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A series of cationic sterol lipids with gene transfer and bactericidal activity

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Abstract

A family of cationic lipids was synthesized via direct amide coupling of spermine to the C-24 position of cholic acid analogs. Four monosubstituted spermines and a *bis*-substituted spermine were evaluated as plasmid transfection reagents, as bacteriostatic agents, and as bactericidal agents. The incorporation of a double bond in the sterol moiety enhanced transfection efficiency significantly and produced two compounds with little cytotoxicity and transfection potency comparable to Lipofectamine2000. Inclusion of the double bond had no effect on the general trend of increasing bactericidal activity with increasing sterol hydrophobicity. Co-formulation of the most hydrophilic of the compounds with its *bis*-substituted analogue led to enhancement in transfection activity. The *bis*-substituted compound, when tested alone, emerged as the most bacteriostatic compound in the family with minimum inhibitory concentrations (MIC) of 4 μM against *B. subtilis* and 16 μM against *E. coli* and therapeutic indexes (minimum hemolytic concentration/minimum inhibitory concentration) of 61 and 15, respectively. Cationic lipids can be optimized for both gene delivery and antibacterial applications by similar modifications.

1. INTRODUCTION

Cationic lipids were first reported over 20 years ago to facilitate liposomal gene delivery[1] and many are commercially available.[2] Lipofection efficiency remains low compared to viral-based gene delivery. Less than 8% of clinical gene therapy trials have used cationic lipid vectors.[3] In reviewing common cationic lipid classes, such as *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride (DOTMA) [1,4] or 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol)[5] (Figure 1), it is notable that similar structures identified as cationic lipid amphiphiles, synthetic ionophores, and cationic steroid antibiotics, are cited throughout anti-microbial literature. Squalamine, isolated from the stomach tissue of the dogfish shark *Squalus acanthias*, was the first of these steroid derivative amphiphiles proven to have significant antibiotic activity against a broad-spectrum of organisms.[6] A number of synthetic analogues mimicking the functionality of squalamine in simplified forms also have bactericidal activity.[7,8,9,10]

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The potential for dual functionality[8,11] has led us to design a family of cationic lipids with varying structures to investigate their membrane activity in two different contexts of gene transfer and antimicrobial activity. We chose the naturally occurring spermine, spermidine, and putrescine, known for their abilities to aid in DNA condensation as well as cell proliferation and differentiation.[12,13,14] Further, free spermine and putrescine have been shown to associate in the minor groove of B-DNA and the major groove of A-DNA.[15]

Inspired by the success of DC-Chol which pioneered the use of cholesterol in place of aliphatic chains,[5] we elected to use cholesterol-derivatives as our hydrophobic tails. The added rigidity of the structure provided by cholesterol favors a large cross-sectional area on the hydrophobic tail.

Consideration of the linkage strategy led us to select a bile acid subset of cholesterol derivatives that could be linked to polyamines by amide coupling. These bile acid derivatives characteristically display a carboxylic acid moiety on their alkyl chain that reacts with amines through a carbodiimide-assisted mechanism to form amides.[28] Conjugating the sterols at this position as opposed to on the ring structure, as seen in DC-Chol and many others in the literature, further increases the cross-sectional area of the hydrophobic tail. The susceptibility of amides to hydrolysis in acidic environments facilitates the eventual degradation of the compounds in physiological environments such as the late endosome.

In this work, we present a synthesis strategy and promising results for both *in-vitro* transfection and antibacterial applications for a unique family of cationic lipids. Structural variations across the family provide an opportunity to consider the relationships between these features and their functional impacts.

Our approach began with a focus on design for transfection activity. Generally, correlations between lipid structure, hydration and *in-vivo* transfection activity have shown that maximizing the imbalance between the large cross sectional area occupied by the hydrophobic tail and the small cross sectional area occupied by the cationic head promotes transfection activity, potentially due to improvements in membrane fusion.[17]

2. RESULTS

2.1 Chemistry

In an effort to span a range of hydrophobicities on the sterol tail group, compounds **1-4** were synthesized from a variety of bile acid derivatives (Figure 2). Compounds **2** and **3** incorporate double bonds in the base sterol structure. The octanol-water partition coefficients (logD) calculated for each of the compounds provide a quantitative comparison of the hydrophobicities across the family in Table 1.

Molecular weights and ^{1}H NMR were determined for each compound:

2.2 Lipofection: Efficiency and Toxicity

All transfection results were normalized against the positive control of Lipofectamine2000™ at its optimal charge ratio (CR) to DNA within the sample set (typically CR = 1). Cell viability results were normalized against the negative control of naked plasmid DNA. Each condition was tested as n = 8 with the average results and error bars of one standard deviation represented in Figure 3.

Insets in each of the subplots show fluorescence images of the optimal ratios for each compound formulated with pEGP-N3 reporter plasmid transfecting sub-confluent BAEC's in 24-well plates.

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At all charge ratios, we included liposome formulations of the compounds that did not include the neutral helper lipid DOPE. In all cases, transfection was not achieved for these formulations.

The most successful transfections in Figure 3 were achieved with compounds **2** and **3**. The optimal mole ratios with DOPE were vastly different, however, at 3.5 for compound **2** and 0.5 for compound **3**. Additionally, compound **2** performed best at a charge ratio of 12, while compound **3** was better at CR = 8. These two compounds both incorporate a single double bond in the base sterol structure as shown in Figure 2.

Aside from the compound **2** without DOPE formulation, none of the lipoplexes appeared to introduce significant toxicity to the cells as compared to the introduction of naked plasmid even up to a charge ratio of 12 with DNA (Figure 4).

2.3 Liposome Size

DLS measurements were taken for aliquots of the liposomal formulations as they were being combined with the plasmid DNA for transfection and are shown in Figure 5. The ranges shown are representative of the polydispersities of each sample taken. To verify that the method of preparation of our liposomes provided a reasonably uniform dispersion, light scattering measurements were also conducted with several commercially available transfection reagents including Lipofectamine™ 2000, Lipofectamine™ (Invitrogen™), and Transfectam® (Promega). These formulations, when prepared exactly as the manufacture recommended, gave similarly large polydispersity ranges (data not shown).

Compounds **1** and **4** formed generally smaller liposomes than **2** and **3**, with average radii under 150 nm. Interestingly, though, the most successful formulation with **3** was under 100nm and with **2** was over 300 nm. Further, similarly sized liposomes did not produce the same results even when made with different formulations of the same compound, as shown by mole ratios 3.5 and 1.5 of compound **2** (Figure 5B) and mole ratios 1.5 and 2.5 of compound **3** (Figure 5C).

2.4 Cocktail Formulations

Bis-substitution of spermine on both of the two primary amine positions is an expected byproduct detected in the crude reaction mixtures (Figure 2). Intentional synthesis of this *bis*-substituted byproduct (**5**) was achieved by including just 0.5 equivalents of spermine in the reaction mixture and working up as usual. Mass-selective semi-preparative chromatography was employed for final purification of **5** as in the case of compounds **1-4**.

Mole percent compositions of 0 to 30 with respect to compound **4**, as well as 100% compound **5**, were considered in this doping study of transfection activity and toxicity. Liposome and lipoplex formulations were carried out in the same manner as for compounds **1-4**.

As figure 6 shows, the progressive addition of compound **5** and the use of compound **5** alone produced no appreciable drop in cell viability, but had a significant impact on transfection. Inclusion of 20 mol% **5** at a mole ratio of 2.0 with respect to DOPE and a charge ratio of 12, resulted in a three-fold improvement in the efficiency of the transfection. DLS measurements with polydispersity ranges are included as well in figure 6E and 6F.

2.5 Antibacterial and Hemolytic Activity

Compounds **1-4** were evaluated as bacteriostatic agents against representative strains of gram-positive (*B. subtilis* ATC6051) and gram-negative (*E. coli* MG1655) bacteria and compared to LL-37 and polymyxin B in Table 2. Minimum inhibitory concentrations (MIC) were determined for each strain. Compound **5** was the most effective inhibitor for both strains.

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Coupling the MIC data for each compound with minimum hemolytic concentrations (MHC) allowed for the calculation of therapeutic indexes (TI) as the ratio of hemolytic to inhibitory activity as shown in Table 2. The most favorable indices were achieved with compounds **5** and **2** for both the gram-positive and gram-negative strains.

Compounds **1** and **3** were the most bactericidal against both strains with activity in bacteria killing assays (Figure 7A–B) at concentrations as low as 20 μM for gram-positive and 50 μM for gram-negative bacteria.

The hemolytic activity of compounds **1**-**4** against freshly isolated red blood cells from whole blood donor samples was also evaluated in a dose response manner. Figure 7C shows that compound **5** was the most lytic with nearly complete hemolysis at 2 mM, while the rest of the compounds displayed less than 30% lysis at 1–2 mM.

3. DISCUSSION

Cationic lipids have received attention as both gene delivery vehicles and as antibacterials. In this work, we set out to synthesize a family of cationic lipids bearing structural features potentially beneficial for both applications and evaluate the effects of varying these features on both gene delivery and antibacterial function. The synthesis of our compound family employed a facile amide coupling that produced a clean product with few significant impurities. A dicyclohexylurea was generated as a side product along with *bis*-substituted spermine. Both impurities could be removed by semi-preparative reverse phase chromatography.

Formulation of the compounds as liposomes and subsequently lipoplexes revealed the necessity to incorporate a neutral helper lipid to achieve transfection (Figure 3). As has been proposed by other groups in the literature, this is likely due to the apparent fusogenic properties of DOPE that enhance endosomal escape.[18]

The most active transfection reagents (Figure 3b–3c), compounds **2** and **3**, included a double bond in their hydrophobic tail sterol structure (Figure 2). We included this feature both to more closely resemble the structure of cholesterol (Figure 1) found abundantly in the bilayers of cell membranes, and to potentially mimick the trend of increasing transfection efficiency with increasing degrees of unsaturation found in long aliphatic chain cationic lipids.[19]

The optimal liposomal formulation mole ratios for each of these compounds with the neutral helper lipid DOPE were very different at 3.5 for compound **2** and 0.5 for compound **3** (figure 3b-3c). DLS measurements confirmed the formation of liposomes in all cases (Figure 5), but showed no correlation between the size of the liposomes as formulated in sterile water and ultimate transfection efficiency.

None of the compound formulations with DOPE exhibited any appreciable toxic effect on the cells (Figure 4). Only compound **2**, when formulated without DOPE, appeared significantly toxic to the BAEC cells.

Investigation of the impact of impurities on gene delivery potential showed that incorporating the *bis*-substituted spermine (compound **5**) version of compound **4** in the liposomal formulations increased the transfection efficiency three-fold without increasing the toxicity to eukaryotic cells (Figure 6).

This idea of multiple hydrophobic tails connected to a cationic head group helping facilitate transfection is further supported by the study of “molecular umbrellas” for DNA binding. Polyamine scaffolds are decorated at multiple positions with cholic acid and have been found to have increasing affinity for DNA with the addition of more cholic acid groups.[20]

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In considering the antibacterial potential for these compounds, we investigated both their ability to prevent colony growth (Table 2) and to kill bacteria (Figure 7). Compound **1**, the most hydrophobic, was the most effective bactericidal agent but was much less effective in the MIC assay for bacteriostatic potential. Likewise, compounds **3** and **4** appeared more effective in killing both strains of bacteria than in inhibiting their growth. This is likely due to the inclusion of 50% v/v Mueller-Hinton broth in the MIC experiments, designed to make the conditions more physiologically relevant. This effect is not obvious in the case of compounds **2**, since its activity in the killing assays within this concentration range was quite low.

Compounds **1**, **2** and **4** have been synthesized (although by a different method) and reported by Regen and co-workers[7]. In their work, minimum inhibitory concentrations were reported for a range of gram-positive, gram-negative and fungal organisms. For compound **1**, the gram-positive MIC data reported here (32 μg/ml for *B. subtilis ATC6051*) is greater than values reported by Regen ranging from 0.39 μg/ml to 3.13 μg/ml for their gram-positive organisms. Compounds **2** and **4** also have greater gram-positive MIC values here (32 μg/ml and greater than 256 μg/ml, respectively) than those ranges reported by Regen (1.56 μg/ml to 6.25 μg/ml and 3.13 μg/ml to 50 μg/ml, respectively).

The gram-negative MIC data for compound **1** (128 μg/ml for *E. coli MG1655*) is consistent with Regen's reported range of 0.78μg/ml to greater than 100μg/ml MICs for gram-negative organisms. The gram-negative MIC values for compounds **2** (64μg/ml) and **4** (greater than 256 μg/ml) were also consistent with Regen's reported values ranging from 1.56 μg/ml to greater than 100 μg/ml for compound **2** and from 25 μg/ml to greater than 100 μg/ml for compound **4**.

The trend of increasing activity with increasing hydrophobicity among these compounds is consistent between the data shown here and that reported by Regen.

Minimum hemolytic concentrations reported by Regen are lower than those reported here, at 50μg/ml, 100μg/ml, and greater than 100μg/ml for compounds **1**, **2** and **4**, perhaps owing to variances in the experimental conditions (Regen employed sheep erythrocytes as opposed to isolates from human blood samples). Compound **2** was, in this work, the least hemolytic, requiring milli-molar concentrations to elicit red blood cell lysis.

Compound **5**, which incorporates two hydrophobic tail structures on one cationic head, was the most active in inhibiting both the gram-positive and gram-negative bacteria with MIC's of 4μM and 16μM, respectively. This is consistent with the work of several other groups in developing the previously mentioned "umbrellas" of cholic acid derivatives tethered to polyamines for use as antimicrobials.[9,21] On a molar basis, these results were nearly comparable to those achieved with polymyxin B. The hemolytic activity, however, was the greatest in the set.

4. CONCLUSIONS

We employed a single-step amide coupling to synthesize a family of cationic lipids with varying functionality that allowed us to study performance in both gene delivery and antibacterial applications. We found that inclusion of a single double bond in the hydrophobic tail sterol structure significantly enhanced transfection efficiency without exhibiting significant cytotoxicity over naked plasmid delivery.

A positive effect when including the double bond was not observed in our antibacterial studies, however, where the hydrophobicity of the compounds seemed to control the ability to kill bacteria and inhibit colony growth. The most hydrophobic compounds were the most active against both gram-positive and gram-negative bacteria. Co-formulation of our compound **4**

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with is head-tail-head *bis*-substituted analogue (compound **5**) provided significant enhancement to both transfection activity and antibacterial activity. Further, this *bis*-substituted emerged as the most active antibacterial compound tested.

The investigation of this compound family has provided some insights in to the design of cationic lipids for gene delivery and as antibacterial agents. The compounds presented here were reached through a direct synthesis from readily available starting materials. Transfection results particularly for compounds **2** and **3** showed strong performance and significantly decreased toxicity as compared to Lipofectamine™ 2000. The co-formulation of compounds **4** and **5** was interesting with respect to transfection, and could merit further consideration for future development.

5. EXPERIMENTAL

5.1 Chemistry

The carboxylic acid starting materials for the amide couplings to make compound **1-3** were purchased from Steraloids Inc. (Newport, RI). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO) and used as received. Solvents were of HLPC grade.

Compounds **1-4** were synthesized in a similar manner, starting with the carboxylic acid sterol derivatives. The carboxylic acid was charged to a clean, dry 3-neck round bottom flask equipped with magnetic stirring. Dicyclohexylcarbodiimide was added in 1.1 equivalents along with enough anhydrous tetrahydrofuran to afford a 2.7mM solution. 0.2 equivalents of 1-hydroxybenzotriazole hydrate and 1.1 equivalents of spermine were added to the reaction mixture and stirred overnight at room temperature. Solvents were removed by rotary evaporation at 40°C and 0mbar to produce an oily residue. The residue was dissolved up in a 1:1 v/v mixture of dimethylsulfoxide and acetonitrile (0.6mM with respect to starting material). The resulting clear solution was filtered and purified on a semi-preparative C18 column (Shimadzu Premier 10mm × 150mm) equipped with LC/MS (Shimadzu LC2010) positive mode electrospray ionization and an acetonitrile and water solvent system buffered with 0.05% formic acid. Mass-selected fractions were concentrated to a residue as the formic acid salt and then dissolved in methanol for storage at −20°C. Final product yields of 30–40% were typical.

Compound **5** was prepared in the same manner except with 0.5 equivalents of spermine.

Reactions were also monitored by LC/MS with the Shimadzu C18 Premier 4.6mm × 0mm column. ^{1}H NMR was conducted on a Bruker DMX300 spectrometer.

5.1.1 5β-cholanyl-spermide-3α-ol 1—MS *m/z* 561 $[M+H]^+$. ^{1}H NMR (CD_3OD, 300MHz) δ(ppm) = 4.96 (t, 1 H, NH), 4.76 (m, 4 H, 2x NH 1x NH_2), 3.37 (m, 1 H, CH), 3.37 (d, J = 0.66 Hz, 2 H, CH_2-N), 3.24 (d, J = 1.45 Hz, 8 H, 4x CH_2-N), 3.23 (t, 2 H, CH_2-N), 3.17 (s, 2 H, CH_2-CO), 3.08 (s, 4 H, 2x CH_2), 3.07 (s, 2 H, CH_2), 3.06 (d, J = 2.01 Hz, 1 H, OH), 2.66 (s, 2 H, CH_2), 2.65 (s, 2 H, CH_2), 1.34 (t, 2 H, CH_2), 1.33 (d, J = 0.57 Hz, 2 H, CH_2), 1.30 (m, 14 H, 7x CH_2), 0.94 (m, 6 H, 6x CH), 0.20 (s, 9 H, 3x CH_3).

5.1.2 5-cholenyl-spermide-3β-ol 2—MS *m/z* 560 $[M+H]^+$. ^{1}H NMR (CD_3OD, 300MHz) δ(ppm) = 4.96 (t, 1 H, NH-CO), 4.90 (t, 1 H, CH), 4.75 (m, 4 H, 2x NH, NH_2), 3.37 (m, 1 H, CH), 3.37 (d, J = 1.07 Hz, 2 H, CH_2-N), 3.24 (d, J = 1.21 Hz, 8 H, 4x CH_2-N), 3.23 (t, 2 H, CH2-N), 3.17 (s, 2 H, CH2-CO), 3.07 (s, 4 H, 2x CH_2), 3.06 (s, 2 H, CH_2), 3.05 (d, J = 1.53 Hz, 1 H, OH), 2.65 (s, 2 H, CH_2), 2.64 (s, 2 H, CH_2), 2.08 (d, J = 0.89 Hz, 2 H, CH_2-CO), 1.54 (t, 2 H, CH_2), 1.43 (d, J = 0.57 Hz, 2 H, CH_2), 1.28 (m, 10 H, 5x CH_2), 1.02 (m, 2 H, 2x CH), 0.72 (m, 3 H, 3x CH), 0.07 (d, J = 0.3 Hz, 3 H, CH_3), 0.06 (s, 6 H, 2x CH_3).

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5.1.3 8-cholenyl-spermide-3α,12α-diol 3—MS *m/z* 576 $[M+H]^+$. ^{1}H NMR (DMSO-d6, 300MHz) δ(ppm) = 3.51 (s, 2 H, 2x OH), 3.32 (d, *J* = 39.89 Hz, 12 H, 6x CH_2-N), 3.11 (m, 5 H, 3x NH, NH_2), 1.27–2.30 (m, 20 H, 10x CH_2), 1.24 (s, 10 H, 5x CH_2), 1.15 (s, 6 H, 2x CH_3), 0.82–1.09 (m, 5 H, 5x CH), 0.76 (d, *J* = 4.62, 3 H, CH_3).

5.1.4 5β-cholanyl-spermide-3α,7α,12α-triol 4—MS *m/z* 594 $[M+H]^+$. ^{1}H NMR (DMSO-d6, 300mHz) δ(ppm) = 3.51 (s, 3 H, 3x OH), 3.34 (d, *J* = 39.81, 12 H, 6x CH2-N), 2.71–2.75 (m, 5 H, 3x NH, NH_2), 1.56–1.90 (m, 20 H, 10x CH_3), 1.24 (s, 10 H, 5x CH_2), 1.15 (s, 6 H, 2x CH_3), 0.93 (d, *J* = 6.57, 3 H, CH_3), 0.74–0.91 (m, 8 H, 8x CH).

5.1.5 *bis*-cholanyl-spermide-triol 5—MS *m/z* 984 $[M+H]^+$. ^{1}H NMR (DMSO-d6, 300mHz) δ(ppm) = 3.51 (s, 6 H, 6x OH), 3.34 (d, *J* = 39.81, 12 H, 6x CH2-N), 2.71–2.75 (m, 4 H, 4x NH), 1.56–1.90 (m, 40 H, 20x CH_3), 1.24 (s, 10H, 5x CH_2), 1.15 (s, 12 H, 4x CH_3), 0.93 (d, *J* = 6.57, 6 H, 2x CH_3), 0.74–0.91 (m, 16 H, 16x CH).

5.2 Liposome Formulation

Formulations of liposomes and lipoplexes were designed to span ranges of both molar ratio with respect to the neutral helper lipid DOPE (Avanti® Polar Lipids, Inc. Alabaster, AL) and charge ratio with respect to the plasmid DNA. Liposome formulations were prepared carefully so as to promote small unilamellar vesicle formation. Solutions of the lipids dissolved in organic solvent are combined and dried under vacuum overnight to ensure full solvent removal. The lipid films were then hydrated with sterile nuclease-free water and resuspended by vortexing for 5 minutes and sonication for one to two hours. The liposomes were then held overnight at 4°C and the vortexing and sonication were repeated immediately before combination with DNA. Dynamic light scattering analysis (DynaPro™ Protein Solutions) of each formulation after sonication provided a method of determining the size distribution of the liposomes. A minimum of 20 measurements were taken for each sample and average radii were determined on a mass % basis. Polydispersities were also noted.

5.3 Transfection

Bovine aortic endothelial cells (BAEC) of passage 4–15 were seeded and maintained in T75 flasks prior to use in these experiments. A full growth media of 87% Dulbecco's modified eagle media (D-MEM) (Gibco®), 10% fetal bovine serum(Gibco®), 2% penicillin streptomycin(Gibco®), and 1% L-glutamine(Gibco®) was changed out every 2–3 days and the cells were passaged every 5–10 days. 1–2 days prior to transfection, cells were seeded on 96-well plates for pGL4.75 transfections and 24-well plates for pEGFP-N3 transfections.

High throughput screening techniques were used to identify optimal liposome and lipoplex formulations for each compound based on the maximization of transfection efficiency and minimization of cytotoxicity. 96-well plates were used to formulate 12 different liposomal conditions per experimental set (including controls), each prepared in replicates of eight. Lipoplexes were generated in one 384-well plate for each 96-well liposome plate such that 4 charge ratios could be screened for each set of liposomes. The luciferase reporter plasmid, pGL4.75 (Promega) was complexed with each of the test liposomes, a positive control gene delivery vector Lipofectamine™ 2000 (Invitrogen™), and left as naked DNA. This vector provides a luminescent signal when the expressed *Renilla* luciferase reacts with coelenterazine-h which is generated by the digestion of EnduRen™ Live Cell substrate (Promega) by intracellular esterase. The reporter plasmid pEGFP-N3 (Promega) was also used to form lipoplexes in the same manner. Successful delivery of this plasmid results in a fluorescent signal on expression which can be imaged directly. All of the lipoplex formulations were prepared, pipette-mixed, and allowed to incubate for 30 minutes prior to delivery. Lipoplexes were transferred to well plates of confluent bovine aortic endothelial cells (BAEC) in

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OptiMem™ (Gibco®) serum-free media. After two hours of incubation (37°C, 5% CO_2), the media in the cell well plates were replaced with full growth media and the cells were incubated overnight. 20 hours post-transfection, full growth media was aspirated from the cell well plates and EnduRen™ Live Cell substrate diluted in full growth media was added to each well. Following incubation for at least 1.5 hours, each well plate was analyzed for luminescent intensity in an Envision plate reader equipped with luminescent filter.

5.4 Toxicity Assay

To evaluate the cytotoxicity of the lipoplexes, the reagent CellTiter-Glo® Luminescent Cell Viability Assay (Promega) was added to each of the wells and incubated for 30 minutes. This assay facilitated a determination of the number of viable cells in each well based on a linear relationship with the quantity of ATP present on cell lysis (i.e. total ATP content reflects number of living cells). The reagent contains beetle luciferin and a recombinant firefly luciferase engineered to prevent ATPases and other endogenous enzymes released during cell lysis from interfering with the quantification of the ATP in the sample. A luminescent signal is produced when the luciferin is mono-oxygenated on catalysis by the luciferase in the presence of Mg^+, ATP and O_2.

5.5 Minimum Inhibitory Concentrations

Exponential phase *B. subtilis* ATC6051 and *E. coli* MG1655 were suspended in Mueller-Hinton broth (Fluka) at 10^5 to 10^6 CFU/ml. Serial dilutions of compounds **1-5**, LL-37, and polymyxin B (Sigma-Aldrich) were prepared in PBS and combined 1:1 by volume with the cells in 96 well plates. The samples were incubated for 20 hours at 37°C.

5.6 Bacterial Killing Assays

Exponential phase *B. subtilis* ATC6051 and *E. coli* MG1655 were suspended in phosphate buffered saline (PBS) (Gibco®) at 10^5–10^6 CFU/ml and incubated at 37°C for one hour. Compounds **1-5** were added to each suspension and incubated for 4 hours. Samples were extracted from each, prepared in 4 ten-fold dilutions with PBS, plated on agar gels and incubated at 37°C for overnight. Colonies were then manually counted and the averages were plotted on a log scale.

5.7 Hemolysis Assays

Donor whole blood samples were drawn in to solutions of RC-heparin sulfate/ACD and chilled to 4°C. The samples were centrifuged to a pellet at 1000g and washed three times with 5mM phosphate buffer/140mM sodium chloride/10mM HEPES (Gibco®). The resulting pellet was suspended in PBS and chilled at 4°C. Serial dilutions of compounds **1-5** were prepared in PBS and combined with the cell solution 1:1 by volume. After 1 hour of 37°C incubation, the samples were centrifuged for 10 seconds at 2000g and 100uL of the supernatant from each was combined with 900uL of PBS. The samples were analyzed at 540nm and compared against buffer alone. 1% Triton X-100 (Invitrogen™) was used as the positive control reference to calculate the percent lysis.

Minimum hemolytic concentrations were determined as concentrations resulting in hemolysis at ≥2%.

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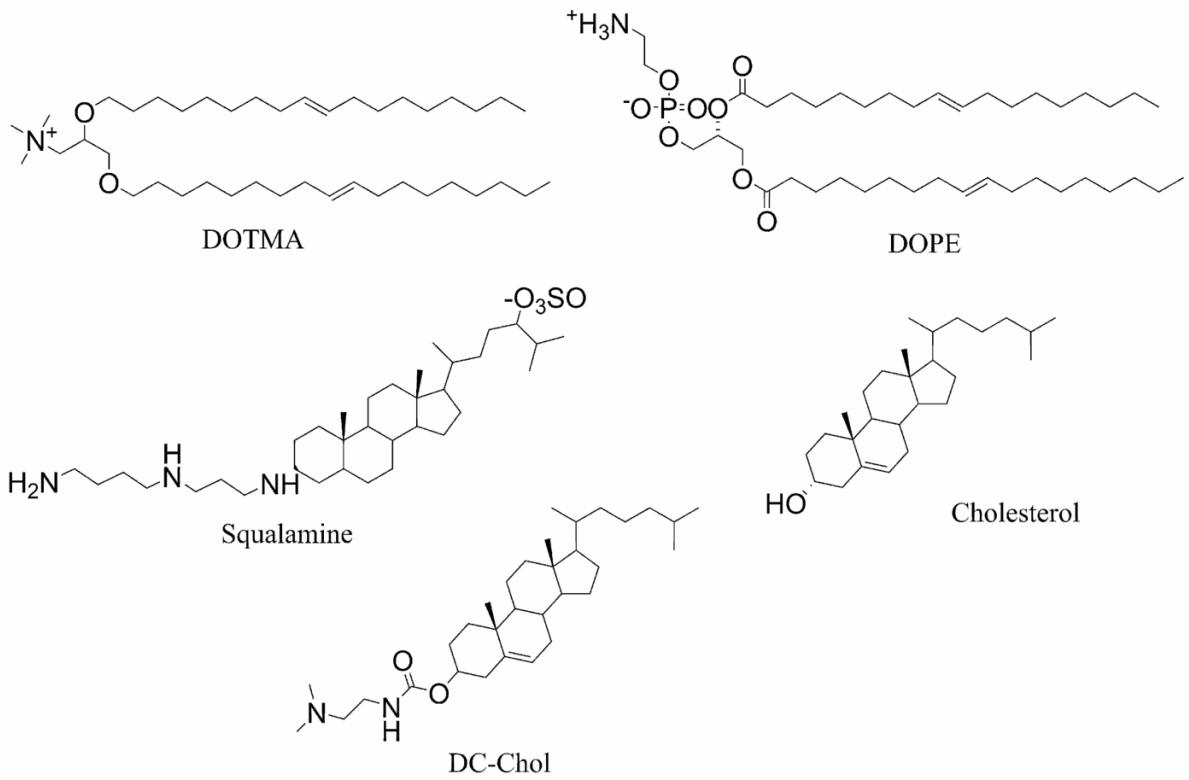

Figure 1. Referenced Lipid Structures

DOTMA was the first cationic lipid used in formulation with DOPE for lipofection by Felgner in 1987. Squalamine was isolated from the dogfish shark by Moore in 1993 and proved to be a potent broad-spectrum antibiotic. The basic lipid structure of cholesterol provides a framework for the hydrophobic tail portions of our compound family. The success of DC-Chol as a transfection reagent directed our design strategy for the hydrophobic tail.

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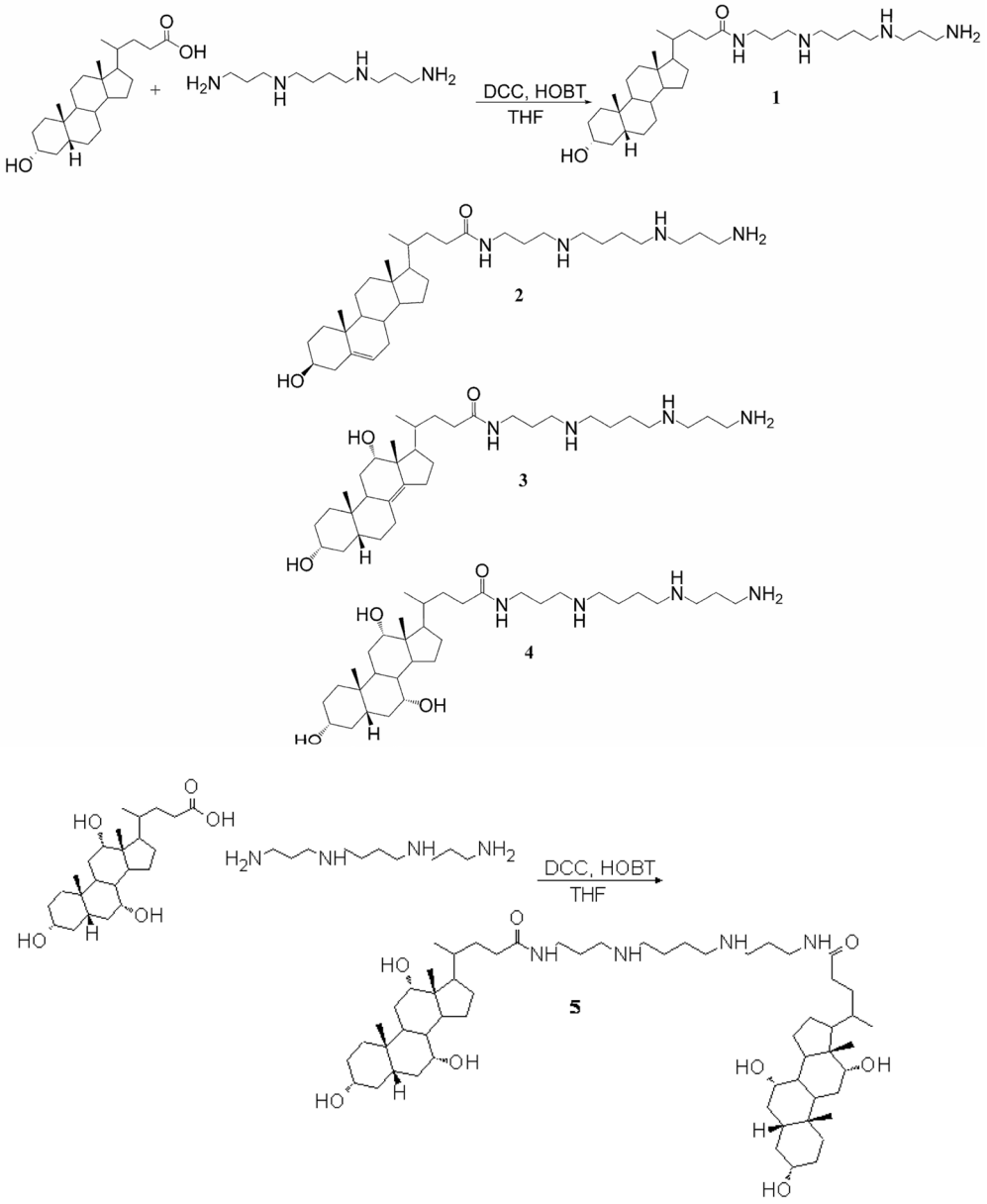

Figure 2. Cationic Lipid Family Structures

Compounds **1** through **4** were synthesized from the parent bile acid derivative by carbodiimide-assisted amide formation with spermine. Compound **5** was a product of the same chemistry with altered stoichiometry, such that one bile acid derivative was added on each of the two primary amines.

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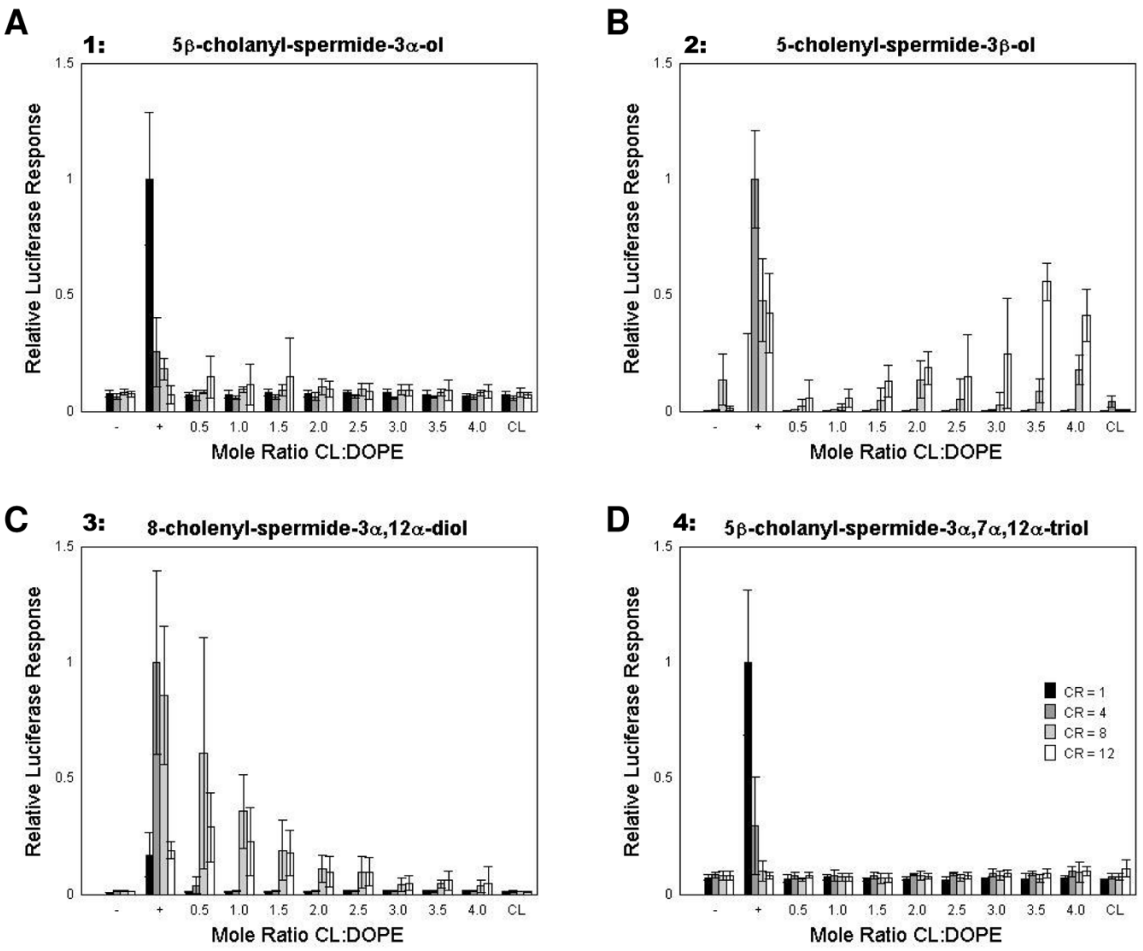

Figure 3. Transfection Activity

BAEC cells were transfected with the luciferase reporter pGL4.75 prepared as a lipoplex with each cationic lipid in varying mole ratio with DOPE and at varying charge ratio with the plasmid DNA. The mol ratios marked − and + correspond to naked plasmid and Lipofectamine2000™, respectively. CL is the cationic lipid formulated without DOPE. Results were all normalized to the positive control Lipofectamine2000™. Each condition was tested as n = 8.

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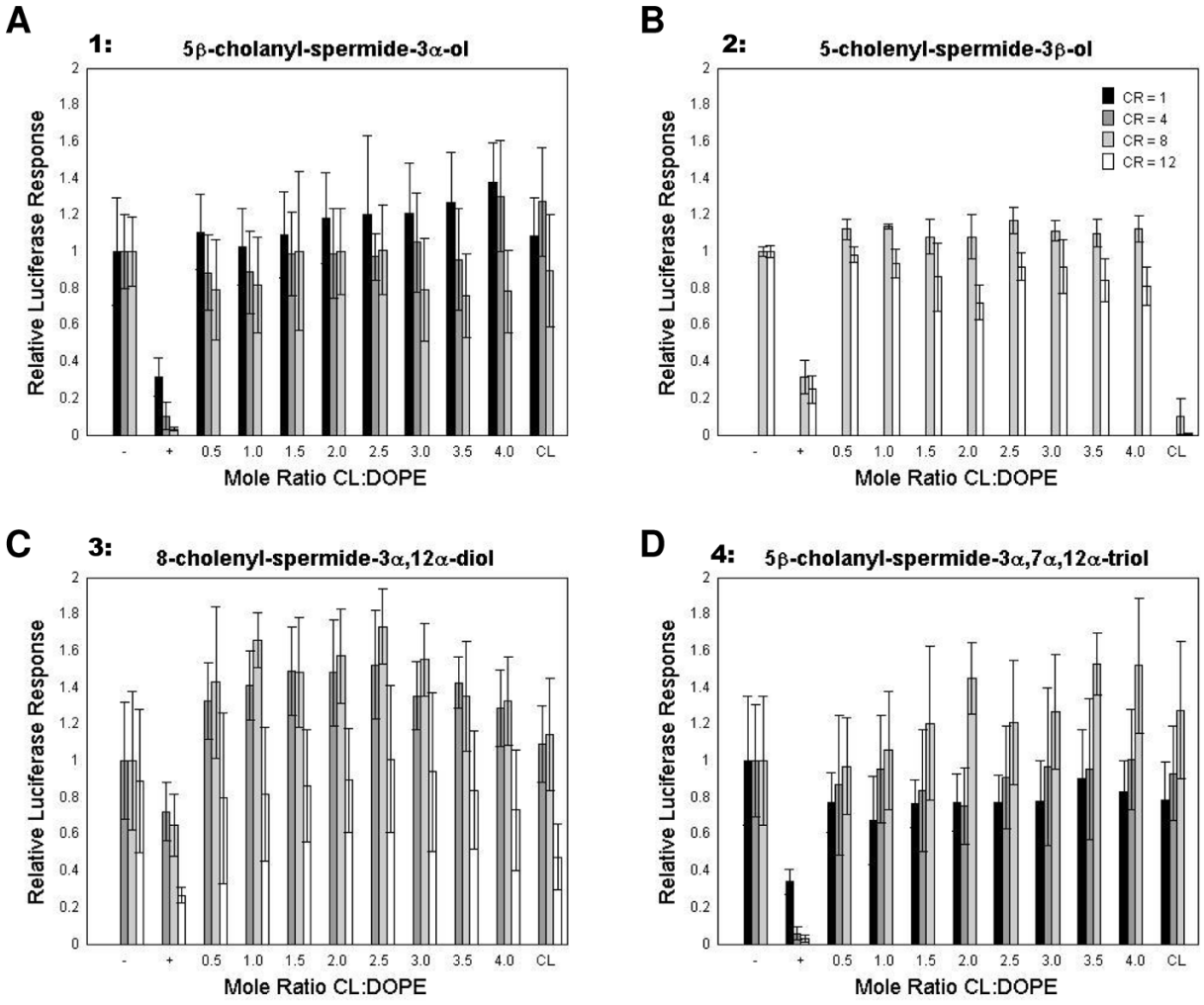

Figure 4. Cell Viability Post-Transfection

The same transfection preparations from figure 2 were dosed with CellTiterGlo™ 24 hours after transfection and a luciferase response proportional to the level of ATP was generated as a measure of viability. The mole ratios marked − and + correspond to naked plasmid and Lipofectamine2000™, respectively. CL is the cationic lipid formulated without DOPE. Results were normalized against the naked plasmid control response. Each condition was tested as n = 8.

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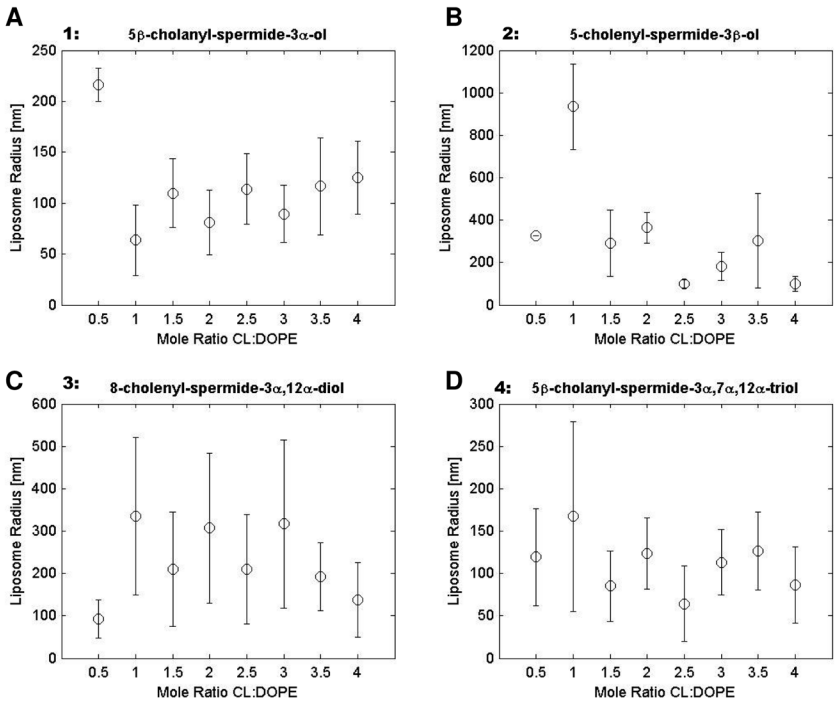

Figure 5. Liposome Size

Dynamic light scattering measurements were taken to determine the size of the formulated liposomes for each cationic lipid. Circles mark the average radius and the ranges indicate the polydispersity. A minimum of 20 measurements were taken for each sample.

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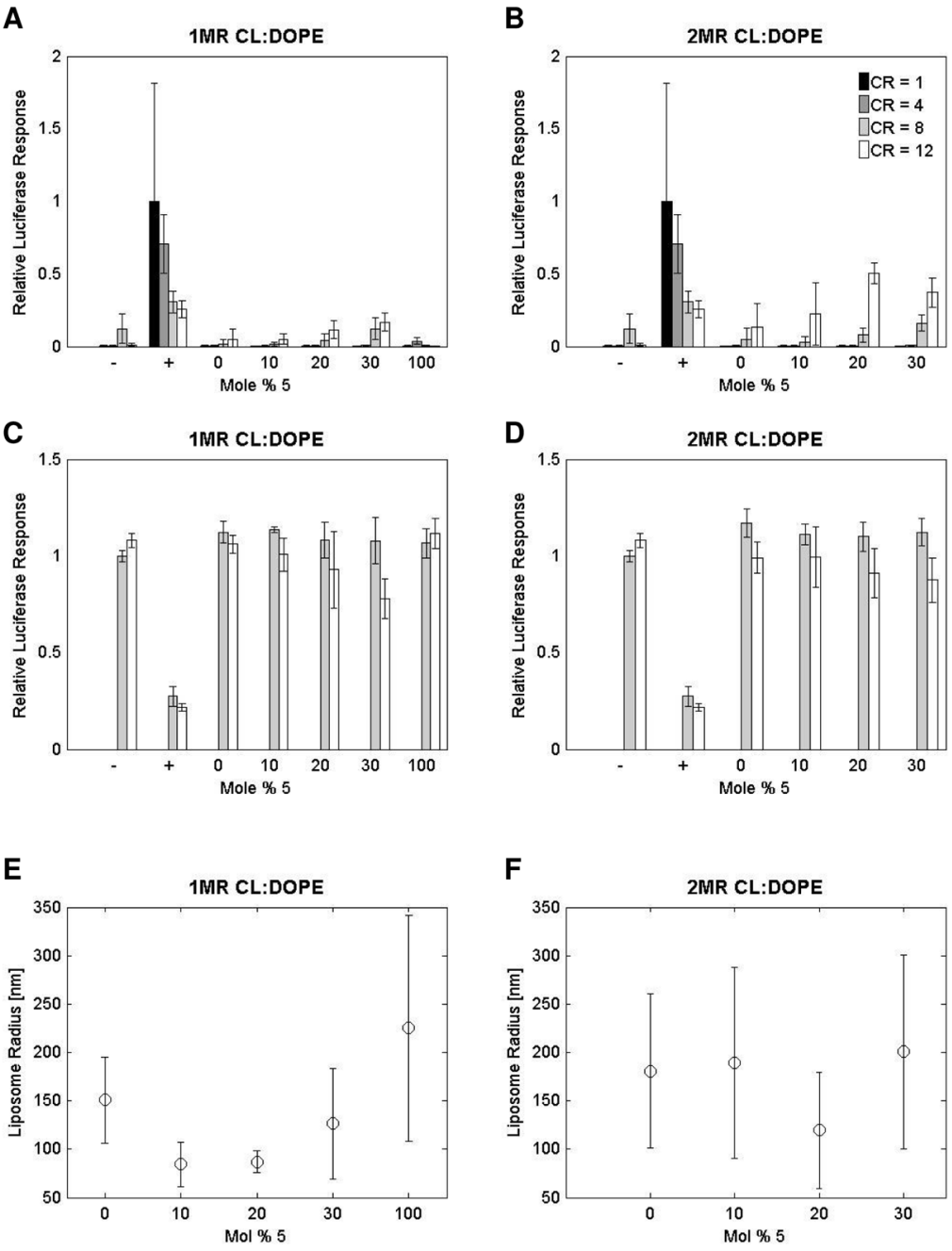

Figure 6. Compound 4 and 5 Co-formulations

Compound **5** was co-formulated with compound **4** prior to liposomal formulation with DOPE. Plots A and B show transfection activity with BAEC cells of the formulations at mole ratios of 1.0 and 2.0 of cationic lipid to DOPE and at varying charge ratio. Plots C and D show cell viability at 24 hours for the same preparations. These experiments were each performed as n = 8. Dynamic light scattering measurements of the liposomal formulations are shown in plots E and F with the circles marking the average radii and the ranges indicating the polydispersity of each sample. At least 20 measurements were taken for each.

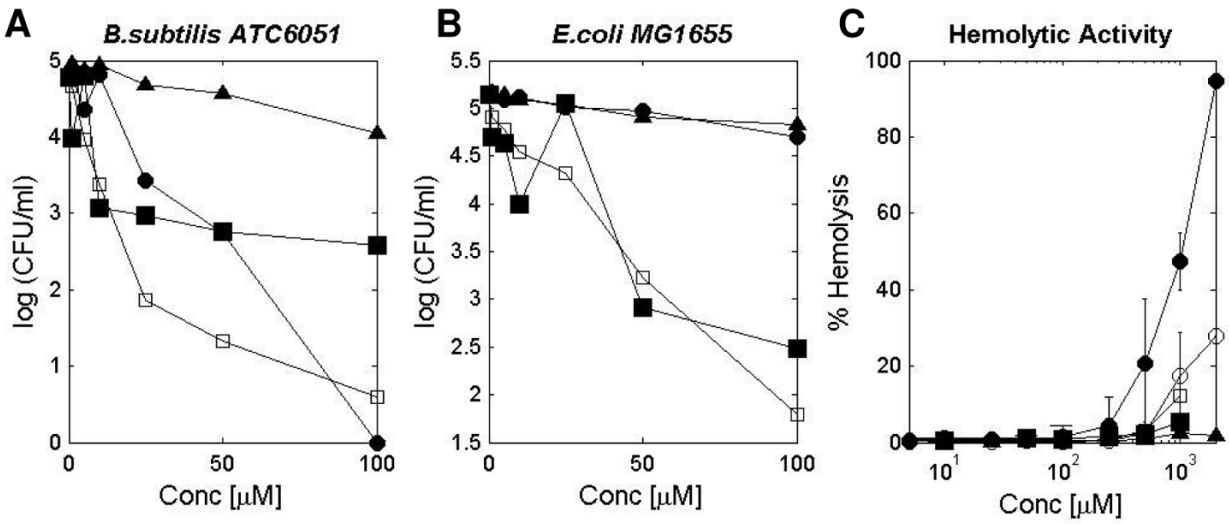

Figure 7. Antibacterial and Hemolytic Activity

Plots A and B show concentration dependent bactericidal activity against both gram-positive (*B. subtilis ATC6051*) and gram-negative (*E. coli MG1655*) strains for each compound. Plot C reflects concentration dependent lysis of red blood cells isolated from whole blood samples. All experiments were performed with a minimum of n = 3 and errors for colony counts > log 3 were less than 20%. Error bars are omitted for the clarity of the figure. ■ represents results for compound **1**, 5β-cholanyl-spermide-3α-ol; ▲ represents results for compound **2**, 5-cholenyl-spermide-3β-ol; □ represents results for compound **3**, 8-cholenyl-spermide-3α,12α-diol; ○ represents results for compound **4**, 5β-cholanyl-spermide-3α,7α,12α-triol; and ● represents results for compound **5**, *bis*-spermide-triol.

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Table 1

Cationic Lipids

Molecular weights were confirmed by electrospray mass spectrometry and octanol–water partition coefficients (logD) were calculated with ADMET Predictor™ (Simulations Plus, Inc.).

#	Compound Name	mw	logD
1	5β-cholanyl-spermide-3α-ol	560.9	2.27
2	5-cholenyl-spermide-3β-ol	558.9	2.09
3	8-cholenyl-spermide-3α, 12α-diol	574.9	1.33
4	5β-cholanyl-spermide-3α, 7α, 12α-triol	592.9	0.64
5	*bis*-cholanyl-spermide-triol	983.5	4.04

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Table 2

MIC and Therapeutic Indexes of Cationic Lipids

Minimum inhibitory concentrations (MIC) were determined for both strains and the ratios with minimum hemolytic concentrations (MHC) were calculated as the therapeutic index (TI = MHC/MIC).

	B. subtilis ATC6051			E. coli MG1655			
Compound	**MIC [μg/ml]**	**MIC [μM]**	**TI**	**MIC [μg/ml]**	**MIC [μM]**	**TI**	**MHC [μM]**
1	32	57	9	128	228	2	500
2	32	57	18	64	115	9	1000
3	16	28	18	64	111	5	500
4	>256	>432	<1	>256	>432	<1	500
5	4	4	61	16	16	15	250
LL-37	16	0.4	ND	8	0.4	ND	ND
Polymyxin B	2	1	ND	8	6	ND	ND