1.75 than at N/P=1. However, the expression of pT7Luc was optimal with the lipid-to-DNA charge ratio around N/P=1 (Figure 2b). Above this ratio, no benefit was obtained: the luciferase activity was lower at N/P=1.75. An excess of cationic charges is likely to increase interactions between liposomes and DNA. In the case of the nuclear gene expression system (pCMVLuc), it would prevent a rapid dissociation of the lipoplexes inside the cell, protecting DNA from degradation and thereby favoring its nuclear delivery. Conversely, in the case of the cytosolic gene expression system (pT7Luc), a high lipoplexes stability could be a limiting factor. Therefore, a decrease of the amount of cationic liposomes would reduce the stability of lipoplexes, facilitating their dissociation and the accessibility of the DNA to the cytoplasmic expression machinery.

Cholesterol in combination with lipid **1** with a molar ratio of 2:1 formed liposomes (Chol/lipid **1**) with a size of 78 ± 24 nm. Under the same conditions, the sizes of commercially available liposomes such as Lipofectin, LipofectAMINE, DOTAP and DC-Chol were found to be 68 ± 20, 77 ± 25, 51 and 170 ± 50 nm, respectively. The ξ potential (the global charge) of Chol/lipid **1** was 32 ± 1.5 mV. This value was similar to that of Lipofectin and LipofectAMINE but, higher than that of DC-Chol (16 ± 1.5 mV) and lower than that of DOTAP (55 ± 2 mV). When mixed with 5 μg of plasmid DNA, Chol/lipid **1** (2:1) formed lipoplexes with a size of 490 ± 20 nm and exhibited a negative ξ potential of -2.5 mV. Electro-

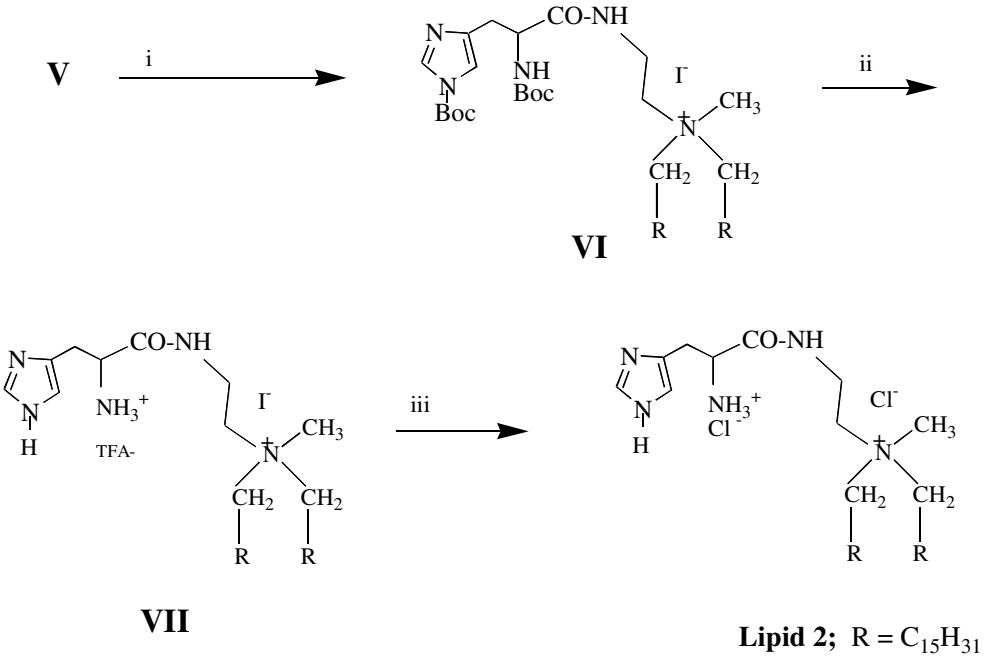


Scheme 1 Reagents*: (i) Br CH_2CH_2NHBoc, EtoAc, 60°, 36 h; (ii) TFA, DCM, 12 h; (iii) 1 N NaOH, DCM, 2 h; (iv) DCC, HOSu, DMF/DCM, 24 h; (v) HCl in dioxane, 12 h; (vi) Amberlyst A-26 chloride ion-exchange resin.*

phoretic agarose gel analysis showed that all the DNA was completely retarded under these conditions (data not shown).

The transfection efficiency of the present lipoplexes was compared with that of FuGENE™6 and DC-Chol/DOPE, two widely used commercially available nonviral transfection kits, in four different cultured cell lines namely, A549, 293T7, HeLa and HepG2 (Figures 3 and 4). The most impressive transfection efficiency was obtained for HepG2 cells. The luciferase activity upon transfection with Chol/lipid **1** lipoplexes was two order of magnitude higher than that obtained using FuGENE™6 and DC-Chol/DOPE. The transfection efficiency of Chol/lipid **1** lipoplexes into A549, 293T7 and HeLa cells was similar to that obtained upon transfection with FuGENE™6 and DC-Chol/DOPE.

Transfection data obtained with liposomes made with lipid **1** indicated that they were more efficient than those made with lipid **2**. Representative comparative transfection results in HepG2, HeLa and 293T7 cells are summarized in Figure 5. At a cholesterol-to-lipid molar ratio of 2:1, the transfection efficiency of Chol/lipid **2** was observed to be lower than that of Chol/lipid **1**. With HepG2 and HeLa cells, Chol/lipid **1** lipoplexes (at a molar ratio of 2:1 and 1:1) exhibited a superior transfection efficiency to Chol/lipid **2** across the varying cholesterol-to-lipid **2** molar ratios of 2:1 to 1:2 (Figure 5a and b). With 293T7 cells, the transfection efficiency of Chol/lipid **2** (at a molar ratio of 1:2) was superior to Chol/lipid **1** (at a molar ratio of 2:1) (Figure 5c). The transfection efficiency of Chol/lipid **2** was significantly higher than that of DOTAP, another widely used commercially available simple cationic transfection lipid. Interestingly, the transfection efficiency of Chol/lipid **2** (at a molar ratio of 1:2) using the cytoplasmic expression plasmid (pT7Luc) was 10-fold higher than that of Chol/lipid **1** across the varying cholesterol-to-lipid **1** molar ratios of 2:1–1:2 (Figure 5d). Such better transfection efficiency of Chol/lipid **2** in cytoplasmic expression system may originate from an enhanced release of the plasmid and/or its better accessibility by the T7 RNA polymerase. Clearly, further mechanistic investigations are needed to throw more light into the origin of such differential relative transfection profile of Chol/lipid **1** and Chol/lipid **2** in cytoplasmic and nuclear expression systems. It is noticeable that the cellular toxicity of Chol/lipid **2** was found to be very low (data not shown).



Scheme 2 Reagents: *(i) CH_3I, DCM, 2 h; (ii) HCl in dioxane, 24 h; (iii) Amberlyst A-26 chloride ion-exchange resin.*

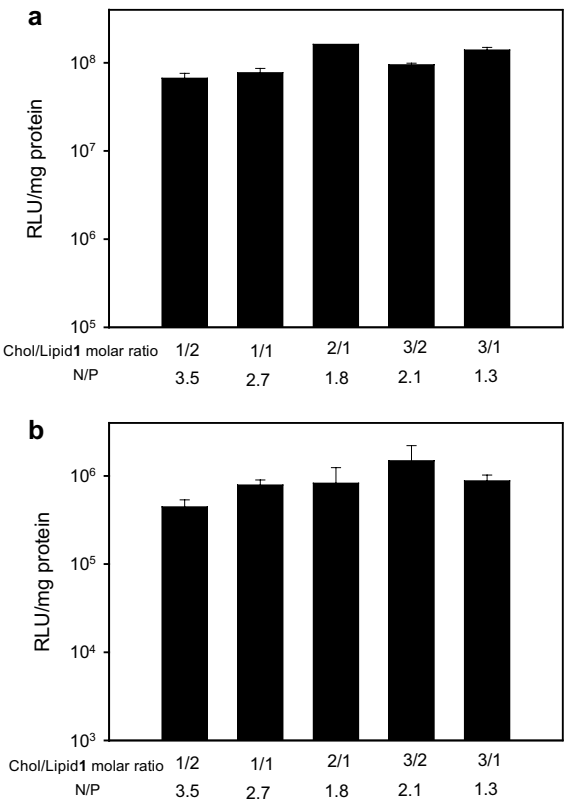


Figure 1 *Transfection efficiency of Chol/lipid **1**. 293T7 cells were transfected with 2.5 μg of either pCMVLuc (a) or pT7Luc (b). The luciferase activity was measured upon 24 h culture. N/P is the charge ratio between the number of cationic charges of the liposomes (two positive charges for lipid **1** at pH 7.4) and the negative charges of the plasmid. The transfection values shown are average of three independent experiments.*

Membrane-disrupting capacity of histidylated cationic liposomes

Toward addressing the membrane-disrupting capacity of the presently described histidylated cationic liposomes for endosomal membranes, we have exploited the widely used fluorescence resonance energy transfer (FRET) technique developed by Struck *et al*[30] This technique depends upon the interactions that occur between two fluorophores when the emission band of one, the energy donor, overlaps with the excitation band of the second, the energy acceptor, because the two probes are in close physical proximity. These conditions are satisfied within a liposome made of both donor and acceptor fluorophore lipids such as *N*-(7-nitro-2-oxa-1,3-diazol-4-yl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (NBD-PE) and Rhodamine Red™-x1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (Rho-PE), respectively. In such liposomes, the energy from a photon absorbed by the energy donor, NBD-PE, is transferred to the energy acceptor, Rho-PE, causing the latter to fluoresce as if it is excited directly. Since the efficiency of FRET between two such fluorophores is dependent upon their spatial separation, any fusion event of such double-fluorophore-containing liposome with a second liposome devoid of any fluorophore decreases the efficiency of resonance energy transfer. Thus, any decrease in FRET efficiency provides evidence for membrane fusion.

Keeping in view the acidity (pH 5–6) of the lumen of endosomes, membrane fusion measurements using FRET technique were carried out in the pH range 5.0–7.4 in the present investigation. When egg yolk phosphatidyl choline (PC)/NBD-PE/Rho-PE liposomes containing both the fluorescent donor and acceptor phospholipid analogs were mixed with Chol/lipid **1** (lipid molar ratio of 2:1 with no added fluorophore) at

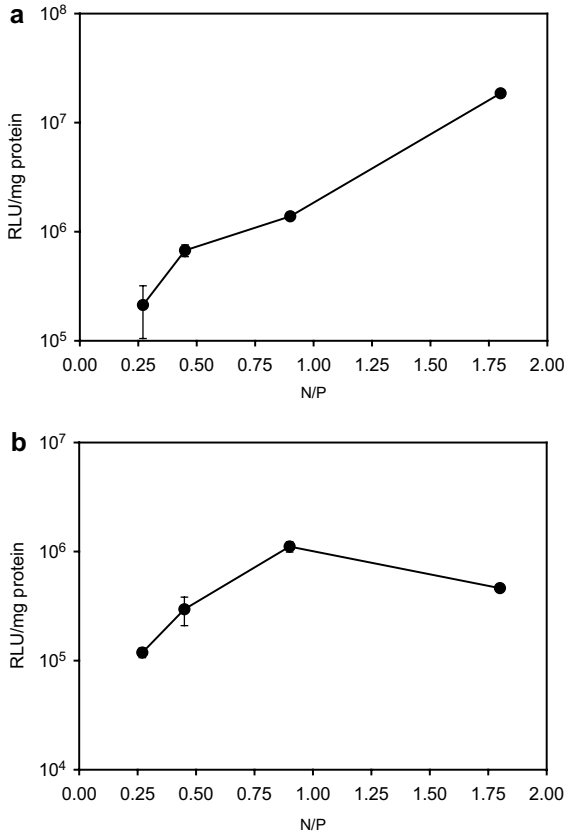


Figure 2 *Transfection efficiency of Chol/lipid* ***1*** *(lipid molar ratio of 2:1) versus the liposome/DNA charge ratio. 293T7 cells were transfected with 2.5 μg of either pCMVLuc (a) or pT7Luc (b) complexed with various amounts of liposomes. N/P is the charge ratio between the number of cationic charges of the liposomes (two positive charges for lipid* ***1*** *at pH 7.4) and the negative charges of the plasmid. The luciferase activity was measured upon 24 h culture. The transfection values shown are average of three independent experiments.*

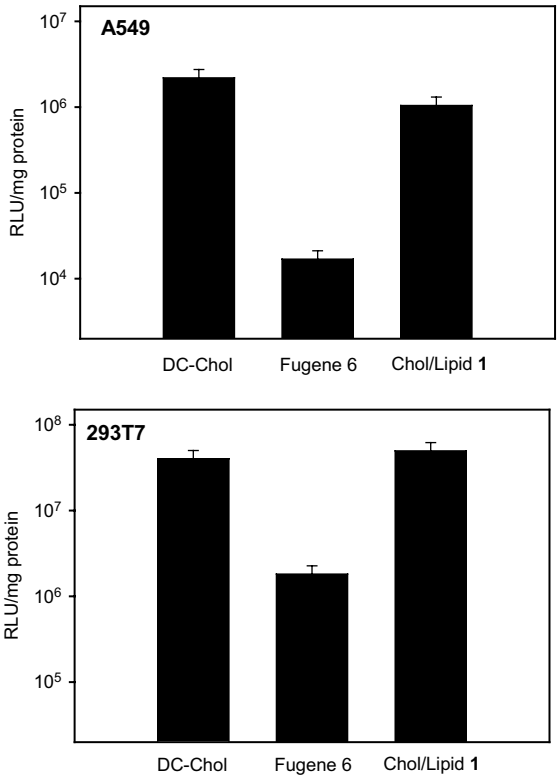


Figure 3 *Transfection efficiency of Chol/lipid* ***1*** *into A549 and 293T7 cells. The cells were transfected with 2.5 μg pCMVLuc complexed with Chol/lipid* ***1*** *(lipid molar ratio of 2:1). The luciferase activity was measured upon 24 h culture. DC-Chol/DOPE was used at a lipid molar ratio of 3:2. The transfection values shown are average of three independent experiments.*

pH 7.4, no significant loss of FRET efficiency was observed, indicating that no fusion occurred (Figure 6). However, when the fusion was induced at pH 5.0 or 6.0, a significant decrease of FRET efficiency was observed. The extent of the acid-induced fusion depended upon the amount of histidylated liposomes added, and 50% of FRET efficiency was obtained in the presence of about 0.1 μM lipid (Figure 6 inset). Similar results were obtained by using liposomes made with PC, L-α-phosphatidylethanolamine (PE), L-α-phosphatidyl-L-serine (PS) and cholesterol (PC/NBD-PE/Rho-PE/PE/PS/cholesterol; 44%:5%:5%:16%:10%:20%), a lipid composition close to that of the plasma membrane (data not shown). Presumably, the histidine head-group of lipid **1** destabilizes lipid bilayers in a slightly acidic medium and thereby induces membrane fusion upon protonation of the imidazole groups by increasing interactions between lipid **1** and the membrane phospholipids. Given the low acidity of endosomes, such enhanced decrease in FRET efficiency at pH values 5 and 6 is consistent with the endosome-fusion property of the presently described mono-histidylated cationic transfection lipids. In addition, the transgene expression results summarized in Figures 1b, 2b and 5d using the cytoplasmic expression vector (pT7Luc) convincingly demonstrate the cytosolic delivery of DNA from endosomes. Such cytosolic delivery indirectly supports the endosome-disrupting role of the presently described mono-histidylated cationic transfection lipids.

Conclusion

In summary, we show that covalent grafting of a single histidine amino acid residue to suitable twin-chain hydrophobic compounds is sufficient to impart remarkable transfection properties on the resulting mono-histidylated cationic amphiphile presumably via the endosome-disrupting characteristics of the histidine functionalities. To date, reports on the use of mono-histidylated cationic lipid for *in vitro* gene delivery are few. The only other mono-histidylated cationic lipid that we are aware of is a recent structure–activity report by Heyes *et al*[31] in which only two mono-histidylated cationic compounds were included in a library of 61 cationic lipids with various amino acid head-groups and varying chain length aliphatic hydrophobic anchors connected via glycerol linkers. Thus, the endosome fusion properties of the presently described lipids indicate that undertaking of systematic structure–activity investigation involving a series of mono-histidilyted cationic lipids with varying anchor lengths and diverse

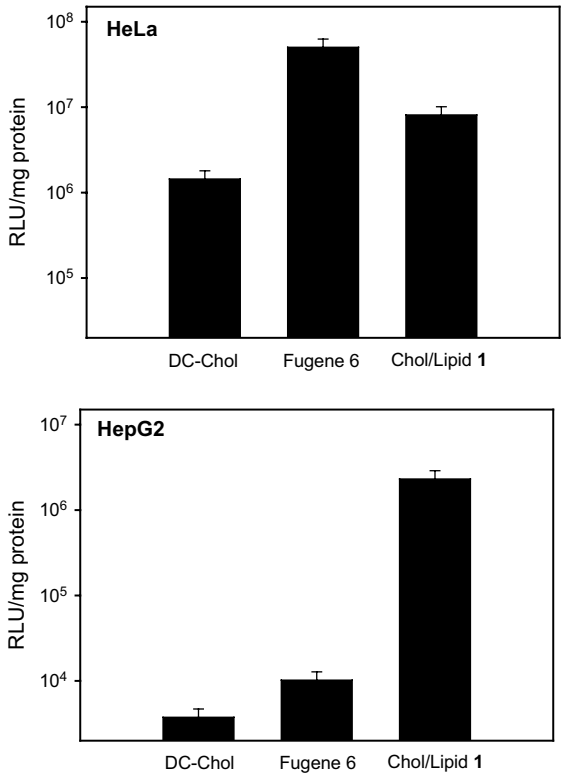


Figure 4 *Transfection efficiency of Chol/lipid* ***1*** *into HeLa and HepG2 cells. The cells were transfected with 2.5 μg pCMVLuc complexed with Chol/lipid* ***1*** *(lipid molar ratio of 2:1). The luciferase activity was measured upon 24 h culture. DC-Chol/DOPE was used at a lipid molar ratio of 3:2. The transfection values shown are average of three independent experiments.*

hydrophobic skeletons is likely to be rewarding. Toward this end, work is under progress in our laboratories.

Materials and methods

Synthesis

N,N-di-n-hexadecylamine (I). A mixture of 8 g of *n*-hexadecylamine (33.2 mmol), 10.2 g of *n*-hexadecyl bromide (33.2 mmol) and 1.8 g of anhydrous potassium carbonate (13.1 mmol) in 4 ml dimethyl sulfoxide was kept under stirring for 12 h at 80°C. The reaction mixture was taken in 100 ml chloroform, washed with water (3×100 ml), dried over anhydrous magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue, using 60–120 mesh silica gel size and 1–2% methanol in chloroform (v/v) as the eluent, afforded 3.5 g (23% yield) of the intermediate secondary amine (R_f=0.6 in 10% methanolic chloroform, v/v, as the TLC developing solvent).
^{1}H NMR (200 MHz, $CDCl_3$): δ/ppm=0.9 [t, 6H, $C\underline{H}_3$-$(CH_2)_{12}$-]; 1.2–1.3 [m, 52H, -$(C\underline{H}_2)_{13}$-CH_3]; 1.5 [m, 4H, N(-CH_2-$C\underline{H}_2$-)$_2$]; 2.4 [t, 4H, N($C\underline{H}_2$-CH_2-)$_2$].

N-[2-tert-butyloxycarbonylamino ethyl]-N,N-di-n-hexadecylamine (II). A mixture of 2.2 g (4.7 mmol) of *N,N* di-*n*-hexadecylamine (**I**) and 1.1 g (5.2 mmol) of *N*-tert-butyloxycarbonyl-2-bromoethylamine (prepared conventionally by reacting 2-bromoethylamine hydrobromide with di-tert-butyl pyrocarbonate in the presence of triethylamine followed by usual work up) was refluxed in EtoAc in the presence of anhydrous potassium carbonate (1.4 g; 10 mmol) for 36 h. The reaction mixture was taken in 100 ml chloroform, washed with water (2×100 ml), dried over anhydrous magnesium sulfate and filtered. Chloroform was removed from the filtrate on a rotary evaporator. Silica gel column chromatographic purification of the resulting residue (using 60–120 mesh silica gel size and 0.5% methanol in chloroform, v/v, as the eluent) afforded 2 g (light yellow solid, 71% yield) of the title tertiary amine (R_f=0.6 in 5% methanolic chloroform, v/v, as the TLC developing solvent).
^{1}H NMR (200 MHz, $CDCI_3$): δ/ppm=0.9 [t, 6H, $C\underline{H}_3$-$(CH_2)_{13}$-]; 1.2–1.4 [m, 52H, -$(C\underline{H}_2)_{13}$-CH_3]; 1.5 [s, 9H, -O-CO-C($C\underline{H}_3$)$_3$]; 2.4 [t, 4H, -N($C\underline{H}_2$-$(CH_2$-$)_{13}$]; 2.5 [t, 2H, -N($C\underline{H}_2$-CH_2-NHBoc]; 3.1 [m, 2H, N(CH_2-$C\underline{H}_2$-NHBoc)]; 4.9 [bm, 1H, N$\underline{H}$Boc].

N-2-aminoethyl-N,N-di-n-hexadecylamine (III). The tertiary amine (**II**) obtained above (1.9 g; 3.2 mmol) was dissolved in 4 ml of anhydrous dichloromethane (DCM), and 2 ml of neat trifluoroacetic acid (TFA) was added. The reaction mixture was kept under stirring overnight at room temperature. The TFA was removed by flushing with nitrogen and the remaining residue was kept under vacuum for 15 min. The resulting trifluoroacetate salt of the title compound was dissolved in 25 ml of DCM, and 25 ml of aqueous 1 N NaOH was added. The resulting biphasic mixture was stirred at room temperature for 2 h. The organic layer was washed with water (3×25 ml), dried over anhydrous sodium sulfate, filtered and the solvent from the filtrate was evaporated under rotary evaporator. Column chromatographic purification of the residue (using 60–120 mesh size silica gel and 2–3% methanol in chloroform, v/v, as the eluent) afforded 1.5 g of the title compound (93% yield, R_f=0.4 using 10% methanolic chloroform, v/v, as the TLC developing solvent).
^{1}H NMR (200 MHz, $CDCl_3$): δ/ppm=0.9 [t, 6H, $C\underline{H}_3$-$(CH_2)_{12}$-]; 1.2–1.4 [m, 52H, -$(C\underline{H}_2)_{13}$-]; 1.5 [m, 4H, N(-$C\underline{H}_2$-$C\underline{H}_2$-)$_2$]; 2.5 [m, 4H, N($C\underline{H}_2$-$C\underline{H}_2$-)$_2$]; 2.6 [t, 2H, -N($C\underline{H}_2$-$C\underline{H}_2$-NH_2)]; 2.9 [t, 2H, -N(CH_2-$C\underline{H}_2$-NH_2)]; 3.5–3.7 [bm, 2H, N$\underline{H}_2$].

N$^{\alpha}$,N^{im},di-butyloxycarbonyl-L-histidine (IV). A mixture of *N,N*-dimethylformamide (2 ml), water (2.5 ml), triethylamine (2.5 ml; 17.9 mmol) and L-histidine (770 mg; 5 mmol) was taken in a 50 ml round-bottomed flask and the mixture was cooled to 0°C. Di-tert-butylpyrocarbonate (2.5 ml; 10.9 mmol) was added dropwise to this cold solution. The reaction mixture was stirred at room temperature for 48 h and washed with hexane (20 ml). The hexane layer was extracted with saturated aqueous $NaHCO_3$ (20 ml). The combined aqueous extracts were acidified with solid potassium hydrogen sulfate to pH 2 and extracted with EtoAc (3×20 ml). The EtoAc extract was washed with water (4×20 ml), dried with anhydrous sodium sulfate, filtered and the filtrate concentrated on a rotary evaporator to give 1.25 g of the title compound as a white gummy solid (71% yield).

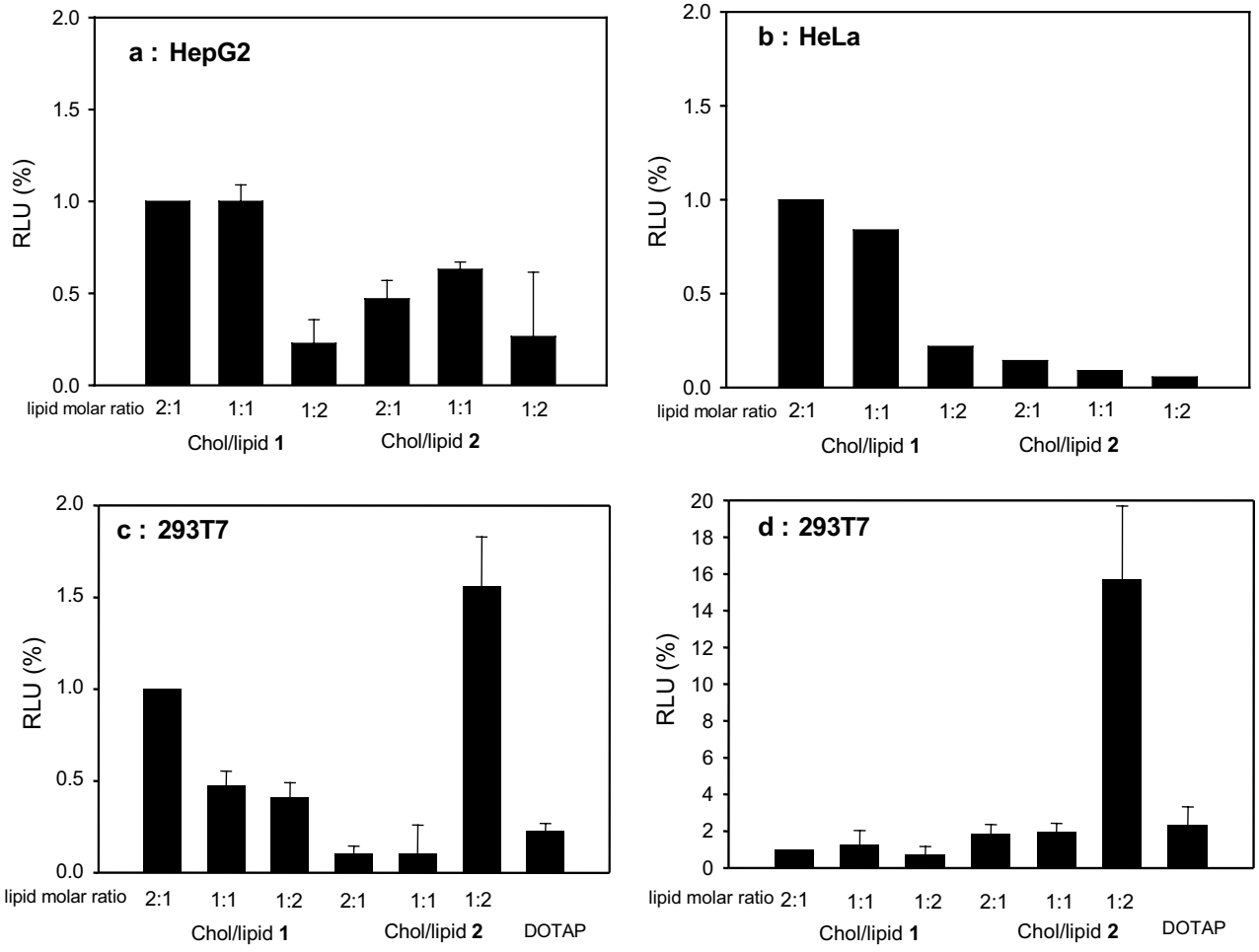


Figure 5 *Comparative transfection efficiencies of Chol/lipid* ***1*** *and Chol/lipid* ***2****. Cells were transfected with 2.5 μg pCMVLuc (a, b and c) or pT7Luc (d) complexed with the liposomes. The luciferase activity was measured upon 24 h culture. The transfection efficiency is expressed as the percentage relative to Chol/lipid* ***1*** *(lipid molar ratio of 2:1). The transfection values shown are average of three independent experiments.*

^{1}H NMR ($CDCl_3$, 200 MHz): δ/ppm=1.4–1.5 [s, 18H, -CO-O-($C\underline{H}_3$)$_3$]; 3.0 [dd, 2H, -$C\underline{H}_2$-CH(NHBoc)COOH]; 4.1 [m, 1H, -$C\underline{H}$(NHBoc)COOH]; 6.1 [s, 1H, -N$\underline{H}$-CO-O-(CH_3)$_3$]; 6.9 [s, 1H, -NBoc-C$\underline{H}$=C-]; 7.7 [s, 1H, -NBoc-C$\underline{H}$=N-].

N^{α},N^{im},di-butyloxycarbonyl-L-histidine(N,N-di-n-hexadecylamine)ethylamide (V). A mixture of N^{im},N^{α},Di-Boc-L-histidine (0.68 g; 1.9 mmol), dicyclohexylcarbodiimide (0.41 g; 2 mmol), *N*-hydroxysuccinimide (0.271 g; 2.3 mmol) was dissolved in 8 ml of dry *N*,*N*-dimethylformamide and stirred for 1 h. *N*-2-aminoethyl-*N*,*N*-di-*n*-hexadecylamine (1 g; 2 mmol) dissolved in 2 ml of dry DCM was added slowly to the mixture at 0°C and stirred for 24 h. In total, 50 ml of DCM was added to the reaction mixture, and the mixture was washed with water (3 × 100 ml) and dried with anhydrous sodium sulfate. Silica gel column chromatography (using 60–120 mesh size silica and 0.5–1% methanol in chloroform, v/v, as the eluent) of the residue afforded 1.1 g of pure coupled product (yield=66%, R_f=0.6 using 5% methanolic chloroform, v/v, as the TLC developing solvent).
^{1}H NMR ($CDCl_3$, 200 MHz): δ/ppm=0.9 [t, 6H, $C\underline{H}_3$-(CH_2)$_{13}$-]; 1.2–1.3 [m, 52H, CH_3-($C\underline{H}_2$)$_{13}$]; 1.4–1.6 [2s, 18H, -CO-O-($C\underline{H}_3$)$_3$]; 2.5–2.6 [m, 6H, -N-$C\underline{H}_2$-(CH_2)$_{14}$-CH_3; -N-$C\underline{H}_2$-CH_2-NH-CO-]; 2.9–3.0 [2 dd, 2H, -$C\underline{H}_2$-CH-NH-CO-]; 3.3 [m, 2H, -CH_2-$C\underline{H}_2$-NH-CO-]; 4.4 [m, 1H, -$C\underline{H}$(NHBoc)-CO-]; 6.1 [s, 1H, -N$\underline{H}$-CO-O-(CH_3)$_3$]; 7.0 [s, 1H, N-C$\underline{H}$=C-]; 8.0 [s, 1H, N-C$\underline{H}$=N-]:
fast atom bombardment mass spectrometry FABMS: *m*/*z*: 846 (36%).

L-histidine-(N,N-di-n-hexadecylamine)ethylamide (lipid 1). Compound **V** (0.1 g; 0.12 mmol) was dissolved in 1 ml of 2 N HCl in dioxane and the solution was kept under stirring at room temperature overnight. The solvent was removed with nitrogen flush and the residue was kept under vacuum for 1 h. Silica gel column chromatography (using 60–120 mesh size silica and 10–15% methanolic chloroform, v/v, as the eluent) of the dried residue afforded 40 mg of pure title compound (53% yield).
^{1}H NMR ($CDCl_3$, 200 MHz): δ/ppm=0.9 [t, 6H, $C\underline{H}_3$-(CH_2)$_{13}$-]; 1.2–1.3 [m, 52H, CH_3-($C\underline{H}_2$)$_{13}$-]; 1.6–1.7 [m, 4H, -$C\underline{H}_2$-CH_2-N^+-]; 3.0 [m, 5H, -$CH_2C\underline{H}$($N\underline{H}_3^+$)CO-]; 3.3 [m, 4H, -N^+($C\underline{H}_2$-(CH_2-)$_{14}$-CH_3]; 3.5 [m, 2H, -N^+-$C\underline{H}_2$-CH_2-NH-CO]; 3.7 [m, 2H, -N^+-CH_2-$C\underline{H}_2$-NH-CO-]; 4.3 [m, 1H, -$C\underline{H}$(NH_3^+)CO-]; 7.2 [s, 1H, -N-C$\underline{H}$=C-], 8.3 [s, 1H, -N-

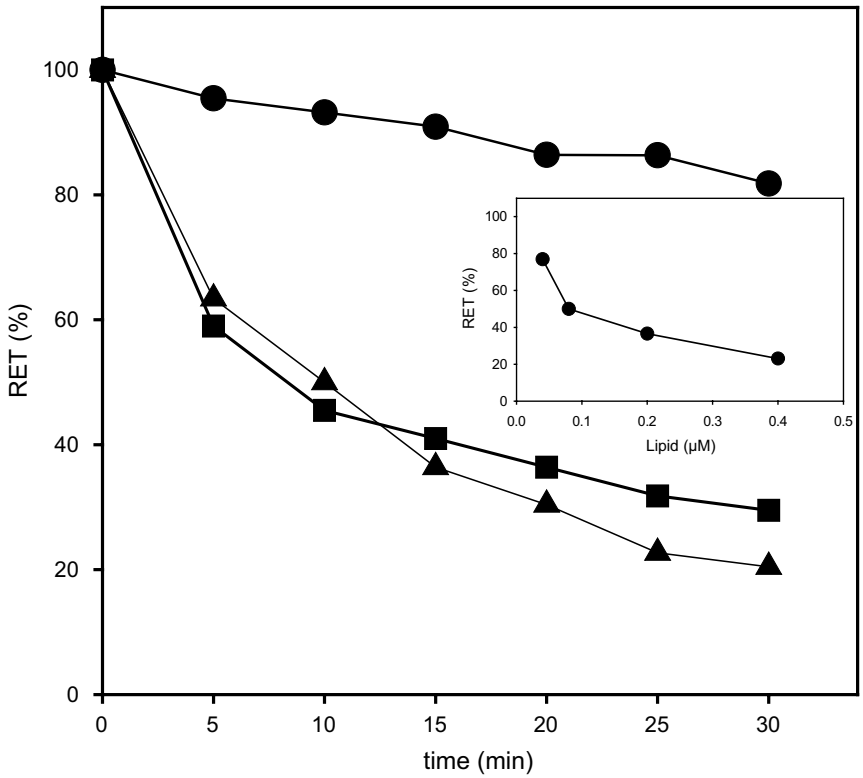


Figure 6 *The membrane fusion activity of Chol/lipid* ***1****. Fusion* versus *pH. Fusion was induced by adding PC/NBD-PE/Rho-PE liposomes (0.01 μmol) to Chol/lipid* ***1*** *(lipid molar ratio of 2:1) (0.4 μmol in 0.6 ml of 10 mM Hepes buffer at ● pH 7.4, ■ pH 6.0, and ▲ pH 5.0) directly in the cuvette. Insert: Fusion* versus *the amount of Chol/lipid 1. Fusion was induced by adding PC/NBD-PE/Rho-PE liposomes (0.01 μmol) to various amounts of Chol/lipid* ***1*** *(lipid molar ratio of 2:1) (in 0.6 ml of 10 mM Hepes buffer, pH 7.4) directly in the cuvette. The fluorescence intensity of rhodamine was monitored at 30°C. The values shown are representative of three independent measurements.*

CH=N-]; 9.3 [s, 1H, -NH-CO-].
FABMS: *m/z*: 647 (40%).

N^{α},N^{im},di-butyloxycarbonyl-L-histidine(N,N-di-n-hexadecylamine,N-methyl)ethylamide (VI). Compound **V** (1 g, 12 mmol) was dissolved in 3 ml DCM, and 2 ml methyl iodide was added. The solution was stirred for 1 h, solvent removed on rotary evaporator and silica gel column chromatography (using 60–120 mesh size silica and 2% methanolic chloroform, v/v, as the eluent) of the residue afforded 0.42 g of the title compound (35% yield).
^{1}H NMR($CDCl_3$, 200 MHz): δ/ppm=0.9 [t, 6H, CH_3-$(CH_2)_{13}$-]; 1.2–1.3 [m, 52H, CH_3-$(CH_2)_{13}$-]; 1.4–1.6 [2s, 18H, CO-O-C$(CH_3)_3$]; 1.6–1.7 [m, 4H, -CH_2-CH_2-N^+-]; 3.0 [m, 2H, -CH_2-CH(NHBoc)CO-]; 3.2 [S, 3H, CH_3-N^+-]; 3.4 [m, 4H,-N^+-CH_2-$(CH_2)_{14}$-CH_3]; 3.6–3.7 [bm, 4H, -N^+-CH_2-CH_2-NHCO-]; 4.3 [m, 1H, -CH(NHBoc)CO-]; 6.0 [s, 1H, -NH-CO-O-$(CH_3)_3$]; 7.2 [s, 1H, -N-CH=C-]; 8.0 [s, 1H, N-CH=N-]; 8.5 [s, 1H, - CH_2-NH-CO-].
FABMS: *m/z*: 861 (40%).

L-histidine(N,N-di-n-hexadecylamine, N-methyl)ethylamide (lipid 2). Compound **VI** (0.4 g, 0.46 mmol) was dissolved in 2 ml of 2 N HCl in dioxane and the solution was kept under stirring for 24 h. The solvent was removed with nitrogen flush and the residue was kept under vacuum for an additional 1 h. Silica gel column chromatography (using 60–120 mesh size silica and 10–15% methanolic chloroform, v/v, as the eluent) followed by chloride ion-exchange chromatography (using Amberlyst-A 26 chloride ion-exchange resin) of the dried residue afforded 90 mg of pure title compound (29% yield).
^{1}H NMR ($CDCl_3$, 200 MHz): δ/ppm=0.9 [s, 6H, CH_3-$(CH_2)_{13}$-]; 1.2–1.3 [m, 52H, CH_3-$(CH_2)_{13}$-]; 1.6–1.7 [m, 4H, CH_3-$(CH_2)_{13}$-CH_2-CH_2-N^+-]; 3.0 [2 dd, 2H, -CH_2-CH(NH_3^+)CO-]; 3.2 [S, 3H, CH_3-N^+-CH_2-CH_2-]; 3.4–3.7 [m, 8H, (CH_3-$(CH_2)_{14}$-CH_2)$_2$-N^+(CH_3)-CH_2-CH_2-NHCO-]; 4.1 [m, 1H, -CH(NH_3^+)CO-]; 6.9 [s, 1H, -N-CH=C-]; 7.7 [s, 1H, -N-CH=N-]; 7.4 [s, 1H, -CH_2-CH_2-NH-CO-].
FABMS: *m/z*: 661 (80%).

ξ potential and size measurements

The charge and the size of liposomes and lipoplexes were measured by electrophoretic mobility and quasi-elastic laser light scattering (QELS), respectively, using Zeta Sizer 3000 (Malvern Instruments, Orsay, France). The size was measured in 10 mM Hepes buffer, pH 7.4, 10 times with a sample refractive index of 1.59 and a viscosity of 0.89. The system was calibrated using the 200±5 nm polystyrene polymer (Duke Scientific Corps Palo Alto, CA, USA). The diameter of liposomes and lipoplexes was calculated by using the automatic mode. The ξ potential was measured in 10 mM Hepes buffer, pH 7.4, with the following parameters: viscosity, 0.891 cP; dielectric constant, 79; temperature, 25°C; *F*(Ka), 1.50 (Smoluchowsky); maximum voltage of the current, 15 V. The system was calibrated using DTS 5050 standard from Malvern. Measurements were done 5 times with the zero

field correction. The ξ potential was calculated using the Smoluchowsky approximation.

Cells and Cell Culture

Human hepatocarcinoma HepG2 cells (8055 HB, ATCC, Rockville, MD, USA), human epithelial ovary carcinoma HeLa cells (CCL21, ATCC), human carcinoma lung A549 cells (CCL185, ATCC) and human embryo kidney 293T7 cells[25] were cultured in MEM containing 10% heat-inactivated fetal bovine serium (Life Technologies, Cergy Pontoise, France), 2 mM L-glutamine (Life Technologies), 100 U/ml penicillin (Life Technologies) and 100 U/ml streptomycin (Life Technologies). 293T7 cells were cultured in the presence of geneticin (400 μg/ml). Cells were mycoplasma-free as evidenced by bisbenzimidazole (Hoechst 33258, Molecular Probes).[32]

Plasmids

pCMVLuc (pUT650, 5.15 kb, Cayla, Toulouse, France) and pT7Luc (kindly given by Dr M Brisson) were plasmid DNA encoding the firefly luciferase under the control of the human cytomegalovirus and the bacteriophage T7 RNA polymerase promoter, respectively. Supercoiled plasmid DNA was isolated by a standard alkaline lysis method and purification was carried out with the QIAGEN® Plasmid Mega kit (QIAGEN, Courtaboeuf, France).

Transfections

Two days prior to transfection, cells were seeded at 2×10^5 cells in 2 ml culture medium in a 12-wells plate. At the time of the experiment, cell cultures were 80% confluent and lipofections were performed as described below. Liposomes were prepared by ethanol injection method: 15 μl of an ethanol solution of the lipid mixture – Chol/lipid1, Chol/lipid2 or DC-Chol/DOPE (DC-Chol and DOPE from Sigma) – at 2.7 mM or 5.4 mM was injected rapidly into 200 μl of 10 mM Hepes buffer, pH 7.4. After 15 min at room temperature, the liposomes were mixed with the plasmid (5 μg in 20 μl of 10 mM Hepes buffer, pH 7.4). After 15 min at room temperature, the lipoplexes solution was completed at 1 ml with serum-free medium and the NaCl concentration was adjusted at 0.15 M with a 5 M NaCl solution. Before incubation with 0.5 ml lipoplexes, cells were washed 2 times with serum-free culture medium. Cells were incubated for 4 h at 37°C, the medium was removed and cells were cultured for 48 h at 37°C in complete culture medium without any additives.

LipofectAMINE™ (Life Technologies) or Lipofectin™ (Roche Diagnostic, Meylan, France), DOTAP (Roche Diagnostic), FuGENE™6 (Roche Diagnostic) were used according to the manufacturer's procedure. Briefly, 25 μl of Lipofectin or Lipofectamine was added to 100 μl of OPTIMEM, left for 15 min at room temperature and then mixed with DNA (2.5 μg in 100 μl OPTIMEM). After 15 min, the lipoplexes solution was completed at 1 ml with OPTIMEM. DOTAP liposomes were made by adding 30 μl of DOTAP in 100 μl of 20 mM Hepes buffer, pH 7.4. After 15 min at room temperature, liposomes were mixed with DNA (5 μg in 100 μl of 20 mM Hepes buffer, pH 7.4). Upon 15 min complexation at room temperature, the lipoplexes solution was completed at 2 ml with serum-free MEM. FuGENE™6 liposomes were prepared by adding 6 μl of FuGENE™6 in 100 μl of DMEM. After 15 min at room temperature, the liposomes were mixed with DNA (2.5 μg in 100 μl DMEM). Then, the lipoplexes solution was completed at 1 ml with serum-free DMEM. The cell transfection procedure using these reagents was similar as described above.

Luciferase assay

Luciferase gene expression was measured by monitoring the luminescence activity according to De Wet *et al*.[33] The medium was discarded and cells were washed 3 times with PBS. The homogenization buffer (200 μl of 8 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM ethylenetetra acetic acid, 1% Triton X-100, 15% glycerol, 25 mM Tris-phosphate buffer, pH 7.8) was added into each well and tissue culture plates were kept for 15 min at 20°C. The solution was recovered and spun down (5 min at 800 g). A total, 95 μl of a 2 mM ATP solution in the homogenization buffer without Triton X-100 was added to 60 μl supernatant and the solution was shaken with a vortex. The luminescence was recorded for 4 s in a Lumat LB 9501 luminometer (Berthold, Wildbach, Germany) upon addition of 150 μl of a 167 mM luciferin solution in water. Measurements were done in duplicate. The number of relative light units (RLU) of 1 pg/ml of luciferase was 2000 under these assay conditions. The data shown correspond to the number of RLU per mg proteins. Proteins were determined on each sample using the modified bicinchoninic acid (BCA) colorimetric assay.[34,35]

Membrane fusion

The membrane fusion activity of Chol/lipid **1** liposomes was measured with the FRET assay as previously described.[30] NBD-PE (Molecular Probes, Eugene, OR, USA) and Rho-PE (Molecular Probes) were used as donor and acceptor fluorescent lipids, respectively. PC (1.14 mg; 1.8 μmol in chloroform) (Sigma), NBD-PE (0.05 mg; 0.052 μmol in ethanol) and Rho-PE (0.14 mg; 0.096 μmol in ethanol) were mixed and dried under reduced pressure. The dried lipid films were hydrated overnight at 4°C in 4 ml of 10 mM Hepes buffer, pH 7.4. The suspension was vigorously vortexed for 2–5 min at room temperature and sonicated for 15 min in cold bath sonicator at 35 kHz (Bioblock Scientific, Strasbourg, France). Fusion was induced by adding PC/NBD-PE/Rho-PE liposomes (0.01 μmol) to Chol/lipid **1** (lipid molar ratio 2:1) liposomes (0.4 μmol in 0.6 ml of 10 mM Hepes buffer, pH 7.4) directly in the cuvette. The fluorescence intensity of rhodamine was monitored with a spectrofluorometer (Jobin-Yvon Fluoromax-2). The excitation was at 465 nm and emissions were at 530 and 580 nm for NDB and Rho, respectively. All experiments were done at 30°C. Fusion (100%) was determined from the rhodamine fluorescence intensity of PC/NBD-PE/Rho-PE liposomes in the presence of 0.1% Triton X100.

Acknowledgements

We express our gratitude to Dr JP Behr (Strasbourg, France) for initiating this Indo-French collaboration. We thank warmly Drs L Huang and M Brisson (University of Pittsburgh, Pittsburgh, PA, USA) for 293T7 cells and pT7Luc, and Miss Laëtitia Million for her help in FRET measurements. This work was supported by grants from

Agence Nationale de Recherche sur le Sida (ANRS) and Association pour la Recherche sur le Cancer (ARC). AC and VVK gratefully acknowledge the financial support received from the Department of Biotechnology, Government of India, New Delhi. PM is Research Director at INSERM. CP is Professor Assistant at the University of Orléans.

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