Solid-Phase Synthesis of 89 Polyamine-Based Cationic Lipids for DNA Delivery to Mammalian Cells

Boon-ek Yingyongnarongkul,[a] Mark Howarth,[b] Tim Elliott,[b] and Mark Bradley*[a]

Abstract: The ability of non-viral gene delivery systems to overcome extracellular and intracellular barriers is a critical issue for future clinical applications of gene therapy. In recent years much effort has been focused on the development of a variety of DNA carriers, and cationic liposomes have become the most common non-viral gene delivery system. Solid-phase synthesis was used to produce three libraries of polyamine-based cationic lipids with diverse hydrophobic tails. These were characterised, and structure-activity relationships were determined for DNA binding and transfection ability of these compounds when formulated as cationic liposomes. Two of the cationic lipids produced high-efficiency transfection of human cells. Surprisingly, these two compounds were from the library with two headgroups and one aliphatic tail, a compound class regarded as detergent-like and little investigated for transfection. These cationic lipids are promising reagents for gene delivery and illustrate the potential of solid-phase synthesis methods for lipoplex discovery.

Keywords: cationic lipids · combinatorial chemistry · gene therapy · non-viral vector · solid-phase synthesis

Introduction

Gene therapy represents an important advance in the alternative treatment of a variety of diseases of both genetic and acquired origin. In this process, the corrected exogeneous genes or portions of a gene are introduced into target cells to replace defective DNA sequences. The most efficient method for transfer of DNA into cells is the use of viral vectors.[1] However, there are growing concerns about both the short- and the long-term risks of viral vectors, the key problems being the immune response they provoke, which can even be lethal,[2] the limit on the size of the DNA that can be introduced, and the difficulty of large-scale production of virus. There have hence been great efforts to develop a range of non-viral vectors for gene therapy applications.[3] A broad range of non-viral delivery systems have been described to date, including microinjection,[4] electroporation,[5] and chemical-based systems such as calcium phosphate,[6] DEAE-dextran[7] and cationic lipid-mediated transfection.[8] Of all the non-viral vectors, cationic lipids have shown the most promise for in vivo applications, based on a combination of efficiency, stability and lack of toxicity. Since the first application of cationic lipids in DNA delivery,[9] numerous cationic lipids have been synthesised. Some of these have been used in gene therapy clinical trials,[10,11] while many others have established cationic lipids as the most common method for the transfection of cell lines in the laboratory.

Transfection of DNA into mammalian cells involves numerous steps.[12] The DNA is first compacted by the cationic transfection agent; the positively charged transfection complex may then undergo electrostatic interactions with the negatively charged cell surface. Uptake into the cell seems to be principally by endocytosis, and a small fraction of the DNA taken up into endosomes then escapes into the cytosol,[12] where a small fraction enters the nucleus. It is in the nucleus that the DNA can be transcribed to produce transient gene expression insert into host chromosomes to produce stable gene expression or to switch off gene expression/transcription. The presence of so many steps in the transfection pathway means that it has been very difficult to generate strong structure-activity relationships for transfection compounds, necessitating an empirical approach, which makes the use of the combinatorial library approach employed here highly advantageous. One key feature of transfection compounds, however, seems to be the ability to change the structure of the liposomes from a lamellar phase, with DNA between lipid bilayers, to an inverted hexagonal

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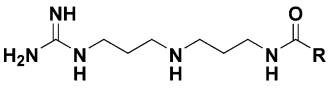
phase, with DNA at the centre of hexagonal arrays of lipid tubes,[13] and this phase transition is believed to promote the escape of the DNA from the endosomes into the cytosol. Dioleoyl-L-α-phosphatidylethanolamine (DOPE) is included as a co-lipid with the cationic lipids used in this study because it helps to promote this phase transition.[13]

While there are no absolute features for the structure of a cationic lipid, the cationic lipid generally contains one or more cationic headgroup(s) attached to one or more hydrophobic tail(s), the headgroup often consisting of a guanidinium functionality or an amine (primary, secondary, tertiary or quaternary). The hydrophobic domain is generally a single- or double-chain hydrocarbon of 12–18 carbon units in length and can be a straight chain, saturated or unsaturated, or a steroidal derivative. Combinatorial solid-phase synthesis allows highly efficient production and purification of diverse libraries of compounds in the search for new therapeutic agents.[14] The first reported use of a solid-phase synthesis approach to generate[15] and study structure–activity relationships[16] of DNA delivery compounds was by Byk. However, the protected polycationic building blocks had to be cleaved from the solid support prior to attachment of the hydrophobic tails. Our work reported here has an advantage that polyamines are directly attached to—and the cationic head groups and hydrophobic tails coupled—on the solid phase. Here we report the synthesis and testing of 89 polyamine-based cationic lipids and the isolation of two compounds with high transfection activity and low toxicity. The two most effective compounds in the library had transfection activity superior to that of the commercial lipid transfection reagent effectene and merit further investigation.

Results and Discussion

In the search for new efficient cationic liposomes as DNA carriers, three libraries of transfection agents **1**–**3** were synthesised. The guanidinium group was selected as the hydrophilic, positively charged section of the transfection agent, since it has been shown that compounds containing the guanidinium group are highly efficient for transfection of a variety of mammalian cell lines.[17] In order that the potential of these guanidinium-containing headgroups could be explored fully, they were conjugated to a wide range of hydrophobic tails: straight-chain and branched-chain tails of 5–18 carbon units, as well as steroidal bile acids (Table 1).



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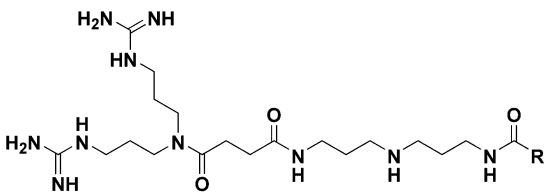

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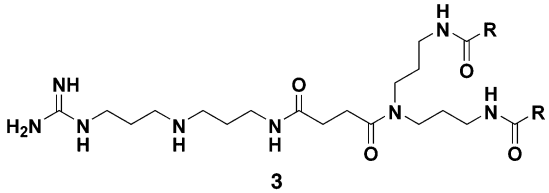

3

Synthesis of the polyamine scaffold: The first step was the construction of a polyamine scaffold, which was used in the synthesis of all three libraries. An unsymmetrical polyamine (**5**) was synthesised, with trifluoroacetyl (Tfa) and (4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde) protecting groups, because of their relative orthogonality (Scheme 1). The Tfa group has been used specifically to protect primary over secondary amines[18] and can be removed with aqueous ammonia[19] or aqueous methanolic K_2CO_3 solution.[20] The Dde group was chosen for its ability to react selectively with primary amines even when used in large excess, while it is stable to both acidic and basic conditions and can be removed under mild conditions.[21] The initial synthetic strategy was to protect one of the primary amino groups of polyamine **4** selectively by treatment with ethyl trifluoroacetate (1 equiv) (Scheme 1). The crude product was subjected to treatment with DdeOH (1.2 equiv) to afford, after careful column chromatography, polyamine **5** (61% yield). This orthogonally protected polyamine **5** was treated with linker **6** to afford **7** in high yield. To attach the polyamine scaffold **7** onto aminomethyl resin, the Aloc protecting group was cleaved by treatment with $[Pd(PPh_3)_4]$, with thiosalicylic acid as the nucleophilic scavenger.[22] This afforded the corresponding acid **8**, which was attached onto aminomethyl resin by a standard DIC coupling strategy to give **9**.

Synthesis of guanidinium-containing transfection agents

Library 1: The first library contained compounds with one guanidinium polar headgroup and one hydrophobic tail (**1**) and was synthesised as shown in Scheme 2. Starting from polyamine scaffold-bound resin **9**, the Dde protecting group was removed by treatment with hydrazine in DMF (5%). Several methods and reagents for the conversion of an amine to a guanidine to generate the guanidine functionality were investigated. The most commonly used reagents include *S*-alkylisothiouronium salts,[23] *N*,*N*′-bis(alkoxycarbonyl)-protected *S*-alkylisothiourea derivatives,[24] pyrazole-1-carboxamidine hydrochloride,[25] di(benzotriazol-1-yl)methanimine[26] and di(imidazol-1-yl)methanimine.[27] In this case, the *S*-alkylisothiouronium salt was observed to be the most reactive. Resin **9** was thus deprotected before treatment with *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv) to afford **10** (a ninhydrin test was negative, showing that there was no primary amine group present). The Tfa group was removed with 1 M KOH/THF/MeOH (4:3:1), and various aliphatic carboxylic acids were coupled to the free amine, to give compounds **11**. The products were obtained in yields of 76–100% after cleavage from the resin with a cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1).

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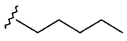
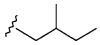
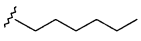
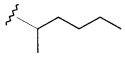
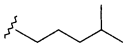
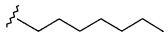
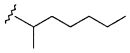
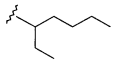
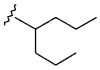
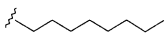
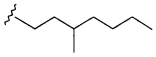
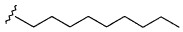
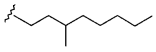
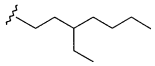
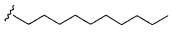
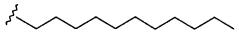
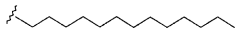
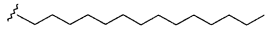
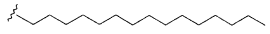
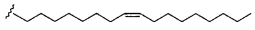
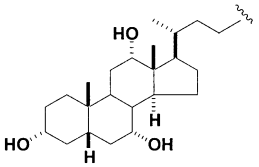
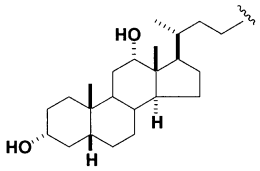
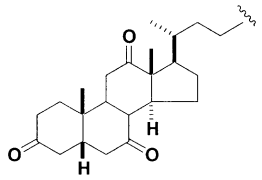
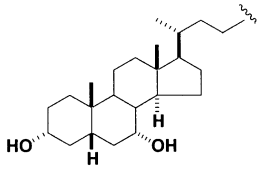
Table 1. Structures of the hydrophobic tails used in the library of cationic lipids.

Compound	R group	Compound	R group
x.1		**x.2**	
x.3		**x.4**	
x.5		**x.6**	
x.7		**x.8**	
x.9		**x.10**	
x.11		**x.12**	
x.13		**x.14**	
x.15		**x.16**	
x.17		**x.18**	
x.19		**x.20**	
x.21		**x.22**	
x.23		**x.24**	
x.25		**x.26**	
x.27		**x.28**	
x.28		**x.30**	
x.31			

Spectroscopic characterisation of these groups of compounds was achieved by NMR (^{1}H, ^{13}C, ^{1}H–^{1}H COSY, ^{1}H–^{13}C COSY and HMBC), to provide the connections of the polyamines to the guanidine and carboxylic acids. Thus, H-8 showed a cross-peak with the guanidine carbon, whereas H-2 showed a cross-peak with the carbonyl carbon, which also correlated to H-2′ and H-3′ (Figure 1).

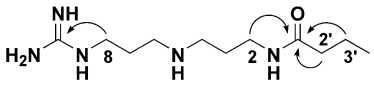


Figure 1. Arrows showing observed ^{1}H–^{13}C correlation from HMBC spectra of compounds from library 1.

Library 2: The second library contained compounds with two guanidinium polar headgroups and one hydrophobic tail (**2**) and was synthesised as shown in Scheme 3 and Scheme 4. Acid **13** was synthesised in high yield (95%) by treatment of N^1,N^9-bis-(Dde)-norspermidine **12** with succinic anhydride (Scheme 3). Compound **12** was a by-product from the preparation of the asymmetric protected polyamines **5** (Scheme 1) or was obtained simply by treatment of norspermidine **4** with DdeOH (2.2 equiv). Resin **14**, obtained by treatment of resin **9** with hydrazine in DMF (5%), was treated with acid **13** (2 equiv) in the present of DIC (2 equiv), HOBt (2 equiv) and DMAP to give polyamine scaffold **15** (Scheme 4). The Dde protecting groups were selectively removed as described above. The two free amine groups were then converted into the guanidinium functionality as described for library 1. After removal of the trifluoroacetyl protecting group with aqueous methanolic base, a range of acids were coupled to the free amino group. The products were obtained in good yield (54–98% pure) after cleavage from the solid support (Scheme 4).

Structure elucidation of **2** was achieved as follows (Figure 2). The methylene protons (H-8″) correlated with the guanidine carbons by HMBC. The protons H-8″, H-7″ and H-6″ were assigned by ^{1}H–^{1}H COSY. The H-2′ and H-3′ proton signals exhibited cross-peaks to the carbonyl signal (C-1′), which also correlated with H-2 in the HMBC experiment. Protons H-2, H-3 and H-4 were confirmed by a ^{1}H–^{1}H

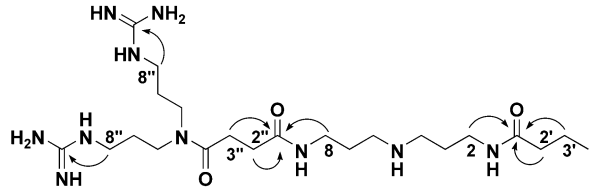


Figure 2. The arrows show ^{1}H–^{13}C correlation from HMBC spectra from compound library 2.

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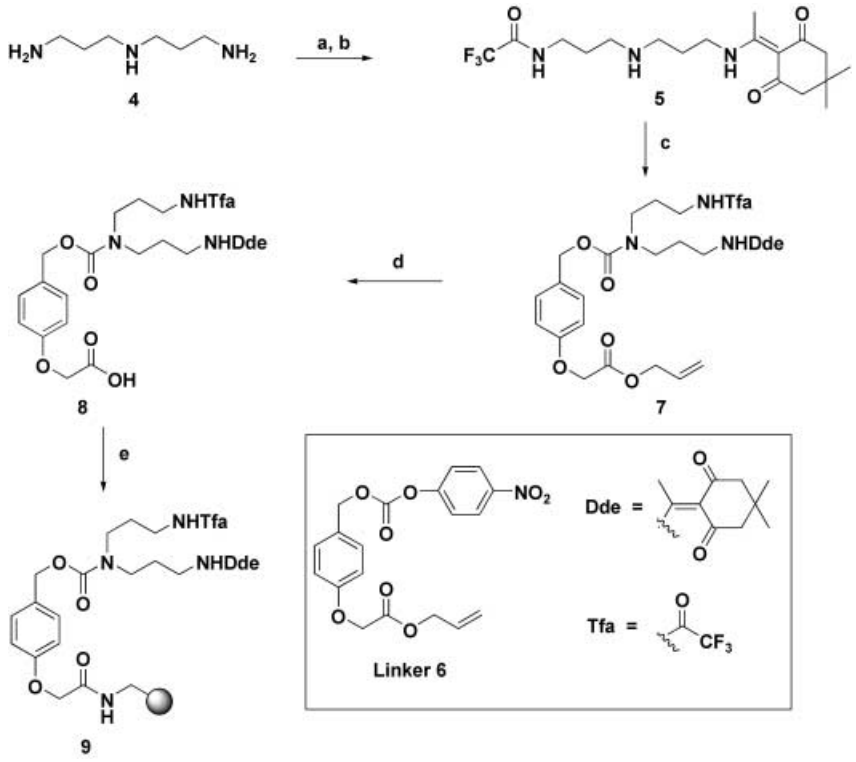
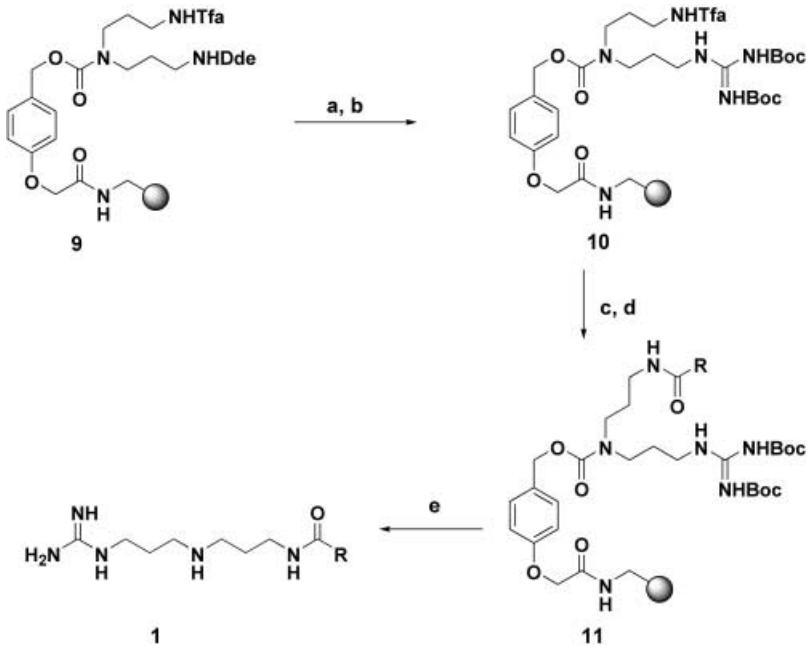
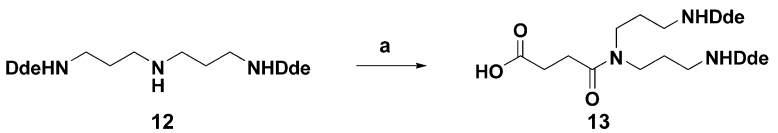


Scheme 1. Reagents and conditions: a) Ethyl trifluoroacetate (1 equiv), MeOH, −78 °C, 1 h, 0 °C, 4 h; b) 2-acetyldimedone (1.2 equiv), CH_2Cl_2, 12 h, two steps 61 %; *c*) linker **6** (1.2 equiv), DMF, 12 h, 88 %; d) $[Pd(PPh_3)_4]$ (0.1 equiv), thiosalicylic acid (4 equiv), CH_2Cl_2/THF (1:1), 2 h, 72 %; *e*) aminomethyl polystyrene resin, DIC (1.5 equiv), HOBt (1.5 equiv), CH_2Cl_2, 12 h.



Scheme 2. Reagents and conditions: a) Hydrazine/DMF (5 %), 2 h; b) *N,N′*-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv), pyridine, THF, 12 h; c) 1 M KOH/THF/MeOH (4:3:1), 2 h; d) carboxylic acids (4 equiv), DIC (4 equiv), HOBt (4 equiv), CH_2Cl_2, 2 h; e) TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1), 2 h.



Scheme 3. Reagents and conditions: a) Succinic anhydride (1.1 equiv), pyridine (1.1 equiv), CH_2Cl_2, 1 h, 95 %.

COSY experiment. Proton H-8 showed a cross-peak to C-1″, while the H-2″ and H-3″ signals also correlated with this carbonyl carbon.

Library 3: The third library contained compounds with one guanidinium polar head group and two hydrophobic tails (**3**) and was synthesised as shown in Scheme 5. The trifluoroacetyl group on compound **10** (see Scheme 2) was removed (1 M KOH/THF/MeOH 4:3:1) and the resulting resin was treated with acid **13** to give resin **17**. The two Dde protecting groups were removed (N_2H_4/DMF, 5 %) and the resulting product was coupled with a range of acids to afford compounds **18**. Compound **3** was obtained after treatment of **18** with a solution of TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1). The structure of **3** was again confirmed by 1D and 2D NMR techniques. The hydrophobic tails of all the transfection agents prepared are shown in Table 1.

Derivatives with tails 1 to 31 were prepared for Libraries 1 and 2. However, for Library 3 only derivatives with tails 1 to 27 were synthesised, as treatment with the steroidal groups produced multiple products.

Data for all library members is given in the Supporting Information.

DNA binding affinities: The relative binding affinities of the transfection agents for DNA were evaluated to determine whether transfection activities correlated with DNA binding. Relative binding affinities were assessed in two ways: i) a gel retardation assay[28] (Figure 3) and ii) an ethidium bromide displacement assay[29] (Table 2).

For the gel retardation assay, compounds were mixed with plasmid DNA at ratios of 1:5 and 1:20 (DNA/sample, w/w) and loaded onto an agarose gel (Figure 4). All compounds con-

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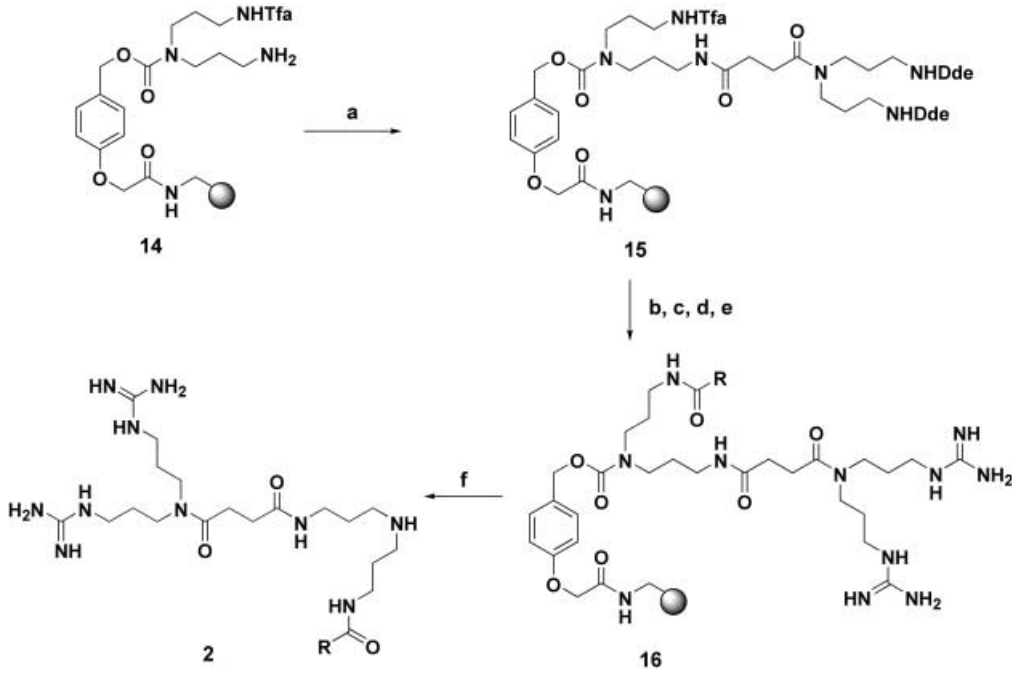
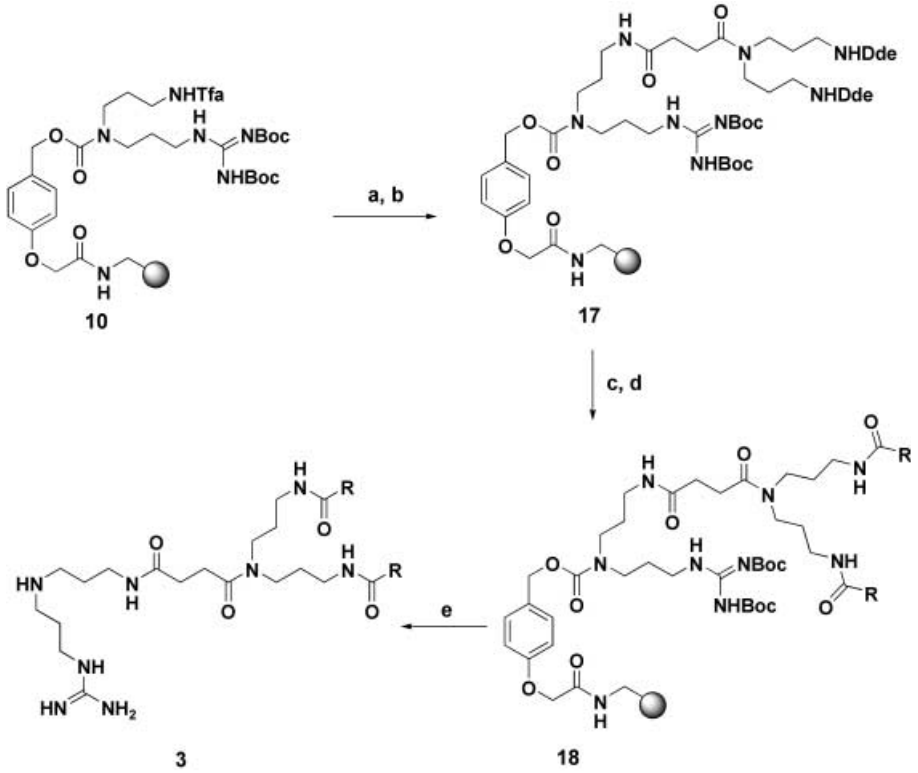


Scheme 4. Reagents and conditions: a) Acid **13** (2 equiv), DIC (2 equiv), HOBt (2 equiv), CH_2Cl_2, DMAP (0.1 equiv), 2 h; b) hydrazine/DMF (5 %), 2 h; c) *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv), pyridine, THF, 2 h; d) 1 M KOH/THF/MeOH (4:3:1), 2 h; e) carboxylic acids (4 equiv), DIC (4 equiv), HOBt (4 equiv), CH_2Cl_2, 2 h; f) TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1), 2 h.



Scheme 5. Reagents and conditions: a) 1 M KOH/THF/MeOH (4:3:1), 2 h; b) acid **13** (1.7 equiv), DIC (2 equiv), HOBt (2 equiv), CH_2Cl_2, 2 h; c) hydrazine/DMF (5 %), d) carboxylic acids (4 equiv), DIC (4 equiv), HOBt (4 equiv), CH_2Cl_2, 2 h; e) TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1), 2 h.

taining a tail of fewer than nine carbons bound poorly to DNA. In libraries 2 and 3, the chain length of the hydrophobic tail required for DNA binding (9–18 carbons) was less than that of library 1 (14–18 carbons). In library 2, in contrast with library 1, the steroids were able to bind DNA. The steroids bearing hydroxy groups in library 2 (**2.28**, **2.29** and **2.31**) bound DNA more efficiently than the steroid bearing carbonyl groups (**2.30**).

The ethidium bromide displacement assay was based on the displacement of ethidium bromide (0.125 μg) from its intercalation site in DNA (0.5 μg) by the cationic lipids, and the decrease in fluorescence measured (λ_{excit} = 485 nm, λ_{emiss} = 590 nm) after 1 minute of equilibration, and the weight ratio (lipid/DNA) at which 50 % of the ethidium bromide was displaced from the DNA (WR_{50}) determined (Table 2). WR_{50} values greater than 3 indicate weak binding to DNA and these compounds were excluded from the table. There was good correspondence between DNA binding affinity from ethidium bromide displacement and from the gel electrophoresis assay (Figure 3). Again, compounds with a hydrophobic tail of fewer than nine carbons bound weakly to

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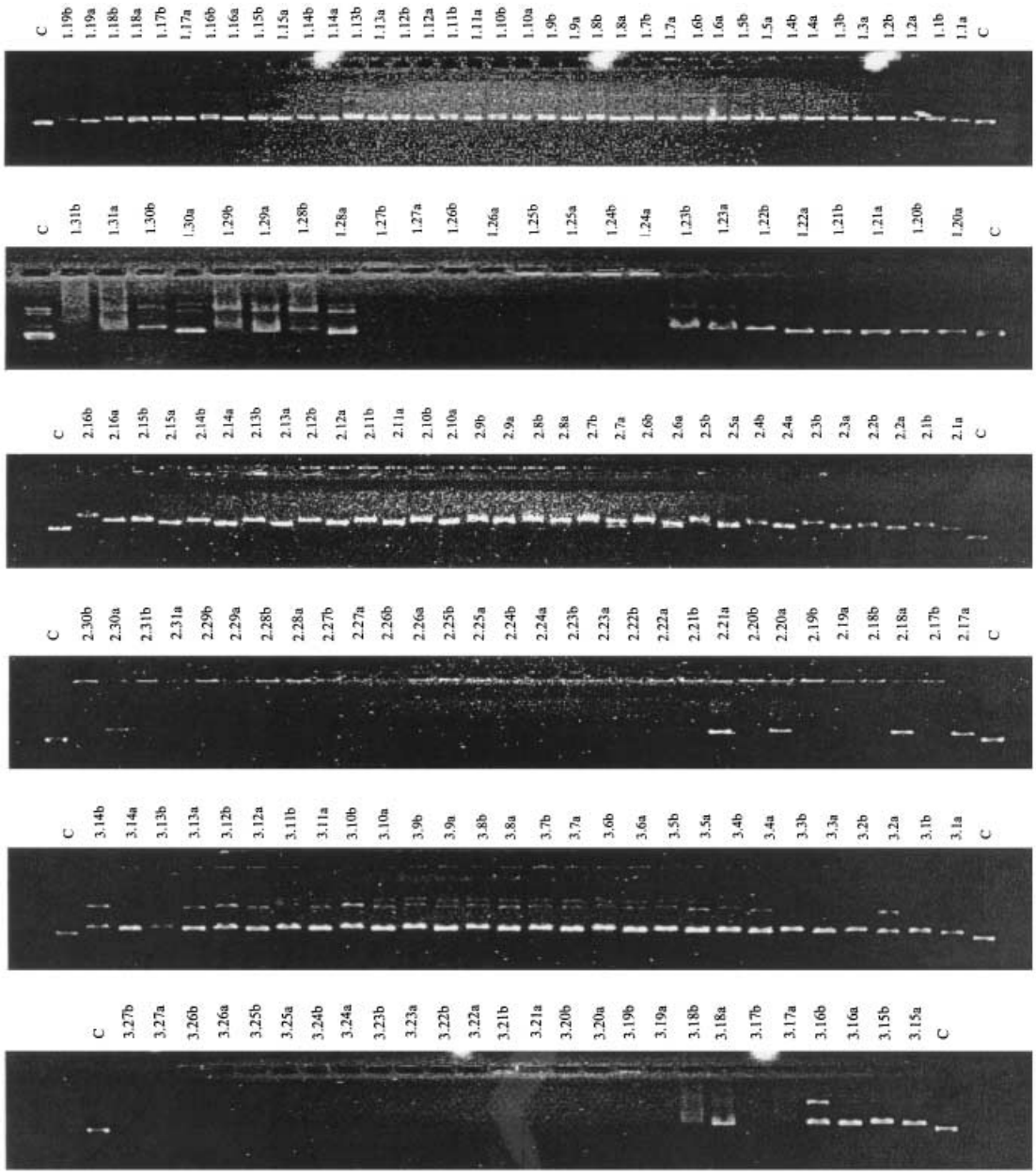

Figure 3. Gel electrophoresis assay of the mixtures of plasmid DNA and cationic lipids at 1:5 (a) and 1:20 (b) weight ratios. Lanes marked C contained DNA alone and were used as a control. The presence of a lower band shows that the DNA has migrated and so has not been bound by the transfection compound.

Table 2. WR_{50} values from the ethidium bromide displacement assay. Values for all other compounds were greater than 3, indicating poor DNA binding.

Compound	WR_{50} (lipid/DNA)	Compound	WR_{50} (lipid/DNA)	Compound	WR_{50} (lipid/DNA)
1.24	1.08	**2.20**	2.16	**3.17**	0.73
1.25	0.67	**2.21**	1.14	**3.20**	2.36
1.26	1.69	**2.22**	1.25	**3.21**	2.43
1.27	0.95	**2.23**	0.81	**3.22**	2.10
1.31	1.70	**2.24**	0.63	**3.23**	1.88
2.16	1.91	**2.25**	0.77	**3.24**	2.45
2.17	1.71	**2.26**	0.78	**3.25**	2.41
2.18	2.59	**2.27**	0.51	**3.27**	1.98
2.19	2.54	**2.31**	1.32		

DNA. Library 2 generally had higher binding affinity than libraries 1 and 3. Nearly all the steroid-containing samples showed poor DNA binding. Compounds containing a straight chain were more effective at binding DNA than compounds containing a branched chain of the same length (**3.17** versus **3.18**, **2.19** versus **2.20** or **2.21**). Conflicting data about the ideal lipid chain length have been obtained previously,[30–32] but the consensus is that tails shorter than 12 carbons are unable to form lipid bilayers and thus do not interact well with DNA.

Transfection results: The cationic lipids that bound DNA (from the binding affinity assays) were used to study transfection of mammalian cell lines. Typically, cationic liposomes

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50 μL (20 μg μL^{-1} in ethanol) and DOPE 50 μL (20 μg μL^{-1} in chloroform) were mixed and the organic solvent was removed under a stream of nitrogen. The resulting thin film was dried under high vacuum (>2 h). The thin film was hydrated with 100 μL phosphate-buffered saline (PBS). The soution was vortexed (1 min) and sonicated (2×15 min) in a bath-type sonicator to form the liposomes. The resulting solution was stored at 4 °C for 24 h before use. The transfection activities of cationic liposomes were evaluated in comparison with the commercially available transfection reagent effectene by use of β-galactosidase as a reporter gene, the transfection activity of each cationic liposome being reported as a percentage of that of the effectene control. Figure 4 displays the data generated by employment of a plasmid encoding β-galactosidase (100 ng/well) at DNA/cationic lipid ratios (w/w) of 1:10 and 1:20. Most of the compounds in library 1 and 3 bearing one guanidinium group did not mediate transfection under these conditions. However, several compounds from library 2, containing two guanidinium headgroups, caused much higher levels of protein expression. Compounds **2.24** and **2.27** gave transfection levels of 117% and 105%, respectively. Compounds **2.23**, **2.25**, **2.26**, **3.23** and **3.27** were also identified as active transfection agents, but the levels of gene expression were lower than those for **2.24** and **2.27**. DNA–liposome complexes need a net positive charge for high transfection efficiencies,[33] although the activity decreases when the lipid ratio is too high, due to cytotoxicity[9,33] Most of the compounds in library 2 had charge ratios lower than 15. DNA/cationic lipid of lower charge ratios (<15) gave better transfections than those of higher charge ratios (Figure 5). The steroidal compound **2.30** was an exception, with transfection activity only at the higher charge ratios (>20). These compound libraries did not show significant transfection activity when DOPE was omitted (data not shown).

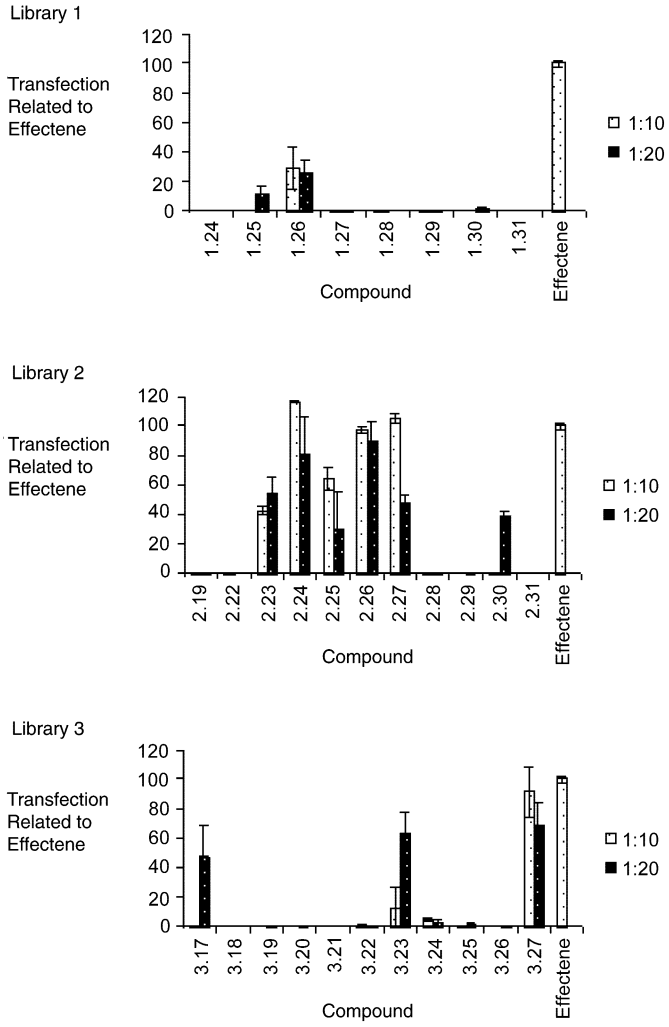

Figure 4. Relative transfection activities (%) of each library of cationic lipids relative to effectene. Cationic lipids were mixed with plasmid DNA (β-Gal, 0.1 μg μL^{-1} per well) at weight ratios (DNA/cationic lipid) of 1:10 and 1:20 and used for transfection of 293T cells.

The most active compounds in transfection, **2.24** and **2.27**, were two of the compounds with the highest affinity for DNA as measured by the ethidium bromide displacement assay. No correlation was found between the measured transfection activity or DNA binding and the hydrophobicity of the compound (cLogP) (as estimated from ChemDraw Ultra, data not shown).

Transfection compound toxicity: To assess the relationship between cytotoxicity and gene expression efficiency, the toxicity of the two cationic lipids (**2.24** and **2.27**) most active in transfection was examined by measuring changes in cell metabolic activity (MTT assay)[34] and by induction of cell death by trypan blue exclusion[35] after transfection (Figure 6). The IC_{50} values of most complexes were 30 μM, two times higher than the concentrations used for transfection. The exception was compound **2.27** which had an IC_{50} of approximately 8 μM in the presence of DNA (but in the absence of DOPE). Effectene was slightly more toxic than compounds **2.24** and **2.27** in the MTT assay (data not shown). The effect of the transfection compounds on cell death, as assessed by trypan blue assay, was much less than their effect on metabolic activity (Figure 6b). The complexes from **2.24** and **2.27** exhibited minimal toxicity and this was not greatly changed by inclusion of DOPE as a colipid. Thus, overall, compounds **2.24** and **2.27** can produce high levels of transfection without inducing significant cell death, although there is a reduction in metabolic activity. This reduction in metabolic activity is also observed in other commercial transfection reagents and should not limit the application of these compounds.

Conclusions

It is surprising that the compounds in library 2, with two headgroups and one tail, showed the highest transfection activities, since most cationic lipid transfection compounds (e.g., *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulfate (DOTAP), *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA), (+)-*N*-(2-hydroxyethyl)-*N*,*N*-dimethyl-2,3-bis(tetradecyloxy)-1-propanaminium bromide (DMRIE)) have two aliphatic tails.[8]

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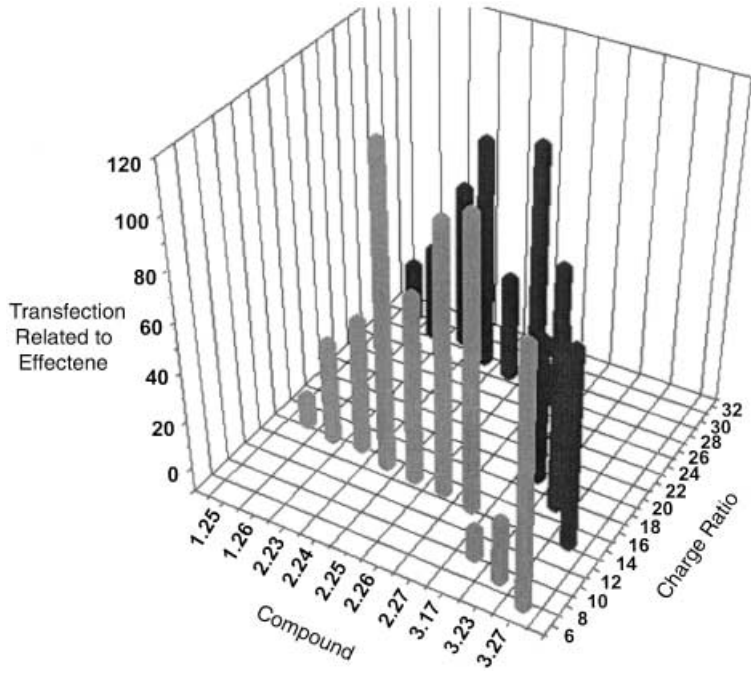
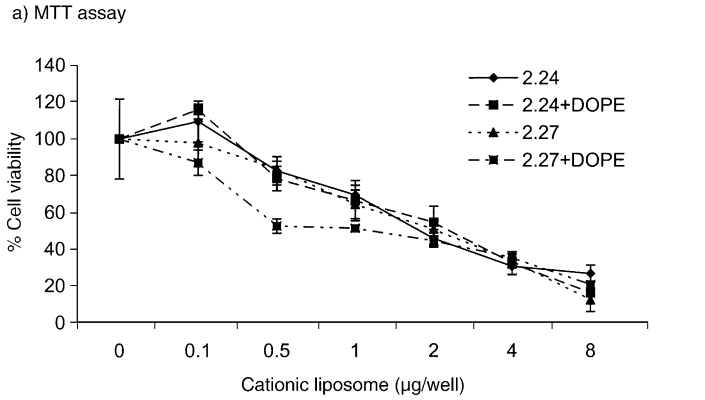


Figure 5. Relative transfection activities (%) of cationic lipids (selected from Figure 4) at lower (grey bar) and higher (black bar) charge ratios.



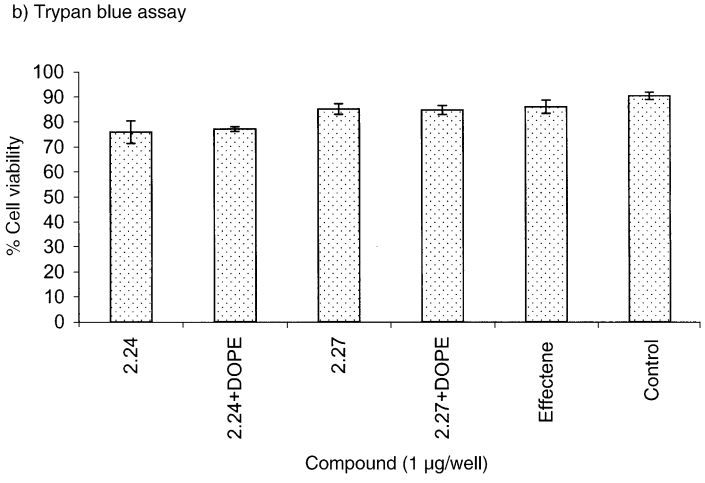


Figure 6. Toxicity of the two most active compounds used for transfection of 293T cells. These two compounds were tested for reduction in metabolic activity (MTT assay) and induction of cell death (trypan blue). Determinations were performed after 24 h incubation with medium containing the complexes (0.1 μg DNA/100 μL).

Cationic detergents have also been shown to condense DNA.[36] Detergents have a much higher water solubility than cationic lipids, release their DNA rapidly and kill cells and consequently cannot be used as transfection agents.[37] A previous study with liposomes made of DOPE combined with a compound containing a single aliphatic tail produced a high degree of toxicity even after three hours of exposure,[38] which was not observed with any of the compounds in library 2, suggesting that investigation of further compounds containing one aliphatic tail for their transfection potency is warranted.

The generation of compounds with transfection abilities similar to or greater than those of a widely used commercial reagent suggests that this class of compound has significant potential for overcoming gene transfer difficulties in vitro and possibly in vivo. Further work will investigate the effect of compound formulation and examine the varying susceptibilities of different cell types (e.g., B cells, dendritic cells, fibroblasts) to transfection by different members of these libraries.

Experimental Section

General information: NMR spectra were recorded on a Bruker AC 400 spectrometer operating at 400 MHz for ^{1}H and 100 MHz for ^{13}C. All coupling constants (J values) were measured in Hz. ES mass spectra were recorded with a VG Platform Quadrupole Electrospray Ionisation mass spectrometer. High-resolution electrospray mass spectra were recorded on a Bruker Apex III FT-ICR mass spectrometer. Infra-red spectra were recorded on a BioRad Golden Gate FTS 135 Paragon 1000. Reversed-phase analytical HPLC (RP-HPLC) was performed on a Hewlett Packard HP1100 Chemstation, with a Phenomenex C_{18} prodigy 5μ (150 mm × 3.0 mm i.d.) column. Thin layer chromatography (TLC) was performed on Alugram SIL G/UV/254 precoated plates. Bands were visualised under UV light. Column chromatography was carried out on SiO_2. All reactions were carried out at room temperature unless otherwise stated.

***N*^{1}-(Trifluoroacetyl)-*N*^{9}-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (5) and *N*^{1},*N*^{9}-bis-1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-norspermidine (12)**: A solution of ethyl trifluoroacetate (1 equiv, 5.30 mL, 44.54 mmol) in MeOH (20 mL) was added dropwise at −78 °C over 1 h to a stirred solution of norspermidine (**4**) (1 equiv, 5.85 g, 44.58 mmol) in MeOH (30 mL) and the system was left to stir at 0 °C for 4 h. The solvent was removed under reduced pressure and the residue was co-evaporated with CH_2Cl_2 (2 × 50 mL) to remove trace amounts of MeOH. The crude product was dissolved in CH_2Cl_2 (50 mL), 2-acetyldimedone (1.2 equiv, 9.75 g, 53.5 mmol) was added, and the reaction mixture was left to stir overnight. The solvent was evaporated to dryness and the crude product was purified by chromatography on silica gel, with elution with CH_2Cl_2/MeOH (95:5 to 93:7), to afford **12** (3.89 g, 19 %) and **5** (10.68 g, 61 %) as viscous oils.

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Compound 12: R_f = 0.30 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.05 (s, 12H; 2×($C(CH_3)_2$), 1.87 (tt, ^3J(H,H) = 7, 7 Hz, 4H; $CH_2CH_2NHCH_2CH_2$), 2.37 (s, 8H; $COCH_2$), 2.57 (s, 6H; C=CCH_3), 2.76 (t, ^3J(H,H) = 7 Hz, 4H; CH_2NHCH_2), 3.51 (m, 4H; 2×CH_2NH–Dde), 13.44 (brs, 2H; NH–Dde) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 18.3 (C=CCH_3), 28.6 ($C(CH_3)_2$), 29.7 ($CH_2CH_2NHCH_2CH_2$), 30.5 ($C(CH_3)_2$), 41.7 (CH_2NH–Dde), 47.2 (CH_2NHCH_2), 53.2 ($COCH_2$), 108.3 (C=CCH_3), 174.0 (C=CCH_3), 198.4 (C=O) ppm; IR (CH_2Cl_2): $\tilde{\nu}$ = 1628 cm^{-1} (C=O); MS (ES$^+$): *m*/*z* (%): 460.4 (100) [*M*+H]$^+$; HRMS (ES$^+$): *m*/*z*: calcd for $C_{26}H_{42}N_3O_4$ [*M*+H]$^+$: 460.3170; found: 460.3172.

Compound 5: R_f = 0.26 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 1.05 (s, 6H; $C(CH_3)_2$, 1.75–1.92 (m, 4H; $CH_2CH_2NHCH_2CH_2$), 2.39 (s, 4H; $COCH_2$), 2.59 (s, 3H; C=CCH_3), 2.77 (t, ^3J(H,H) = 7 Hz, 2H; $CH_2CH_2CH_2NHDde$), 2.84 (t, ^3J(H,H) = 6 Hz, 2H; TfaNHCH$_2$CH$_2$CH_2), 3.52 (m, 4H; NH($CH_2CH_2CH_2$)$_2$), 5.03 (brs, 2H; NH), 13.45 (brs, 1H; NH–Dde) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 18.2 (C=CCH_3), 27.8 (TfaNHCH$_2$CH_2), 28.6 ($C(CH_3)_2$); 29.4 (CH_2CH_2NHDde); 30.5 ($C(CH_3)_2$); 39.9 (TfaNHCH_2); 41.6 (CH_2NHDde), 47.1 ($CH_2CH_2CH_2NHDde$); 48.6 (TfaNHCH$_2$CH$_2$CH_2); 53.2 ($COCH_2$); 108.4 (C=CCH_3); 116.5 (q, ^1J = 288 Hz; CF_3–CONH), 157.6 (q, ^2J = 36 Hz, CF_3–CO–NH), 174.0 (C=CCH_3), 198.5 (CO–Dde) ppm; IR (CH_2Cl_2): $\tilde{\nu}$ = 1717 cm^{-1} (C=O); MS (ES$^+$): *m*/*z* (%): 392.3 (16) [*M*+H]$^+$; HRMS (ES$^+$): *m*/*z*: calcd for $C_{18}H_{29}F_3N_3O_3$ [*M*+H]$^+$: 392.2156; found: 392.2145.

N^1-1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(4-(allyloxycarbonylmethoxy)phenyl-methoxycarbonyl)-(N^9-trifluoroacetyl)-norspermidine (7): Compound **5** (1 equiv, 5.42 g, 13.85 mmol) was dissolved in DMF (10 mL), linker **6** (1.2 equiv, 6.43 g, 16.62 mmol) was added, and the reaction mixture was stirred overnight. It was then poured into $KHSO_4$ (1 M, 100 mL) and extracted with EtOAc (2×100 mL). The combined organic layers were washed with water, dried over Na_2SO_4 and evaporated to dryness. The crude product was purified on silica gel, with elution with CH_2Cl_2 to CH_2Cl_2/MeOH (97:3), to afford the desired product **7** (7.77 g, 88%). R_f = 0.37 (CH_2Cl_2/MeOH 20:1); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 0.96 (s, 6H; $C(CH_3)_2$), 1.69 (brs, 2H; TfaHNCH$_2$CH_2), 1.83 (brs, 2H; DdeHNCH$_2$CH_2), 2.29 (s, 4H; 2×$COCH_2$–Dde), 2.42 (s, 3H; C=CCH_3), 3.18–3.35 (m, 8H; N($CH_2CH_2CH_2$)$_2$), 4.59 (s, 2H; $OCH_2C_6H_4$), 4.63 (m, 2H; OCH_2CH=CH_2), 5.02 (s, 2H; $C_6H_4OCH_2CO$), 5.18–5.30 (m, 2H; OCH_2CH=CH_2), 5.85 (m, 1H; OCH_2CH=CH_2), 6.81 (d, ^3J(H,H) = 9 Hz, 2H; ArH), 7.22 (d, ^3J(H,H) = 9 Hz, 2H; ArH), 7.93 (brs, 1H; NH–Tfa), 13.44 (brs, 1H; NH–Dde) ppm; ^{13}C NMR ($CDCl_3$, 100 MHz): δ = 18.3 (C=CCH_3), 27.3 (TfaNHCH$_2$CH_2), 28.3 (DdeHNCH$_2$CH_2), 28.6 ($C(CH_3)_2$), 30.5 ($C(CH_3)_2$), 36.4 (TfaHNCH_2), 41.1 (TfaHNCH$_2$CH$_2$CH_2), 44.3 (DdeHNCH_2), 44.5 (DdeHNCH$_2$CH$_2$CH_2), 53.2 ($COCH_2$–Dde), 65.7 ($OCH_2C_6H_4$), 66.3 (OCH_2CH=CH_2), 67.8 ($C_6H_4OCH_2CO$), 108.4 (C=CCH_3), 115.2 (CH_{arom}), 115.8 (OCH_2CH=CH_2), 119.6 (OCH_2CH=CH_2), 129.8 (C_{arom}), 130.6 (CH_{arom}), 131.8 (C_{arom}), 158.3 (C=O amide), 168.8 (C=O ester), 174.1 (C=CCH_3), 198.4 (C=O–Dde) ppm; IR (CH_2Cl_2): $\tilde{\nu}$ = 1757, 1718 cm^{-1} (C=O); MS (ES$^+$): *m*/*z* (%): 639.9 (100) [*M*+H]$^+$, 661.9 (70) [*M*+Na]$^+$; HRMS (ES$^+$): *m*/*z*: calcd for $C_{31}H_{40}F_3N_3O_8Na$ [*M*+Na]$^+$: 662.2659; found: 662.2641.

N^1-1-(4,4-Dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(4-(carboxymethyl)phenyl-methoxy-carbonyl))-N^9-(trifluoroacetyl)-norspermidine (8): Compound **7** (1 equiv, 6.34 g, 9.91 mmol) in CH_2Cl_2/THF (1:1, 30 mL each) was purged with N_2 (g) for 1 h. [$Pd(PPh_3)_4$] (0.1 equiv, 1.14 g, 0.99 mmol) and thiosalicylic acid (2 equiv, 3.06 g, 19.82 mmol) were added in one portion, and the reaction mixture was left to stir for 2 h. The reaction mixture was evaporated to dryness and the crude product was purified on silica gel, with elution with EtOAc to 50% MeOH/EtOAc, to give **8** (4.29 g, 72%). R_f = 0.23 (CH_2Cl_2/MeOH 2:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 0.96 (s, 6H; $C(CH_3)_2$), 1.75–1.90 (m, 4H; $CH_2CH_2NHCH_2CH_2$), 2.36 (s, 4H; $COCH_2$–Dde), 2.55 (s, 3H; C=CCH_3); 3.20–3.40 (m, 8H; HN($CH_2CH_2CH_2$)$_2$), 4.33 (s, 2H; $OCH_2C_6H_4$), 5.07 (s, 2H; OCH_2CO_2H), 6.90 (d, ^3J(H,H) = 9 Hz, 2H; ArH), 7.32 (d, ^3J(H,H) = 9 Hz, 2H; ArH), 9.57 (brs, 1H; NH–Tfa), 13.38 (brs, 1H; NH–Dde) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 17.7 (C=CCH_3), 27.3 (TfaHNCH$_2$CH_2), 28.3 (DdeHNCH$_2$CH_2+C(CH_3)$_2$), 30.2 ($C(CH_3)_2$), 37.5 (TfaHNCH$_2$), 41.5 (TfaHNCH$_2$CH$_2$CH_2), 45.0 (DdeHNCH$_2$), 49.0 (DdeHNCH$_2$CH$_2$CH_2), 52.8 ($COCH_2$–Dde), 66.6 ($OCH_2C_6H_4$), 67.7 (OCH_2CO_2H), 107.4 (C=CCH_3), 114.8 (CH_{arom}), 128.5 (C_{arom}), 129.6 (CH_{arom}), 131.8 (C_{arom}), 159.1 (C=O amide), 173.3 (C=CCH_3+CO_2H), 196.9 (C=O–Dde) ppm; IR (CH_2Cl_2): $\tilde{\nu}$ = 1691, 1570 cm^{-1} (C=O); MS (ES$^+$): *m*/*z* (%): 600.3 (20) [*M*+H]$^+$, 622.3 (5) [*M*+Na]$^+$; HRMS (ES$^+$): *m*/*z*: calcd for $C_{28}H_{37}F_3N_3O_8$ [*M*+H]$^+$: 600.2527; found: 600.2539.

Synthesis of polyamine scaffold resin 9: Compound **8** (3 equiv, 6.43 mmol) was dissolved in CH_2Cl_2 (15 mL), and then DIC (3.5 equiv, 7.63 mmol) and HOBt (3.5 equiv, 7.63 mmol) were added. After 10 min, this solution was added to aminomethyl resin (1 equiv, 2.18 mmol) and the suspension was shaken overnight. The resulting resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×15 mL each) and dried under high vacuum. The resin gave a negative ninhydrin test.

Solid-phase synthesis of transfection agent library: synthesis of transfection library 1

Full experimental data for all library compounds 1,2, and 3 is given in the Supporting Information.

General procedures

Dde deprotection: Resin (1 equiv) was pre-swollen in DMF for 30 min and filtered. Hydrazine in DMF (5% v/v) was added to this resin and the suspension was shaken for 2 h. The resin was filtered and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (positive ninhydrin test).

Guanidation: The resulting resin was pre-swollen in THF for 30 min and filtered. A solution of *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (4 equiv, 1 M) in THF and a few drops of pyridine were added and the suspension was shaken overnight. The solution was collected and the resin was washed as above (negative ninhydrin test).

Tfa deprotection: A mixture of 1 M KOH/THF/MeOH (4:3:1) was added to the resin (pre-swollen in THF and filtered). This suspension was shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (positive ninhydrin test).

Coupling of carboxylic acid: The resin was pre-swollen for 30 min in CH_2Cl_2 and filtered. A solution of carboxylic acid (4 equiv, 0.5 M), DIC (4 equiv, 0.5 M) and HOBt (4 equiv, 0.5 M) in CH_2Cl_2 was added to this resin. The suspension was shaken for 2 h and the resin was washed as described above (negative ninhydrin test).

Cleavage of the product from the resin: The resin was pre-swollen in CH_2Cl_2 for 30 min and filtered. A cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1) was added and the suspension was shaken for 2 h. The resin was filtered and the solution was collected. The solvent was removed under vacuum, and the crude product was re-dissolved in H_2O and extracted with CH_2Cl_2. The aqueous layer was evaporated under reduced pressure to afford the pure products in 76–100% yield (% yield based on the loading of the aminomethyl resin and product as 4·TFA salts).

Synthesis of N^1,N^9-bis-1,1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl-N^5-(3-carboxypropanoyl)-norspermidine (13): Succinic anhydride (0.48 g, 4.74 mmol) was added to a solution of **12** (1.98 g, 4.31 mmol) in CH_2Cl_2 (5 mL) and the mixture was stirred for 1 h. The reaction mixture was poured into water (200 mL) and extracted with EtOAc (2×100 mL). The combined organic layers were dried over anhydrous Na_2SO_4 and evaporated under reduced pressure to afford the title compound as an amorphous solid (2.22 g, 95%).

R_f = 0.48 (CH_2Cl_2/MeOH 9:1); ^{1}H NMR (400 MHz, [D$_6$]DMSO): δ = 1.08 (s, 12H; 2×$C(CH_3)_2$–Dde); 1.87 (m, 2H; $NCH_2CH_2CH_2NH$), 1.99 (m, 2H; $NHCH_2CH_2CH_2N$), 2.39 (s, 8H; $COCH_2$), 2.50 (m, 2H; $NCOCH_2$), 2.56 (s, 3H; C=C–CH_3–Dde), 2.62 (m, 5H; *m*, C=C–CH_3+CH_2CO_2H), 3.47 (m, 6H; $CH_2NCH_2CH_2CH_2NH$), 3.58 (m, 2H; $NHCH_2CH_2CH_2N$), 13.34 (brs, 1H; NH), 13.42 (brs, 1H; NH) ppm; ^{13}C NMR (100 MHz, [D$_6$]DMSO): δ = 20.0 (C=C–CH_3), 29.8 ($NCH_2CH_2CH_2NH$), 30.2 (CH_2CO_2H), 33.6 ($C(CH_3)_2$+$NHCH_2CH_2CH_2N$), 32.4 (NCH_2), 43.0 ($NHCH_2CH_2CH_2N$), 43.4 ($NCH_2CH_2CH_2NH$), 45.1 ($NCH_2CH_2CH_2NH$), 46.9 ($NHCH_2CH_2CH_2N$), 55.1 (2×CH_2–Dde), 109.7 (C=C–CH_3), 174.0 (C=C–CH_3), 175.5, 175.7 ($NCOCH_2CH_2CO_2H$), 199.1, 199.2 (C=O) ppm; IR (film): $\tilde{\nu}$ = 1725, 1635 cm^{-1} (C=O); MS (ES$^+$): *m*/*z* (%): 528.4 (100) [*M*+H]$^+$; HRMS (ES$^+$): *m*/*z*: calcd for $C_{30}H_{46}N_3O_7$ [*M*+H]$^+$: 560.3330; found: 560.3341.

Synthesis of compound library 2: Hydrazine in DMF (5%, v/v) (10 mL) was added to the resin **9** (1.18 g, 0.76 mmol, pre-swollen in DMF and filtered) and the suspension was shaken for 2 h. The resin was washed with

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CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×10 mL each). The resulting resin (positive ninhydrin test) was pre-swollen in CH_2Cl_2 and filtered. A solution of acid **13** (0.89 g, 1.65 mmol) in CH_2Cl_2 (8 mL), DIC (0.26 mL, 1.65 mmol) and HOBt (0.22 g, 1.65 mmol) were added to this resin. The suspension was shaken for 2 h and the resin was filtered and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3× 10 mL each). Resin **15** was dried under vacuum and gave a negative ninhydrin test. The Dde protecting groups were removed by treatment with N_2H_4 in DMF (5% v/v) for 2 h. The resulting resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×10 mL each). The resin gave a positive ninhydrin test. A solution of the *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (0.99 g, 3.41 mmol) in THF (5 mL) and pyridine (5 drops) were added to this resin. The resulting suspension was shaken overnight. The solution was filtered to recover the guanylating reagent and the resin was washed as above. The resin gave a negative ninhydrin test. A solution of 1 M KOH/THF/MeOH (4:3:1, 10 mL) was added to this resin (pre-swollen in THF and filtered) and the suspension was shaken for 2 h. The resin was washed with MeOH/H_2O (1:1), MeOH, DMF and CH_2Cl_2 (3×10 mL each) and the resin gave a positive ninhydrin test. The resulting resin (35–50 mg per carboxylic acid) was pre-swollen in CH_2Cl_2 for 30 min and the resin was filtered. A solution of commercially available carboxylic acid (4 equiv) in CH_2Cl_2 (2 mL) and 3–5 drops of DMF, DIC (4 equiv) and HOBt (4 equiv) were added. The suspension was shaken for 2 h. The resin was washed with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×10 mL each). The resulting resin **16** gave a negative ninhydrin test. A cleavage cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1, 2 mL) was added to this resin and the suspension was shaken for 2 h. The resin was filtered and washed with CH_2Cl_2 (1 mL) and the combined solutions were collected. The solvent was removed in vacuum and the crude product was re-dissolved in H_2O (20 mL) and extracted with CH_2Cl_2 (20 mL). The aqueous layer was evaporated under reduced pressure to afford the final products **2** (34–77% as **7**·TFA salts).

Synthesis of compound library 3: Resin **9** (1.19 g, 0.76 mmol, 0.64 mmol g^{-1}) was pre-swollen in DMF for 30 min and filtered. Hydrazine in DMF (5% v/v, 10 mL) was added and the suspension was shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×10 mL each). The resulting resin (positive ninhydrin test) was pre-swollen in THF and filtered. A solution of the *N*,*N*′-bis(*tert*-butoxycarbonyl)-*S*-methylisothiourea (5 equiv, 1.14 g, 3.93 mmol) in THF (5 mL) and pyridine were added to this resin. The suspension was shaken overnight. The solution was collected and the resin was washed as described above. The resin gave a negative ninhydrin test. A solution of 1 M KOH/THF/MeOH (4:3:1, 10 mL) was added to resin **10** (pre-swollen in THF and filtered) and the suspension was shaken for 2 h. The resin was washed as described above and gave a positive ninhydrin test. The resulting resin was pre-swollen in CH_2Cl_2 and filtered. A solution of acid **13** (0.75 g, 1.34 mmol) in CH_2Cl_2 (8 mL), DIC (0.26 mL, 1.65 mmol) and HOBt (0.22 g, 1.65 mmol) were added to this resin. The suspension was shaken for 2 h, and the resin was filtered and washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×10 mL each). Resin **17** was dried under vacuum and gave a negative ninhydrin test.

Hydrazine in DMF (5% v/v, 10 mL) was added to resin **17** (pre-swollen in DMF and filtered). This suspension was shaken for 2 h. The resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH, CH_2Cl_2 and Et_2O (3×10 mL each) and dried under vacuum. The resin gave a positive ninhydrin test. The resulting resin (35–50 mg per carboxylic acid) was pre-swollen in CH_2Cl_2 for 30 min and filtered. A solution of carboxylic acid (4 equiv) in CH_2Cl_2 (2 mL) and 3–5 drops of DMF, DIC (4 equiv) and HOBt (4 equiv) were added to this resin. The suspension was shaken for 2 h, and then the resulting resin was washed successively with CH_2Cl_2, MeOH, DMF, MeOH and CH_2Cl_2 (3×3 mL each). The resin gave a negative ninhydrin test. A cleavage cocktail of TFA/CH_2Cl_2/H_2O/thioanisole (8:0.5:0.5:1, 2 mL) was added to this resin (pre-swollen in CH_2Cl_2 and filtered) and the suspension was shaken for 2 h. The resin was filtered and washed with CH_2Cl_2 (1 mL) and the combined solution was collected. The solvent was removed under vacuum, and the crude product was re-dissolved in H_2O (20 mL) and extracted with CH_2Cl_2 (20 mL). The aqueous layer was evaporated under reduced pressure to afford the final products **3** as 4·TFA salts (55–100%).

DNA binding affinities

Electrophoresis assay: DNA binding affinities of all samples were measured at two DNA/sample ratios (w/w), 1:5 and 1:20, by electrophoresis. DNA/sample complexes were formed at a ratio of 1:5 (w/w) by transferal of 12.5 μL (30 μg μL^{-1}) of sample into an Eppendorf tube. Each sample was further diluted with 37.5 μL of acetate buffer (20 mM, pH 7.4). To this solution, an aqueous solution of plasmid DNA (50 μL, 3 μg/25 μL), purified from *E. coli* HMS174 by a Qiagen Maxiprep, was added to each sample, and the solutions were successively mixed. The DNA/sample complex 1:20 (w/w) was prepared as above except that the 50 μL of stock sample (30 μg μL^{-1}) was used without dilution. DNA complexes were incubated at room temperature for 30 min. Bromophenol blue-free gel-loading buffer (100 μL, 13.3% w/v sucrose in water) was added to 100 μL of this complex. The solution was inverted to mix, and each sample (10 μL) was loaded onto a 1% agarose gel (0.5×TBE buffer). The gel was run at 200 V, 400 mA for 2 h. DNA bands were viewed under UV light by ethidium bromide staining.

Ethidium bromide displacement assay: A fluorescence microplate reader was used to measure the fluorescence intensity of eight samples with different charge ratios in a 96-well PS plate. A stock solution of DNA of 0.5 μg/5 μL was prepared in low salt buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4). Ethidium bromide and synthetic compounds were weighed, and stock solutions were prepared with a final concentration of 0.125 mg mL^{-1} in water and 0.1 μg/5 μL in ethanol, respectively. Each sample was prepared as shown in Table 3. Fluorescence was measured (ex filter = 485 nm, em filter = 590 nm) after 1 minute of equilibration. The fluorescence was calculated as a percentage of the maximum fluorescence intensity when ethidium bromide was bound to DNA in the absence of the transfection compound. The WR_{50} value is the lipid/DNA weight ratio that gives a 50% reduction in the fluorescence intensity of the solution containing DNA (0.5 μg) and ethidium bromide (0.125 μg).

Liposome preparation: Dioleoyl-L-α-phosphatidylethanolamine (DOPE) (Sigma) 50 μL (20 μg μL^{-1} in chloroform) and cationic lipid 50 μL (20 μg μL^{-1} in ethanol) were mixed (weight ratio 1:1). The organic solvents were evaporated under a stream of nitrogen gas and further dried under high vacuum (>2 h.). The resulting thin film was hydrated with phosphate buffered saline (PBS, pH 7.4) 100 μL and hydrated at room temperature for one hour. The mixture was vortexed for one minute and sonicated (2×15 min) with one hour rests between sonications in a bath-type sonicator, producing small unilamellar vesicles.[29] The liposomes were stored at 4 °C for 24 h prior to use.

Transfection procedure: 293T (human embryonic kidney) cells were grown in DMEM supplemented with 10% heat inactivated foetal calf serum, penicillin (100 units mL^{-1}), streptomycin (100 μg mL^{-1}) and L-glutamine (4 mM) at 37 °C under 5% CO_2. For transfection, 1.8×10^4 cells/well were seeded in medium (140 μL) in a 96-well culture plate, to give 50–70% confluence for use the next day. The growth medium was removed and replaced with 100 μL of serum-free medium (AIM-V, Sigma). DNA/cationic liposome complexes were prepared as follows. An appropriate volume of each cationic liposome (1 μg μL^{-1}) was added to the plasmid DNA 0.4 μL (0.5 μg μL^{-1}) and the system was incubated at room temperature for 30 min before being diluted with phosphate-buffered

Table 3. Amounts of compounds used in the ethidium bromide displacement assays in a 96-well plate format.

Well	1	2	3	4	5	6	7	8	9	10	11	12
buffer [μL]	190	185	180	175	170	165	160	155	150	145	140	115
DNA [μL]	5	5	5	5	5	5	5	5	5	5	5	5
Et Br [μL]	5	5	5	5	5	5	5	5	5	5	5	5
lipid [μL]	0	5	10	15	20	25	30	35	40	45	50	75

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saline to make a final DNA concentration of 0.1 μg/10 μL. DNA/cationic liposome complexes (10 μL) were added to the cells and left to incubate at 37 °C under 5% CO_2 for 48 h. For effectene (Qiagen) transfections, enhancer (1.6 μL) was added to plasmid DNA (0.2 μg) in buffer EC (60 μL). The mixture was vortexed for 2 s and left on bench for 3 min. Effectene was added to this mixture (5 μL), which was vortexed for 10 seconds and left for 7 min. Serum-free medium (350 μL) was added and mixed by pipetting up and down twice. DNA/effectene complex (50 μL) was added to the cells. β-Galactosidase expression was measured with the FluoReporter LacZ/Galactosidase Quantitation kit (Molecular Probes) according to the manufacturer's instructions, with the reaction developed for 10 min at room temperature. Transfection efficiency was calculated as a percentage relative to effectene transfection, after subtraction of the value of untransfected cells.

Cytotoxicity assay: Cytotoxicity was evaluated by use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent (MTT test) and by trypan blue exclusion. Cells were seeded in 96-well plates at 1×10^4 cells per well. The growth medium was removed next day and replaced with 100 μL of serum-free medium (AIM-V, Sigma). Cationic liposome/DNA complexes and effectene were prepared as described in the transfection procedure. For the MTT assay, an increasing amount of cationic lipid/DNA (1:10 wt/wt) and cationic liposome/DNA (1:10 wt/wt) was added to triplicate wells and the system was incubated at 37 °C under 5% CO_2 for 24 h. The medium was then removed and replaced with a phenol red-free medium (90 μL). MTT (3 mg mL^{-1}) was added (10 μL/well) to the cells and formazan crystals formed in the incubator over 3 h. MTT solubilization solution (Sigma, 100 μL) was added to dissolve the resulting crystals. Absorbance was measured at 570 nm on a microplate reader (Bio-Rad). Reduction in metabolic activity was calculated as (A_{570} with compound/A_{570} untreated). For trypan blue exclusion, 10 μL (1 μg/10 μL) of cationic lipid/DNA (1:10 wt/wt) and 10 μL cationic liposome/DNA (1:10 wt/wt) were added to cells in duplicate wells, and the system was incubated at 37 °C under 5% CO_2 for 24 h. The cells were detached from the well by pipetting, and the suspension (10 μL) was mixed with 10 μL of 0.4% trypan blue (Sigma). Live and dead cells were counted within four microscopic fields of a haemocytometer. The viability was calculated as a percentage relative to untreated cells.

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[1] R. G. Amado, I. S. Y. Chen, *Biomedicine* **1999**, *285*, 674–676.
[2] N. Somia, I. M. Verma, *Nat. Rev. Genet.* **2000**, *1*, 91–99.
[3] P. L. Felgner, *Adv. Drug Delivery Rev.* **1990**, *5*, 163–187.
[4] R. Harland, H. Weintraub, *J. Cell Biol.* **1985**, *101*, 1094–1099.
[5] W. C. Heiser, *Methods Mol. Biol.* **2000**, *130*, 117–134.
[6] E. T. Schenborn, V. Goiffon, *Methods Mol. Biol.* **2000**, *130*, 135–145.
[7] J. S. Pagano, *Prog. Med. Virol.* **1970**, *12*, 1–48.
[8] a) A. D. Miller, *Angew. Chem.* **1998**, *110*, 1862–1880; *Angew. Chem. Int. Ed.* **1998**, *37*, 1768–1785.
[9] P. L. Felgner, T. R. Gadek, M. Holm, R. Roman, H. W. Chan, M. Wenz, J. P. Northrop, G. M. Ringold, M. Danielsen, *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 7413–7417.
[10] G. J. Nabel, E. G. Nabel, Z. Y. Yang, B. A. Fox, G. E. Plautz, X. Gao, L. Huang, S. Shu, D. Gordon, A. E. Chang, *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 11307–11311.
[11] N. J. Caplen, E. W. Alton, P. G. Middleton, J. R. Dorin, B. J. Stevenson, X. Gao, S. R. Durham, P. K. Jeffery, M. E. Hodson, C. Coutelle, L. Huang, D. J. Porteous, R. Williamson, D. M. Geddes, *Nat. Med.* **1995**, *1*, 39–46.
[12] J. Zabner, A. J. Fasbender, T. Moninger, K. A. Poellinger, M. J. Welsh, *J. Biol. Chem.* **1995**, *270*, 18997–19007.
[13] I. Koltover, T. Salditt, J. O. Raedler, C. R. Safinya, *Science* **1998**, *281*, 78–81.
[14] G. Jung, *Combinatorial Chemistry Synthesis, Analysis, Screening*, Wiley-VCH, Weinheim, Germany, **1999**.
[15] G. Byk, M. Ferderic, D. Scherman, *Tetrahedron Lett.* **1997**, *38*, 3219–3222.
[16] G. Byk, C. Dubertret, V. Escriou, M. Frederic, G. Jaslin, R. Rangara, B. Pitard, J. Crouzet, P. Wils, B. Schwartz, D. Scherman, *J. Med. Chem.* **1998**, *41*, 224–235.
[17] J.-P. Vigneron, N. Oudrhiri, M. Fauquet, L. Vergely, J.-C. Bradley, M. Basseville, P. Lehn, J.-M. Lehn, *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 9682–9686.
[18] a) M. C. O'Sullivan, D. M. Dalrymple, *Tetrahedron Lett.* **1995**, *36*, 3451–3452; b) D. Q. Xu, K. Prasad, O. Repic, T. J. Blacklock, *Tetrahedron Lett.* **1995**, *36*, 7357–7360; c) I. S. Blagbrough, A. J. Geall, *Tetrahedron Lett.* **1998**, *39*, 439–442.
[19] M. Imazawa, F. Eckstein, *J. Org. Chem.* **1979**, *44*, 2039–2041.
[20] R. J. Bergeron, J. S. McManis, *J. Org. Chem.* **1988**, *53*, 3108–3111.
[21] a) B. W. Bycroft, W. C. Chan, S. R. Chhabra, P. H. T. Spittle, P. M. Hardy, *J. Chem. Soc. Chem. Commun.* **1993**, 776–77; b) S. R. Chhabra, A. N. Khan, B. W. Bycroft, *Tetrahedron Lett.* **1998**, *39*, 3585–3588.
[22] S. Lemaire-Audoire, M. Savignac, J. P. Genet, *Tetrahedron Lett.* **1995**, *36*, 1267–1269.
[23] J. Wityak, S. J. Gould, S. J. Hein, P. A. Keszler, *J. Org. Chem.* **1987**, *52*, 2179–2183.
[24] a) R. J. Bergeron, J. S. McManis, *J. Org. Chem.* **1987**, *52*, 1700–1703.; b) M. Moroni, B. Koksch, S. N. Osipov, M. Crucianelli, M. Frigerio, P. Bravo, K. Burger, *J. Org. Chem.* **2001**, *66*, 130–133.
[25] M. A. Exposito, B. Lopez, R. Fernandez, M. Vazquez, C. Debitus, P. Bravo, K. Burger, *Tetrahedron* **1998**, *54*, 7539–7550.
[26] A. R. Katritzky, B. V. Rogovoy, C. Chassaing, V. Vvedensky, *J. Org. Chem.* **2000**, *65*, 8080–8082.
[27] Y.-Q. Wu, S. K. Hamilton, D. E. Wilkinson, G. S. Hamilton, *J. Org. Chem.* **2002**, *67*, 7553–7556.
[28] D. M. Lynn, D. G. Anderson, D. Putnam, R. Langer, *J. Am. Chem. Soc.* **2001**, *123*, 8155–8156.
[29] a) H. Gershon, R. Ghirlando, S. B. Guttman, A. Minsky, *Biochemistry* **1993**, *32*, 7143–7151; b) Y. Xu, F. C. Szoka, *Biochemistry*, **1996**, *35*, 5616–5623.
[30] J. H. Felgner, R. Kumar, C. N. Sridhar, C. J. Wheeler, Y. J. Tsai, R. Border, P. Ramsey, M. Martin, P. L. Felgner, *J. Biol. Chem.* **1994**, *269*, 2550–2561.
[31] R. P. Balasubramaniam, M. J. Bennett, A. M. Aberle, J. G. Malone, M. H. Nantz, R. W. Malone, *Gene Ther.* **1996**, *3*, 163–172.
[32] J. A. Heyes, D. Niculescu-Duvaz, R. G. Cooper, C. J. Springer, *J. Med. Chem.* **2002**, *45*, 99–114.
[33] J.-P. Behr, B. Demeneix, J.-P. Loefeler, J. Perez-Mutul, *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 6982–6986.
[34] F. Denizot, . R. Lang, *J. Immunol. Methods* **1986**, *89*, 271–277.
[35] H. Niu, R. D. Simari, E. M. Zimmermann, G. M. Christman, *Gene Ther.* **1998**, *91*, 735–740.
[36] J.-P. Behr, *Tetrahedron Lett.* **1986**, *27*, 5861–5864.
[37] J.-P. Behr, *Acc. Chem. Res.* **1993**, *26*, 274–278.
[38] P. Pinnaduwage, L. Schmitt, L. Huang, *Biochim. Biophys. Acta* **1989**, *985*, 33–37.

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