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RESEARCH ARTICLE

Cationic corticosteroid for nonviral gene delivery

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Delivery of plasmid DNA for gene therapy often provokes an inflammatory response that reduces transgene expression. Cationic lipids for lipofection lack pharmacological activity despite the hydrophobicity of many drug candidates that could be exploited. We report a one-step synthesis of a water-soluble, dexamethasone–spermine (DS) cationic lipid that has potent gene transfer capability in confluent endothelial cells when used with the neutral lipid, dioleoylphosphatidylethanolamine (DOPE). In contrast, unconjugated mixtures of dexamethasone, spermine, and/or DOPE have essentially no gene transfer activity. DS retains partial corticosteroid character as quantified by the rapid translocation of glucocorticoid receptor to the nucleus and by dose-dependent transactivation from a glucocorticoid response element. DS has anti-inflammatory activity in vivo in the mouse thioglycollate model of inflammation. In a mouse lung model, DS:DOPE resulted in significantly less interferon-γ production at Day 1 and elevated transgene expression at Days 1 and 7 postintranasal instillation compared to DC-Chol:DOPE (sterol:DOPE:phosphate molar ratio of 1:1:1). Cationic pharmacophores such as DS represent a new approach to gene delivery and localized therapy.
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Introduction

Immunological responses following nonviral DNA delivery decrease the magnitude and duration of transgene expression, and reduce the effectiveness of frequent dosing.[1–4] Unmethylated CpG motifs on plasmid DNA,[1] cationic lipid formulations,[2] and lipid/DNA lipoplexes[2–4] contribute to the deleterious immunological responses. The addition of free glucocorticoids to lipofection formulations has been shown to increase the transfection efficiency over lipoplexes alone by modulating the immunological response to gene delivery.[1,5,6] Although cationic lipids for lipofection condense plasmid, facilitate endosome escape, neutralize charge of DNA, and/or shield DNA from nucleases,[7] no pharmacologically active cationic lipids have been reported to possess anti-inflammatory activity.

We report a new approach to nonviral gene delivery that uses a novel, pharmacologically active cationic glucocorticoid. The rationale was to engineer gene delivery activity into an approved pharmaceutical agent as well as to develop a cost-effective and easily scaleable synthesis. Therefore, we conjugated dexamethasone, a potent glucocorticoid recognized to enhance transgene expression *in vivo* by its anti-inflammatory activities,[1,6] with spermine via iminothiolane in a one-pot reaction to synthesize the dexamethasone–spermine (DS) conjugate. Reminiscent of the clinically relevant cationic lipids,[7] DC-Chol and CTAP, DS has the added functionality of glucocorticoids to improve gene delivery. To our knowledge, this is the first reported example of a cationic lipid that both displays pharmacological activity as well as facilitates DNA packaging and delivery.

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Results

Synthesis of cationic dexamethasone

The 21-hydroxy group of dexamethasone is not required for anti-inflammatory activity,[8] and therefore is an ideal choice for conjugation to a polycation (Figure 1a). A one-pot reaction between spermine, 2-iminothiolane (Traut's reagent) and dexamethasone mesylate yielded the DS conjugate, **1** as the major product (Figure 1b). Traut's reagent is selectively ring-opened by the primary amines[9] on spermine, forming a hydrolytically sensitive amidine bond[9] between spermine and iminothiolane and a reactive thiolate anion that reacts with the α-keto mesylate on the 21 position of dexamethasone mesylate[10] to form an α-keto thioether between spermine and iminothiolane. The conjugation reaction was complete in 45 min by HPLC. ^{1}H NMR of DS (data not shown) confirmed the presence of the 3-*bis*-enone and 11- and 17-hydroxy groups required for glucocorticoid activity (Figure 1a) as well as ^{1}H signals from the conjugated spermine and the 21-α-keto thioether group. Hydrolysis of DS in 1 M NaOH for 20 min resulted in the cleavage of the amidine linkage between spermine and iminothiolane, forming **2**, a *d*examethasone-*a*mide (DA) (Figure 1b), which has a 21-substituted butyl thioether amide side chain on dexamethasone. For **2**: Calc, C: 63.26; H:

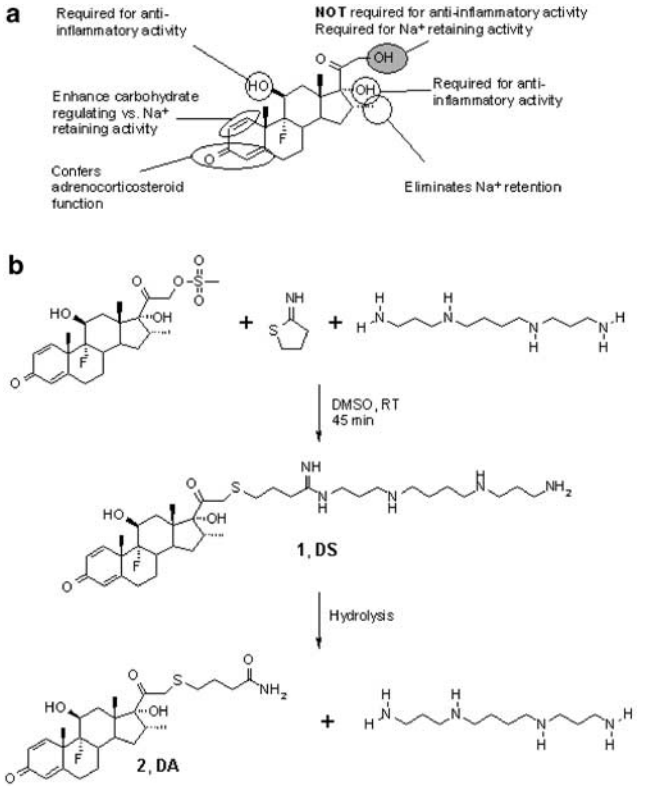

Figure 1 *Synthesis of a cationic steroid for gene delivery and anti-inflammatory activity. (a) The 21-hydroxy position of dexamethasone can be conjugated with a cationic group without loss of glucocorticoid function. (b) The use of 2-iminothiolane (Traut's reagent) as a coupling reagent does not consume a cation on spermine during the synthesis of the cationic steroid, DS (1). After 20 min in 1 M NaOH, DS undergoes hydrolysis, releasing spermine and a DA (2).*

7.35; N: 2.84; and Found, C: 63.44; H: 7.27; N: 2.83. Water solubility: DS > 100 000 mg/l; DA 60 mg/l; (dexamethasone 100 mg/l).

In vitro gene delivery with cationic steroids

Using the neutral lipid dioleoylphosphatidylethanolamine (DOPE) and 1 μg plasmid/well, we measured the gene expression obtained with varying amounts of DS (0–20 μg) and varying amounts of DOPE (0–20 μg) (Figure 2a). Lipofectamine was used (6 μg/μg DNA) as a benchmark. The mass ratio of 1:2 of DS:DOPE (yellow bars, Figure 2a) provided a high EGFP expression, while minimizing the use of the DS conjugate and minimizing the total lipid load below 10 μg total lipid/μg DNA. In a separate set of experiments maintaining the DS:DOPE mass ratio constant at 1:2, the amount of DS relative to DNA was systematically varied from 1 to 10 charge equivalents (0.9 μg DS/μg DNA), assuming an average charge of the spermine of DS to be 3.8/molecule,[11] and no net charge contribution from DOPE. The optimal lipofection plateaued at a charge ratio of 6 DS:1 DNA, giving a more than 10-fold increase in the amount of transgene expression relative to Lipofectamine reagent. Increases beyond a charge ratio of 6:1 DS:DNA provided no further increase in expression (Figure 2b). To test if gene transfer activity of DS was merely associated with the DNA-binding ability of spermine and the hydrophobic character of dexamethasone, we mixed DNA with spermine, dexamethasone, and DOPE (all unconjugated) using the same molar ratios and concentrations as in the conjugated DS/DOPE transfection (Figure 2d). Only when the dexamethasone was conjugated with spermine was EGFP expression detected (Figure 2e). Spermine alone or dexamethasone alone had no detectable gene transfer activity (data not shown). Using a flow cytometry cutoff of 100 FI to define percent transfection,[12] we observed a 4.3-fold increase in percent transfection over Lipofectamine from 5.9 to 25.5% (Figure 3). Lipofection of subconfluent (proliferating) BAEC with DS/DOPE yielded a 4.6-fold increase in percent transfection over Lipofectamine from 16.0% with Lipofectamine to 73.8% lipofection with DS/DOPE (data not shown). The amidine bond in DS appeared to be relatively stable in neutral pH when formulated with DOPE, since DS/DOPE formulations stored for 6 months at 4°C in water retained their lipofection activity.

Pharmacological activity of cationic steroid in vitro

The delivery of dexamethasone or DS/DOPE at 10–1000 nM, of steroid pharmacophore, caused rapid nuclear localization of a glucocorticoid-competent GFP-GR chimeric protein (Figure 4a) stably expressed in 3T3 cells. As soon as 5 min after the addition of DS/DOPE cells, GFP-GR fluorescence increased within the nucleus with complete localization occurring in less than 1 h similar to the localization induced by dexamethasone. Partial nuclear import of GFP-GR by 10 nM DS/DOPE and complete nuclear import at 100 nM suggested that the effective K_D for DS was approximately 10-fold higher than that of dexamethasone ($K_D \sim 3$ nM),[13] potentially due to reduced access to the cytosolic GR caused by endosome sequestration or association of DS with anionic elements in the cytosol. While nuclear localization of GR is one test of glucocorticoid activity, we also employed a second test of pharmacological activity, the ability to induce gene expression from a glucocorticoid-responsive promoter. DS induced dose-dependent transcription from a GRE promoter construct (pGRE-SEAP), displaying an EC_{50} of ~10–100 nM relative to dexamethasone in 293 cells. DS induced statistically significant levels of SEAP transcription from a GRE promoter at or above 100 nM concentrations. At high dose, DS may be a partial antagonist of corticosteroid-mediated transactivation of GRE promoters.

In vivo activity of DS in thioglycollate (TG) model of inflammation

Given the known benefits of using dexamethasone to enhance nonviral gene transfer *in vivo*, we designed experiments to address specifically the central issue of whether DS had anti-inflammatory activity. Male BALB/c mice were pretreated with vehicle, dexamethasone (2 mg/kg), or an equimolar amount of DS (5.85 mg/kg), for 30 min prior to intraperitoneal (i.p.) TG challenge. At 4 h postchallenge, DS significantly ($P = 0.0058$, $n = 6$) inhibited the accumulation of neutrophils in the i.p. space *versus* vehicle (1% ethanol in phosphate-buffered saline (PBS)), resulting in a 60% reduction in neutrophil immigration (Figure 5). Similar results were obtained with groups pretreated with dexamethasone or DS 48 h

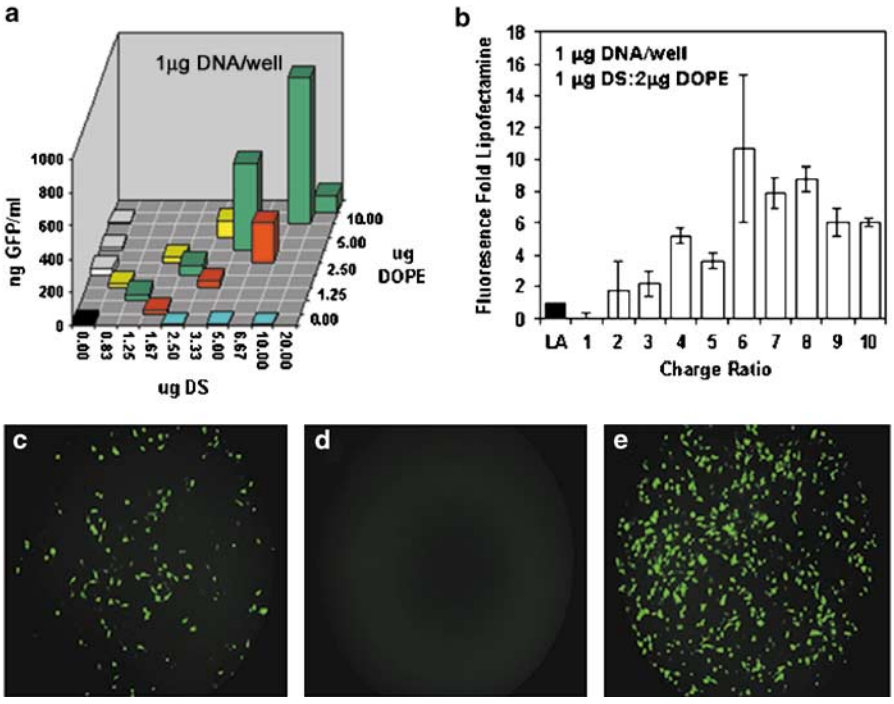

Figure 2 *Optimization of cationic steroid lipoplex formulation for transfection of confluent BAECs. (a) Lipofections of 1 µg plasmid/well (2-cm²/well at ~4 × 10⁵ cells/well) were carried out at increasing amounts of total lipid (0–40 µg)/µg DNA and various DS:DOPE mass ratios of 1:0 µg/µg DS:DOPE (blue); 2:1 µg/µg DS:DOPE (red); 1:1 µg/µg DS:DOPE (green); 1:2 µg/µg DS:DOPE (yellow); 0:1 µg/µg DS:DOPE (white); as well as for Lipofectamine at 6 µg lipid:1 µg DNA when neither DS nor DOPE was present (black). (b) Optimization of the lipid:DNA ratio at 1:2 µg/µg DS:DOPE led to a 10-fold enhancement of EGFP production relative to Lipofectamine. EGFP expression was normalized to the expression obtained with Lipofectamine. GFP-positive BAEC imaged by epifluorescence microscopy were lipofected with Lipofectamine (c), unconjugated dexamethasone/spermine/DOPE (d), or DS/DOPE (e), indicating that dexamethasone must be conjugated to spermine to have gene transfer activity.*

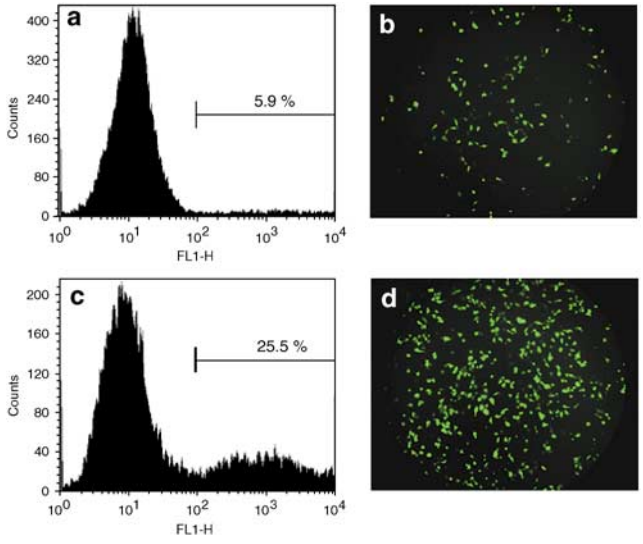

Figure 3 *FACS analysis of lipofections using Lipofectamine or DS/DOPE. Confluent BAEC cells were lipofected with 6:1 Lipofectamine:DNA (a, b) or 6:12:1 DS:DOPE:DNA (c, d) and subjected to flow cytometry (a, c) or epifluorescence microscopy (b, d). FACS analysis showed a 4.3-fold increase in percent transfection using DS/DOPE versus Lipofectamine, consistent with the increased transfection observed by direct visualization of EGFP in attached living cells.*

prior to TG challenge (not shown). All animals remained healthy in all groups, indicating that DS at the tested dose was not lethal.

Mouse pulmonary DNA transfer

Having demonstrated the anti-inflammatory activity of DS at a relevant pharmacological dose (no DOPE present) in the TG challenge model, a set of experiments was conducted with lipoplexes to evaluate gene transfer activity and the anti-inflammatory action of DS. DC-Chol was chosen as a commercially available reference lipid since it is sterol-based and has been used in animal and human clinical trials and is commonly formulated with the neutral lipid DOPE. This avoids referencing DS:DOPE against other nonsterol cationic lipids used with cholesterol, for example, as the neutral lipid. At a molar ratio of cationic sterol lipid:DOPE:base of 1:1:1 used to deliver 100 µg of plasmid (50 µl intranasal instillation), DS:DOPE resulted in a statistically significant 64% reduction ($P = 0.08$, $n = 4$) of interferon-γ (IFN-γ)/g of homogenized lung at Day 1 compared to DC-Chol:DOPE (Table 1). DS lipoplexes resulted in less tumor necrosis factor-α (TNF-α) expression on average compared to DC-Chol lipoplexes at Day 1, but did not reach statistical significance in this small cohort study due to the high variability of TNF-α expression in the DC-Chol group. At Day 1, DS:DOPE resulted in a statistically significant increase of 3.22-fold ($P = 0.029$) over DC-Chol:DOPE in the expression of the alkaline phosphatase (alk. phos.) transgene. At Day 7, one animal of the DS:DOPE cohort expressed >15 times more alk. phos. than the other three members of the cohort. By eliminating this overexpressor the DS:DOPE cohort resulted in a statistically significant 6.78-fold increase

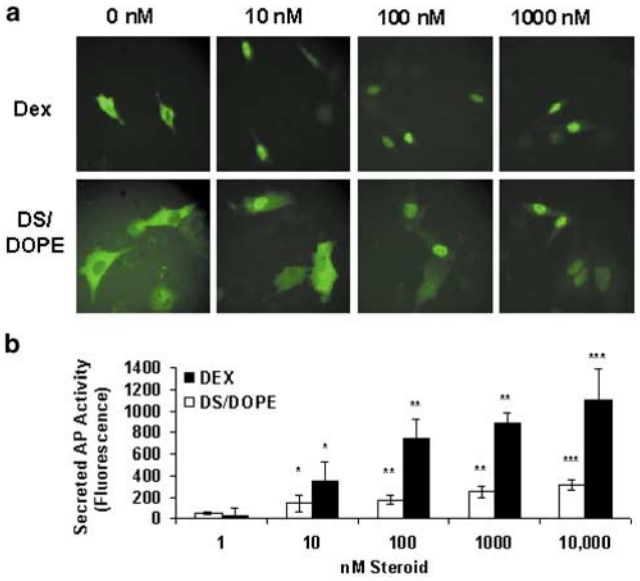

Figure 4 *Cationic steroids are pharmacologically active. A 30 min treatment of 3T3 cells expressing GFP-GR protein with dexamethasone or DS/DOPE caused nuclear localization of GFP-GR at doses from 10 to 1000 nM (a). Untreated cells displayed predominantly cytosolic localization of the fluorescent glucocorticoid receptor protein (0 nM). Dexamethasone induced nuclear localization at 10 nM concentrations, while DS/DOPE had a slightly less activity at this dose. (b) Dexamethasone and DS caused dose-dependent induction of SEAP transcription from a GRE-SEAP reporter plasmid as indicated by a fluorogenic assay for secreted alk. phosph. activity. Promoter construct experiments were carried out in triplicate (***$P<0.001$; **$P<0.005$, *$P<0.05$).*

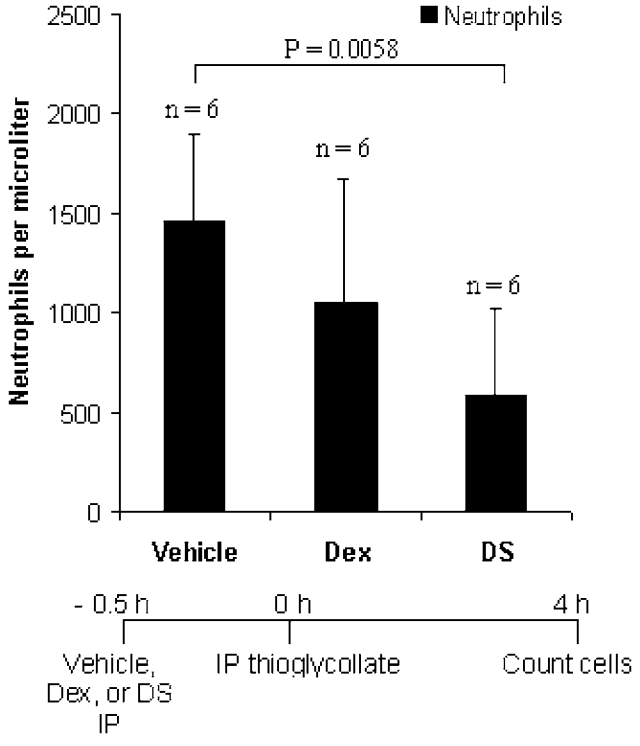

Figure 5 *Intraperitoneal (i.p.) injection of dexamethasone or DS in mice prior to thioglycollate. At a dose of 5.85 mg/kg, DS significantly inhibited the accumulation of neutrophils in the i.p. space versus vehicle (1% ethanol in PBS), resulting in a 60% reduction ($P=0.0058$, $n=6$) in neutrophil immigration.*

($P=0.0015$, $n=3$) in transgene expression compared to the DC-Chol cohort (Table 1). No member of the DC-Chol:DOPE group expressed more transgene than the lowest expressor of the DS:DOPE group at Day 1 or Day 7 postinstillation. Transgene expression actually increased from Days 1 to 7 in the DS:DOPE cohort. As expected for lipoplexes, the transgene expression level obtained with DS:DOPE was ~150-fold less than that achieved with intranasal instillation of 2.5×10^{11} adenoviral (Ad5.CB.AP) particles expressing alk. phosp. from a matched promoter (169 ± 152 ng alk.phos./g at Day 1 postinstillation, $n=3$).

Discussion

Cationic prodrug vehicles for gene transfer represent an attractive approach for simultaneous local gene and drug delivery. Many pharmacologically active agents are hydrophobic and can be converted to cationic prodrugs using chemistries similar to those shown in Figure 1b. We used dexamethasone since it is a well-studied hydrophobic drug with potent immunomodulating effects and has a K_D with GR of ~3 nM.[8,13] At the optimal *in vitro* formulation, at a charge ratio of 6:1 DS:DNA, each plasmid can deliver DS to a cell to result in about 30 nM final intracellular concentration of drug, well above the K_D. Moreover, DS has anti-inflammatory activity *in vivo* and may be useful in its own right as a drug for inflammation, asthma, arthritis, or other inflammatory diseases.

Distinct from other approaches that conjugate steroid directly to oligonucleotides[14,15] or plasmids,[16] the delivery of plasmid with DS resulted in pharmacologically relevant amounts of steroid inside each lipofected cell. It is unlikely that steroid covalently linked to DNA will allow the normal functioning of the glucocorticoid receptor, whereas DS activates nuclear translocation of GR and gene induction from a glucocorticoid response element (Figure 4). In addition, DS delivers DNA but is not conjugated to the DNA and therefore does not inactivate transcription of the transgene (Figure 2b) as has been observed with the chemical conjugation of steroid[16] or classical NLS to DNA. In addition to its benefits as an anti-inflammatory agent (Figure 4), DS serves as the cationic lipid itself for high efficiency transfection (Figure 3), whereas chemical modification of DNA with steroid still requires the use of a cationic delivery vehicle.[16] Furthermore, the pharmacokinetic clearance of DS is expected to be markedly different from dexamethasone, thus providing a depot effect of the anti-inflammatory drug in the local tissue site of DNA administration, which cannot be achieved by simply adding dexamethasone to the lipoplex, or by systemic or local administration of dexamethasone.

The approach described here builds upon these known benefits of glucocorticoids to gene transfer[1,5,6] as well as providing evidence that DS conjugates are efficient gene delivery molecules themselves when used with DOPE. DS/DOPE/DNA lipoplexes resulted in 25.5 and 73.8% transfection (4.3- and 4.6-fold enhancement in percent transfection over Lipofectamine) in confluent and subconfluent BAEC, respectively (Figure 3). DS was designed for broad applicability to nonviral gene delivery applications, except those requiring an

Table 1 Detection of IFN-γ antigen, TNF-α antigen, and the transgene alk. phos. in a mouse lung tissue after nasal instillation of 100 μg plasmid at Days 1 and 7 postinstillation

	INF-γ Day 1 (pg/g)	*TNF-α Day 1 (pg/g)*	*Alk. phos.*	
			Day 1 (pg/g)	*Day 7 (pg/g)*
DC-Chol/DOPE	513±247	653±674	359±92	502±514
DS/DOPE	188±58 ($P=0.08$)	403±119 NS	1159±537 ($P=0.029$)	3407±721 ($P=0.0015$)

increased immune/inflammatory response (eg DNA vaccines). DS/DOPE for gene delivery may work well for diseases that show palliation by glucocorticoids such as asthma, arthritis, allergies, and other inflammatory diseases. Alternatively, the choice of pharmacophore could be made in conjunction with the selection of the transgene and the route of delivery to achieve a particular therapeutic effect.

Dexamethasone has been shown to help gene delivery by blocking inflammation and regulating the immune response to gene delivery. The major inflammatory cytokines upregulated after gene delivery are TNF-α, IL-1, IL-6, IL-12, and IFN-γ, all of which are transrepressed by corticosteroids. A major cell type in initiating inflammation is the neutrophil, and DS worked as good as or better than dexamethasone to inhibit neutrophil immigration in a mouse model of inflammation (Figure 5). In addition, DS also gives good gene delivery results *in vitro* relative to the standard gene delivery agent Lipofectamine. Therefore, DS may offer unique benefits to gene delivery *in vivo* because it both delivers DNA well and inhibits the inflammatory response that reduces the level and duration of transgene expression.

In vivo analysis of the anti-inflammatory activity of DS *versus* dexamethasone was performed with the i.p. injection of TG into mice (Figure 5), since it is a simple model to implement for screening purposes, and because it is an injection into the body of the animal as opposed to topical application of the drug (eg sephadex-induced lung edema in the rat, or the mouse ear punch assay), thereby mimicking *in vivo* injection of DNA. Figure 5 illustrates that DS inhibits neutrophil immigration. Although the formulation of DS with DOPE and DNA may significantly alter its pharmacodynamics and pharmacokinetics, these data illustrate the proof of principal that DS has anti-inflammatory activity.

The intent of this study was to demonstrate that pharmacophores can be modified through one-step chemistries to become lipofection reagents that display *in vitro* pharmacological activity. We also demonstrated that this compound has anti-inflammatory action in two different animal models. While GL67 has a powerful gene transfer activity that exceeds DC-Chol in pulmonary gene transfer studies,[17] issues of toxicity persist. The potential exists to use DS in formulations with GL67 (or even with adenovirus) to reduce undesired inflammatory reactions. While DS:DOPE resulted in less IFN-γ expression at Day 1 and higher transgene expression at Day 7 compared to DC-Chol:DOPE, it remains to be shown that the reduction of inflammation was the cause of the >6-fold increase in transgene expression at Day 7 (Table 1) seen with DS:DOPE. Such studies would require dexamethasone analogs lacking corticosteroid activity.

We note that for gene delivery applications, at an optimal *in vitro* charge ratio of 6:1 DS:DNA, each plasmid can deliver DS to a cell to result in about 30 nM final intracellular concentration of the drug, well above the K_D. Hundreds to thousands of lipoplexes would deliver significantly more DS to each cell, while DS may be useful in its own right as a depot-like agent for inflammatory disease.

Materials and methods

Synthesis and hydrolysis of cationic steroids

Dexamethasone and dexamethasone mesylate (Steraloids, NH), Traut's reagent (Pierce), spermine (Sigma), and DMSO (Aldrich) were used as received. A total of 105 mg (223 μmol) of dexamethasone-mesylate, 800 μl of DMSO, and 28.4 mg (206 μmol) of Traut's reagent were dissolved together prior to the addition of 31.9 μl (145 μmol) of spermine at room temperature. After 45 min, the reaction was complete by TLC. HPLC purification (60/40 0.1%TFA/acetonitrile, Hamilton PRP-1 column) and freeze-drying yielded DS trifluoracetic acid salt as a white powder. Yield: 15.3 mg (14.0%). DA was isolated from hydrolyzed DS by semipreparative HPLC. For all studies using DS, DS was purified by semipreparative scale HPLC as a tetrafluoroacetate salt. DS had no contamination with free dexamethasone or dexamethasone mesylate, and less than 1% contamination with DA.

Cell culture and Lipofection

Bovine aortic endothelial cells (BAECs, passages 4–13) were passed at a 1:3 split to 24-well culture plates, and then grown to dense confluence before lipofection. The growth medium was Dulbeccos's modified Eagle's medium containing 10% heat-inactivated charcoal-filtered (to remove steroid hormones) fetal calf serum (Hyclone), 0.30 mg/ml glutamine, 150 U/ml penicillin, and 0.15 mg/ml streptomycin (Gibco). The 293 cells were grown under identical conditions. The plasmids pEGFP-N3 and pGRE-SEAP (Clontech) were used for the expression of EGFP or secreted alk. phosp. under the regulation of CMV or GRE promoters, respectively. Lipofectamine reagent (Gibco) containing 3:1 (μg:μg) mixture of polycationic lipid 2,3-dioleoyloxy-*N*-[2(sperminecarboxamido)ethyl]-*N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA):DOPE was used according to the manufacturer's instructions.

Preparation and characterization of lipid assemblies and lipoplexes

To form the DS:DOPE lipid solution, 67 μl of a 10 mg/ml solution of DS in ethanol and 27 μl of a 50 mg/ml

solution of DOPE in ethanol were mixed and the solvent was removed in a nitrogen stream. The resulting lipid film was hydrated with 1 ml of sterile Millipore water and sonicated for 10 min. The solution (2 μg of lipid/μl of water) was stored at 4°C and retained lipofection activity for over 6 months (data not shown). The hydrodynamic diameter of DS/DOPE (1 μg/2 μg) obtained by dynamic light scattering (DynaPro 99 instrument) was 70–150 nm without DNA, while lipoplexes with 1 μg DNA and 0–20 equivalents of DS and 0–20 equivalents of DOPE had sizes ranging from 200 to 500 nm.

Fluorescence GFP measurement of transgene expression

For lipofection, a solution of DS/DOPE in 125 μl OptiMEM I (Gibco) was mixed with a solution of DNA in 125 μl OptiMEM I, incubated for 30–60 min, and then overlaid on BAEC cells for 2 h, followed by PBS rinse and addition of growth media. Fluorescence was measured after 48 h. At lipid concentrations exceeding charge ratios of 6:1 DS:DNA, the formulations began to display cytotoxicity in BAEC as indicated by altered morphology, similar to Lipofectamine. EGFP expression of lipofections was monitored in duplicate (Figure 2a) or triplicate (Figure 2b) with a fluorescent plate reader (Labsystems Fluoroskan Ascent; 485 nm/538 nm filter pair) with background subtraction using the autofluorescence of nontransfected BAEC. EGFP expression was calibrated using 0–200 ng recombinant GFP (Clontech) in 750 μl PBS. In assays of EGFP in cell lysates, lipofected cells were trypsinized, pelleted (200 g, 8 min), resuspended in 150 μl PBS, and subjected to 3 × freeze/thaw at −78°C. The lysate was centrifuged at 13 500 r.p.m. (5 min) in an Eppendorf Minifuge. The supernatant was collected and pooled with the supernatant from PBS washed and pelleted cells (total volume 750 μl), and EGFP fluorescence was measured (Ex 485, Em 515; SLM fluorometer). In separate experiments, flow cytometry of lipofected and trypsinized cells expressing EGFP was performed at the University of Pennsylvania Flow Cytometry Core Facility using a FACSCalibur instrument.

Glucocorticoid receptor localization assay

The 3T3 cell line 3676 expressing green fluorescent protein-glucocorticoid receptor chimeric protein from a tetracycline-regulated promoter[18] was used to image GR receptor localization into the nucleus. Cells were maintained in growth media supplemented with 100 μg/ml geneticin. The cells were incubated for 30 min with dexamethasone or DS/DOPE ranging in concentration from 10 to 1000 nM, and nuclear translocation of GFP-GR was visualized at 40 × using a Hamamatsu CCD camera and Leica DM IRBE fluorescent microscope.

Induction of transcription from GRE

The levels of GRE induction using DS/DOPE was compared with dexamethasone using a GRE-SEAP promoter construct assay (Clontech). The 293 cells were lipofected with GRE-SEAP plasmid in triplicate using 1 μg plasmid and 6 μg Lipofectamine per well in 24-well plates. After 120 min, the lipofection media were replaced with 500 μl of growth media, and the cells were treated with dexamethasone or DS/DOPE over a range from 1 to 10 000 nM. After 24 h of induction, 50 μl aliquots of growth media were analyzed for alk. phosph. activity using the SEAP fluorogenic assay following the manufacturer's instructions.

Thioglycollate mouse model of inflammation

Male, 6- to 8-week-old (20 g) BALB/c mice, six per group were used. Mice were injected i.p. with either vehicle (200 μl of 1% ethanol in PBS), dexamethasone (Dex, a dose of 2 mg/kg in 200 μl of 1% ethanol in PBS), or an equimolar amount (102 nmol of drug/animal) of DS (5.85 mg/kg in 200 μl of 1% ethanol in PBS). After 30 min, mice were injected i.p. with 1.5 ml of 3% TG in PBS containing 1% ethanol. At 4 h post-TG, the i.p. fluid was collected and neutrophils were counted. All work performed with animals was conducted in compliance with the Animal Welfare Act (CFR 9) and its amendments.

Mouse pulmonary DNA transfer

For studies of *in vivo* gene delivery, mice (C57/B6) were anesthetized with ketamine/zylazine (i.p.). A total of eight mice per group received 50 μl of lipid/plasmid (100 μg pCB-AP (6075 bp) in 10% sucrose) by nasal instillation during inspiration following the approach of Lee *et al*.[17] Either DS or DC-Chol (Avanti Polar) was used at a molar ratio of one sterol per base and one cationic lipid per DOPE, resulting in a cationic lipid:DOPE:phosphate molar ratio of 1:1:1. Mice were weighed and euthanized at 1 or 7 days postinstillation. The lung from each mouse was harvested and weighed. One half of each lung was homogenized in 2 ml of 1 × Reporter Gene Assay Lysis Buffer (Roche Diagnostics) and clarified by centrifugation (10 min, 3000 r.p.m.). Supernatants were stored at −80°C until assayed in triplicate using the SEAP Chemiluminescent kit (Roche Diagnostics). Antigenic levels of TNF-α and IFN-γ were quantified against mouse standards using ELISA (Pierce Endogen) at Day 1 based on peak inflammatory responses expected at this time.[3] All work performed with animals was conducted in compliance with the Animal Welfare Act (CFR 9) and its amendments.

Acknowledgements

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References

1 Tan Y *et al*. The inhibitory role of CpG immunostimulatory motifs in cationic lipid vector-mediated transgene expression *in vivo*. *Hum Gene Ther* 1999; **10**: 2153–2161.

2 Tousignant JD *et al*. Comprehensive analysis of the acute toxicities induced by systemic administration of cationic lipid:plasmid DNA complexes in mice. *Hum Gene Ther* 2000; **11**: 2493–2513.
3 Scheule RK *et al*. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum Gene Ther* 1997; **8**: 689–707.
4 Li S *et al*. Effect of immune response on gene transfer to the lung via systemic administration of cationic lipidic vectors. *Am J Physiol* 1999; **276**: L796–804.
5 Wiseman JW, Goddard CA, Colledge WH. Steroid hormone enhancement of gene delivery to a human airway epithelial cell line *in vitro* and mouse airways *in vivo*. *Gene Therapy* 2001; **8**: 1562–1571.
6 Braun S *et al*. *In vitro* and *in vivo* effects of glucocorticoids on gene transfer to skeletal muscle. *FEBS Lett* 1999; **454**: 277–282.
7 Lasic D. *Liposomes in Gene Delivery*. CRC Press: Boca Raton, FL, 1997.
8 Schimmer BP, Parker KL In: Hardman JG, Limbird LE (ed) *The Pharmacological Basis of Therapeutics*. McGraw-Hill: New York, 1996, pp 1459–1485.
9 Hermanson G. *Bioconjugate Techniques*. Academic Press: London, 1996.
10 Simmons SJ, Pons M, Johnson DF. Alpha-keto mesylate: a reactive, thiol-specific functional group. *J Org Chem* 1980; **45**: 3084–3088.
11 Geall AJ *et al*. Synthesis of cholesteryl polyamine carbamates: p*K*(a) studies and condensation of calf thymus DNA. *Bioconjug Chem* 2000; **11**: 314–326.
12 Subramanian A, Ranganathan P, Diamond SL. Nuclear targeting peptide scaffolds for lipofection of nondividing mammalian cells. *Nat Biotechnol* 1999; **17**: 873–877.
13 Ashwell JD, Lu FW, Vacchio MS. Glucocorticoids in T cell development and function. *Ann Rev Immunol* 2000; **18**: 209–345.
14 Chidambaram N, Zhou L, Cohen JS. Targeting of antisense: synthesis of steroid-linked and steroid-bridged oligodeoxyribonucleotides. *Drug Deliv* 1996; **3**: 27–33.
15 Acedo M *et al*. Preparation of oligonucleotide–dexamethasone conjugates. *Bioorg Med Chem Lett* 1995; **5**: 1577–1580.
16 Rebuffat A *et al*. Selective enhancement of gene transfer by steroid-mediated gene delivery. *Nat Biotechnol* 2001; **19**: 1155–1161.
17 Lee ER *et al*. Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum Gene Ther* 1996; **7**: 1701–1717.
18 Walker D, Htun H, Hager GL. Using inducible vectors to study intracellular trafficking of GFP-tagged steroid/nuclear receptors in living cells. *Methods* 1999; **19**: 386–393.