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Novel Cationic Lipids with Enhanced Gene Delivery and Antimicrobial Activity

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ABSTRACT

Cationic lipids facilitate plasmid delivery, and some cationic sterol-based compounds have antimicrobial activity because of their amphiphilic character. These dual functions are relevant in the context of local ongoing infection during intrapulmonary gene transfer for cystic fibrosis. The transfection activities of two cationic lipids, dexamethasone spermine (DS) and disubstituted spermine (D_2S), were tested as individual components and mixtures in bovine aortic endothelial cells and A549 cells. The results showed a 3- to 7-fold improvement in transgene expression for mixtures of DS with 20 to 40 mol% D_2S. D_2S and coformulations with DS, dioleoyl phosphatidylethanolamine, and DNA exhibited potent bactericidal activity against *Escherichia coli* MG1655, *Bacillus subtilis*, and *Pseudomonas aeruginosa* PAO1, which was maintained in bronchoalveolar lavage fluid. Complete bacterial killing was demonstrated at ~5 μM, including gene delivery formulations, with 2 orders of magnitude higher tolerance before eukaryotic membrane disruption (erythrocyte hemolysis). D_2S also exhibited lipopolysaccharide (LPS) scavenging activity resulting in significant inhibition of LPS-mediated activation of human neutrophils with 85 and 65% lower interleukin-8 released at 12 and 24 h, respectively. Mixtures of DS and D_2S can improve transfection activity over common lipofection reagents, and D_2S has strong antimicrobial action suited for the suppression of bacterial-mediated inflammation.

Introduction

Given the persistent bacterial infection associated with several diseases targeted by gene therapy such as cystic fibrosis (Boucher, 2007) and the potential consequence of infections on the efficacy of gene delivery administration, antibacterial activity exhibited by the gene delivery vehicle could offer a therapeutic benefit. Several novel steroidal dimers have shown activity against certain pathogens, and some compounds have been used to facilitate both in vitro transfection and bactericidal activity (Blagbrough et al., 2003; Salunke et al., 2004; Kichler et al., 2005). Facially amphiphilic lipid structures are believed to interact with membranes by an analogous mechanism to naturally occurring peptide antibiotics, which are active against both Gram-positive and Gram-negative bacteria. These findings motivate a new area for characterization of amphiphilic nonviral vectors with combined gene delivery and bactericidal activity.

Cationic lipids are commonly used nonviral vectors for gene delivery because of their ability to condense plasmid DNA (Hirko et al., 2003). After synthesis of *N*-[1-(2,3-dioleyloxy)propyl]-*N*,*N*,*N*-trimethylammonium chloride for lipofection (Felgner et al., 1987), optimization of the molecular structures of cationic lipids has been an active area of research, including head group (Narang et al., 2005; Obata et al., 2008), linker (Aissaoui et al., 2004; Rajesh et al., 2007; Bajaj et al., 2008), and hydrophobic domain modifications (Remy et al., 1994; Heyes et al., 2002). Important modifications have included the use of multivalent polyamines (Behr

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ABBREVIATIONS: DOPE, dioleoyl phosphatidylethanolamine; DS, dexamethasone spermine; D_2S, disubstituted spermine; CR, charge ratio; GFP, green fluorescent protein; BAEC, bovine aortic endothelial cell; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB; IL, interleukin; TNF, tumor necrosis factor; TLR, toll-like receptor; LC-MS, liquid chromatography-mass spectrometry; PBS, phosphate-buffered saline; LB, Luria broth; RBC, red blood cell.

et al., 1989), which improve DNA binding and delivery via enhanced surface charge density (Martin et al., 2005), and the use of sterol-based hydrophobic groups such as 3*B*-[*N*-(*N*′,*N*′-dimethylaminoethane)-carbamoyl] cholesterol, which limits toxicity (Gao and Huang, 1991). Helper lipids such as dioleoyl phosphatidylethanolamine (DOPE) are used to improve transgene expression via enhanced liposomal hydrophobicity and hexagonal inverted-phase transition to facilitate endosomal escape (Karanth and Murthy, 2007). Studies of mixed lipids are less common; however, recent studies involving mixtures of cationic lipid derivatives have shown promise and represent an interesting new area for optimization (Wang and MacDonald, 2004, 2007; Caracciolo et al., 2007).

In addition to the molecular structures of cationic lipids, transfection efficiency has been linked to physicochemical characteristics and morphology of structures formed after complex formation with DNA (Ma et al., 2007). Critical factors influencing transfection activity include lipoplex charge ratio (lipid/DNA), solution ionic strength, and residual net surface charge of lipoplexes (liposome-DNA complex). Although it is generally accepted that correlation of lipoplex structural changes with gene delivery activity is important, specific structure-morphology relationships are difficult to develop.

It is noteworthy that several findings have indicated that inflammatory cytokines can inhibit gene transfer in vitro with a decrease in both transcription and transgene activity of ~50% (Baatz et al., 2001; Bastonero et al., 2005). This inhibitory effect was prevented by glucocorticoid treatment indicating the blocking the NF-κB pathway, which is known to control the up-regulation of numerous inflammatory cytokines, including IL-8 and TNF-α (Kulms and Schwarz, 2006), may play a critical role between induced inflammation and efficiency of gene transfer. In addition to the pathogenesis associated with infection, bacterial membrane bound molecules, such as lipopolysaccharide (LPS), are known to activate a strong inflammatory response in eukaryotic cells via toll-like receptors (TRLs), especially TRL4 (Schnare et al., 2006); therefore, prevention of bacterial-mediated inflammation may also have a direct affect on gene delivery efficiency.

The present study assesses the activities of two sterol-based cationic lipids, dexamethasone-spermine (DS) (Gruneich et al., 2004) and disubstituted spermine (D_2S), resulting from the conjugation of dexamethasone to the polyamine spermine. DS has been shown previously to exhibit anti-inflammatory activity in an in vivo mouse intraperitoneal thioglycollate challenge model based on neutrophil infiltration and has been shown to condense and deliver plasmid DNA enabling in vitro transfection of plasmid DNA. DS has also been shown to improve airway targeting, attenuate vector-induced inflammation, and facilitate readministration in vivo when formulated with adenovirus vectors (Price et al., 2005, 2007). D_2S has not been examined previously; therefore, lipofection activity was assessed as an individual component and with DS to establish potential synergistic activity in mixtures. Antibacterial activity and LPS binding were also studied to determine additional therapeutic potential for these molecules.

Materials and Methods

Synthesis of Cationic Glucocorticoids. DS and D_2S were prepared as described previously (Gruneich et al., 2004). In brief, dexamethasone mesylate (Steraloids, Newport, RI), Traut's reagent (Sigma-Aldrich, St. Louis, MO), and spermine (Sigma-Aldrich) were reacted in a 1:1:1 M ratio in a one-step reaction in ethanol at 40°C. The reaction was monitored by analytical LC-MS until a steady state was achieved (~1 h) and was quenched with trifluoroacetic acid (Sigma-Aldrich). Ethanol was evaporated under vacuum, and the reaction products were resuspended in water before separation.

Instrumentation/Semipreparative Purification. The LC-MS system consisted of an LC-20AB solvent delivery system and SIL-20A autosampler coupled to a SPD-20A dual wavelength UV-Vis detector and LCMS 2010EV mass spectrometer (all from Shimadzu, Columbia, MD). Purification was adapted from the method described previously (Gruneich et al., 2004). The semipreparative separation system consisted of the Shimadzu instrument coupled to a Hamilton (Reno, NV) PRP-1 column (150 × 10 mm i.d., 10-μm particle size). The mobile phase flow rate was 4 ml/min with a starting ratio of 90% mobile phase A (water) and 10% mobile phase B (acetonitrile). The elution profile consisted of an isocratic step to 16% phase B for 30 min, and 30% phase B for 30 min to separate the reaction products. Fractions were collected as either trifluoroacetic acid or formate salts followed by complete solvent removal by lyophilization. Final products were dissolved in either nuclease-free water or methanol/chloroform (50:50 vol%) at 5 to 10 mg/ml.

Analytical Characterization. Analytical characterization was performed with the Shimadzu instrument coupled to a Hamilton PRP-1 column (150 × 2.1 mm internal diameter, 5 μm particle size). The mobile phase flow rate was 0.25 ml/min with a starting ratio of 90% mobile phase A (water) and 10% mobile phase B (acetonitrile). The elution profile consisted of an isocratic step to 16% phase B for 60 min, and 30% phase B for 60 min to quantify purity with mass spectrometry performed on the eluent. ^{1}H and ^{13}C NMR analyses were performed with an AVANCE III 500 MHz instrument (Bruker, Newark, DE) using a dual 5-mm cryoprobe or a Bruker DMX 600 using a 5-mm TXI three-axis grad probe.

Preparation of Liposomes and Lipoplexes. To form the liposomes, DOPE (Avanti Polar Lipids, Alabaster, AL) was added to a glass tube in chloroform, and the solvent was removed under vacuum to generate a lipid film. Cationic lipids were added to the lipid film in a 1:1 M ratio in either sterile water or reduced serum medium (Opti-MEM; Invitrogen, Carlsbad, CA) to achieve the various charge ratios (cationic lipid/DNA) tested. After hydration, the lipid mixtures were probe-sonicated for 30 s and briefly vortexed before use. Lipoplexes were formed by diluting plasmid DNA in Opti-MEM to achieve a concentration yielding the desired charge ratio upon equal volume mixing with the cationic/DOPE lipid mixture. Lipoplexes were formed 15 min before use in all experiments.

Size Distribution and ζ-Potential Measurements. Particle sizes were determined by dynamic light scattering with a ZetaPlus (Brookhaven Instruments Corporation, Holtsville, NY) with particle sizing option equivalent to the Brookhaven 90Plus. The measured autocorrelation function (90Plus) is analyzed using a cumulant analysis, with the first cumulant yielding an effective diameter, a type of average hydrodynamic diameter. Monodisperse polystyrene microsphere size standards (Polysciences, Warrington, PA) were used to validate the DLS instrument. ζ Potential was calculated from the electrophoretic mobility using the ZetaPlus. The Doppler shifted frequency spectrum at a 15° scattering angle, and 25°C yielded an average Doppler shift that was measured five times and averaged to determine an electrophoretic velocity. The mobility was calculated by dividing the velocity by the electric field strength.

Transfection. Transfection experiments were performed with bovine aortic endothelial cells (BAEC) from the American Type Culture Collection (Manassas, VA) and A549 cells (a gift from Penn Vector Core, Philadelphia, PA). Both cell lines were cultured at 37°C and 5%

CO_2 in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen), 2% penicillin/streptomycin (Mediatech, Inc., Manassas, VA), and 1% L-glutamine (Mediatech) before transfection, which was carried out in Opti-MEM. All experiments were executed with cells seeded 24 h before transfection at 50 to 75% confluence. Lipofectamine 2000 (Invitrogen) was used as a positive control for all transfection experiments and optimized independently for transfection efficiency with minimal toxicity for each cell line. A 1:1 (w/w) ratio with plasmid was used for BAECs and a 3:1 (w/w) ratio with plasmid was used for A549 cells according to the manufacturer's instructions. Although higher ratios of Lipofectamine 2000 to DNA were tested (data not shown), a ratio of 1:1 was used for BAECs and 3:1 for A549 cells because these ratios maximized transfection efficiency whereas minimizing toxicity for this product under these conditions in each cell line. Plasmid DNA without any cationic lipid was used as the negative control. Because of the observed shift in net surface charge resulting from different compositions of the experimental cationic lipids, three charge ratios were tested to assess how excess lipid/cationic charge affected transgene expression. For fluorescence microscopy and flow cytometry on BAECs, pEGFP-N_3 plasmid (Clontech, Mountain View, CA) was used to generate GFP as the fluorescent reporter transgene protein. BAECs were transfected in six-well plates with each condition in duplicate. One day after GFP transfection, cells were imaged and then harvested in 500 μl of PBS and kept on ice until analysis. A BD Biosciences (San Jose, CA) FACSCalibur flow cytometer was used to obtain fluorescence data with 50,000 counts recorded per condition. For the luminescence assays, pGL4.75 plasmid (Promega, Madison, WI) was used to generate *Renilla reniformis* luciferase as the reporter transgene protein. Cells were transfected in 96-well plates with 8 replicates of each condition. To measure transgene expression, EnduRen Live Cell Substrate (Promega) was added, and luminescence was measured 90 min after the addition of the reagent. Cell viability was determined by adding an equal volume of Cell Titer Glo (Promega) and measuring luminescence. Luminescence in both assays was measured with an EnVision Multilabel Plate Reader (PerkinElmer Life and Analytical Sciences, Waltham, MA).

Antimicrobial Activity. A single colony of *Escherichia coli* MG1655, *Bacillus subtilis,* subspecies (Ehrenberg) Cohn, or kanamycin-resistant *Pseudomonas aeruginosa* PAO1 was selected from an LB or *P. aeruginosa* isolation agar plate and grown to mid-log phase (optical density at 600 nm ~0.3) in 2 ml of LB medium (BD Biosciences). One milliliter of the bacterial suspension was centrifuged at 5000 rpm for 5 min at room temperature, and the bacterial pellet was resuspended in PBS. Serial dilutions of DS, D_2S, LL-37 peptide, and the ceragenin CSA-13 were mixed with the diluted bacterial suspension in 0.1-ml aliquots. The tubes were then incubated at 37°C for 1 h and transferred to ice. Duplicate 10-μl aliquots of 10-fold dilutions (undiluted, 1:10, 1:100, 1:1000) of these mixtures were plated on sectors of LB agar or *P. aeruginosa* isolation agar plates, and plates were incubated overnight at 37°C. The number of colonies in the duplicate samples at each dilution was counted the following morning, and the colony-forming units of the individual mixture were determined from the dilution factor. Coformulations of D_2S with DOPE and/or DNA were presented to bacteria cells as specified in the figure legends. Liposome and lipoplex formulations were prepared as described for transfection experiments. Excess bronchioalveolar lavage was obtained from material collected for diagnostic purposes from patients attending the Department of Pulmonology. Specimen collection was performed in accordance with an approved protocol by the Medical University of Białystok Ethics Committee for Research on Humans and Animals (written consent was obtained from all subjects). To assess D_2S scavenging binding potential, LPS from *E. coli* (Sigma) was added to the bacterial suspensions and incubated for 1 h.

Red Blood Cell Hemolysis. The hemolytic activity of D_2S against human red blood cells (RBCs) was tested using PBS suspension prepared from fresh blood (hematocrit ~5%). D_2S dissolved in PBS was added to RBC suspensions, and the incubation was continued for 1 h at 37°C. The samples were then centrifuged at 1300*g* for 10 min for hemoglobin release analysis. Relative hemoglobin concentration in supernatants was monitored by measuring the absorbance at 540 nm. The 100% hemolysis was taken from samples in which 1% Triton X-100 was added to disrupt the membrane. Liposome and lipoplex formulations were prepared as described for transfection experiments.

Human Neutrophil Activation. Human neutrophils (3×10^6 cells/ml) suspended in RPMI 1640 buffer containing 2% human albumin were activated with highly purified LPS from *E. coli* (0.1 μg/ml; Sigma). When required, D_2S was added to neutrophil suspension as liposome or lipoplex formulations and prepared as described for transfection. Cell-free neutrophil supernatants were collected by centrifugation at 5000*g* for 5 min and stored at −80°C until cytokine determination. IL-8 was measured using a sandwich enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Thermo Fisher Scientific, Waltham, MA). The detection limit was 30 pg/ml.

Results

Molecular Structure, Particle Size, and ζ Potential. Molecular structures of both DS (1) and D_2S (2) are shown in Fig. 1. Particle size was measured as a function of the mixed

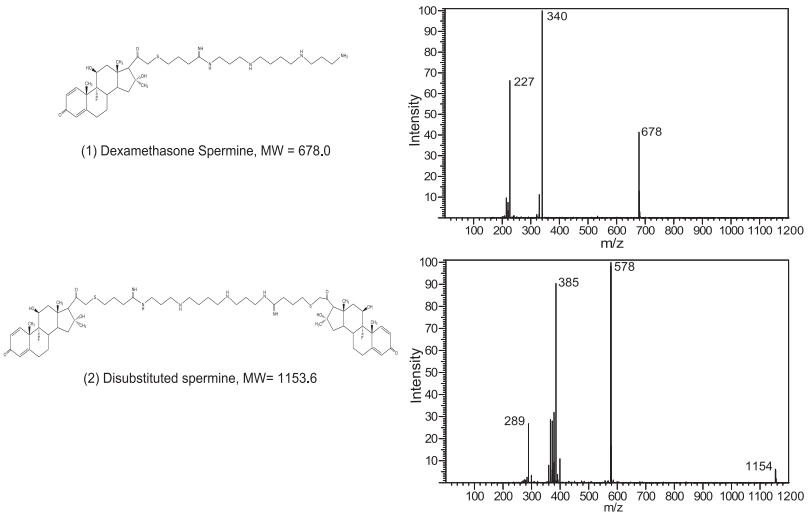

Fig. 1. Structures of DS (1) and D_2S (2) with mass spectrometry data showing multiple ionizations. Single quadruple mass spectrometry was performed using electrospray ionization in positive ion mode with a scanned *m/z* range from 160 to 2000.

lipid composition because it has been found to correlate with gene transfer activity (Ross and Hui, 1999). Both liposome and lipoplex (liposome-plasmid DNA complex) size were measured with the composition varied from 100 mol% DS to 100 mol% D_2S with a 1:1 M ratio of DOPE. The mean effective diameter for these liposomes in water was nearly constant across the entire series as shown in Fig. 2a, and the addition of plasmid DNA resulted in an increase in particle size for all compositions. Particle size in reduced-serum medium is shown in Fig. 2b and demonstrated a transition to relatively larger particles for mixtures containing 20 to 60 mol% D_2S, which correlated directly with the observed peaks in transfection activity in both cell types tested under the same solution conditions. The polydispersity indices of the particle sizes did not change significantly (data not shown), indicating uniform size distributions for all measurements.

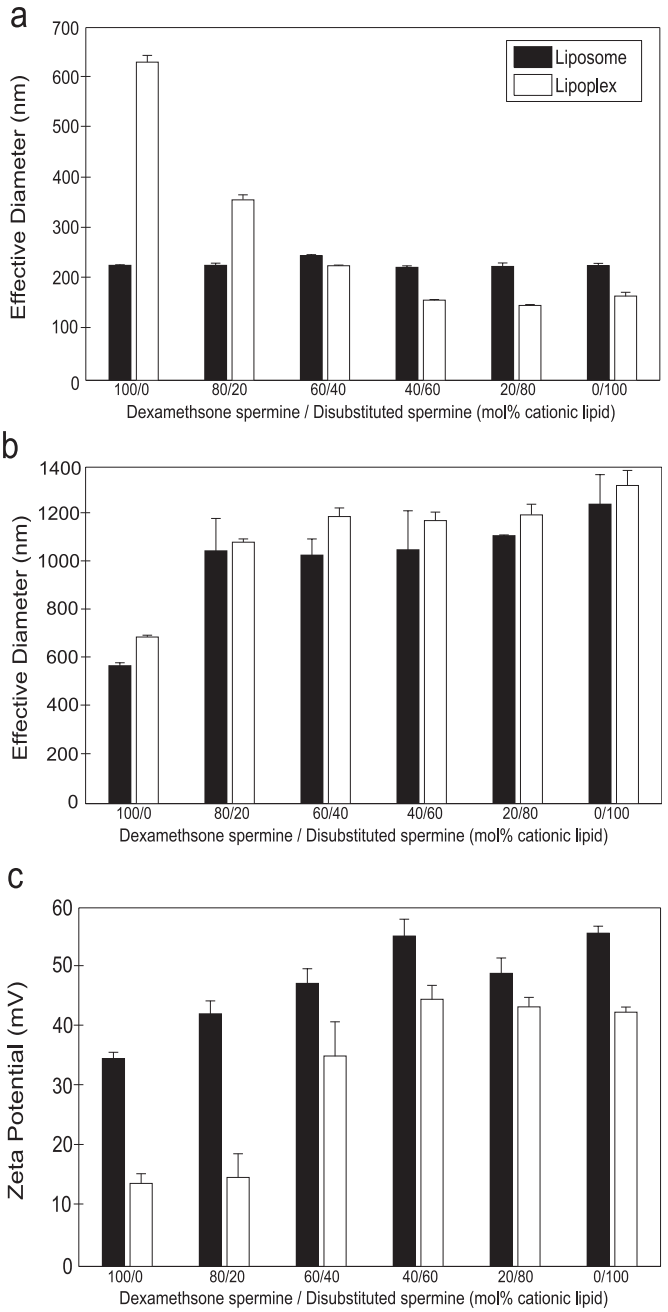

Fig. 2. Average effective diameter of mixtures of DS and D_2S in water from dynamic light scattering. All lipoplexes were formulated to achieve a net charge ratio of 6:1. Error bars represent standard error from two replicates of 1-min runs for each condition a, average effective diameter of mixtures of DS and D_2S in Opti-MEM from dynamic light scattering. Error bars represent standard error from two replicates of 1-min runs for each condition (b). Average ζ potential of mixtures of DS and D_2S in water. Error bars represent standard error from five replicates of each condition (c). Open bars indicate liposomes and solid bars indicate lipoplexes.

The net surface charge of liposomal particles is also an important physicochemical property because it can affect complex formation with nucleic acids and interaction with cellular membranes (Salvati et al., 2006). Electrophoretic mobility was measured for each lipid mixture and used to calculate the ζ potential as a measure of the surface charge for the liposomes and residual surface charge after complex formation between lipid and plasmid DNA as shown in Fig. 2c. The ζ potential was positive for all conditions as expected because all of the measurements were carried out in excess cationic lipid, and a transition to higher ζ potential was noted with the addition of 20 to 60 mol% D_2S. The shift to higher ζ potential correlated with the formation of larger particles and with the peaks in transfection activity in both cell types.

Luminescent Reporter Transfection. Transfection activity of mixtures of DS and D_2S showed a clear dependence on cell type as shown in Fig. 3. Lipofection of BAECs and A549 cells, a human carcinoma alveolar epithelial cell line, both showed peaks in transfection activity with mixtures of DS and D_2S but at different charge ratios and lipid compositions in each cell type. Peak activity in BAECs was 3-fold greater than control (charge ratio = 12 and 20 mol% D_2S), whereas A549 cell transfection showed a maximum 7-fold increase in transgene expression (charge ratio = 3 and 40 mol% D_2S). Cell viability decreased to some extent with D_2S concentration and charge ratio (12–15% maximum) compared with negative controls.

Fluorescent Reporter Transfection. A second transfection experiment was performed with BAEC using GFP as the reporter as shown in Fig. 4. Fluorescent images and flow cytometric data confirm the finding from the luminescent assay of a peak in transfection with a mixture of DS and D_2S. The additional mixture at 10 mol% D_2S demonstrated intermediate transfection activity to the 0 to 20 mol% D_2S mixtures proving that the peak in transfection for this charge ratio was defined. In this experiment, the total number of positively transfected cells was approximately the same for the maximal lipid mixture and the Lipofectamine 2000 control with more than 70% of cells expressing GFP at these conditions.

Antimicrobial Assays. The amphipathic nature of lipids used for transfection can have deleterious effects at high concentrations because of disruption of host cell membranes but also potentially beneficial effects because of preferential disruption of bacterial membranes, which, unlike eukaryotic membrane, expose highly anionic lipids at their surface. The addition of the cationic lipid mixtures to suspensions of the Gram-negative bacterium *E. coli* MG1655 was performed to evaluate antimicrobial activity, and differences were observed across the series of compositions tested as shown in Fig. 5a. The number of colony-forming units was determined using a conventional killing assay and showed higher antibacterial activity for D_2S compared with DS. No bacterial growth was observed upon

the addition of 5 μM D_2S, which was equivalent to the activity of the cathelicidin peptide LL37 and the ceragenin CSA-13 (data not shown) used as positive controls in the assay. Formulation of D_2S liposomes (D_2S/DOPE or 20% D_2S/80% DS/DOPE) and lipoplexes (liposome + DNA), which were effective formulations as gene-delivery vectors, exhibited similar antibacterial activity against *E. coli* MG1655 compared with the observed activity of the pure compound (Fig. 5b), as indicated by a lack of any outgrowth after 1 h of incubation at a concentration of 5 μM. D_2S displayed equally strong antibacterial activity against Gram-positive *B. subtilis* and Gram-negative *P. aeruginosa* PAO1 as shown in Fig. 5c, including gene delivery formulations against *P. aeruginosa* PAO1 as demonstrated by the lack of any outgrowth after 1 h of incubation at a concentration of $\leq$5 μM (data not shown). In addition, bactericidal activity of D_2S was found to be compromised in the presence of purified LPS (*E. coli*), indicating a specific interaction between those two molecules as shown in Fig. 5c. Complexation of LPS with D_2S may potentially result in the inhibition of LPS-mediated bacterial toxicity. Similar to natural cationic antibacterial peptides, such as cathelicidin LL-37, or their cholic acid based mimics such as ceragenin CSA-13, the antibacterial activity of D_2S was inhibited in the presence of human plasma (Fig. 5d). However, the addition of human bronchioalveolar lavage (maximally 50%) had very little inhibitory effect (<2%), indicating that D_2S and both liposome/lipoplex formulations may effectively kill bacteria locally at the surface of lung epithelium.

Measurement of hemoglobin release from human RBCs was used to determine DS and D_2S potential toxicity toward eukaryotic cell membranes. The results are shown in Fig. 6a and demonstrate a trend identical with the bacterial killing assay. The amount of hemoglobin released from RBC suspension in PBS (hematocrit ~5%), increased with D_2S concentration in the lipid mixtures with ~100% lysis measured at 500 μM for 100 mol% D_2S, a concentration 100 times greater than needed for complete killing of bacteria. In addition, D_2S formulation as liposomes and lipoplexes slightly reduced (~10%) the observed hemolytic activity (Fig. 6b). It is noteworthy that hemolytic activity of D_2S in liposome or lipoplex formulations was not observed after its addition to a 1:1 dilution of whole human blood in PBS, indicating that D_2S interaction with blood lipoproteins or other blood components prevents its insertion into RBC membrane (data not shown).

Human Neutrophil Activation. Quantitative measurement of IL-8 released from human neutrophils after activation with bacterial LPS is shown in Fig. 7. All treatment

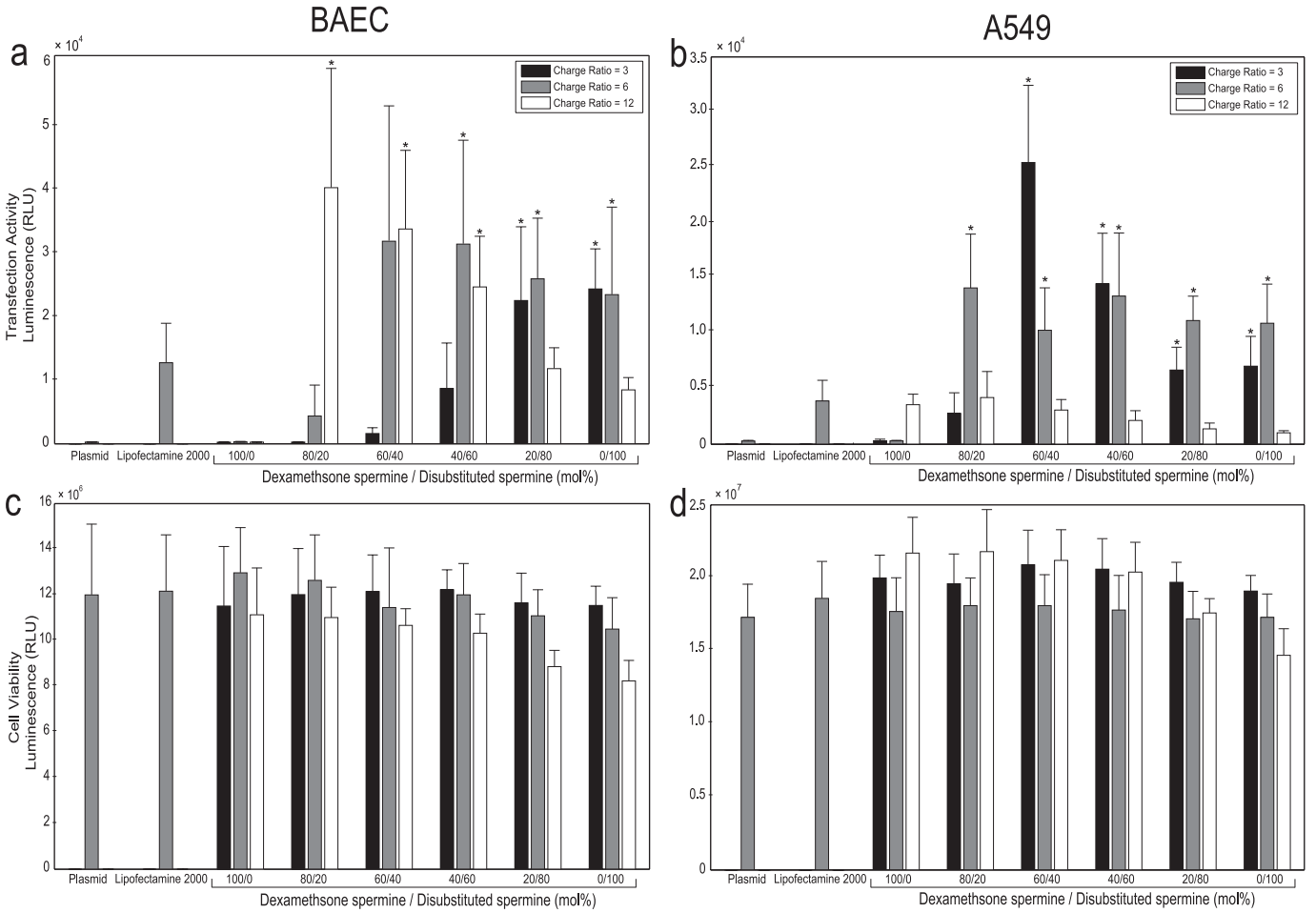

Fig. 3. Transfection activity of BAECs (a) and A549 cells (b) with DS and D_2S at three charge ratios with *R. reniformis* luciferase transgene 24 h after exposure to lipoplexes. Cell viability for BAECs (c) and A549 cells (d) measured 30 min after transfection assessment. Error bars represent standard deviations from eight replicates of each condition. Significant increases in luminescence from transfection with respect to the positive control (Lipofectamine 2000) were calculated using the Mann-Whitney *U* test (*, $P \leq 0.05$). RLU, relative light unit.

conditions were sampled at 12 and 24 h. LPS-treated neutrophils were used as a positive control and showed significant increases in IL-8 at 12 (15-fold increase) and 24 h (6-fold increase). An 85% reduction in IL-8 was observed for neutrophils treated with LPS in the presence of D_2S at 12 h, and a 65% reduction was measured at 24 h compared with the positive controls (Fig. 7a). Inhibition of IL-8 secretion at 24 h was also observed with D_2S liposomes, 20% D_2S/80% DS liposomes or 20% D_2S/80% DS lipoplexes (5 μM) compared with the negative controls. However, the level of inhibition was lower compared with unformulated D_2S activity, indicating that physicochemical mechanisms governing D_2S interaction with LPS packed in the bacterial cell wall and LPS aggregates in extracellular space are different, because D_2S formulation as liposomes and lipoplexes did not affect antibacterial activity.

Discussion

Nonviral gene delivery and expression have been the focus of many studies, and the fundamental parameters affecting efficiency have largely been discovered empirically. Because of the number of variables that can affect liposome-mediated gene delivery, physical properties are commonly studied to determine whether they correlate with the resulting activity and to further develop structure-activity relationships for vector design (Ma et al., 2007). In the current work, we designed transfection experiments to challenge the gene transfer efficiency of novel cationic lipids by using two distinct cells lines with an optimized commercial product as a positive control. The transfection experiments were carried out under identical conditions in both BAEC and A549 cells and showed that the two cell lines responded more effectively

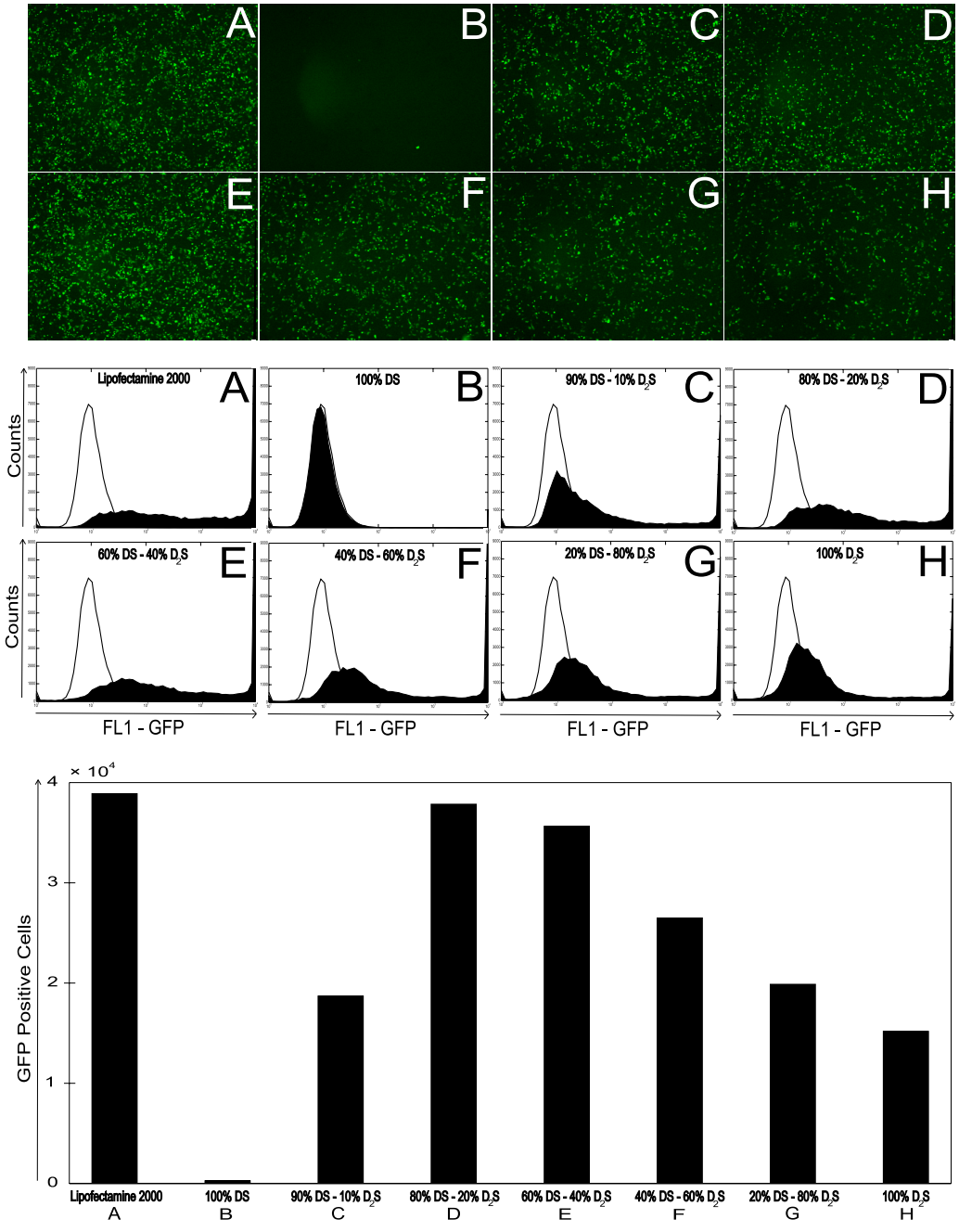

Fig. 4. Transfection of BAECs with DS and D_2S using GFP transgene at a charge ratio of 6:1 at 24 h. Fluorescent images and flow cytometry data shown with experimental data (black area) over negative control (white area) for each condition. Histogram of number of gated positive transfected cells based on negative control. Lipofectamine 2000 control (A), 100:0 mol% DS/D_2S (B), 90:10 mol% DS/D_2S (C), 80:20 mol% DS/D_2S (D), 60:40 mol% DS/D_2S (E), 40:60 mol% DS/D_2S (F), 20:80 mol% DS/D_2S (G), and 0:100 mol% DS/D_2S (H).

to mixtures of DS and D_2S. Peak transfection activity was observed at different lipid compositions and charge ratios; however, both cell types displayed higher transgene expression with mixtures of the two experimental lipids than either independently. It is important to note that the maximum enhancement in transgene expression observed in both cell lines was achieved without compromising cell viability and that the 3- to 7-fold increase over a common lipofection reagent indicates a significant improvement.

It was also determined that a correlation exists between shifts in both particle size and ζ potential to the peak transfection activity. This apparent association between physical parameters and transfection activity could be due to a number of factors, including the morphology of the lipoplexes, heterogeneity of lipids between the liposomal surfaces, or packaging of the plasmid DNA; however, a clear relationship was determined to exist between size, ζ potential, and transfection activity over the experimental range. Enhancement in gene delivery and optimal biophysical parameters were observed in both cell lines with mixtures of DS and D_2S, indicating that improvement in vector design can be achieved without the generation of new structures.

Similarity in the structure of D_2S to recently reported steroidal dimers (Salunke et al., 2004) and other membrane-active cationic steroid antibiotics called ceragenins (Chin et al., 2007) indicates that this compound may have bactericidal activity resulting from amphiphilic structure, mimicking the charge characteristic of natural cationic antibacterial peptides such as cathelicidin LL37. Based on preliminary data, it was theorized that D_2S could be a more effective destabilization agent of cellular membranes than DS, which would explain how moderate concentrations in combination with DS could lead to optimal transfection activity. These theories were tested by measuring antimicrobial activity to determine the relative membrane disruption potential for DS, D_2S, and mixtures of these compounds. A Gram-negative strain of bacteria was chosen to test the range of lipid mixtures because this is typically a more challenging test for antimicrobial killing because of the permeability barrier of the second bacterial membrane. The results of the bactericidal activity against *E. coli* MG1655 demonstrate that D_2S is the most active destabilization agent, because mixtures with increasing concentration of DS led to decreased activity. D_2S antibacterial activity against the Gram-positive *B. subtilis* and an additional Gram-negative bacterium *P. aeruginosa* PAO1 demonstrated killing activity at the same (*P. aeruginosa*) or lower (*B. subtilis*) concentrations compared with *E. coli*, indicating that both Gram-positive and Gram-negative bacteria are in the spectrum of bactericidal activity. In addition, liposomes and lipoplexes of D_2S, which demonstrated optimal transfection activity, were also effective antibacterial formulations, indicating that membrane destabilization is not compromised after preparation for gene delivery.

The effectiveness of D_2S in the bacterial killing assays, with complete killing demonstrated at 5 μM, was comparable with ceragenin CSA-13 and cathelicidin LL37 and prompted an investigation into eukaryotic membrane permeabilization (Chin et al., 2007). Red blood cells were used to determine toxicity based on the extent of hemoglobin release after exposure to the cationic lipids. According to previous observations, membrane asymmetry and the absence of anionic lipids in the outer leaflet of eukaryotic cells account for lower lytic activities of antibacterial peptides compared with bacteria (Bucki and Janmey, 2006). In the present study, complete rupture in the RBC hemolysis assay was only reached at a concentration 100 times greater (500 μM) than in the antimicrobial assay for D_2S, indicating a high therapeutic

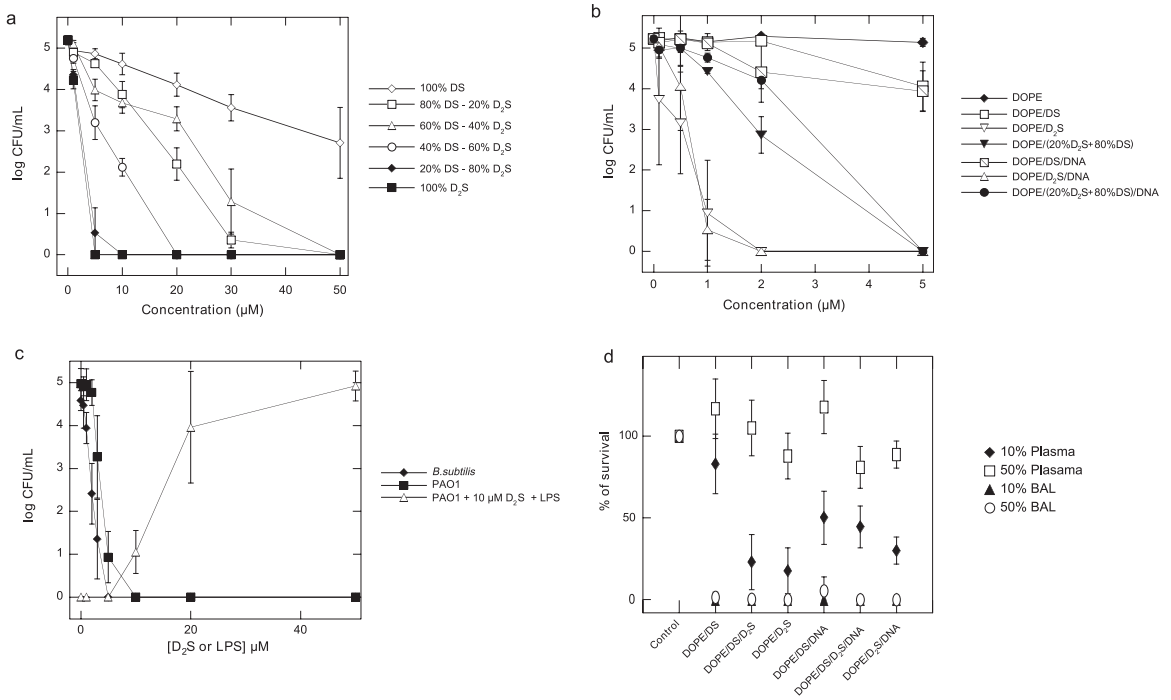

Fig. 5. Concentration kill curves for *E. coli* MG1655 with mixtures of DS and D_2S (a) and formulated in liposome and lipoplex formulations (b). Concentration kill curves for *B. subtilis*, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO1 in the presence of purified LPS (c). Antibacterial activity of D_2S (20 μM) liposomes and lipoplex formulations against *P. aeruginosa* PAO1 in the presence of human plasma and bronchoalveolar lavage fluid (d).

index and possible application of this component as a bactericidal agent (Bucki et al., 2007).

In the present work, we have demonstrated the ability of D_2S to both bind and inactivate bacterial LPS. The addition of LPS to suspensions of *P. aeruginosa* PAO1 resulted in inactivation of D_2S, probably because of an electrostatic interaction between the cationic lipid and the negatively charged bacterial endotoxin. In this context, LPS inactivation of bactericidal activity showed that D_2S can act as a scavenger of LPS preventing the binding of its target eukaryotic pattern recognition receptors (TRLs) potentially interfering with the inflammatory signaling pathway. We have also shown that D_2S effectively prevented LPS from causing up-regulation of IL-8 expression in human neutrophils, which are a primary source of production of proinflammatory cytokines (including IL-8 and TNF-α) and can be induced by bacterial LPS (Bucki et al., 2008). This suppression of bacterial-mediated inflammation is probably because of the interaction of D_2S with LPS; however, because this lipid has a base glucocorticoid structure, it may be expected that pharmacological activity is providing additional anti-inflammatory activity through cortisol receptor activation (Price et al., 2005). This is evidenced by the complete inactivation of a highly inflammatory LPS concentration by D_2S, but additional studies are needed to separate pharmacological activity from the prevention of LPS-induced inflammation.

It is important to note that the combined activity of mixtures of DS and D_2S in gene delivery and LPS inactivation represents an important connection in light of the recent findings indicating that inflammatory cytokines can directly inhibit gene transfer (Baatz et al., 2001; Bastonero et al., 2005). Binding of bacterial wall membrane bound LPS to TRL4 initiates signal transmission through the adapter protein myeloid differentiation factor 88, ultimately resulting in the up-regulation of NF-κB-controlled transcription of cytokines and chemokines (Schnare et al., 2006). Because activation of NF-κB is also responsible for increased levels of IL-4 and TNF-α, bacterial activation of TRLs could lead to the inhibition of gene transfer, which is particularly relevant in disease targets with known bacterial colonization (i.e., cystic fibrosis). Therefore, prevention of bacterial induced inflammation and cytokine production may further improve the efficiency of gene transfer.

The results of this study show that liposomes composed of both DS and D_2S can exhibit improved transfection activity. Several studies have reported optimal activities upon mixing lipids with different hydrophobic domains (Wang et al., 2006; Wang and McDonald, 2007); therefore, the current results support the principle that optimal liposomal properties can be obtained from mixing two distinct lipids instead of pro-

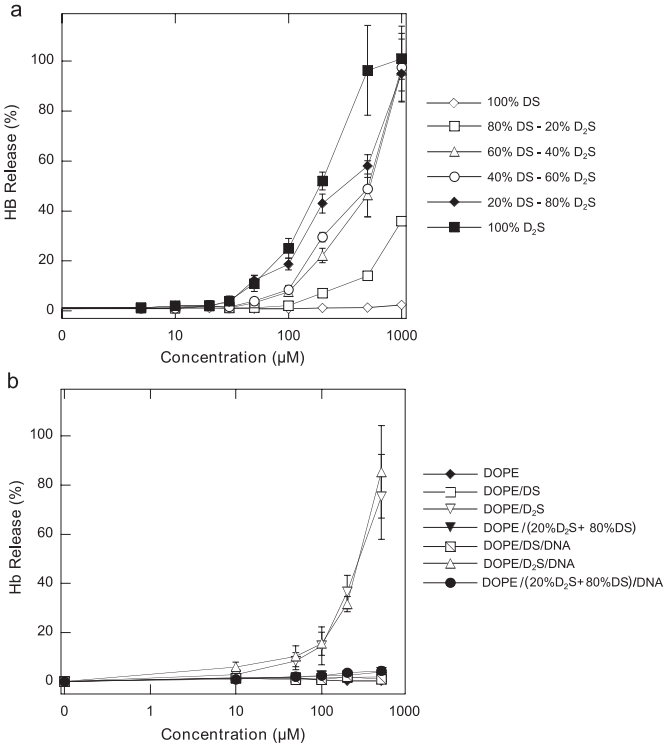

Fig. 6. Red blood cell lysis in response to treatment with DS and D_2S (a) and in response to treatment with liposomes or lipoplex formulations (b). Error bars represent standard deviations from three to five replicates for each condition.

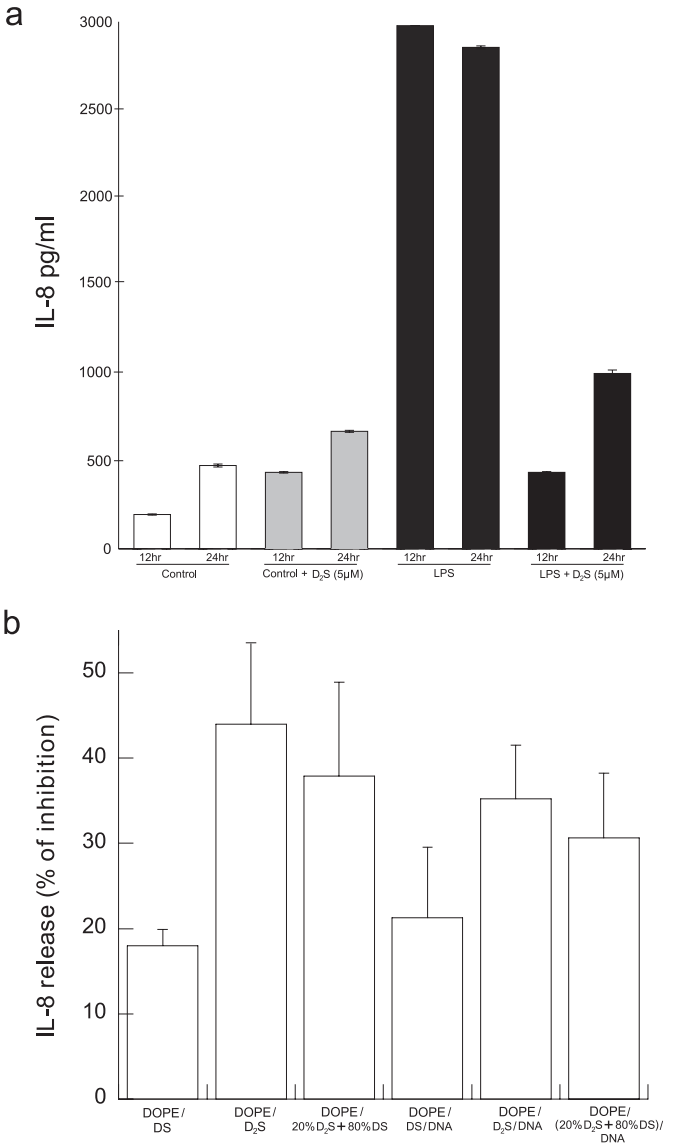

Fig. 7. IL-8 release from human neutrophils (3×10^6 cells/ml) after activation with LPS (0.1 μg/ml) at 12 and 24 h. D_2S (a) or liposome and lipoplex formulations (b) significantly prevented release of IL-8 from neutrophils in the presence of LPS. Error bars represent standard deviations from two replicates for each condition.

gressively engineering new lipids by altering chemical structure. Antimicrobial activity of D_2S against both Gram-positive and Gram-negative bacteria represents an important area of focus for this molecule because of the large relative difference in effective concentrations between the bacterial and eukaryotic membrane disruption, which indicates a favorable therapeutic index. The ability of D_2S to bind and inactivate LPS, effectively suppressing of bacterial-mediated inflammation, may prove to have therapeutic potential in certain diseases targeted by gene therapy and characterized by persistent infection, especially considering the evidence that inflammatory cytokines can inhibit of gene transfer.

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