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DODAG; a versatile new cationic lipid that mediates efficient delivery of pDNA and siRNA

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ABSTRACT

We report the syntheses of novel cationic lipids comprised of cholesteryl-moieties linked to guanidinium functional groups, and also cationic lipids comprising a dialkylglycylamide moiety conjugated with a polyamine or a guanidinium functional group. In plasmid DNA (pDNA) transfection studies, these cationic lipids were formulated into cationic liposomes with the neutral co-lipid dioleoyl-L-α-phosphatidylethanolamine (DOPE) or with a recently reported neutral lipophosphoramidate derivative of histamine (MM27). We observe that cationic liposomes prepared from the cationic lipid *N′,N′*-dioctadecyl-*N*-4,8-diaza-10-aminodecanoylglycine amide (DODAG) and DOPE frequently mediate the highest levels of transfection *in vitro* in all three different cell lines studied (OVCAR-3, IGROV-1 and HeLa) both in the presence or absence of serum. In addition, *in vitro* cellular toxicity was found to be minimal. Alternatively, we observe that DODAG alone forms lipoplex nanoparticles with small interfering RNA (siRNA) that are able to mediate the functional delivery of two previously validated anti-hepatitis B virus (HBV) — siRNAs to murine liver *in vivo* with minimal observable liver toxicity and immune stimulation. Specific knock-down of HBV infection parameters (virion and hepatic mRNA levels) is observed that is at least equivalent to the impact of extensive treatment with lamivudine (a licensed antiviral drug).

1. Introduction

Gene delivery has numerous potential applications both clinically and for basic science research. Typically, the term gene therapy refers to the delivery of nucleic acids by means of a vector to a patient for some therapeutic purpose. The vector is the means of carriage of the nucleic acid from site of administration to site of action. Most current vector technologies can be divided into two groups; viral and nonviral, with both having their intrinsic advantages and disadvantages. Despite the high transfection efficiency of viral vectors, safety concerns have been raised in clinical trials because of their highly toxic nature [1]. Nonviral vectors, such as cationic liposome and polymer-based systems, are considered to be less toxic, less immunogenic, and easier to prepare than viral vectors and are potentially more attractive vectors for clinical applications [2–4].

Cationic liposomes/micelles and cationic polymers have been utilized since Felgner et al.[5] and Wu et al.[6] respectively demonstrated their use for nucleic acid delivery *in vitro*. Over the

Abbreviations: pDNA, plasmid DNA; DOPE, dioleoyl-L-α-phosphatidylethanolamine; DODAG, *N′,N′*-dioctadecyl-*N*-4,8-diaza-10-aminodecanoylglycine amide; HBV, hepatitis B virus; siRNA, small interfering RNA; Chol, cholesterol; CTAP, N^{15}-Cholesteryloxycarbonyl-3,7,12-triazapentadecane-1,15-diamine; CDAN, N^1-cholesteryloxycarbonyl-3,7-diazanonane-1,9-diamine; CEAG, N^1-cholesteryloxycarbonyl ethylene-1-amino-2-guanidinium chloride; CAPG, N^1-cholesteryloxycarbonyl-3-aza-pent-1-amino-5-guanidinium chloride; DOAG, *N′,N′*-dioctadecylamido-*N*-methyl-guanidinium chloride; DOGS, dioctadecylamidoglycylspermine; Z, benzyloxycarbonyl; *t*BOC, *t*-Butyloxy-carbonyl; HBTU, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; DMAP, 4-dimethylaminopyridine; TFA, trifluoroacetic acid; DIPEA, diisopropylethylamine; PCS, photon correlation spectroscopy; LDH, lactate dehydrogenase; GFP, green fluorescent protein; PEG, polyethylene glycol; ALT, alanine transaminase; VPEs, Viral Particle Equivalents; HBsAg, hepatitis B s-antigen; OAS-1, oligoadenylate synthase-1; IFN-β, interferon-β; RNAi, RNA interference; HCC, hepatocellular carcinoma; IFN-α, interferon-α; AAVs, adeno-associated viruses; shRNA, small hairpin RNA sequences; shRNAi, small hairpin interfering RNA; SNALPs, stabilized nucleic acid-lipid particles.

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last twenty years various cationic liposome/micelle formulations have been formulated using a succession of novel cationic lipids to assist nucleic acid delivery *in vitro* and even *in vivo* with generally disappointing results [7–16]. Most cationic liposome/micelle systems for nucleic acid delivery involve the formulation of a cationic lipid (cytofectins) and a neutral co-lipid such as dioleoyl-L-α-phosphatidylethanolamine (DOPE) **1** or cholesterol **2** (Fig. 1). The role of the cationic lipid is to facilitate nucleic acid binding and condensation leading to cationic liposome/micelle–nucleic acid complex (lipoplex) nanoparticles. The cationic lipids also facilitate nanoparticle interactions with cell wall membranes to trigger internalization (typically by endocytosis) and if well designed, endosomolysis (endosome escape) in order that nucleic acids may enter the cytosol post-nanoparticle internalization [15,16]. The neutral co-lipid DOPE **1** is a natural fusogenic lipid with a tendency to adopt the inversion hexagonal phase, H_{II}, over a wide range of temperatures, a characteristic typically expected to aid endosomolysis and improve intracellular trafficking of nucleic acids post nanoparticle internalization [17–24]. However, DOPE **1** has potentially limited utility *in vivo* because although the fusogenic nature helps to promote transfection *in vitro*, this also renders lipoplex nanoparticles unstable with respect to aggregation in more complex *in vivo* environments [25]. In order to overcome this problem, fluorinated phosphatidylethanolamine co-lipids were recently developed by Boussif et al. [26,27]. Alternatively, a series of dialkynoyl DOPE analogues has been developed [28,29]. Most recently, Jaffrès and Midoux et al. described the synthesis of a new neutral co-lipid lipophosphoramidate derivative of histamine (MM27) **3** that appears to enhance transfection up to 100-fold above levels achieved using cationic liposome/micelles formulated with DOPE **1** (Fig. 1) [30,31]. This effect may be due to the low pK_a (approximately 6) value of the imidazole ring nitrogen atoms [14,29,32]. As a result of this low pK_a value, imidazole rings should be unprotonated at pH 7 prior to nanoparticle entry into cells, but would be expected to become protonated when endosome compartments are acidified to around pH 5.5, post endocytosis. Protonation is associated typically with an increase in endosome ionic strength that is widely considered to cause endosomolysis by an osmotic shock type mechanism assisted by co-lipid fusogenic characteristics [32].

In previous research, cationic lipids have been prepared frequently by the conjugation of a variety of hydrophobic moieties to a variety of polyamine structures. This approach to cationic lipid design has been used widely. The polyamine employed can have a small and fully defined structure such as spermine–lipid conjugates [33,34] or spermine–lipophosphoramides [35]. Conversely, lipidic moieties may also be conjugated with monodisperse polymers such as PEI [36,37] or PLL [38]. In our case, two cationic lipids each with a cholesteryl hydrophobic moiety, namely N^{15}-Cholesteryloxycarbonyl-3,7,12-triazapenta-decane-1,15-diamine (CTAP) **4** and N^{1}-cholesteryl-oxycarbonyl-3, 7-diazanonane-1, 9-diamine (CDAN) **5**, have proven particularly useful for *in vitro* and *in vivo* delivery of plasmid DNA (pDNA) and small interfering RNA (siRNA) [32,39–44]. However, in spite of the apparently high level of utility in both cases, we have considered that **4** and **5** may in fact not be optimal structures for all requirements. Therefore, we became interested in exploring the structures of other cationic lipids in an attempt to find cationic lipids with potentially further improved and/or more versatile properties. In particular we were intrigued to investigate the effects of substituting the polyamine functional head group with guanidinium functional

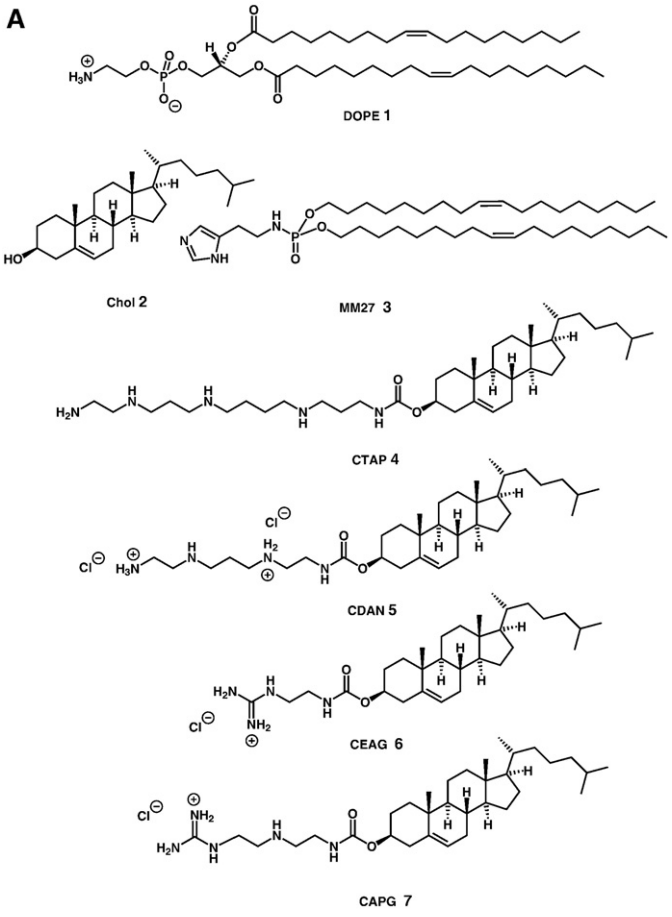


Fig. 1. Schematic illustration of structures of cationic lipids and neutral co-lipids constituents synthesized or used in transfection studies.

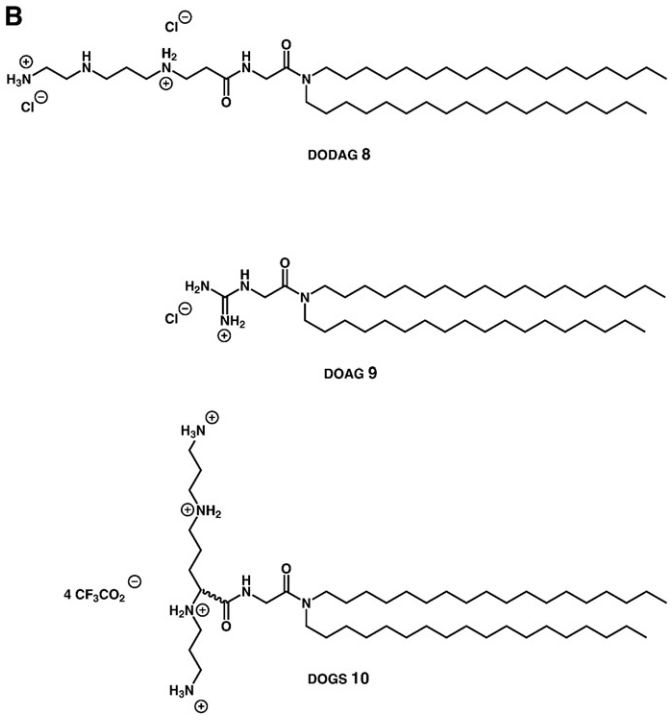


Fig. 1 (*continued*).

moeities. Conversely we wished to explore the consequences of replacing the cholesteryl hydrophobic moiety of **5** with a long-chain hydrocarbon moiety. Guanidinium functional groups have a pK_a value of approximately 14, and so are easily protonated at physiological pH. Different complexes between guanidinium groups and anionic phosphate have already been observed [45,46] that can facilitate the formation of lipoplex nanoparticles with pDNA. Therefore, besides applications in the anti-trypanosome agent synthaline [47] guanidinium groups have also been used by a number of different groups to originate cationic lipids for pDNA transfection [48–51].

Accordingly, we now report on the syntheses and nucleic acid delivery properties of novel cationic lipids comprised of cholesteryl hydrophobic moieties linked to guanidinium functional groups (**6** and **7**). At the same time we report on the syntheses and nucleic acid delivery properties of novel cationic lipids with a polyamine or guanidinium functional group conjugated (**8** and **9** respectively) to a long-chain hydrocarbon dialkylglycylamide moiety of the type that was first described in the structure of the spermine–lipid conjugate dioctadecylamido-glycylspermine (DOGS) **10** (Fig. 1). We observe the important co-lipid characteristics of the lipophosphoramidate derivative of histamine (MM27) **3** in partnering guanidinium functional group containing cationic lipids. We also observe the surprising versatility of the dialkyl glycylamide polyamine cationic lipid, *N′,N′*-dioctadecyl-*N*-4, 8-diaza-10-aminodecanoylglycine amide (DODAG), **8** in helping to mediate functional pDNA delivery to cells *in vitro* and siRNA delivery to liver *in vivo*.

2. Materials and methods

2.1. General synthesis

All chemicals and reagents were purchased from Sigma Aldrich (Dorset, UK) or Avanti Lipids (USA) or Invitrogen (UK) unless stated otherwise. Solvents were typically prepared as freshly distilled from appropriate drying agents (CH_2Cl_2 was distilled over P_2O_5, diisopropylethylamine (DIPEA) was distilled over NaOH) and reactions were run under a nitrogen atmosphere. Other solvents were purchased predried or as required from Sigma-Aldrich (Dorset, UK) or BDH Laboratory Supplies (Poole, UK). HPLC-grade acetonitrile was purchased from Fisher Chemicals (Leicester, UK) and other HPLC-grade solvents from BDH Laboratory Supplies (Poole, UK). Thin layer chromatography (TLC) was performed on pre-coated Merck–Kieselgel 60 F_{254} aluminium backed plated and revealed with ultraviolet light, iodine, acidic ammonium molybdate (IV), acidic ethanolic vanillin, or other agents as appropriate. Flash column chromatography was accomplished on Merck–Kieselgel 60 (230–400 mesh). Mass spectra were recorded using Bruker Esquire 3000, VG-7070B or JEOL SX-102 instruments. ^{1}H NMR spectra were recorded on an Advance Brucker 400 Ultrashield ^{TM} machine using residual isotopic solvent as an internal reference. Coupling constants *J* are given in Hz (The following abbreviations were used: s = singlet, d = doublet, t = triplet, q = quartet, quin = quintet, br = broad singlet). Analytical HPLC (Hitachi-LaChrom L-7150 pump system equipped with a Polymer Laboratories PL-ELS 1000 evaporative light scattering detector) was conducted with a Vydac C4 peptide column and HPLC gradient mixes assigned as follows: gradient mix A = H_2O/0.1%TFA; mix B = MeCN/0.1%TFA; mix C = MeOH. Liposomes were sized using a DynaPro particle sizer (Protein Solutions; Piscataway, NJ) and employing the Dynamics data collection and analysis software. Detailed syntheses are reported in supplementary information.

2.2. Formulation of cationic liposomes

All lipids were stored as stock solutions in anhydrous organic solvents ($CHCl_3$, MeOH, or a mixture of both), at −20 °C under argon. Liposomes were made with cationic lipids and co-lipids (DOPE **1** or MM27, **3**) at a standard ratio of 1:1 (molar ratio) to give a cationic liposome suspension with a fixed total lipid concentration of 3 mg mL^{-1} in water. Fluorescent labeled liposomes always contained 0.5 mol % of DOPE-Rhodamine (Avanti Polar Lipids Inc., USA). Appropriate volumes of each lipid stock were placed in a round-bottom flask (typically 5 mL). The solvent was slowly removed *in vacuo* to ensure production of an even lipid film. The film was rehydrated with water at a defined volume (hydration time of 5 min). The resulting

solution was sonicated for 30 min at 30 °C to form liposomes of appropriate size. The size of the liposomes was determined by photon correlation spectroscopy (PCS). In each experiment the liposomal preparation (20 μL) was diluted with PBS to a final volume of 200 μL. The mean average diameter of the vesicles was determined at 25 °C as the result of at least three reliable readings.

2.3. Preparation of lipoplex nanoparticles

The plasmid pEGFP-Luc, which encodes for an enhanced green fluorescence protein (GFP), was amplified in *Escherichia coli* and purified using GIGA prep Purification protocol. Purity of the plasmid was checked by electrophoresis on 1.0% agarose gel [pDNA], and was estimated spectroscopically by measuring the $A^{0.1}{}_{260}$ and confirmed by gel electrophoresis. The plasmid preparations showing a value of $A_{260}/A_{280} > 1.8$ were considered essentially free of background protein contamination and used immediately. Cationic liposome-pDNA complexes (lipoplex nanoparticles) were prepared at 1:2, 1:4, 1:8 and 1:12 (pDNA:lipid w/w) with pDNA; pDNA (1 μg) was diluted with sterile pyrogen-free deionized water and added to an aliquot of cationic liposome (2, 4, 8 or 12 μg) in a vial. Lipofectamine 2000 was used as per manufacturers instructions (Invitrogen) and lipoplex nanoparticles were prepared at a pDNA:lipid ratio of 1:2.5 (w/w). After vortex mixing (30 s), the resulting samples of lipoplex nanoparticles were kept at room temperature for 30 min before being used in transfection experiments. Lipoplex nanoparticle sizes were determined in parallel by PCS. In each experiment the nanoparticle suspensions (20 μL) were diluted with PBS to a final volume of 200 μL. The mean average diameter of the vesicles was determined at 25 °C as the result of at least three reliable readings.

2.4. Formulation of siRNA-DODAG nanoparticles

Anti-HBV and control siRNA [42] was dispersed in deionized water (1 mg/ml) at ambient temperature, and then combined with an appropriate aliquot of DODAG **8** (2 mg/ml) in deionized water under conditions of rapid vortex mixing; followed by bath sonication (15 min) in the presence of the osmolyte trehalose (5% w/v final concentration). This procedure typically resulted in the formation of siRNA-DODAG **8** nanoparticles (70 ± 20 nm) with a final siRNA:lipid ratio was 1:3 (w/w).

2.5. Transfection experiments in vitro

Cationic liposome mediated transfection studies were performed using three cancer cell lines: HeLa, OVCAR-3 and IGROV-1. These cells were grown in D-MEM media (Gibco-BRL, UK) and supplemented with 10% fetal calf serum (FCS) (Gibco-BRL, UK) and 100 U/mL of penicillin/streptomycin. All the cells were maintained in a humidified incubator with 5% CO_2 at 37 °C. Cells were seeded 24 h before transfection onto a 24-well plate at a density of 60,000 cells per well and incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. The cells were grown until 80% confluence was reached, then washed twice with PBS solution before incubation with lipoplex nanoparticles. Lipoplex nanoparticles in OptiMem or OptiMem with FCS 10% (w/v) (about 500 μL) were added to each well (with 1 μg of pDNA per well). After 4 h transfection time at 37 °C, the media was removed, cells were washed twice with PBS solution and fresh media was added. Following a further 48 h of incubation at 37 °C, the cells were assayed for luciferase expression using a chemiluminescent assay (Promega). After transfection the medium was aspirated and the wells were washed briefly with PBS solution. Post removal of PBS the cells were lysed by adding 80 μL of lysis buffer (Promega) to each well. The cell lysate was collected and used for luciferase and protein assays. For the luciferase assay, 20 μL of cell lysate was transferred to a test tube and assessed directly by means of a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) using a luciferase assay kit (Promega). The luminescence was recorded for 5 s after addition of luciferin (100 μL). Cellular protein content per well was quantified using a bicinchoninic acid (BCA) assay (Pierce; Rockford, IL). The BCA assay was prepared as specified in its manufacturer's instructions. An amount of 20 μL of cell lysate was treated with 200 μL of BCA reagent. The solution was incubated for 30 min at 37 °C, and absorbance at A_{570} was measured by means of a Beckman DU-600 UV–vis spectrometer (Palo Alto, CA). Thereafter, the protein content was estimated by comparison to BSA standards, and the luciferase activity was normalized by protein content and data was expressed as relative luciferase units per microgram of protein (RLU/μg protein). Results are reported as means ± SEM.

2.6. Cellular toxicity in vitro

The cytotoxicity of different lipoplex nanoparticles was evaluated using three different cancer cell lines, HeLa, OVCAR-3 and IGROV-1. Cells were grown in the same conditions as described before, and then seeded for 24 h prior to experimentation on a 96-well plate at a density of 15,000 cells per well and incubated overnight in a humidified 5% CO_2 atmosphere at 37 °C. Normal *in vitro* transfections were performed as described above using lipoplex nanoparticles prepared at 1:2, 1:4, 1:8 and 1:12 (pDNA:lipid w/w) with pDNA (pEGFP-Luc, 0.5 μg). All nanoparticles were prepared in OptiMEM. At 24 h after post-transfection an aliquot of supernatant (100 μL) from each well was collected and transferred to a fresh 96 well assay plate. Cells in the original assay plate were then washed two times with PBS after which 5× reporter lysis buffer (Promega) was added (100 μL per well) and the plates were incubated for 30 min at 37 °C. Aliquots (50 μL) of LDH kit solution were added to the wells of the supernatant and cell lysis assay plates. After 30 min of incubation at room temperature an aliquot (50 μL) of stop solution was added to each well. Light absorption at A_{450} was measured by means of a Beckman DU-600 UV–vis spectrometer (Palo Alto, CA). The complete LDH assay was carried out using the CytoTox 96 non-radioactive cytotoxicity assay (Promega, USA).

2.7. Fluorescence microscopy of in vitro transfection

Adherent HeLa cells were seeded then grown for 24 h in a 6-well (2.5×10^5 cells per well, 3 mL of complete medium) plate fitted with plastic microscopy slides, in a wet (37 °C) 10% CO_2/90% air atmosphere. The cells were grown until 80% confluent. Cells were then treated with either naked pDNA, DODAG **8**/DOPE **1** cationic liposome or DODAG **8**/DOPE **1** lipoplex nanoparticles (pDNA:lipid 1:4, w/w) in OptiMEM, and then incubated in a wet (37 °C) 10% CO_2/90% air atmosphere for 4 h. Media was then removed, and the cells were washed with PBS (2×), then treated with paraformaldehyde (PFA, 1× (20 min at 37 °C), PBS (2×), glycine (1 × 20 mM, 20 min at 37 °C), PBS (2×). Thereafter slides were then removed from their wells and fixing was performed using Vectashield (molecular probes, UK). Microscopy images were obtained on an Olympus 251 scope.

2.8. DODAG mediated delivery of anti-HBV siRNAs in vivo

HBV transgenic mice were also used to assess anti viral efficacy of formulations as described previously [42]. The Animal Care Committee at Stanford University, CA USA approved of all these procedures for all these experiments. SiRNA-DODAG **8** nanoparticle formulations were prepared as described above and the siRNA dose for each injection was 1 mg/kg animal body weight at the indicated days. Groups initially comprised 6 animals. In mice receiving lamivudine, the drug was administered IP daily at a dose of 200 mg/kg mouse body weight. The main markers of viral replication (VPEs in serum, HBsAg mRNA in liver) were assayed as described previously [42]. A complete

experimental toxicity was also carried out in exactly the same manner as described previously including analysis of blood [42]. The blood samples were submitted for haematological analysis, urea and electrolyte concentration determination, ALT and LDH activity determination. Assays were performed in the accredited Haematology and Chemical Pathology Department laboratories of the South African National Health Laboratory Services (NHLS) in Johannesburg.

2.9. Statistical analysis

Data are expressed as the mean ± standard error of the mean. Statistical difference was considered significant * when $P<0.05$ and was determined according to the Dunnett's multiple comparison test and calculated with the GraphPad Prism software package (GraphPad Software Inc., CA, USA).

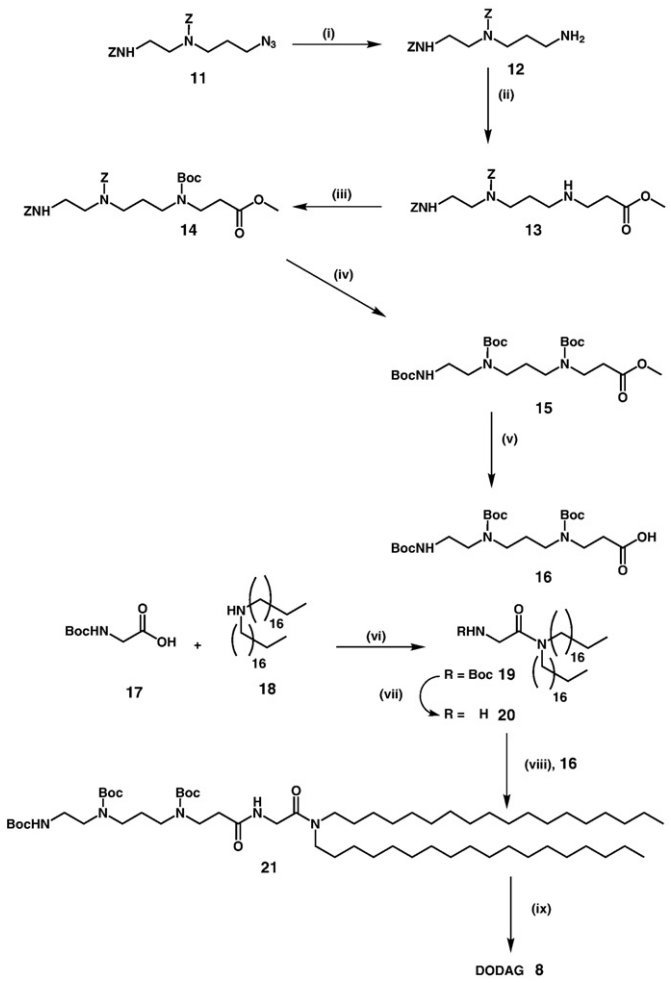
3. Results

3.1. Synthesis

DODAG **8** (Fig. 1) was designed to be a structural chimera involving the CDAN **5** polar head group and the dialkylglycine amide moiety found in micelle forming cationic lipid dioctadecylamido glycylspermine (DOGS) **10** (Fig. 1) [10,14]. The synthesis of DODAG **8** was accomplished in two main stages as shown (Scheme 1). Starting azide **11** was synthesized as reported previously [39] and subsequently smoothly converted into polyamine **12** using a trimethylphosphine adaptation of the Staudinger reaction (Scheme 1). Afterwards, a Michael addition of polyamine **12** to methylacrylate was used to give methyl ester **13** in moderate yield. *N*-Boc protection of the methyl ester then resulted in a fully protected methyl aminodecanoate **14**. This methyl aminodecanoate **14** was then subject to protecting group exchange resulting in a uniformly protected ester **15**, which was then subjected to methyl ester hydrolysis to give the protected aminodecanoic acid **16**. In the second stage of DODAG **8** synthesis, *N*-Boc-glycine **17** was converted, using dioctadecylamine **18**, into tertiary *N*-Boc-glycine amide **19** and then α-*N*-deprotection was carried out to give the key tertiary glycine amide intermediate **20**, in excellent yield. Conjugation of this final intermediate with uniformly protected aminodecanoic acid **16** gave fully protected DODAG **21**. Final deprotection resulted in >98% pure DODAG **8** in excellent yield. This was lyophilized in several cycles from aqueous acetonitrile giving the desired compound as a mixed acid/free base. Thereafter, we synthesized two cholesteryl guanidinum lipids N^1-cholesteryloxycarbonyl ethylene-1-amino-2-guanidinium chloride (CEAG) **6** and N^1-cholesteryloxycarbonyl-3-aza-pent-1-amino-5-guanidinium chloride (CAPG) **7** in the following way. Cholesteryl chloroformate **22** was combined with large excesses of ethylenediamine or diethylentriamine yielding cholesteryl amine intermediates **23** and **24** respectively (Scheme 2). Next, the resulting intermediate compounds **23** and **24** were each treated using the procedure of Matsueda et al. [52] that makes use of ^{1}H-pyrazole-1-carboxamidine monohydrochloride in ethanol as a guanidinylation reagent (Scheme 2) to produce compounds CEAG **6** and CAPG **7** in 70% and 63% yields. Finally desired compound *N*′,*N*′-dioctadecylamido-*N*-methylguanidinium chloride (DOAG) **9** was also synthesized from key tertiary glycine amide intermediate 20 using the same conditions with 58% yield (Scheme 2).

3.2. Cationic liposome/micelle formulations

Liposome formulations were prepared using the thin-film technique and sonicated [53]. The formulations were made in the ratio of 50 mol% cationic and 49.5 mol% neutral helper lipid each time (0.5 mol% of DOPE-Rhodamine was also included for fluorescence microscopy) at a total concentration of 3 mg mL^{-1} in water. The size

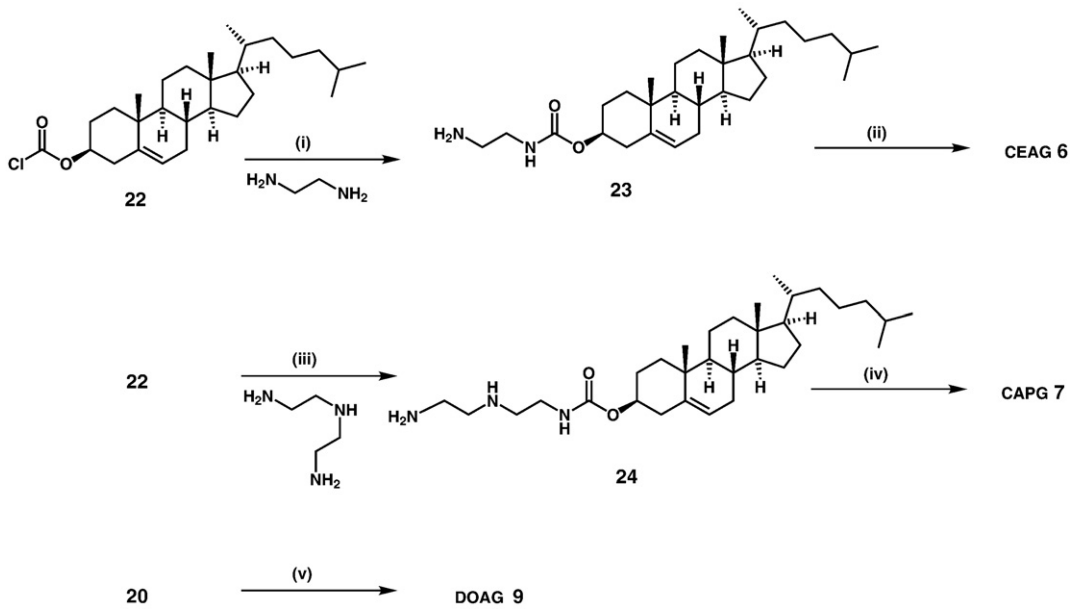


Scheme 1. Reagents and conditions: i) PMe_3 plus H_2O in TMF, r.t., 2.5 h., 82%; ii) Methylacrylate, MeOH, r.t., 12 h., 54%; iii) Boc_2O, NEt_3, CH_2Cl_2, r.t., 12 h, 70%; iv) Pd/C-10%, Boc_2O, MeOH, H_2, r.t., 12 h, 92%; v) LiOH, THF, 0 °C, 12 h., 97%; vi) HBTU, DMAP, $CHCl_3$, r.t., 12 h, 70%; vii) TFA, CH_2Cl_2, N_2, r.t., 2 h, 92%; viii) HBTU, DMAP, $CHCl_3$, r.t., 24 h, 62%; ix) TFA, CH_2Cl_2, r.t., 3 h, 97%.

of the liposomes and the lipoplexes was measured by PCS (Table 1). Suitable lipoplexes were not formed with DOAG **9** when formulated with either DOPE **1** or the novel co-lipid MM27 **3**. PCS size measurements for these particles were around 1000 nm with both co-lipids and as such transfection results were equally poor. The other liposomes were formulated with sizes between 200 and 500 nm, and for their corresponding lipoplexes their size was measured to be between 155 and 423 nm. DODAG (50 nm particle size) were prepared in water at pH 7 by dispersal of DODAG **8** alone in water.

3.3. Plasmid DNA transfection in vitro

Transfections of three cancer cell lines OVCAR-3, IGROV-1 and HeLa were performed with a range of cationic liposomes [either (1:2), (1:4), (1:8), (1:12) pDNA:lipid w/w (Lipofectamine 2000, pDNA:lipid 1:2.5, w/w)] in the absence and presence (10% v/v) of serum. OVCAR-3 transfection data (Fig. 2(A)) are interesting. DODAG **8**/DOPE **1** or DODAG **8**/MM27 **3** cationic liposomes were the most effective agents of transfection at a pDNA:lipid 1:4 w/w ratio, over and above commercially available Lipofectamine 2000, with and without the presence of serum. The DODAG **8**/MM27 **3** cationic liposome combination was also prominent at a pDNA:lipid 1:8 w/w ratio, with and without the presence of serum. In the case of IGROV-1 cell



Scheme 2. i) Excess of amine (20.2 equivalents), H_2O, 8 h, 65%, ii) 1H-pyrazole-1-carboxamidine.mono chlorohydrate, DIPEA, EtOH, 78 °C, 16 h, 70%, iii) excess of amine (10 equivalents), H_2O, 8 h, 54%, iv) 1H-pyrazole 1-carboxamidine.monochlorohydrate, DIPEA, EtOH, 78 °C, 16 h, 67%, v) 1H-pyrazole-1-carboxamidine.monochlorohydrate, DIPEA, EtOH, 78 °C, 48 h, 58%.

line transfection (Fig. 2(B)), DODAG **8**/DOPE **1** cationic liposomes were comparable agents of transfection with Lipofectamine 2000 in the presence of serum, at pDNA:lipid ratios of 1:4 and 1:8 w/w. In the absence of serum, CAPG **7**/MM27 **3** and CDAN **5**/DOPE **1** cationic liposomes combined at pDNA:lipid ratios of 1:12 w/w, and DODAG **8**/DOPE **1** cationic liposomes combined at a pDNA:lipid ratio of 1:4 w/w, appeared modestly more effective than Lipofectamine 2000. Finally in the case of HeLa transfection (Fig. 2(C)), DODAG **8**/DOPE **1** cationic liposome mediated pDNA-transfection was the most effective at low pDNA:lipid 1:4 or 1:2 w/w ratios in the presence of serum, closely followed by DODAG **8**/MM27 **3** cationic liposome mediated transfection. In the absence of serum, DODAG **8**/DOPE **1** cationic liposomes formulated at low pDNA:lipid 1:4 or 1:2 w/w ratios were also found more effective than Lipofectamine 2000 for transfection, alongside CAPG **7**/MM27 **3** and CEAG **6**/MM27 **3** cationic liposomes formulated at high pDNA:lipid ratios of 1:12 w/w. These data in total suggest that DODAG **8** /DOPE **1** and DODAG **8** /MM27 **3** cationic liposomes formulated at pDNA:lipid 1:4 w/w ratios may have utility as a broad-band transfection agent, comparable with Lipofectamine 2000, for different cell lines *in vitro* (suitable for use both with and without serum in cell growth medium). There are also indications that CAPG **7**/MM27 **3** cationic liposomes formulated at a pDNA:lipid ratios of 1:12 w/w, may be an alternative niche transfection system.

Table 1
Liposome and lipoplex nanoparticle sizes with included polydispersity indices.

Liposomes	DOPE 1 Size (nm)	Polydispersity index	MM27 3 Size (nm)	Polydispersity index
CDAN 5	183.3 ± 78.1	0.66	204.2 ± 69.5	0.216
CEAG 6	160.1 ± 64.9	0.481	378.8 ± 160.6	0.678
CAPG 7	190.4 ± 72.6	0.347	218.4 ± 63.5	0.128
DODAG 8	233.6 ± 60.2	0.23	516.8 ± 178.3	0.227
DOAG 9	623.1 ± 274.2	0.859	430.1 ± 177.4	0.531
Lipofectamine 2000	Liposome size: 114.8 ± 32.1		Polydispersity index: 0.29	
Lipoplex nanoparticles				
CDAN 5	290.7 ± 105	0.290	155.4 ± 64.3	0.542
CEAG 6	233.4 ± 94.3	0.460	423.7 ± 150.8	0.257
CAPG 7	229.9 ± 89.3	0.387	311.2 ± 122.2	0.403
DODAG 8	301.5 ± 111.2	0.298	307.2 ± 107.5	0.240
DOAG 9	1365.6 ± 396.5	0.127	818.9 ± 351.2	0.696
Lipofectamine 2000	Lipoplex size: 442.3 ± 81.2		Polydispersity index: 0.41	

3.4. In vitro cellular toxicity

Our own previously published data [40] and reports of Uchida et al. [54] have described extensively the cytotoxicity of Lipofectamine 2000 that results in upwards of 20% lactate dehydrogenase (LDH) release (equivalent to cell death) in the LDH cytotoxicity assay applied to immortalized adherent cell lines. Therefore, we elected to benchmark the cytotoxicity of the different cationic liposomes and their corresponding lipoplex nanoparticles reported here against the published Lipofectamine 2000 mark using the same LDH cytotoxicity assay. Results are illustrated (Fig. 3 (A–C)). Overall there is a general indication in IGROV-1 and HeLa cells that cytotoxicity increases with pDNA/lipid ratio consistent with an increase in cationic charge mediated cytotoxicity. Also co-lipid MM27 **3** appears to confer more cytotoxicity than DOPE **1** in IGROV-1 and OVCAR-3 cells. Otherwise, DODAG **8**/DOPE **1** — pDNA 1:4 w/w lipoplex nanoparticles were found consistently to exhibit the lowest or close to lowest cytotoxicity (approximately 5% LDH release) in all three cell lines. These data are important and gratifying considering the general effectiveness of DODAG **8**/DOPE **1** cationic liposome mediated transfection reported above with a pDNA:lipid ratio1:4 w/w (see Fig. 2). Hence DODAG **8**/DOPE **1** cationic liposomes appear to be overall the most versatile cationic liposomes for efficient transfection with minimal toxicity. Accordingly we would suggest this cationic liposome system represents a potentially valuable alternative to Lipofectamine 2000 as a broad-band transfection agent for adherent cells *in vitro*.

3.5. Fluorescence microscopy

Transfection of HeLa cells by DODAG **8**/DOPE **1** cationic liposomes which were the most efficient transfecting lipid pair was observed by light microscopy in order to verify the process of transfection. Cells

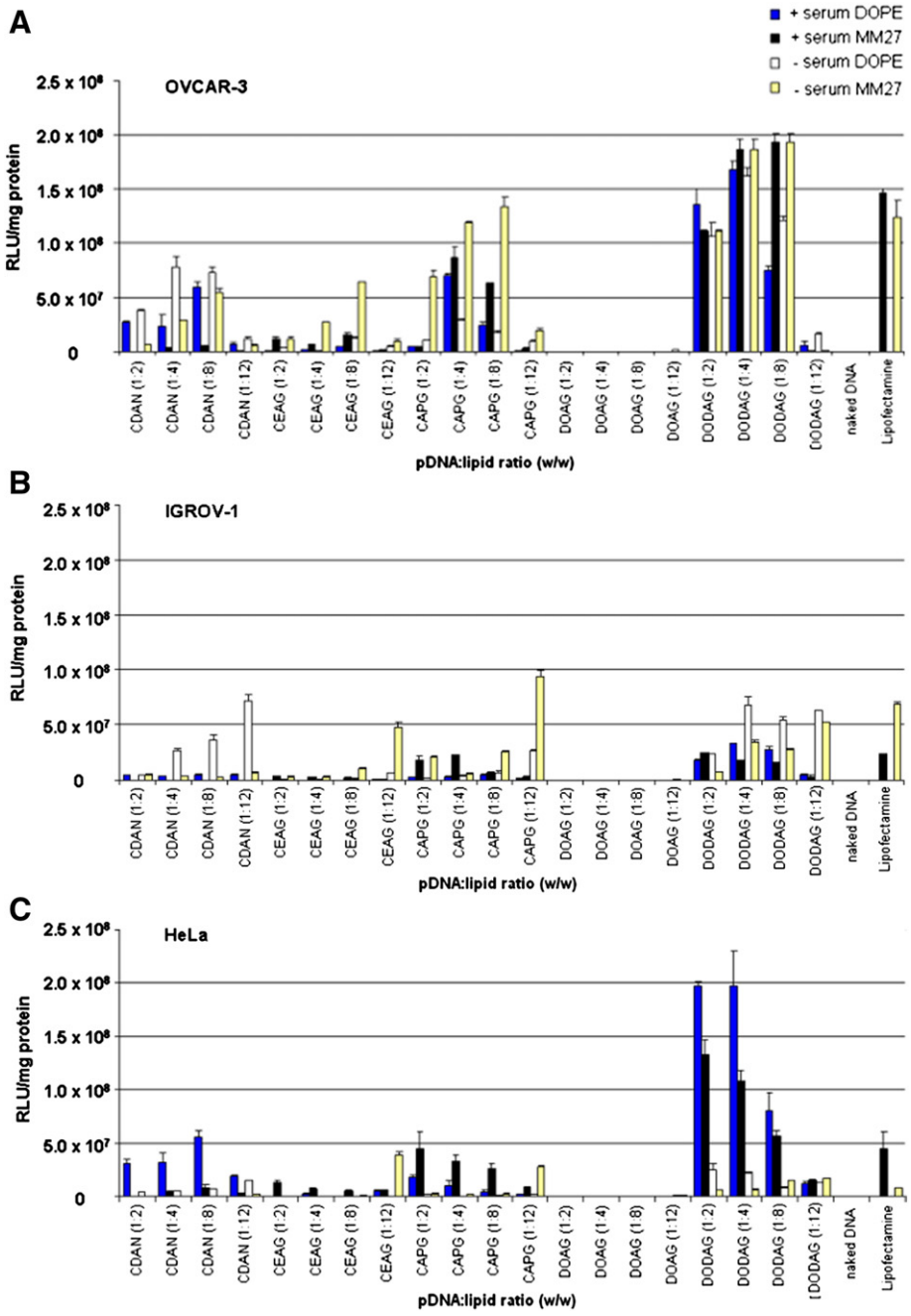


Fig. 2. Transfection data for three cell lines and 20 different lipoplex nanoparticle systems with Lipofectamine2000 transfection as positive control (pDNA:lipid ratio: 1:2.5, w/w). The indicated cationic lipids were mixed with the indicated co-lipids in 1:1 m/m ratio to generate cationic liposomes. Transfections were then performed using pEGFP-luc pDNA expressing both enhanced GFP (EGFP) and luciferase (luc). For each transfection well, different cationic liposomes were combined with pEGFP-luc (1 μg/well) at the indicated pDNA: lipid w/w ratios in order to form lipoplex nanoparticles that were administered to cells for 4 h transfections. The black bars for Lipofectamine represent transfections in the presence of serum and the yellow bars represent lack of serum respectively (A–C).

were incubated with DODAG **8**/DOPE **1** lipoplex nanoparticles for 4 h, washed and fixed for microscopy post incubation. From the images, cell entry and endosomolysis is clearly apparent (Fig. 4), consistent with a high level of efficient transfection. In all other cases of cationic liposome mediated transfection studied here by microscopy, intracellular access was found to be far less effective (as judged by Rhodamine fluorescence microscopy) than that mediated by DODAG **8**/DOPE **1** cationic liposomes. Furthermore, intracellular/nuclear localized green fluorescent protein (GFP) expression was seen at 4 h only post DODAG **8**/DOPE **1** cationic liposome-mediated transfection (Fig. 4) which is itself unusual but indicative of highly efficient transfection.

3.6. Functional delivery of anti-HBV siRNA in vivo

Efficient functional nonviral delivery of pDNA to the liver *in vivo* can only currently be accomplished by physical means using hydrodynamic delivery [55]. However, functional delivery of small interfering RNA (siRNA) to liver appears more tractable although with quite complex multi-component nanoparticles systems as we demonstrated previously [42]. In initial experiments, we were surprised to observe that DODAG **8** alone was able to form nanoparticles with siRNA. The formation of siRNA-DODAG **8** nanoparticles was achieved with relative ease at a lipid:siRNA ratio (3:1, w/w) . This translates into a cationic lipid to nucleotide molar ratio, [cyt]/[nt], of 1.25, and

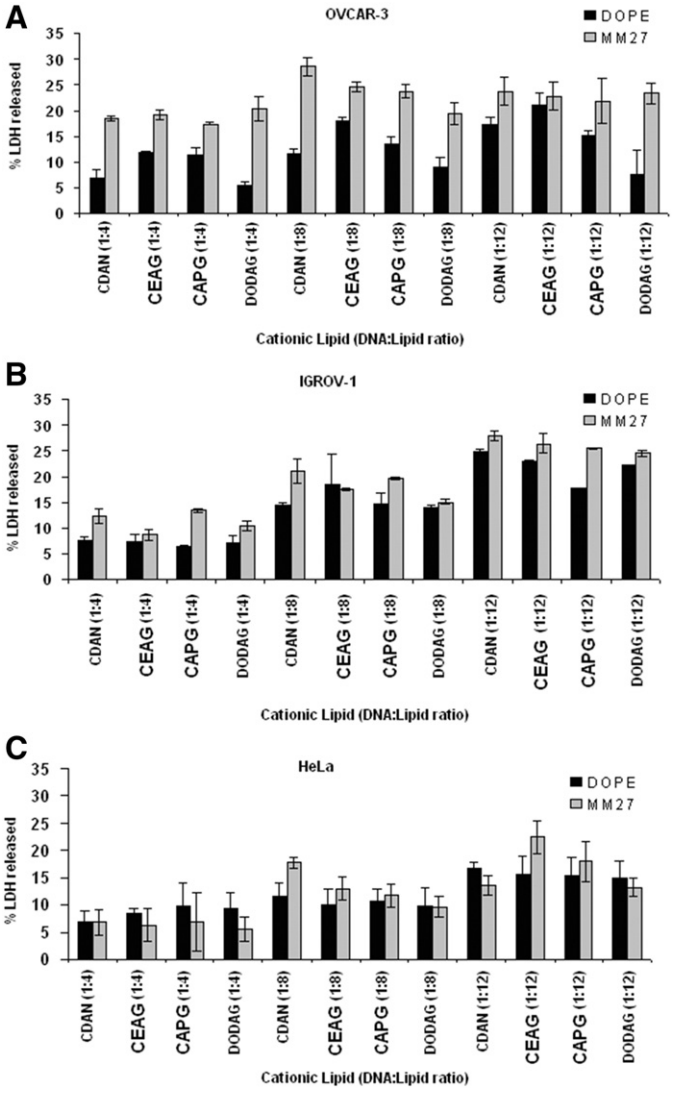


Fig. 3. Percentage LDH cytotoxicity data for lipoplex nanoparticles formulated with cationic lipids DODAG **8**, CDAN **5**, CEAG **6** and CAPG **7** with either DOPE **1** or MM27 **3** as helper co-lipids. The indicated cationic lipids were mixed with the indicated co-lipids in 1:1 m/m ratio to generate cationic liposomes. LDH toxicity experiments were then performed using pEGFP-luc as above. For each toxicity experiment, different cationic liposomes were combined with pEGFP-luc (0.5 μg/well) at the indicated pDNA:lipid w/w ratios in order to form lipoplex nanoparticles that were administered to cells for 30 min toxicity experiments. All toxicity data are normalized to the % LDH release observed with pDNA administration alone (typically near zero).

hence an N/P ratio of approximately 1.8 assuming that each DODAG **8** molecule possesses a cationic charge of approximately 1.5 at neutral pH, in line with the behavior of the original parent molecule CDAN **5** [14,40]. Agarose gel retardation assays showed that at an N/P ratio of 1.8 the movement of siRNA was entirely retarded according to our gel electrophoresis data, consistent with complete interaction with DODAG **8** (Fig. 5A).

Lipoplex siRNA-nanoparticle sizes are dependent on the duration of sonication, but typically a size of 70 ± 20 nm (as determined by photon correlation spectroscopy post preparation) was selected given the target organ preference [42]. Lipoplex siRNA nanoparticles were prepared fresh and then immediately administered in the presence of the trisaccharide osmolyte known as trehalose, intended to shield nanoparticles from immediate serum effects post IV injection thereby giving sufficient time for particles to reach the liver and avoid excessive sequestration by Kupffer cells. Potential toxic side effects of siRNA-DODAG **8** nanoparticles were investigated experimentally *in vivo* (as reported previously) [42] using identical markers of hepatic, renal and haematological damage as previously without the discovery of any obvious signs of toxicity even of LDH and alanine transaminase (ALT) levels (Table 2).

Previously, we reported how anti-HBV siRNA sequences had been screened *in vitro* and two chemically naked sequences were selected and validated for *in vivo* analysis [42,56,57]. Therefore, siRNA-DODAG **8** nanoparticles were prepared as above with the same validated siRNAs, namely siRNA-1407 and siRNA-1794. Lipoplex siRNA-nanoparticles were then administered IV to HBV transgenic mice [58] every 3 days for 4 weeks. The siRNA dose at every IV injection was 1 mg/kg of murine body weight. Compared with controls, nanoparticle-mediated administration of siRNA-1407 or siRNA-1794 resulted in a 2–3 fold fall in viral particle equivalent (VPE) levels in serum, together with a similar decrease in HBV s-antigen (HBsAg) mRNA levels in liver relative to controls after 4 weeks (Fig. 5B, C). Overall, siRNA-DODAG **8** nanoparticle mediated administration of siRNA-1407 or siRNA-1794 appeared to result in comparable inhibitory anti-viral effects to those observed after lamivudine treatment (Fig. 5B, C). Indeed, siRNA-DODAG **8**-nanoparticle administration of siRNA-1407 appeared to promote an even more pronounced anti-viral inhibitory effect than lamivudine. Moreover the antiviral effects were also comparable to the effects produced delivering siRNA-1407 or siRNA-1794 using the much more complex multi-component. pH-triggerable, siRNA-nanoparticle system that we had designed previously for functional delivery of siRNA to liver [42]. As in our previous study, interferon response genes for oligoadenylate synthase-1 (OAS-1) and interferon-β (IFN-β) were found not stimulated by siRNA-DODAG **8** nanoparticle administration suggesting that our observed anti-HBV effects here (Fig. 5B, C) are the consequence of an RNA interference (RNAi) mechanism, as observed previously [42].

4. Discussion

Our premise in this paper was to evolve cationic lipid design through a simple functional group swap of hydrophilic polyamine side chains for guanidinium side chains and/or an exchange of hydrophobic cholesteryl for *N′, N′*-dialkyl glycylamide moieties. In addition, the list of potential co-lipid partners was extended to include the latest

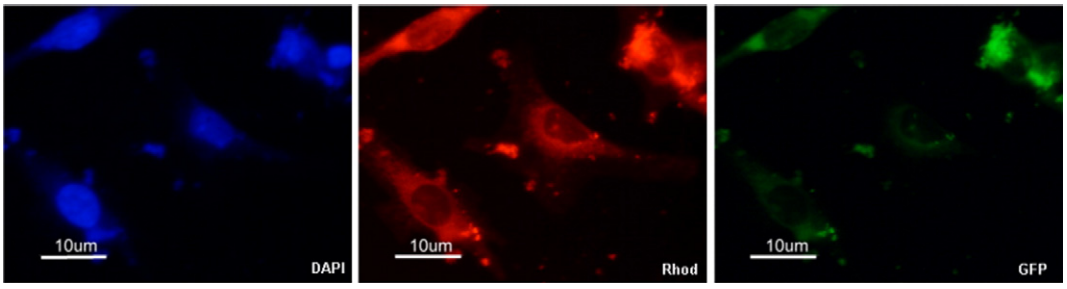


Fig. 4. Fluorescence microscopy of HeLa cells post transfection with DODAG **8**/DOPE **1** cationic liposome–pDNA complexes (lipoplexes, ×400 magnification). Cells are stained with DAPI (left) to illustrate nuclei, visualized for GFP expression (right) and visualized for rhodamine fluorescence (middle).

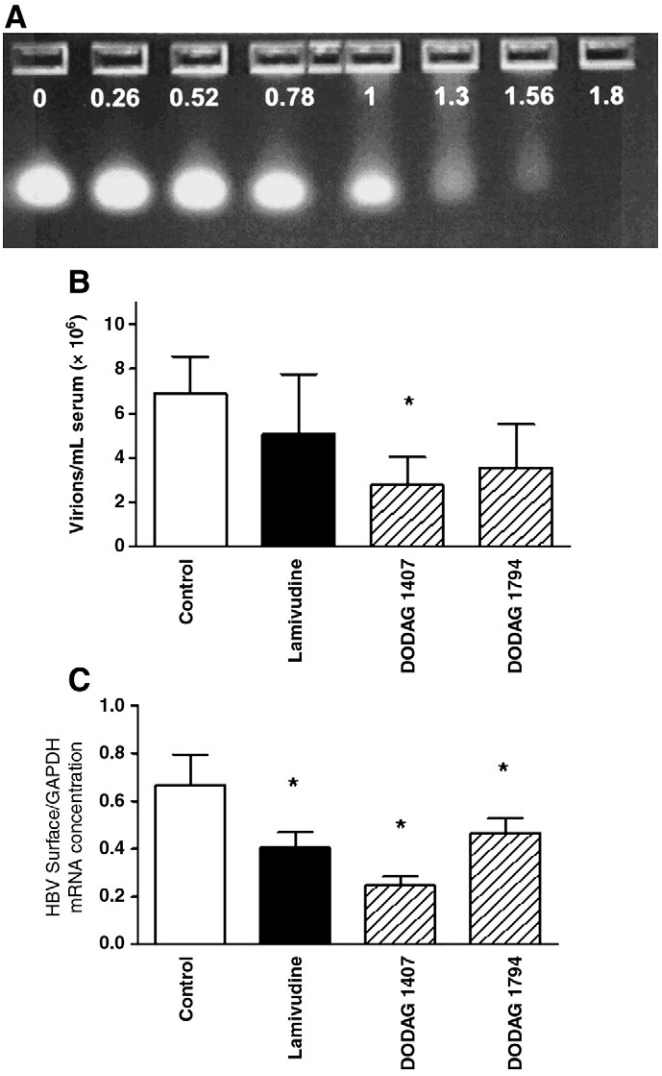


Fig. 5. Formulation and experimental assessment of siRNA-DODAG **8** nanoparticle formulations. (A) Gel retardation experiment showing variation of siRNA (1 μg) retardation viewed by agarose gel electrophoresis as a function of DODAG **8**:siRNA ratio (w/w); Effect of siRNA-DODAG **8** nanoparticle formulations on markers of HBV replication: (B) Circulating VPEs measured at 28 days in HBV transgenic mice treated every 3 days with a siRNA-DODAG **8** nanoparticle formulation delivering non-functional control siRNA (**Control**) [equivalent result obtained with saline injection], or siRNA-DODAG **8** nanoparticle formulations delivering functional siRNA-1407 or siRNA-1794 (**DODAG 1407** or **DODAG 1794** respectively), in comparison with lamivudine. (C) HBsAg mRNA measured at 28 days in HBV transgenic mice treated exactly as in (B).

neutral co-lipid reported, namely the lipophosphoramidate derivative of histamine (MM27) **3** (Fig. 1). Our initial interest was to test out whether the new combinations of structural elements could lead to enhanced *in vitro* pDNA transfections of different cell lines, under different conditions, consistently on a par with or in excess of a lipofectamine 2000 positive control transfection. This was achieved and on balance DODAG **8**/DOPE **1** cationic liposomes formulated at a low pDNA:lipid 1:4 w/w ratio compared well with lipofectamine 2000 both in terms of transfection efficiency (with and without the presence of serum) and cellular cytotoxicity (Figs. 2 and 3). The CAPG **7**/MM27 **3** cationic liposome combination may have some utility at a higher pDNA:lipid 1:12 w/w ratio in the absence of serum and at the lower pDNA:lipid ratios in the presence of serum (Fig. 2).

The utility and mechanism of CDAN **5**/DOPE **1** cationic liposome mediated delivery has been noted and described in detail previously [32,39]. In comparison, the advent of CAPG **7**/MM27 **3** cationic liposomes indicates the explicit sense of pairing a cationic lipid that has a hydrophilic side chain possessing a high pK_a (basic), with a neutral co-lipid with a hydrophilic side chain, that is mildly acidic in character, in order to promote transfection efficiency. In this pairing we would suggest that the cationic lipid provides for nucleic acid binding and cell binding/entry; the co-lipid for endosomolysis and intracellular trafficking (as described in the Introduction above). By contrast, the clear efficacy of the DODAG **8**/DOPE **1** cationic liposome mediated delivery appears to suggest the involvement of other additional physical properties that could contribute towards the observed efficacy. In terms of the cationic lipid and the co-lipid, the hydrophilic head group of DODAG **8** is borrowed from CDAN **5** that has been described to exhibit both basic and mildly acidic characteristics [32,39]. Hence we would suggest by extrapolation that such a head group should have the capacity for involvement in all aspects of nucleic acid delivery including nucleic acid binding itself, cell binding/entry, endosomolysis (possibly involving osmotic shock) and intracellular trafficking in association with DOPE **1**. However, these effects could be further enhanced by cationic liposome and lipoplex nanoparticle metastability. Indeed cationic liposome and corresponding lipoplex metastability with respect to aggregation and colloidal instability has already been reported to be helpful to promote CDAN **5**/DOPE **1** cationic liposome-mediated transfection *in vitro* [39]. In the case of DODAG **8**/DOPE **1** cationic liposomes, we would like to suggest that cationic liposome and lipoplex metastability may also be important for efficient transfection and could be related in this case to considerations about lipid molecular shape.

DOPE **1** is regarded classically as having an inverse-conical molecular shape that is wide at the termini of the dioleoyl fatty acid side chains and narrow at the polar head group. This molecular shape is perceived to be a critical reason that DOPE **1** shows preference for inverse hexagonal, H_{II}, macromolecular lipid phase structures and is more abundantly present into the inner cytoplasmic leaflet of plasma membranes. On the other hand, given the close correlation between the molecular shape of DOGS **10** and DODAG **8**, then DODAG **8** like DOGS **10** should possess a conical molecular shape that is narrow at the termini of the dioleyl alkyl side chains and wide at the polar head group. In which case DODAG **8** like DOGS **10** might also be expected to show a preference for normal hexagonal, H_I, macromolecular lipid phase structures such as normal lipid micelles. Accordingly, in combination with each other in cationic liposomes or in corresponding lipoplex nanoparticles, DODAG **8** and DOPE **1** would thus appear to be of opposite steric character and lamellar phase structures thus formed by both lipids in combination would exhibit inevitable instability with respect to phase change, a characteristic that could be regarded as favorable for efficient transfection of cells *in vitro*. We have some circumstantial evidence in support of this proposed phase metastability in that fluorescence microscope images of cellular transfection using DODAG **8**/DOPE **1** cationic liposomes

Table 2
Experimental toxicity and inflammation markers. Details concerning the measurement of these parameters are described elsewhere [42]. OAS-1 and IFN-β are early interferon (IFN) response genes. Units of expression are IFN response gene/GAPDH mRNA ratio (absolute). Pro-inflammatory poly-IC treatment results in values of approximately 0.2 and 0.25 for OAS-1 and IFN-β respectively. All abbreviations are explained in the main text as appropriate.

Toxicity/ inflammationmarker	Saline	Naked siRNA 1794	Naked siRNA 1407	DODAG 1794	DODAG 1407
ALT (IU/l)	300 ± 200	600 ± 300	900 ± 600	200 ± 50	500 ± 200
LDH (IU/ml)	950 ± 200	1000 ± 100	1250 ± 450	750 ± 50	1300 ± 200
Urea (mmol/l)	9.5 ± 0.5	9.5 ± 0.5	10 ± 1.0	9.0 ± 0.5	9.0 ± 0.5
Creatinine (μmol/l)	8.0 ± 0.5	9.0 ± 0.5	8.5 ± 0.5	7.0 ± 0.5	8.0 ± 0.5
Na+ (mmol/l)	150 ± 5	150 ± 5	150 ± 5	150 ± 5	150 ± 5
Cl− (mmol/l)	115 ± 5	110 ± 5	115 ± 5	115 ± 5	115 ± 5
K+ (mmol/l)	120 ± 5	130 ± 5	120 ± 5	100 ± 5	120 ± 5
OAS-1	<0.01	<0.02	<0.02	<0.02	<0.02
IFN-β	<0.01	<0.02	<0.02	<0.02	<0.02

show that lipoplex nanoparticles have completely disintegrated in the cell post entry and are spread throughout the cellular cytoplasm without a pronounced appearance of endosome entrapment (Fig. 4). Moreover, this ease of entry is reflected in very early GFP expression at 4 h post transfection (instead of a more typical time period of 8–12 h) (Fig. 4), an observation not made readily with any other cationic liposome system studied here.

The efficacy of DODAG **8**/DOPE **1** cationic liposome mediated plasmid DNA delivery *in vitro* appears usefully high and accompanied by only modest cytotoxicity, but the cationic liposome system was expected to have only limited utility *in vivo*, especially for systemic applications, in view of the general metastability of lipoplex nanoparticles [28,29]. However, the implication that DODAG **8** has a conical lipid structure suggested to us that DODAG **8** alone might be able to form lipoplex nanoparticles with siRNA that in turn might be stable enough for limited *in vivo* applications. This proposition was tested here and appears to be the case. Simple siRNA-DODAG **8** nanoparticles were formulated and appeared to be stable when delivered IV in a high concentration of trehalose, to mediate functional delivery of validated anti-HBV siRNAs (chemically unmodified), to livers of HBV-transgenic mice [58] in sufficient quantity to modulate both viral particle equivalents and liver HBV mRNA levels downward by 2–3 fold (Fig. 5). Importantly this siRNA-mediated silencing of HBV replication with nanoparticle delivered anti-HBV sequences (siRNA-1407 or siRNA-1794) was at least as effective as the inhibition of viral proliferation observed with the licensed HBV drug lamivudine. Our observed suppression of HBV parameters (Fig. 5) are significant. Also our data compare well with results reported following the use of higher doses of chemically modified siRNA or expressed RNAi effectors [59,60].

Previously we developed the siFECTamine^{TM} cationic liposome system [40] to deliver siRNA to cells (siFection) and showed that this approach may be used for RNAi-based gene silencing *in vitro*. This cationic liposome system was formulated from CDAN **5** and DOPE **1** in a 45:55 molar ratio (Fig. 1). Highly efficient siRNA-mediated gene knockdown was routinely achievable without causing toxicity in cultured cells [40]. A number of siRNA loaded hepatotrophic formulations have since been generated for *in vivo* use that comprise molecular conjugates involving cholesterol or α-tocopherol conjugation, complexation with apolipoprotein A1 or association with vitamin A-coupled liposomes [61–64]. 'Stabilized nucleic acid-lipid particles' (SNALPs) have also been used to deliver functional siRNA *in vivo* [65,66] and we have recently described the use of multi-component, pH triggered, PEGylated siRNA nanoparticles (also known as siRNA–**ABC** nanoparticles) to treat HBV infection in a severe, chronic animal model of human HBV infection (*i.e.,* the HBV transgenic mouse model) [42]. In this study here, we have extended the range of synthetic, non-viral hepatotrophic vectors to include very simple siRNA-DODAG **8** nanoparticles. To the best of our knowledge, siRNA-DODAG **8** nanoparticles provide a first demonstration of a single cationic lipid-based vector system that can be used for siRNA delivery *in vivo*. The methodology that we describe here would certainly enable large-scale preparation, an essential prerequisite for pharmaceutical applications. Further refinements of nucleic acid-DODAG **8** nanoparticles will be required and nanoparticles will also need to be comprehensively characterized under a variety of different conditions to understand and appreciate fully the mechanisms of delivery. We are also investigating dose optimization, detailed evaluation of non-specific effects, together with an assessment of stability, pharmacokinetic analysis and metabolite profiling. Furthermore, we expect to carry out studies to improve silencing efficiency of the formulations by introducing stabilizing modifications to the siRNAs. Although challenges remain, we believe that our data together with those reported by ourselves and others [59,60,65–68], are encouraging and support the proposition that siRNA-DODAG **8** lipoplex nanoparticles may be of use for the treatment of HBV infection and other hepatic diseases where silencing of pathology-causing symptoms are required for disease treatment.

5. Conclusion

We have synthesized a number of novel cationic lipids of which one, DODAG **8** in combination with neutral co-lipid DOPE **1**, forms DODAG **8**/DOPE **1** cationic liposomes that appear to have the capacity to act as a broad band agent for transfection *in vitro* under various conditions. We suggest that cationic liposome and corresponding lipoplex nanoparticle metastability due to lipid molecular shape differences may be a key factor to ensure highly efficient transfection *in vitro*. In contrast, we observe that DODAG **8** mixed with siRNA forms siRNA-DODAG **8** lipoplex nanoparticles that mediate multiple, efficient delivery of anti-HBV siRNAs *in vivo* to the livers of HBV transgenic mice leading to a partial treatment of HBV infection over a period of 28 days.

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