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RESEARCH ARTICLE

Synthesis and structure-activity relationships of a series of increasingly hydrophobic cationic steroid lipofection reagents

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Abstract

The use of cholesterol-based cationic lipids and the ability of glucocorticoids to reduce local inflammatory response to lipoplexes motivated an investigation of structure-activity relationships for cationic steroids. A one-step synthetic scheme using iminothiolane was developed to link spermine to the 21-OH position of steroids via an amidine linkage. Five steroids (cortisol, dexamethasone, corticosterone, 11-deoxycortisol, and 11-deoxycorticosterone) with increasing hydrophobicity of the parent steroid (Log P_{ster} from 1.51 to 3.01) were conjugated with spermine, formulated with dioleoylphosphatidylethanolamine (DOPE) at DOPE : steroid mole ratios (R) of R = 0.5 to 2, and then complexed with 1 μg enhanced green fluorescent protein (EGFP) plasmid DNA at charge ratios (CR) = 2 to 24 amines per phosphate (0.5 to 6 steroids per phosphate). The resulting 105 different formulations of the cationic steroid series were used to lipofect bovine aortic endothelial cells. Transgene expression data at either 24 or 48 h post-lipofection for all formulations was collapsed onto master curves when plotted against a single empirical dimensionless parameter, the lipofection index (LI) = CR (Log $P_{liposome}$)(Log P_{ster}/|ΔLog P|) [R/(R + 1)] where ΔLog P = Log P_{DOPE} − Log P_{ster} and Log $P_{liposome}$ is a mole-weighted average of the DOPE/cationic steroid liposome hydrophobicity. For 7 < LI < 29, the EGFP expression at 24 or 48 h post-lipofection increased linearly with LI (EGFP ∼0 for LI <7), but did not increase further for LI >29, thus providing a predictive design rule based on Log P of the hydrophobic moiety of the cationic steroid lipid.

Keywords lipofection; steroid; endothelium

Introduction

Cationic lipids, in formulations with neutral helper lipids, help package DNA into lipoplexes for transfection of mammalian cells. Synthetic formulations of packaging agents with DNA offer potentially low-cost, safe, and uniform pharmaceutical-grade material compared to viral-based biologics. Cationic lipids have proven quite versatile and include aliphatic cationic lipids [1–3] such as DOTMA, DMRIE, and DOTAP or cholesterol-based cationic lipids [4–6] such as DC-Chol, CTAP, and GL-67.

For a given cellular target, the predictive basis of efficacy *in vitro* or *in vivo* is limited and formulation optimization is empirical with respect to: lipid cation to DNA anion charge ratio; total lipid to DNA weight ratio; and cationic lipid to neutral lipid mole ratio. Since structure-activity relationships for the cationic head groups have been examined [7] and certain co-lipids (e.g. DOPE) are recognized to facilitate endosome escape [8–10], we investi-

gated the relationships between the hydrophobicity of the lipid moiety of the cationic lipid and the resultant lipofection activity. We hypothesized that the Log P of the lipid moiety may be predictive of transfection of nucleic acid. Log P is the partition coefficient of a molecule in octanol-saturated water and is commonly used to predict the membrane permeability of pharmacological agents [11]. We chose to use sterol-based cationic lipids, specifically cationic pregnanes, since several pregnane derivatives were available that spanned a large range of Log P_{ster} values of the parent steroid (21-OH).

Cationic cholesterol-based lipids have a known safety profile and established *in vivo* efficacy. For example, DC-Chol was the first cationic sterol developed, followed by the guanidinium cholesterols, CTAP and CTAB [12]. While glucocorticoid steroids [13,14] or NF-κB antagonism [15] can enhance nonviral gene transfer, DNA delivery with pharmacologically active cationic lipids has not been extensively studied. Gruneich *et al*. [16] recently reported that *d*examethasone-*s*permine (DS) conjugate was a potent transfection reagent that also triggered glucocorticoid receptor nuclear localization, induced transgene expression from a glucocorticoid response element (GRE), and possessed anti-inflammatory activity in a mouse model. During intranasal delivery of adenovirus to lung, DS/DOPE targeted transduction to the pulmonary epithelium while dramatically reducing cellular infiltrate [17].

Using a synthetic scheme to link spermine to the 21-OH position of a steroid via an amidine linkage (Figure 1), we synthesized a pregnane series **3**–**7** (Figures 1 and 2, Table 1) to study whether systematically varying the -OH substituents on positions 11 and 17 of the parent steroid had an effect on the efficiency of transfection. The 21-hydroxy group of corticosteroids is not required for anti-inflammatory activity [18] and was therefore a logical choice for conjugation to a polycation. Unlike cholesterol-based lipids that have no hydroxy groups, the pregnane series **4**–**7** has an increasing number of hydroxy groups, reducing the Log P_{ster} of the parent steroid from 3.01 to 1.51. The hydroxy groups also confer distinct pharmacological activity to the lipid [18]. For example, a parent steroid with 11-OH and 17-OH groups (e.g. **3** and **7**) has glucocorticoid character, while those without either 11-OH or 17-OH groups (e.g. **5** and **6**) tend to be mineralcorticoids, and **4** has both weak glucocorticoid and weak mineralcorticoid activity. An aim of the present study was to understand the design rules for more optimal lipids (both pregane-based or not) and their formulations using readily available physicochemical data.

Materials and methods

Materials

11-Deoxycorticosterone-21-mesylate, corticosterone-21-mesylate, 11-dexoxycortisol-21-mesylate, cortisol-21-mesylate, and dexamethasone-21-mesylate (Steraloids, NH, USA), 2-iminotholane (Traut's reagent, Pierce), spermine (Sigma), trifluoroacetic acid (TFA) (Sigma), and DOPE (dioleolylphosphatidylethanolamine) (Avanti Polar Lipids) were used as received. HPLC grade solvents

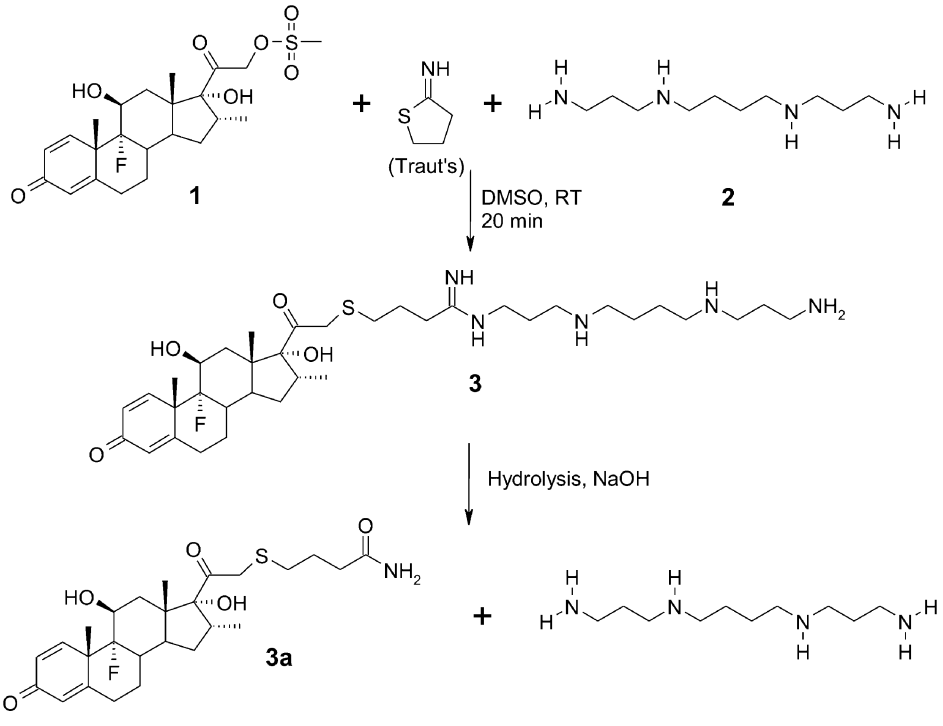

Figure 1. Synthesis of steroid-spermine conjugates using dexamethasone-mesylate (1), Traut's reagent, and spermine (2) to form dexamethasone-spermine (3) via an amidine linkage. Hydrolysis of 3 with 1 M NaOH (20 min) yielded dexamethasone-amide (3a) which had a 21-substituted butyl thioether amide side chain on dexamethasone

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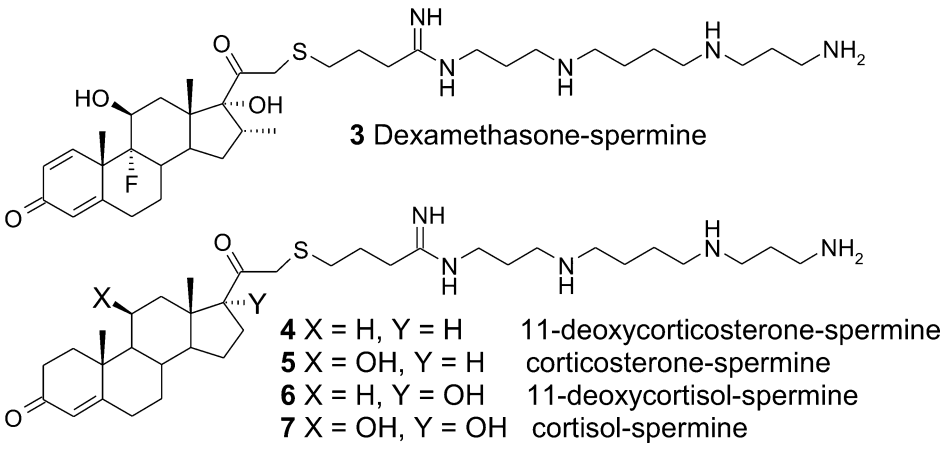

Figure 2. Structures of a series of cationic steroid lipids

Table 1. Parent steroids used for synthesis of cationic steroid-spermine conjugates. Average value of octanol-water portioning coefficient (Log P_{ster}) for each steroid was obtained from the literature [26–30]

Parent steroid	Log P_{ster} (range)	Cationic steroid	MW (calcd/found)
11-deoxycorticosterone	3.01 (2.88–3.25)	4, $C_{35}H_{62}N_5O_2S$: $[M + H]^+$	616.4624/616.4643
corticosterone	2.62 (2.52–2.72)	5, $C_{35}H_{62}N_5O_3S$: $[M + H]^+$	632.4573/632.4599
11-deoxycortisol	1.94	6, $C_{35}H_{62}N_5O_2S$: $[M + H]^+$	632.4573/632.4564
dexamethasone	1.83	3, $C_{38}H_{63}FN_5O_4S$: $[M + H]^+$	678.4428/678.4429
cortisol	1.51 (1.47–1.55)	7, $C_{35}H_{62}N_5O_4S$: $[M + H]^+$	648.4523/648.4555

(Aldrich) were used as received (except dimethyl sulfoxide (DMSO) and methanol were dried and stored over anhydrous sodium sulfate). Sterile phosphate-buffered saline (PBS) (Clonetics) was used as received. Thin-layer chromatography (TLC) was performed on Whatman PK6F silica gel 60 Å TLC plates using 50 : 50 hexane/tetrahydrofuran (THF) elution solvent, visualized at 254 nm wavelength. High-performance liquid chromatography (HPLC) was performed using an HP 1050 HPLC system equipped with a photodiode detector. For analytical scale HPLC, a 4.6 mm × 150 mm Hamilton 5 μ PRP-1 column was used with a 20 μL injection volume, at a rate of 1 mL/min, using an elution profile starting at 70 : 30 TFA buffer/acetonitrile, ramping to 30 : 70 TFA buffer/acetonitrile from 1–4 min and holding at 70 : 30 TFA buffer/acetonitrile from 4–5 min. For semipreparative scale HPLC, a 10 mm × 250 mm Hamilton PRP-1 column with a 1 mL injection volume was used, at a flow rate of 4 mL/min. Mass spectroscopy, elemental analysis, 250 MHz and 500 MHz NMR were performed at the Department of Chemistry, University of Pennsylvania. Lipofectamine reagent (Gibco) containing a 3 : 1 (w/w) 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, at 6 μg-lipid/μg-DNA. The plasmid pEGFP-N3 (Clontech) was purified with an endotoxin-free Maxi-Prep kit (Eppendorf) and used for expression of enhanced green fluorescent protein (EGFP) under the regulation of a cytomegalovirus (CMV) promoter.

Synthesis of *N*-[3-({4-[(3-aminopropyl) amino]butyl}amino)propyl]-4-[(3-oxopregna-1-en-21-yl)sulfanyl] butanimidamide-TFA salt (4, 11-deoxycorticosterone-spermine)

A 25 mL round-bottomed flask equipped with a magnetic stirring bar and rubber septum was purged with nitrogen and charged with 100 mg (245 μmol, 1 eq.) of 11-deoxycorticosterone-21-mesylate, 2 mL of ethanol, and 248 μL spermine (1.10 mmol, 4.5 eq.) and cooled to 0 °C in an ice-bath. The solution was stirred and treated dropwise over 5 min with a solution of 33.8 mg of 2-iminothiolane (Traut's reagent) (245 μmol, 1 eq.) in 240 μL of deionized water. The solution changed color from clear to clear light yellow. The reaction was monitored by TLC and by analytical HPLC. After 45 min, the TLC spots at retention time (R_t) = 0 (the charged species) were maximized. The crude reaction mixture was treated with 20 eq. of TFA (to neutralize spermine amines) and solvent was removed with a rotary evaporator leaving a viscous clear light yellow solution. Deionized water (~1.5 mL) was added to bring the volume of the crude reaction mixture to 2 mL, and the product was purified with preparative scale HPLC (two runs of 1 mL each). The main product (**4**) (R_t = 4.5–8.5 and 3.6–6.0 min) was pooled and acetonitrile was removed with a rotary evaporator leaving ~20 mL of solution. The solution was freeze-dried overnight with a lyophilizer leaving a white hygroscopic powder to which water was added to bring the final volume to 1 mL. The

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product (**4**) was purified with preparative scale HPLC (1 mL injection) and the product at $R_t = 5.7–9.5$ min (preparative HPLC) was pooled, solvent (acetonitrile) was removed with a rotary evaporator, and the solution was transferred to a 50 mL plastic tube and freeze-dried overnight to yield a hygroscopic white powder. TLC (50 : 50 hexane/THF) $R_t = 0$, analytical HPLC $R_t = 0.7$ min. Yield: 64.7 mg (60.4%, calculated as the tetra-TFA salt); ^{1}H NMR (250 MHz, CD_3OD with TMS): $\delta = 6.45$ (d, 1H, $J = 68$ Hz, amidine NH), 5.71 (s, 1H, 4), 3.90 (m, 10H, spermine CH_2N), 3.54 (m2H, 21), 3.38 (t, 2H, $J = 5$ Hz, amidine CH_2N (C25)), 3.12 (m, 2H, CH_2N (spermine methylene adjacent to amidine)), 2.91 (t, 2H, $J = 7$ Hz, C23 (iminothiolane linker)), 2.58 (m, 2H, C24 (iminothiolane linker)), 2.48 (m, 1H, 6b), 2.48 (m, 1H, 17aH), 2.44 (m, 1H, 2b), 2.34 (m, 1H, 6a), 2.27 (m, 1H, 2a), 2.21 (m, 1H, 16b), 2.08 (m, 5H, NH), 2.03 (m, 1H, 1b), 1.92 (m, 1H, 12b), 1.87 (m, 1H, 7b), 1.79 (m, 8H, spermine CH_2), 1.77 (m, 1H, 15a), 1.70 (m, 1H, 1a), 1.69 (m, 1H, 16a), 1.64 (m, 1H, 11a), 1.63 (m, 1H, 12a), 1.61 (m, 1H, 8), 1.46 (m, 1H, 11b H), 1.33 (m, 1H, 15b), 1.23 (s, 3H, 19), 1.17 (m, 1H, 14), 1.10 (m, 1H, 7a), 1.03 (m, 1H, 9), 0.70 (s, 3H, 18); HRMS (FAB$^+$) $C_{35}H_{62}N_5O_2S$: $[M + H]^+$ calcd 616.4624; found 616.4643.

Synthesis of *N*-[3-({4-[(3-aminopropyl) amino]butyl}amino)propyl]-4-[(3-oxopregna-11-hydroxy-1-en-21-yl)sulfanyl]butanimidamide-TFA salt (5, corticosterone-spermine)

Similar to the preparation of **4**, 100 mg (236 μmol, 1 eq.) of corticosterone-21-mesylate, 238 μL spermine (1.06 mmol, 4.5 eq.), and 32.6 mg of 2-iminothiolane (Traut's reagent) (236 μmol, 1 eq. in 326 μL water) gave a hygroscopic white powder. TLC (50 : 50 hexane/THF) $R_t = 0$, analytical HPLC $R_t = 0.7$ min, preparative HPLC $R_t = 5.1–8.5$ min. No melting point was determined. Yield: 80.8 mg (74.3%, calculated as the tetra-TFA salt); ^{1}H NMR (250 MHz, CD_3OD with TMS): $\delta = 6.39$ (d, 1H, $J = 66$ Hz, amidine NH), 5.66 (s, 1H, 4), 4.90 (s, 1H, 11b-OH), 4.35 (m, 1H, 11a), 3.90 (m, 10H, spermine CH_2N), 3.54 (m, 2H, 21), 3.38 (t, 2H, $J = 5$ Hz, amidine CH_2N (C25)), 3.12 (m, 2H, CH_2N (spermine methylene adjacent to amidine)), 2.91 (t, 2H, $J = 7$ Hz, C23 (iminothiolane linker)), 2.58 (m, 2H, C24 (iminothiolane linker)), 2.51 (m, 1H, 6b), 2.45 (m, 1H, 2b), 2.45 (m, 1H, 17aH), 2.34 (m, 1H, 2a), 2.28 (m, 1H, 12b), 2.23 (m, 1H, 6a), 2.18 (m, 1H, 1b), 2.13 (m, 1H, 16b), 2.08 (m, 5H, NH), 2.03 (m, 1H, 7b), 1.95 (m, 1H, 8), 1.87 (m, 1H, 1a), 1.79 (m, 8H, spermine CH_2), 1.75 (m, 1H, 15a), 1.70 (m, 1H, 16a), 1.63 (m, 1H, 12a), 1.46 (s, 3H, 19), 1.36 (m, 1H, 15b), 1.19 (m, 1H, 7a), 1.11 (m, 1H, 14), 1.06 (m, 1H, 9), 0.89 (s, 3H, 18); HRMS (FAB$^+$) $C_{35}H_{62}N_5O_3S$: $[M + H]^+$ calcd 632.4573; found 632.4599.

Synthesis of *N*-[3-({4-[(3-aminopropyl) amino]butyl}amino)propyl]-4-[(3-oxopregna-17-hydroxy-1-en-21-yl)sulfanyl]butanimidamide-TFA salt (6, 11-deoxycortisol-spermine)

Similar to the preparation of **4**, 100 mg (236 μmol, 1 eq.) of 11-deoxycortisol-21-mesylate, 238 μL spermine (1.06 mmol, 4.5 eq.), and 32.6 mg of 2-iminothiolane (Traut's reagent) (236 μmol, 1 eq. in 326 μL water) gave a hygroscopic white powder. TLC (50 : 50 hexane/THF) $R_t = 0$, analytical HPLC $R_t = 0.7$ min, preparative HPLC $R_t = 6.1–9.5$ min. Yield: 79.6 mg (73.2%, calculated as the tetra-TFA salt); ^{1}H NMR (250 MHz, CD_3OD with TMS): $\delta = 6.46$ (d, 1H, $J = 66$ Hz, amidine NH), 5.72 (s, 1H, 4), 3.90 (m, 10H, spermine CH_2N), 3.75 (d, $J = 16$ Hz, 2H, 21), 3.46 (d, $J = 16$ Hz, H, 21), 3.38 (t, 2H, $J = 5$ Hz, amidine CH_2N (C25)), 3.12 (m, 2H, CH_2N (spermine methylene adjacent to amidine)), 2.69 (m, 1H, 16b), 2.66 (m, 2H, C23 (iminothiolane linker)), 2.65 (s, H, 17a-OH), 2.58 (m, 2H, C24 (iminothiolane linker)), 2.50 (m, 1H, 6b), 2.42 (m, 1H, 2b), 2.34 (m, 1H, 6a), 2.30 (m, 1H, 2a), 2.08 (m, 5H, NH), 2.02 (m, 1H, 1b), 1.92 (m, 1H, 15a), 1.89 (m, 1H, 7b), 1.79 (m, 8H, spermine CH_2), 1.75 (m, 1H, 12a), 1.73 (m, 1H, 14), 1.71 (m, 1H, 1a), 1.70 (m, 1H, 11a), 1.63 (m, 1H, 8), 1.58 (m, 1H, 16a), 1.49 (m, 1H, 12b), 1.45 (m, H, 11b H), 1.37 (m, 1H, 15b), 1.23 (s, 3H, 19), 1.13 (m, 1H, 7a), 1.00 (m, 1H, 9), 0.68 (s, 3H, 18); HRMS (FAB$^+$) $C_{35}H_{62}N_5O_3S$: $[M + H]^+$ calcd 632.4573; found 632.4564.

Synthesis of *N*-[3-({4-[(3-aminopropyl) amino]butyl}amino)propyl]-4-[(3-oxopregna-17-hydroxy-1-en-21-yl)sulfanyl]butanimidamide-TFA salt (7, cortisol-spermine)

Similar to the preparation of **4**, 100 mg (227 μmol, 1 eq.) of 11-cortisol-21-mesylate, 229 μL spermine (1.02 mmol, 4.5 eq.), and 31.3 mg of 2-iminothiolane (Traut's reagent) (227 μmol, 1 eq. in 313 μL water) gave a hygroscopic white powder. TLC (50 : 50 hexane/THF) $R_t = 0$, analytical HPLC $R_t = 0.7$ min, preparative HPLC $R_t = 5.6–10.8$ min. No melting point was determined. Yield 82.0 mg (74.3% (calculated as tetra-TFA salt)); ^{1}H NMR (250 MHz, CD_3OD with TMS): $\delta = 6.38$ (d, 1H, $J = 65$ Hz, amidine NH), 5.66 (s, 1H, 4), 4.96 (s, 1H, 11b-OH), 4.39 (m, 1H, 11a), 3.90 (m, 10H, spermine CH_2N), 3.78 (d, 1H, $J = 16$ Hz, 21), 3.43 (d, 1H, $J = 16$ Hz, 21), 3.38 (t, 2H, $J = 5$ Hz, amidine CH_2N (C25)), 3.12 (m, 2H, CH_2N (spermine methylene adjacent to amidine)), 2.73 (m, 1H, 16b), 2.68 (m, 2H, C23 (iminothiolane linker)), 2.65 (s, 1H, 17a-OH), 2.60 (m, 2H, C24 (iminothiolane linker)), 2.50 (m, 1H, 6b), 2.45 (m, 1H, 2b), 2.34 (m, 1H, 2a), 2.29 (m, 1H, 6a), 2.11 (m, 1H, 1b), 2.08 (m, 5H, NH), 2.06 (m, 1H, 8), 2.03 (m, 1H, 7b), 2.01 (m, 1H, 12a), 1.87 (m, 1H, 1a), 1.79 (m, 8H, spermine CH_2), 1.72

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(m, 1H, 15a), 1.66 (m, 1H, 14), 1.51 (m, 1H, 12b), 1.47 (m, 1H, 16a), 1.46 (s, 3H, 19), 1.37 (m, 1H, 15b), 1.14 (m, 1H, 7a), 0.99 (m, 1H, 9), 0.89 (s, 3H, 18), HRMS (FAB^+) $C_{35}H_{62}N_5O_4S$: $[M + H]^+$ calcd 648.4523; found 648.4555.

Synthesis of *N*-[3-({4-[(3-aminopropyl)amino]butyl}amino) propyl]-4-[(9-fluoro-11,17-dihydroxy-16-methyl-3,20-dioxopregna-1,4-dien-21-yl)sulfanyl]butanimidamide-TFA salt (3, dexamethasone-spermine, DS)

A 15×85 mm test tube equipped with a magnetic stirring bar and rubber septum was purged with nitrogen and charged with 105 mg (223 μmol, 3 eq.) of dexamethasone-mesylate, 800 μL of DMSO, and 28.4 mg (206 μmol, 2.8 eq.) of Traut's reagent. After the contents of the solution had dissolved, 31.9 μL (145 μmol, 2 eq.) of spermine were added via pipette over 10 s at room temperature. The solution immediately changed color from clear to clear light yellow. The reaction was monitored by TLC and by analytical HPLC. After 45 min, the TLC spot at $R_t = 0$ (the charged DS) ($R_t = 0.7$ min DS by analytical HPLC) was maximized, and dexamethasone-mesylate (R_t 0.47, 2.8 min HPLC) spot minimized. The crude reaction mixture was diluted with 2 eq. of TFA and water was added to bring the volume of the crude reaction mixture to 1 mL, and purified with preparative scale HPLC. DS ($R_t = 7$ min) was collected with a Pharmacia fraction collector. The tubes containing product were consolidated and acetonitrile was removed under reduced pressure with a rotary evaporator leaving a clear liquid. The resulting DS solution was lyophilized overnight to yield a white powder. Yield: 15.3 mg (14.0%). TLC $R_t =$ 0, 50 : 50 hexane/THF, analytical HPLC $R_t = 0.7$ min. ^{1}H NMR (500MHz, DMSO) $\delta = 9.54$ (s, 1H, spermine NH), 9.16 (s, 1H, spermine NH), 8.75 (s, 2H, spermine NH2), 7.30 (d, J = 0.02, 1H, C1), 6.23 (d, J = 0.023, 1 H, C2), 6.01 (s, 1 H, C4), 5.33 (d, J = 0.01, 1 H, C17-OH), 5.04 (s, 1 H, C11-OH), 4.14 (d, J = 0.02, 1 H, C11-OH), 3.63 (dd, J = 0.39, 0.03,2 H, C21), 3.37 (s, 20 H, spermine), 3.27 (s, 2 H, spermine), 3.93 (s, 4 H, spermine), 2.61 (m, 1 H, C6a), 2.37 (m, 1 H, C6b), 2.33 (m, 1 H, C8), 2.17 (d, J = 0.02, 1 H, C12), 2.1 (q, J = 0.02, 1 H,C14), 1.87 (m, 4 H, spermine), 1.78 (m, J = 0.01, 1 H, C7a), 1.63 (m, 2 H, spermine), 1.58 (m, 1 H, C15a), 1.48 (s, 3 H, C19), 1.46 (s, 1 H, C7), 1.07 (m, 1 H, C15b), 0.87 (s, 3 H, C18), 0.78 (d, 3 H, C22); HRMS (FAB^+) $C_{38}H_{63}FN_5O_4S$: $[M + H]^+$ calcd 678.4428, found 678.4429. For the hydrolysis product: calcd C: 63.26, H: 7.35, N: 2.84; found C: 63.44, H: 7.27, N: 2.83.

Cell culture

Bovine aortic endothelial cells (BAEC, passage 4–13) were grown to confluence, passed at a 1 : 4 split to 24-well plates, and then grown to 90% confluence for 2 days prior to lipofection. Growth medium was Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated charcoal-filtered fetal calf serum (Hyclone, to remove steroids), 0.30 mg/mL glutamine, 150 U/mL penicillin and 0.15 mg/mL streptomycin (Gibco).

Liposome and lipoplex formulation

Each cationic lipid was dissolved in ethanol to make 100 mM stock solutions. The stock solution of the cationic steroid lipid and DOPE (25 mg/mL in chloroform) were added in steroid : DOPE mole ratios of 2 : 1, 2 : 2, and 2 : 4 ratios in an Eppendorf tube, and solvent was evaporated to a lipid film with a nitrogen stream. The resulting lipid film was rehydrated with sterile deionized water (Millipore) and probe-sonicated for 10 min to yield a final concentration of liposomes that were 2 mM in cationic lipid. The solutions were stored at 4 °C and retained lipofection activity for over 6 months (data not shown). Lipoplexes were mixed in 96-well plates (Nunc, nontreated U-bottomed polyethylene) in triplicate as follows. To 150 μL of Optimem solution were added 0.375 to 9 μL of 2 mM cationic liposomes (to give final charge ratio of 2 to 24 cations per DNA phosphate anion). Then 150 μL of a stock solution of pEGFP in Optimem (1 μg/150 μL) were added to each well giving a total volume of lipoplex solution of 300 μL. The complexes were allowed to incubate for 30 min at room temperature before addition to cells or measurement with dynamic light scattering. The hydrodynamic diameter of liposomes and lipoplexes was obtained by dynamic light scattering (DynaPro 99 instrument). Liposomes were measured at a concentration of 2 mM in cationic lipid at room temperature. Lipoplexes were measured at a concentration of 1 μg/mL pEGFP DNA.

Transfection and measurement of EGFP

Media from BAEC in 24-well plates was removed and cells were washed three times with 300 μL of PBS. Transfections were performed in triplicate and each plate included a blank (untreated cells) as negative control and two wells treated with Lipofectamine (6 μg/μg DNA) as positive control. Lipoplexes were added to each well and allowed to incubate at 37 °C for 2 h. Media was then replaced with cell culture media and cells were incubated at 37 °C. EGFP expression was monitored in triplicate with a fluorescent plate reader (Labsystems Fluoroskan Ascent; 485 nm/510 nm filter pair) with background subtraction using the autofluorescence of nontransfected BAEC. EGFP fluorescence was measured after 24 and 48 h by removing media with 3×300 μL washings of PBS replacing with 300 μL of growth media. Full signal (fluorescence intensity (FI) = 30) corresponded to ~75 ng GFP per well using recombinant protein for calibration.

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Results and discussion

Synthesis

We developed a synthetic route requiring no protecting groups, amenable to large-scale coupling of the multivalent cation, spermine, to lipophilic electrophiles. A one-pot reaction between spermine, 2-iminothiolane (Traut's reagent) and steroid-mesylate yielded the steroid-spermine conjugate as the major product (Figure 1). Traut's reagent is selectively ring-opened by the primary amines on spermine, forming a hydrolyzable amidine bond [19] between spermine and iminothiolane and a reactive thiolate anion that reacts with the α-keto mesylate on the 21-position of dexamethasone mesylate [20] to form an α-keto thioether between spermine and iminothiolane. The conjugation reaction was complete in under 45 min as indicated by HPLC. 2-Iminothiolane is unique in that it is one of the few electrophiles that becomes a strong and selective nucleophile during the reaction and requires no protecting groups. The conjugation reaction does not consume a cationic charge when coupled to a cationic amine (e.g. spermine).

^{1}H NMR analysis of all the conjugates confirmed the presence of the 3-enone and, if the parent steroid molecule had them, 11-OH and 17-OH groups [21–25], as well as ^{1}H signals from the conjugated spermine and the 21-α-keto thioether group. NMR evidence of conjugation was visible by the methylene hydrogens on the iminothiolane linkage adjacent to the amidine ($\delta = 3.38$), the methylene hydrogens on the spermine ($\delta = 3.12$) and the protonated amine on the amidine ($\delta \sim 6.4$). In addition, the 21-C methylenes were shifted upfield by about 1 ppm to ~3.4–3.6 due to the thioether bond formed. The four protonated amines were visible via NMR in a nonprotic solvent (e.g. DMSO), but not in protic solvents (e.g. methanol), illustrating that the spermine tail in DS retained four charges (a primary amine was converted into a protonatable amidine) for DNA complexation.

The series was based on analogs with increasing hydrophobicity of the steroid moiety: 11-deoxycorticosterone-spermine (**4**) was the most hydrophobic of the series (Log $P_{ster} = 3.01$) and cortisol-spermine (**7**) was the least hydrophobic (Log $P_{ster} = 1.51$). Averages of several empirical measurements of Log P_{ster} for each steroid were used [26–30] (Table 1).

Hydrolysis of DS in 1 M NaOH for 20 min resulted in the cleavage of the amidine linkage between spermine and iminothiolane, forming **3a**, a *d*examethasone-*a*mide (DA) (Figure 1), which has a 21-substituted butyl thioether amide side chain on dexamethasone. For **3a**: calcd C: 63.26, H: 7.35, N: 2.84; found C: 63.44, H: 7.27, N: 2.83. Moreover, the presence or absence of the cationic tail confers dramatically different physicochemical properties to the steroids. For example, the water solubility of DS (**3**) was greater than 100 000 mg/L, while the solubility of DA (**3a**) was ~60 mg/L (dexamethasone, 100 mg/L). Additionally, although hydrolysis of DS to DA is accelerated in 1 M NaOH, the amidine bond in DS appeared 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.

Structure and function of cationic steroid lipoplexes

The five cationic steroids were formulated with dioleoylphosphatidylethanolamine (DOPE) at cationic steroid : DOPE mole ratios of 2 : 1, 2 : 2, or 2 : 4, and then complexed with 1 μg EGFP plasmid DNA at charge ratios (CR) = 2 to 24 amines per phosphate (0.5 to 6 steroids per phosphate). To explore the role of Log P_{ster}, CR, and lipid : DNA ratio on lipoplex formation and function, we measured the hydrodynamic radii by light scattering and transgene expression (EGFP fluorescence per well) at 24 and 48 h post-lipofection. The liposomes (without DNA) ranged in size from 79 to 179 nm, largely uncorrelated with the tested range of cationic steroid : DOPE ratio or Log P_{ster}. For CR of 2 to 24 and cationic steroid : DOPE ratios of 2 : 1, 2 : 2, and 2 : 4, the lipoplexes with DNA ranged in hydrodynamic radius from 106 to 905 nm, with a mean radius of 390 nm (Figure 3A). In general, the lipoplex size tended to increase from a typical size of ~200 nm to a typical size of about 450 nm as the cationic steroid : DNA charge ratio was increased from 2 to 24 at the various steroid : DOPE ratios tested. However, no striking dependency of lipoplex size on Log P_{ster} was detected over the series of cationic steroids tested.

The EGFP expression in BAEC at 24 and 48 h (Figures 3B and 3C) for each cationic steroid displayed similar qualitative behavior for the various formulations across charge ratios and cationic steroid : DOPE ratios. In general, the more DOPE in the formulation, the higher the expression levels. As expected, the higher the charge ratio, the more active the formulation for lipofection. For the five lipids studied, a threshold CR was required for transfection to occur. Lipofectamine (LA) was used as a benchmark and many of the transfections using cationic steroids exceeded that achieved with this commercially available DOSPA/DOPE formulation.

Structure-activity relationships for cationic steroids

The hydroxyl groups on the steroid clearly had a strong effect on transfection efficiency. From Figures 4A–4C it is apparent that a sufficiently hydrophobic moiety as indicated by Log P_{ster} was required for optimal lipofection. Plotting the EGFP expression data at 48 h as a function of Log P_{ster} organized the EGFP data into clear trends, so that at equal charge ratios and co-lipid ratios, the EGFP expression predictably increased as a function of Log P_{ster}. EGFP expression increased with increasing Log P_{ster} until reaching high combined levels of Log P_{ster}, CR,

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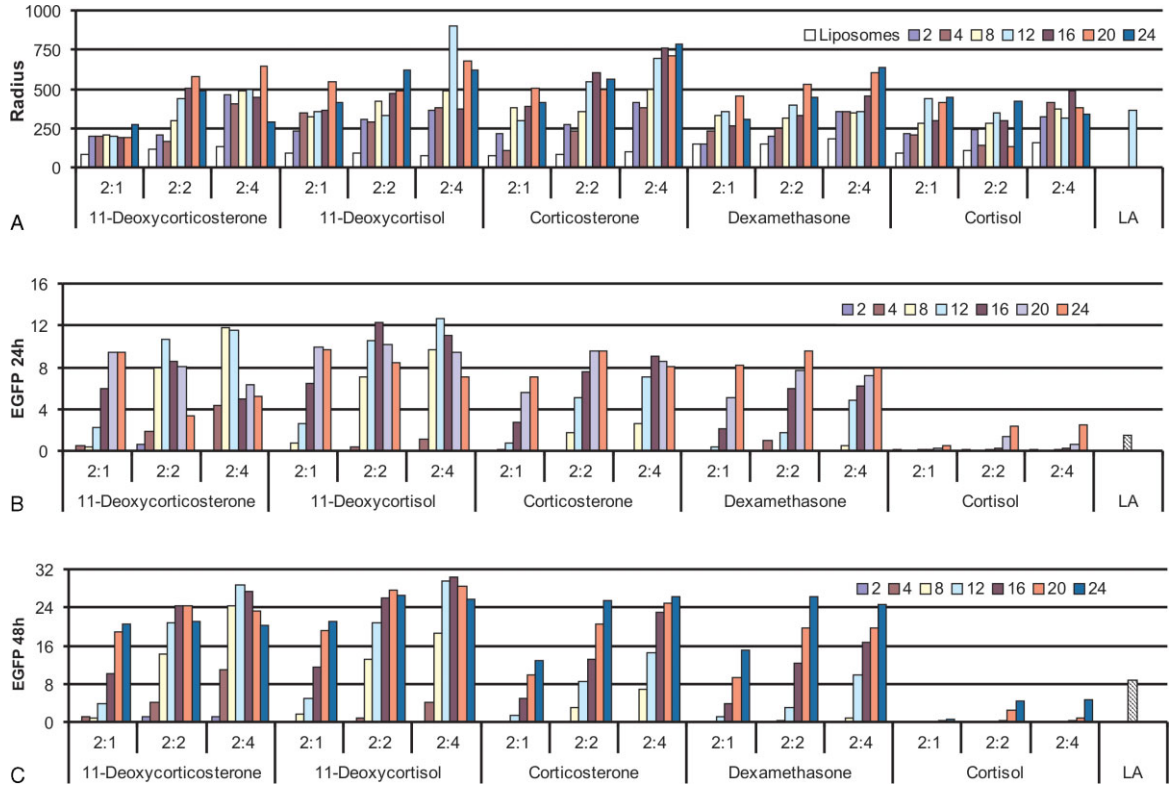

Figure 3. Structure and activity of a series of cationic steroid : DOPE liposomes and lipoplexes and Lipofectamine (LA) for DNA packaging and transfection in confluent BAEC. Dynamic light scattering (A) is given for cationic steroid : DOPE ratios of 2 : 1, 2 : 2, 2 : 4 (R^{-1} = 2, 1, 0.5, respectively) and amine : phosphate ratios (CR) ranging from 2 to 24. EGFP expression (fluorescence intensity, FI) at 24 h (B) and 48 h (C) is given for each lipoplex formulation tested in (A) (FI = 30 corresponded to ~75 ng GFP/well)

and co-lipid ratios, at which point EGFP expression was maximal or began to decrease, particularly for the most hydrophobic cationic lipid **4**.

Plotting EGFP expression versus CR for the series illustrated the general trends of the series and the importance of Log P_{ster} (Figures 5A and 5B). Using this data, we tested empirical dimensionless parameters for correlating the behavior of transfection formulations. Such a parameter should ideally account for the most relevant biophysics and biochemistry: charge ratio (amine/phosphate ratio), hydrophobicity of the steroid (log P_{ster}), hydrophobicity of the neutral lipid, DOPE (log $P_{DOPE} = 6$), the hydrophobicity of the resulting DOPE/cationic lipid formulations, the number of lipids relative to each DNA phosphate, and the match between the hydrophobicity of DOPE and the steroid. We defined the lipofection index (LI) as:

$$LI = CR\ (\text{Log } P_{liposome})(\text{Log } P_{ster}/|\Delta \text{Log } P|)[R/(R+1)] \quad (1)$$

where $\Delta \text{Log } P = \text{Log } P_{DOPE} - \text{Log } P_{ster}$ and Log $P_{liposome}$ is the mole-weighted average of the DOPE/cationic steroid liposome hydrophobicity given by:

$$\text{Log } P_{liposome} = (n_{ster}\text{Log } P_{ster} + n_{DOPE}\text{Log } P_{DOPE})/(n_{ster} + n_{DOPE}) \quad (2)$$

where n_{Ster} and n_{DOPE} are the number of steroid molecules and DOPE molecules per phosphate in the DNA, respectively. The first term in Eqn. (1) is the charge ratio relative to DNA. The second term is the effective Log P of the lipid components of the cationic liposome mixture. The third term accounted for the hydrophobicity of the cationic lipid, i.e. its ability to interact with both DOPE and cellular lipids. The final term accounted for the relative amount of DOPE in the formulation where R is the DOPE : steroid mole ratio which ranged from 1 : 2, 2 : 2, and 4 : 2 (i.e. R = 0.5, 1 and 2, respectively).

Equation (1) was developed by evaluation of the top seven formulations based on the two most hydrophobic lipids (**4** and **5**) where LI ranged from 26.7 to 40.3. The average parameters of the seven best formulations were: Log $P_{steroid} = 2.79$, CR = 12.57, Log $P_{liposome} = 4.7$, DOPE:steroid = 1.57, Log $P_{liposome}$(CR) = 58.8, liposome size R_h = 102 nM, lipoplex size R_h = 499 nm, and viability >75%. In these top performing formulations, the DOPE : steroid ratio was at least 1, suggesting a potential metric for fusion potential. Also, the best formulations had intermediate CR values so that the total dose of lipid was in an intermediate range, thus reducing toxicity. Also, the R_h values of the liposomes were fairly small compared to other less effective formulations, suggesting a benefit in good lipid mixing. An additional insight guided the development of the equation for LI: At the limits of no cationic lipid or no DOPE, the LI predicts no transfection, which accurately reflects the facts that the cationic lipid

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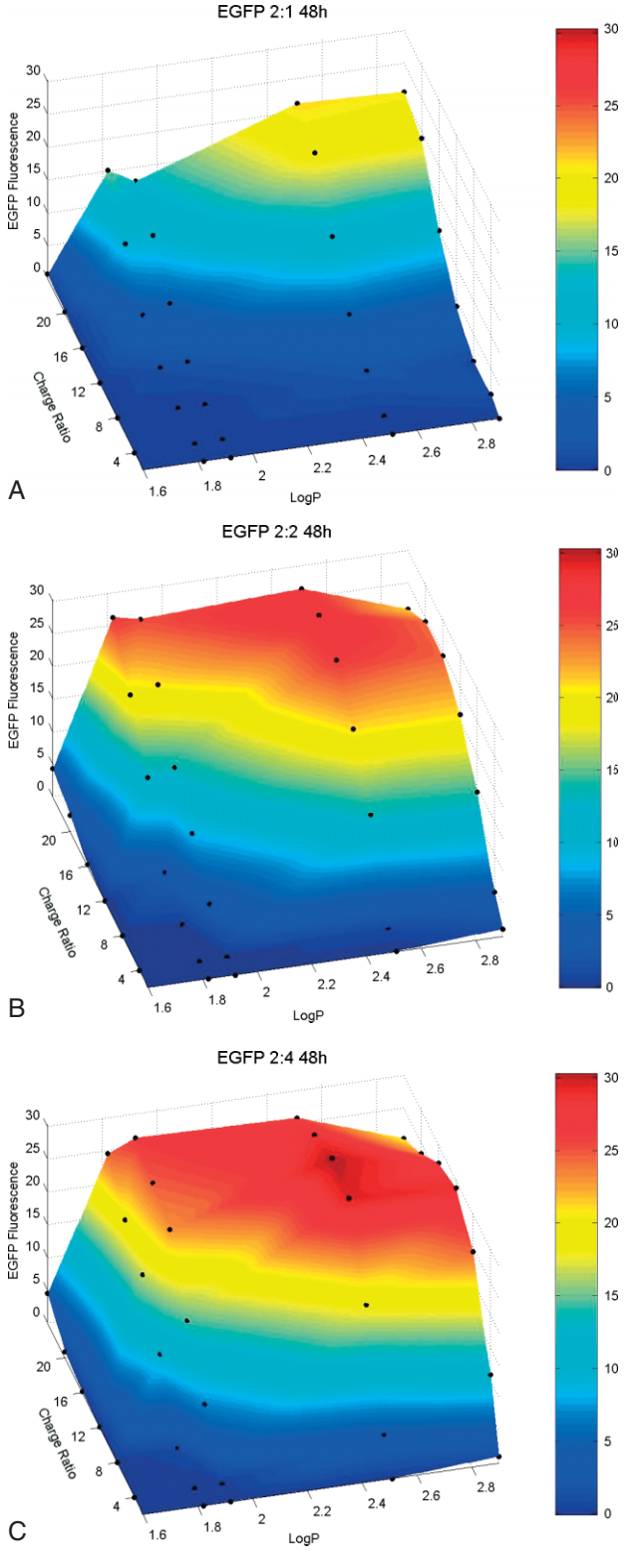

Figure 4. (A) EGFP expression in BAEC at 48 h post-transfection as a function of Log P_{ster} and charge ratio (CR) at steroid cation : DOPE ratio of 2 : 1 ($R^{-1} = 2$). (B) EGFP expression in BAEC at 48 h post-transfection as a function of Log P_{ster} and CR at steroid cation : DOPE ratio of 2 : 2 ($R^{-1} = 1$). (C) EGFP expression in BAEC at 48 h post-transfection as a function of Log P_{ster} and CR at steroid cation : DOPE ratio of 2 : 4 ($R^{-1} = 0.5$)

requires a neutral helper lipid and that DOPE, by itself, is not a good DNA delivery agent.

We observed that particle size increased with charge ratio (Figure 3A) and transfection also increased with charge ratio (Figure 3B). However, we found that transfection was most directly proportional to CR with no statistical gain in the LI correlation by using particle size. For example, 11-deoxycorticosterone-spermine : DOPE at a steroid : DOPE ratio of 2 : 1 produced relatively small lipoplexes ($R_h \sim 175$ nm) but gave high gene expression at CR = 20–24. Increasing the steroid : DOPE ratio produced much larger lipoplexes ($R_h > 500$ nm) but similar amounts of gene transfer. Thus, we found no consistent correlation of size and transfection across all the formulations.

The transfection data at either 24 or 48 h (Figures 5A and 5B) was collapsed onto a single curve when plotted against the lipofection index, LI (Figures 5C and 5D). The LI varied from 0.67 for the least effective lipid (i.e. cortisol-spermine : DOPE at the lowest CR and R tested) to 80.59 for 11-deoxycorticosterone-spermine (**4**) at the highest CR and R tested. At the limits of very low cationic lipid or DOPE (LI < 7), the equation predicted near-zero expression, which accurately reflected the facts that the cationic lipids required DOPE for activity and DOPE by itself is not a good DNA delivery agent. A threshold level of DOPE and cationic lipid to provide for LI = 7 was needed for detection of threshold levels of EGFP expression. In the range of 7 < LI < 29, expression after lipofection with **4**–**7** correlated linearly with LI at both 24 h and 48 h post-lipofection. While LI between 7 and 29 correlates with EGFP expression (Figures 5C and 5D), the LI is only partly predictive of 'percent transfection' since total EGFP expression as measured by fluorescence-activated cell sorting (FACS) increases across a population by increases in both the number of transfected cells (defined at a conservative cutoff of 5× or 10× above cellular autofluorescence [16]) and the transgene expression per cell.

The activity of cortisol-spermine (**7**) was unusually low even when plotted against LI (Figures 5C and 5D). The inactivity of the cortisol lipid was most likely due to both its overall small Log P and the 11-hydroxy group. This may be due to cortisol-spermine formulations not mixing into lipoplexes efficiently, phase separation of the cortisol-spermine lipid from the DOPE, or poor mixing with endosomal or membrane bilayers. Poor transfection with cortical-spermine (**7**) was not due to failure to form lipoplexes. We note that cortisol-spermine formed typically sized liposomes with DOPE (Figure 3A), comparable to the other cationic lipids of the series and that lipoplexes with cortisol-spermine : DOPE (CR = 2 to 24) were not any smaller than those observed with other members of the series (Figure 3A), demonstrating successful formation of lipoplexes.

Examination of EGFP expression at 48 h relative to expression at 24 h provided some insight into the underlying mechanisms of transfection with respect to the series (Figure 6A). EGFP expression at 48 h was always higher than at 24 h. In addition, the ratio of EGFP expression at 48 h to 24 h was at least 2. Three types of

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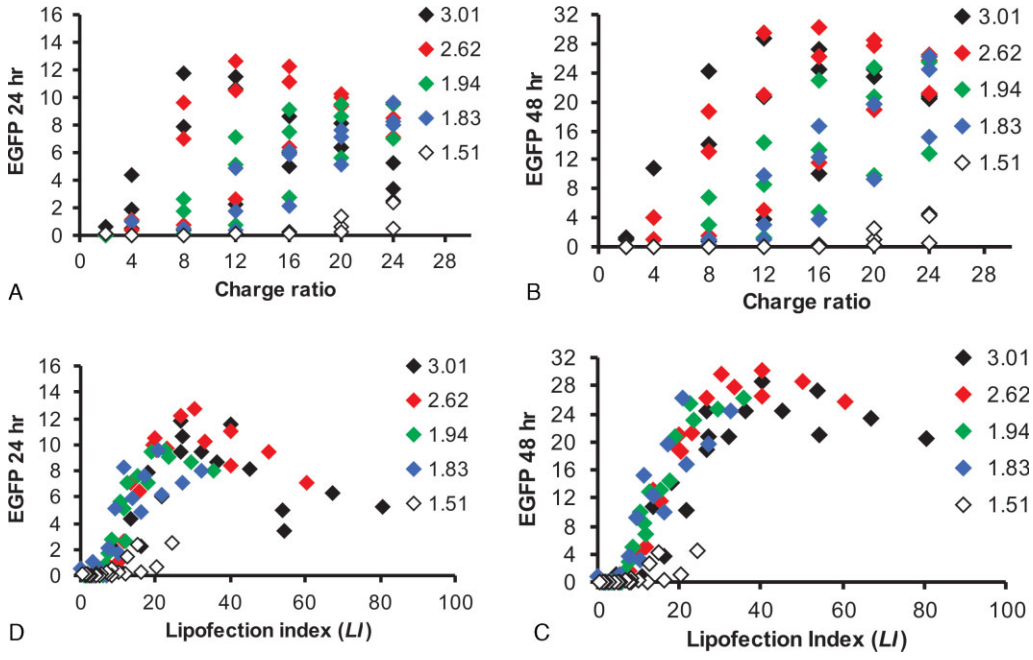

Figure 5. EGFP expression in BAEC at 24 h (A) or 48 h (B) post-transfection for cationic steroid-spermine conjugates. Expression data (A and B) can be remapped to a master curves (C and D) using the dimensionless parameter, lipofection index (LI) = CR (Log $P_{liposome}$)(Log P_{ster}/|ΔLog P|) [R/(R + 1)]

behavior were clustered by LI (Figure 6B). At intermediate LI ($7 < LI < 29$), the ratio of EGFP expression at 48 h to 24 h was 2.1 ($r^2 = 0.96$). This may be due to the formulations possessing good endosome escape capacity with continued and constant expression of plasmid. At low LI < 7, transfection was very low at 24 or 48 h (although expression still doubled from 24 to 48 h post-lipofection). At high LI >29, there was especially strong expression at 48 h relative to that at 24 h; the ratio of EGFP was significantly greater than 2 ($P < 0.01$). This may be due to the increased availability of plasmid for expression over the progress of the experiment from 24 h to 48 h, potentially caused by high lipid load and high Log P_{ster} achieved at LI >29 with consequent effects on endosomal loading, endosome escape, or cell cycle dynamics. However, at values of LI >40, this formulation regime offered little gain in expression at the expense of elevated reagent consumption and lipid load on the cells. Cell viability was measured by the MTT assay for both liposomes and lipoplexes. We found no difference between the viability of cells treated with liposomes vs. lipoplexes. For LI up to 29, the viability was between 70 and 85% across the different formulations. Viability decreased to a level of 50–65% when LI was increased from 30 to 80.

Lipofection reagents that are effective in the presence of serum typically contain pegylated-DOPE. As expected, the cationic sterol : DOPE formulations did not perform well in the presence of serum. We have not studied the formulation of cationic steroid lipid : PEG-DOPE. However, in our earlier studies with intranasal delivery to mouse lung, DS/DOPE functioned properly *in vivo* and more effectively than DC-Chol *in vivo* [16,17].

Conclusions

This report quantifies and correlates the transfection efficiency of lipids with respect to the Log P of the hydrophobic moiety of the cationic lipid. Log P, a central predictive parameter in pharmaceutical development, may also have utility for development of new gene transfer reagents. The higher the Log P of the lipid portion of the cationic steroid lipid, the more effective the transfection. A value of Log $P_{ster} = 1.51$ was too low for transfection (regardless of the amount of DOPE or cationic lipid used). The lipofection index (LI) may be applicable to a wider variety of cationic transfection lipids and may explain the potency of cholesterol-based cationic lipids. Also, LI correlated the transfection activity of a fluorine-containing cationic steroid, dexamethasone-spermine (**3**), amongst the **4**–**7** series. This report also showed that small structural changes in the cationic lipids, e.g. substitution of 11-OH or 17-OH, can lead to major changes in transfection activity.

Horobin and Weissig [31] conducted a quantitative structure-activity relationship (QSAR) analysis of numerous cationic lipids based on structural parameters such as charge, cation type, headgroup size, lipid size, total lipid Log P, amphipathic index and critical chain length. They found that commonly used cationic lipids had certain characteristics that were clustered in the parameter space, with a particular emphasis on mixing potential of the cationic lipid into membrane bilayers. However, their analysis made no effort to rank the series in terms of experimentally measured transgene expression in a given cell line as was done in the present study.

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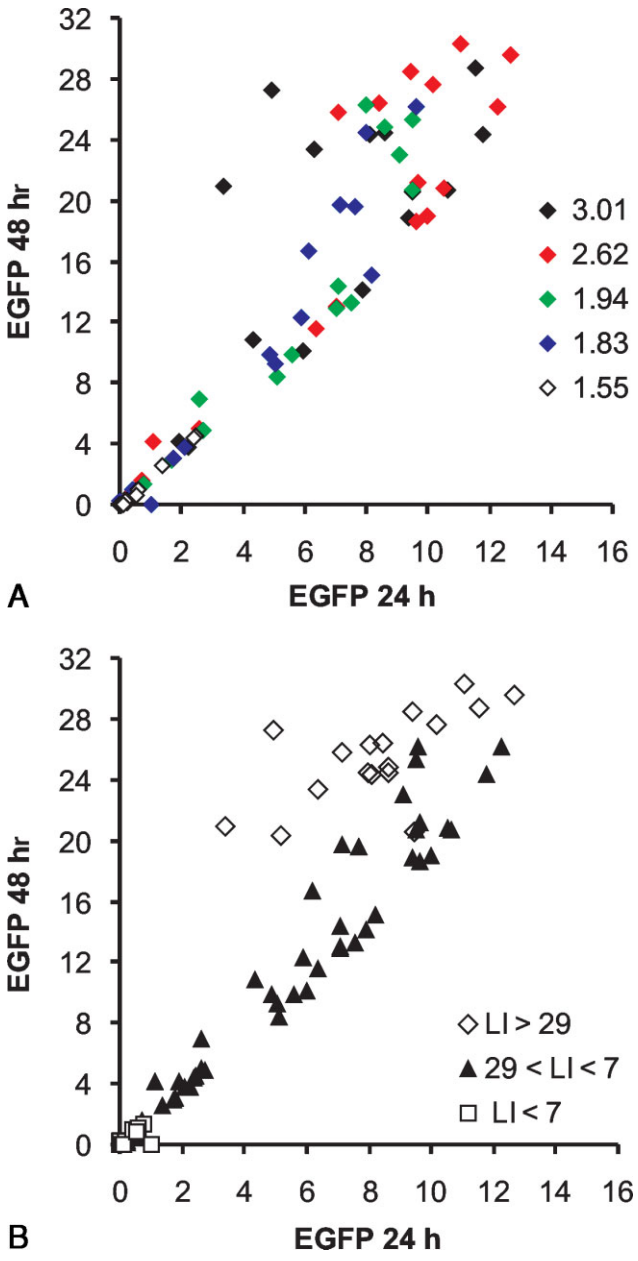

Figure 6. Expression of EGFP at 48 h relative to expression at 24 h as a function of Log P_{ster} (A). The lipofection index (LI) clustered the 48 h vs. 24 h expression data into three major groups. For LI < 7, expression remained low at all times, while for 7 < LI < 29, the EGFP expression at 48 h was simply twice the amount expressed at 24 h (2 : 1 line). For LI >29, expression at 48 h was significantly greater (typically 2.5–5×) than the expression at 24 h

We chose to use ~90% subconfluent BAEC for several reasons. BAEC are a common cardiovascular model cell line and are easy to manipulate in culture. BAEC, as mortal and untransformed cells, are considerably more difficult to lipofect compared to transformed cell lines (e.g. CHO, COS, 293, HeLa, or 3T3 cells) and provide a challenging *in vitro* model. BAEC are also representative of a relevant *in vivo* target, the endothelium. Additionally, BAEC are representative of other endothelial cell types that are frequently used in the pharmaceutical and biotechnology industries for drug development. Since lipofection of confluent BAEC results in decreased expression relative to subconfluent cells, studying the structure-activity relationships in ~90% subconfluent cells provided a higher signal of EGFP expression for analysis.

Dexamethasone-spermine retains glucocorticoid activity *in vitro* and *in vivo* [16,17] and we wanted to address the effect of pharmacological character on transfection performance. DS/DOPE liposomes at 10 to 1000 nM of steroid pharmacophore caused rapid nuclear localization of a glucocorticoid competent GFP-GR chimeric protein stably expressed in 3T3 cells (results not shown). Partial nuclear import of GFP-GR by 10 nM DS/DOPE, and complete nuclear import at 100 nM, suggested that the apparent K_D for DS was approximately 10-fold higher than that of dexamethasone ($K_D \sim 1$ nM) [18]. DS/DOPE induced dose-dependent transcription from a GRE promoter construct (pGRE-SEAP), displaying an EC50 of ~10 to 100 nM relative to dexamethasone in 293 cells (not shown). The fact that mineralcorticoid-based lipids work better than glucocorticoid-based lipids indicated that glucocorticoid potency was not required for good transfection. Thus, activation of the glucocorticoid receptor was not required for transfection. This suggests that the more hydrophobic lipids, which happen to be mineralcorticoids, also aided the function of DOPE in loading and disrupting endosomes. Although the most potent glucocorticoid lipid **3** was better than cortisol-based formulations, this was most likely due to the Log P of dexamethasone being higher than cortisol. This was supported by the fact that pre-treatment or post-treatment of cells with free dexamethasone to localize the GR in the nucleus had little effect on subsequent transfection by DS or lipofectamine (not shown). Thus, neither glucocorticoid activity nor nuclear import of the GR was necessary for high levels of gene delivery or expression when using cationic steroids.

Barriers to lipofection-based gene transfer vary significantly in different cell lines. Thus for each cell line and lipid formulation of study, it is common practice to conduct a multiparameter optimization with respect to DNA/cell, DNA/lipid, and cationic/neutral lipid. Only in cells that present biological or physicochemical barriers similar to BAEC would we expect the LI to be predictive of gene transfer. Even within a chemical series for a single cell line, the LI is not a perfect correlating parameter, as was seen for cortisol-spermine (**7**). Still, the calculation of LI may prove useful in correlating data and optimizing transfection for other types of cells or cationic lipid series.

Pharmacologically active steroids for gene delivery may allow high local levels of drug dosing at the site of DNA delivery. Although glucocorticoid activity is not needed for gene transfer action, the local delivery of steroid may be useful for delivering DNA by combating adverse immunological responses [13,14]. The present study indicates that LI ~30 produced optimal results for expression. This may have implications for delivery of oligonucleotides (e.g. RNAi and antisense oligos), delivery of DNA constructs, and design of new cationic

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lipids and their formulations by facilitating more efficient explorations of a potentially large transfection variable space.

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References

1. Felgner PL, Gadek TR, Holm M, *et al*. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci U S A* 1987; **84**: 7413–7417.
2. San H, Yang ZY, Pompili VJ, *et al*. Safety and short-term toxicity of a novel cationic lipid formulation for human gene therapy. *Hum Gene Ther* 1993; **4**: 781–788.
3. Safinya CR. Structures of lipid-DNA complexes: supramolecular assembly and gene delivery. *Curr Opin Struct Biol* 2001; **11**: 440–448.
4. Cooper RG, Etheridge CJ, Stewart L, *et al*. Polyamine analogues of 3B-[*N*-(*N*′,*N*′-dimethylaminoethane)carbamoyl]-cholesterol (DC-Chol) as agents for gene delivery. *Chem Eur J* 1998; **4**: 137–151.
5. Gao X, Huang L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem Biophys Res Commun* 1991; **179**: 280–285.
6. Scheule RK, St George JA, Bagley RG, *et al*. Basis of pulmonary toxicity associated with cationic lipid-mediated gene transfer to the mammalian lung. *Hum Gene Ther* 1997; **8**: 689–707.
7. Byk G, Dubertret C, Escriou V, *et al*. Synthesis, activity, and structure–activity relationship studies of novel cationic lipids for DNA transfer. *J Med Chem* 1998; **41**: 229–235.
8. Hafez IM, Maurer N, Cullis PR. On the mechanism whereby cationic lipids promote intracellular delivery of polynucleic acids. *Gene Ther* 2001; **8**: 1188–1196.
9. Zelphati O, Szoka FC Jr. Mechanism of oligonucleotide release from cationic liposomes. *Proc Natl Acad Sci U S A* 1996; **93**: 11493–11498.
10. Koltover I, Salditt T, Radler JO, Safinya CR. An inverted hexagonal phase of cationic liposome-DNA complexes related to DNA release and delivery. *Science* 1998; **281**: 78–81.
11. Lipinski CA, Lombardo F, Dominy BW, Feeney PJ. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv Drug Deliv Rev* 2001; **46**: 3–26.
12. Holmen SL, Vanbrocklin MW, Eversole RR, Stapleton SR, Ginsberg LC. Efficient lipid-mediated transfection of DNA into primary rat hepatocytes. *In Vitro Cell Dev Biol Anim* 1995; **31**: 347–351.
13. Nair RR, Rodgers JR, Schwarz LA. Enhancement of transgene expression by combining glucocorticoids and anti-mitotic agents during transient transfection using DNA-cationic liposomes. *Mol Ther* 2002; **5**: 455–462.
14. 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 Ther* 2001; **8**: 1562–1571.
15. Tan Y, Zhang JS, Huang L. Codelivery of NF-kappaB decoy-related oligodeoxynucleotide improves LPD-mediated systemic gene transfer. *Mol Ther* 2002; **6**: 804–812.
16. Gruneich JA, Price A, Zhu J, Diamond SL. Cationic corticosteroid for nonviral gene delivery. *Gene Ther* 2004; **11**: 668–674.
17. Price A, Limberis M, Gruneich JA, Wilson JM, Diamond SL. Targeting viral-mediated transduction to the lung airway epithelium with the anti-inflammatory cationic lipid dexamethasone-spermine. *Mol Ther* 2005; **12**: 502–509.
18. Schimmer BP, Parker KL. In *The Pharmacological Basis of Therapeutics*, Hardman JG, Limbird LE (eds). McGraw-Hill: New York, 1996; 1459–1485.
19. Hermanson G. *Bioconjugate Techniques*, Academic Press: London, 1996.
20. Simmons SJ, Pons M, Johnson DF. alpha-keto mesylate: a reactive, thiol-specific functional group. *J Org Chem* 1980; **45**: 3084–3088.
21. Furuta T, Eguchi N, Yokokawa A, Shibasaki H, Kasuya Y. Synthesis of multi-labeled cortisols and cortisones with (2)H and (13)C for study of cortisol metabolism in humans. *Steroids* 2000; **65**: 180–189.
22. Kouadio L, Bernard P, Sabot JF, Petit-Ramel MM, Barbier CM, Pinatel HJ. Structural analysis of 21-hydroxypregn-4-ene-3,20-dione (deoxycorticosterone) derivative compounds. II. Proton NMR spectra. *Steroids* 1988; **51**: 363–384.
23. Kirk DN, Toms HC, Douglas C, White K. A survey of the high-field 1H NMR spectra of the steroid hormones, their hydroxylated derivatives, and related compounds. *J Chem Soc Perkin Trans* 1990; **2**: 1567–1594.
24. Duddeck H, Rosenbaum D, Hani M, Elgamal A, Fayez MBE. High-field 1H and 13C NMR spectroscopy of some corticosteroids and related compounds. *Mag Res Chem* 1986; **24**: 999–1003.
25. Wong TC, Rutar V, Wang J. Study of 1H chemical shifts and couplings with 19F in 9a-fluorocortisol. Applications of a novel 1H-13C chemical shift correlation technique with homonuclear decoupling. *J Am Chem Soc* 1984; **106**: 7046–7051.
26. Alvarez-Nunez FA, Yalkowsky SH. Correlation between log P and ClogP for some steroids. *J Pharm Sci* 1997; **86**: 1187–1189.
27. Cramer RDI, Patterson DE, Bunce JD. Comparative molecular field analysis (CoMFA). 1. Effect of shape on binding of steroids to carrier proteins. *J Am Chem Soc* 1988; **110**: 5959–5967.
28. Lombardo F, Shalaeva MY, Tupper KA, Gao F, Abraham MH. ElogPoct: a tool for lipophilicity determination in drug discovery. *J Med Chem* 2000; **43**: 2922–2928.
29. Moss GP, Cronin MT. Quantitative structure-permeability relationships for percutaneous absorption: re-analysis of steroid data. *Int J Pharm* 2002; **238**: 105–109.
30. Tomida H, Yotsuyanagi T, Ikeda K. Solubilization of steroid hormones by polyoxyethylene lauryl ether. *Chem Pharm Bull* 1978; **26**: 2832–2837.
31. Horobin RW, Weissig V. A QSAR-modeling perspective on cationic transfection lipids. 1. Predicting efficiency and understanding mechanisms. *J Gene Med* 2005; **7**: 1023–1034.