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Cationic facial amphiphiles: A promising class of transfection agents

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ABSTRACT A promising class of compounds for DNA transfection have been designed by conjugating various polyamines to bile-acid-based amphiphiles. Formulations containing these compounds were tested for their ability to facilitate the uptake of a β-galactosidase reporter plasmid into COS-7 cells. Dioleoyl phosphatidyl ethanolamine (DOPE) formulations of some of the compounds were several times better than Lipofectin at promoting DNA uptake. The most active compounds contained the most hydrophilic bile acid components. The activity is clearly not related to affinity for DNA: the hydrophobic bile acid conjugates were found to form stable complexes with DNA at lower charge ratios than the hydrophilic conjugates. We suggest that the high activity of the best compounds is related to their facial amphiphilicity, which may confer an ability to destabilize membranes. The success of these unusual cationic transfection agents may inspire the design of even more effective gene delivery agents.

Gene therapy is an exciting approach to the treatment of genetic defects, as well as diseases such as cancer and chronic viral infections (1–3). Unfortunately, the enthusiasm initially displayed for gene therapy has been tempered by the realization that there are no easy solutions to the problem of how to get genes into cells. The most efficient methods for transferring DNA across cell membranes involve the use of viral vectors (1, 4, 5); however, there are growing concerns about both the short- and long-term risks of viral vectors. These concerns have prompted a search for other strategies for DNA delivery, and in the past few years, a variety of nonviral gene delivery systems have been investigated (6–9). Although some success in getting DNA into cells has been achieved, gene delivery with nonviral vectors remains an inefficient process. To make gene therapy a reality, more efficient DNA delivery systems are needed. In this paper, we report the design and preliminary evaluation of a promising class of DNA delivery agents.

In designing these delivery agents, we started by considering the properties of existing nonviral delivery systems. Of all the nonviral DNA delivery systems that have been explored, cationic lipids have shown the most promise based on a combination of efficacy, stability, and toxicity. Lipofectin (Fig. 1), a 1:1 mixture of the cationic lipid *N*-[1,2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) and the fusogenic lipid dioleoyl phosphatidylethanolamine (DOPE), was the first cationic lipid formulation to receive widespread attention as a gene delivery agent (10). Since its introduction in 1987, many other cationic lipid formulations have been tested (11–15). The mechanism by which cationic lipid formulations promote DNA uptake is not well understood, but a model for how they function is beginning to emerge from the experimental data (6, 16). It is believed that cationic lipids

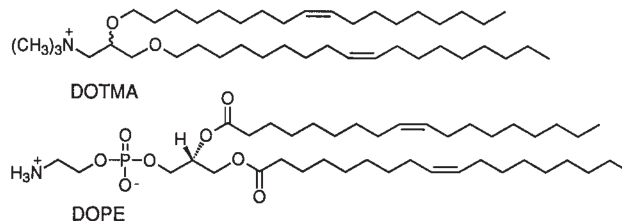

FIG. 1. Components of Lipofectin. DOTMA, *N*-[1,2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride.

interact with the negatively charged phosphate backbone of DNA, neutralizing the charge and promoting collapse of the DNA into a more compact structure. Because the resulting DNA–cationic lipid particles have a net positive charge, they interact with negatively charged biological membranes. What happens next is unclear, but somehow the DNA–lipid particle enters the cell. Entry may occur directly through the plasma membrane or via an intermediate endosome (12, 15, 16). Because many cationic delivery formulations require the presence of a phosphatidylethanolamine capable of destabilizing bilayer membranes and promoting membrane fusion (e.g., DOPE; Fig. 1), it is believed that the DNA–lipid particle must fuse with and/or destabilize the plasma membrane or the endosomal membrane to enter the cytoplasm.

Because there is no clear understanding of what happens to functionally active transfecting particles at the membrane surface, it is difficult to design better chemical delivery agents. Although a cationic component is necessary in any effective gene delivery agent, there is no prescription for what other structural features should be included in such a molecule. It has generally been assumed that the cationic component should be attached to a nonpolar tail, and double- and single-chain lipids as well as cholesterol have been used as nonpolar tails (16). The double-chain lipids have shown the greatest efficacy. Nevertheless, recent evidence suggests that other structural motifs may work as well or better.

For example, Legendre and Szoka (17) have found that a mixture of DOPE and a cationic amphiphilic peptide known to permeabilize membranes facilitates uptake of DNA into some cell types better than Lipofectin. Amphiphilic peptides are unusual as amphiphiles because the hydrophilic and hydrophobic regions are segregated along the long axis of the molecules (Fig. 2*a*). This facially amphiphilic arrangement influences the way in which amphiphilic peptides interact with membranes and is partly responsible for their ability to permeabilize membranes at low concentrations and promote membrane fusion (18). The findings of Legendre and Szoka

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Abbreviations: DOPE, dioleoyl phosphatidylethanolamine; DOPC, dioleoyl phosphatidylcholine; C_{50}, concentration that gives a 50% reduction in fluorescence intensity.
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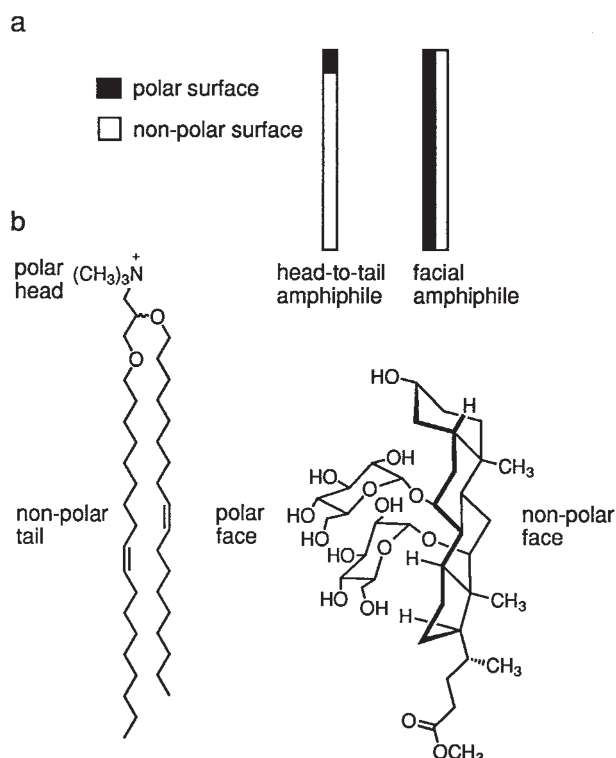


FIG. 2. (*a*) Arrangement of polar and nonpolar domains in different classes of amphiphiles. (*b*) Examples of a head-to-tail amphiphile (DOTMA) and a facial amphiphile (compound **4a′**, Table 1).

(17) suggested that facial amphiphiles might be excellent components of a gene delivery system.

In this paper, we describe the synthesis and preliminary evaluation of a promising class of DNA delivery agents made by conjugating different polyamines to a series of bile-acid-based facial amphiphiles (Fig. 3). The bile acids are a family of natural products consisting of a facially amphiphilic steroid nucleus with a polar side chain. The bile acids and their derivatives are known to interact with and permeabilize membranes (19). Bile acids with different numbers of hydroxyls and hence different degrees of facial amphiphilicity are available. We investigated three natural bile acid skeletons, lithocholic

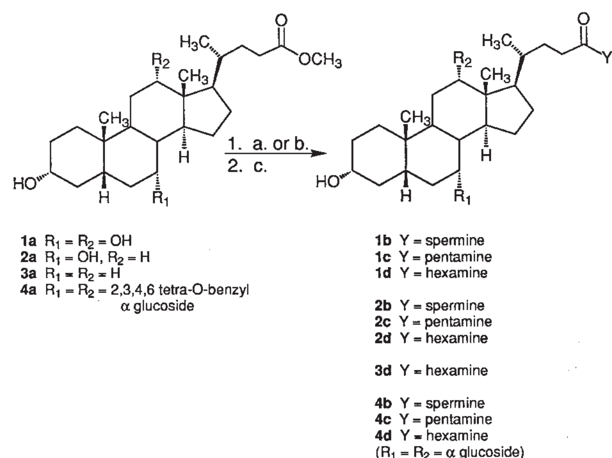


FIG. 3. Schemes for the synthesis of the cationic bile acid conjugates.

acid (one hydroxyl), chenodeoxycholic acid (two hydroxyls), and cholic acid (three hydroxyls), as well as one unnatural skeleton, 7,12-α,α-bisglucosyl cholic acid, which was synthesized recently as an enhanced facial amphiphile (Fig. 2*b*) (20). The results below show that DOPE formulations of several of these gene delivery agents are significantly more effective than Lipofectin for transfecting cultured cells. Moreover, transfection activity correlates with the facial amphiphilicity of the bile acid nucleus. The success of these unusual compounds may inspire the design of additional chemically based gene delivery agents.

MATERIALS AND METHODS

Materials. DOPE and dioleoyl phosphatidylcholine (DOPC) were purchased from Avanti Polar Lipids. The plasmid pSV-β-Gal (6821 bp) coding for β-galactosidase was purchased from Promega and propagated and purified by standard techniques (21). Lipofectin was purchased from GIBCO/BRL. Cholic acid, chenodeoxycholic acid, and lithocholic acid were purchased from Aldrich and esterified with methanolic HCl.

Ten compounds were synthesized for this report by coupling the appropriate polyamine to bile acid derivatives **1a–4a** (Fig. 3). The synthesis of the benzyl-protected 7,12-bisglycosylated cholic acid derivative **4a** from the cholic acid methyl ester has been reported (20, 22). The methyl esters **1a–4a** were converted to the corresponding *N*-hydroxysuccinimide esters or the acylazides and then treated with the desired polyamine (spermine, tetraethylenepentamine, or pentaethylenehexamine) as shown in Fig. 3. Subsequent to polyamine conjugation, the 7,12-bistetra-*O*-benzylglucosyl cholic acid derivatives were debenzylated with $Pd(OH)_2/C$ in the presence of $H_2(g)$ to provide analogs **4b**, **4c**, and **4d**. All final products were purified by passage over CHP-20P reverse-phase column chromatography and were fully characterized by ^1H NMR, IR, MS, and elemental analysis.

Preparation of Cationic Bile Acid Formulations. The cationic bile acid formulations were prepared as 1:1 (wt/wt) mixtures of test compound and phospholipid in deionized water. In a typical preparation, 2.5 mg of DOPE dissolved in ethanol was dried under nitrogen in a glass culture tube. A solution of glycosteroid (2.5 mg/ml) in deionized water was added to the dried DOPE, and the solution was sonicated for 15 min at room temperature in a Branson 3200 sonication bath. Solutions were stored in polyethylene cryotubes at 4°C for a minimum of 48 h prior to use in the transfections.

Transfection Protocol. COS-7 cells were plated at 3×10^4 cells per well in a 24-well plate and incubated at 37°C in Dulbecco's modified Eagle's medium (DMEM)/10% (vol/vol) fetal bovine serum for 24 h prior to transfection. The cells were washed with opti-MEM (GIBCO/BRL) and then overlaid with 200 μl of the transfection mixtures in opti-MEM. The transfection mixtures were prepared as 5× concentrates and allowed to incubate for 15 min prior to dilution with opti-MEM to a final DNA concentration of 1 μg/ml. After 6 h, the transfection mixtures were replaced with DMEM/10% fetal bovine serum and the cells were incubated for another 48 h. The cells were lysed and the β-galactosidase activity in each well lysate was determined by monitoring the hydrolysis of *o*-nitrophenyl galactopyranoside (23). The β-galactosidase activity of Lipofectin-treated cells under optimal conditions was evaluated in parallel, and the transfection activity at the optimal concentration of each cationic lipid formulation is reported as a percentage of the Lipofectin control. Transfection frequency was determined by counting cells stained *in situ* with 5-bromo-4-chloro-3-indolyl β-D-galactoside (23).

In some experiments, chloroquine was added to a final concentration of 100 μM during the transfection.

The toxicity of the lipid formulations was evaluated by comparing the amount of MTT reduced by control COS-7 cells to the amount reduced by COS-7 cells treated with the cationic bile acid formulations (24). IC_{50} values are the concentration of compound that produces 50% cell viability.

Evaluation of Binding Affinity. The relative affinities of the various compounds for DNA were assessed by using an ethidium displacement assay (25). The C_{50} value is the concentration of cationic bile acid that gives a 50% reduction in the fluorescence intensity of a solution containing double-stranded calf thymus DNA (1.32 μM in base pairs) and ethidium bromide (1.26 μM) in 10 mM SHE buffer (8 mM NaCl/2 mM Hepes/0.05 mM EDTA, pH 7.0).

The ability of the cationic compounds to form complexes with DNA in the presence and absence of DOPE was also assessed with a gel retardation assay. Plasmid DNA (0.25 μg) was briefly incubated with various concentrations of each compound or formulation in SHE buffer and then electrophoresed at 100 V in TBE buffer on a 0.9% agarose gel containing ethidium bromide at 5 μg/ml.

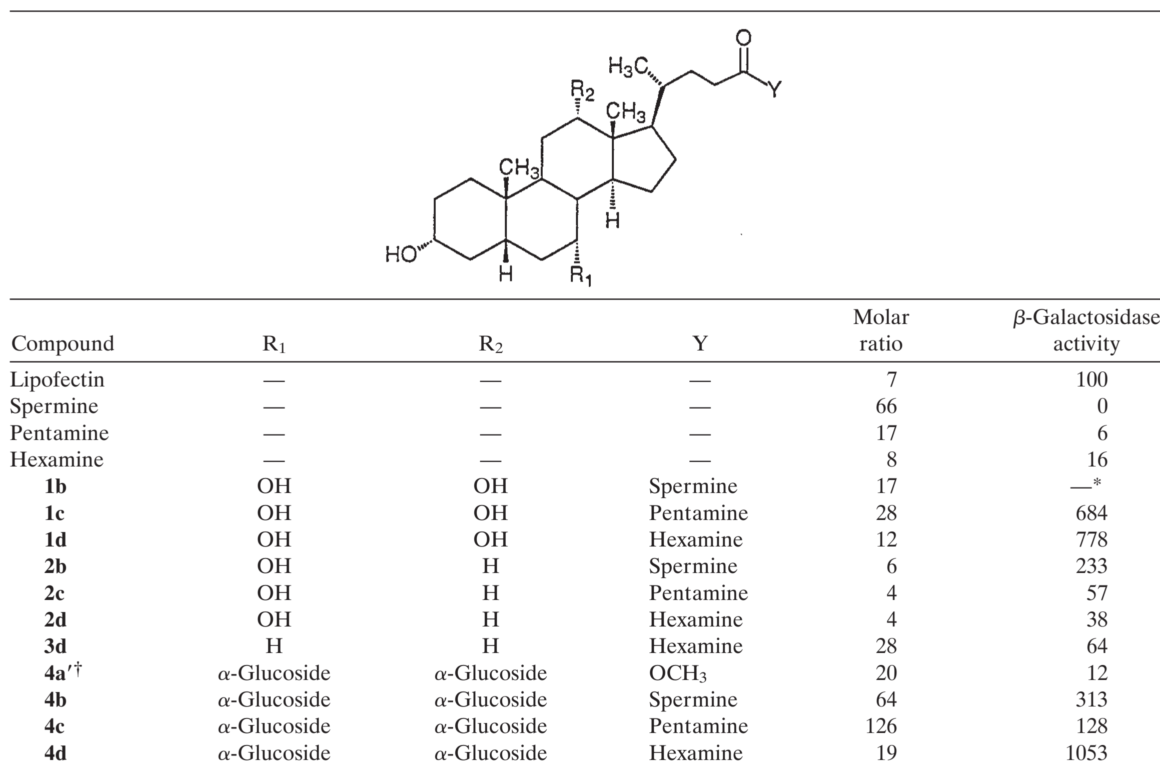
Measurement of Transfecting Particle Sizes. Lipid formulations were prepared as described above and added to plasmid DNA in 100 μl of phosphate-buffered saline at the optimal molar ratio for transfection. The mixtures were vortex mixed and allowed to stand for 15 min at room temperature before dilution with phosphate-buffered saline to a final volume of 3 ml. The hydrodynamic radii of the complexes were determined by dynamic light scattering experiments with a laser light scattering goniometer and BI-2030AT digital correlator (Brookhaven Instruments, Holtsville, NY). Measurements were taken at 25.5°C by using a wavelength of 514.5 nm and an angle of 90°.

RESULTS AND DISCUSSION

Description of the Compounds. We investigated a range of bile acid derivatives, from the very nonpolar lithocholic acid derivative **3a** to the polar bisglycosylated derivative **4a** as components of the gene delivery agents. Lithocholic acid is not a true facial amphiphile because it contains only a single hydroxyl located at one end of the steroid nucleus. The bisglycosylated derivative was designed to have enhanced facial amphiphilicity relative to the natural bile acids (20). The different bile acids were tailored to interact with DNA by attaching various polyamines to the acid side chain to make the corresponding amides (Fig. 3). The side chain is both the simplest position to derivatize and the least likely to affect the amphiphilic properties of the bile acid core. The polyamines investigated were spermine, tetraethylenepentamine, and pentaethylenehexamine. The amines in spermine are protonated in water at pH 7.0, and we have assumed a charge of +3 for the spermine conjugates. The reported pKa values for the amines in triethylenetetramine are 10.0, 9.3, 6.9, and 3.7 (26, 27). The bile acid conjugates of tetraethylenepentamine contain a triethylenetetramine unit and we have assumed a charge of +2.5 for these compounds. The pKa values of tetraethylenepentamine are 10.0, 9.2, 8.2, 4.1, and 2.6, and we have assumed a charge of +3 for the bile acid conjugates of pentaethylenehexamine (26, 27).

Transfection Results. Formulations of each cationic facial amphiphile and DOPE were prepared as described above. Their ability to promote the uptake of a β-galactosidase reporter plasmid was evaluated by measuring the β-galactosidase activity in lysates of transfected cells. Table 1 shows the β-galactosidase activity for each cationic bile acid formulation at its optimum molar ratio expressed as a percentage of the Lipofectin control. The transfection activity of the bile acid conjugates ranged from a low of 38% (**2d**) to >1000% (**4d**) of Lipofectin-treated cells. For the best conjugates, the percentage of cells expressing β-galactosidase was also evaluated by *in situ* staining (21). Protein expression was found to correlate with transfection frequency. For example, 45–85% of cells

Table 1. Transfection results with cationic facial amphiphiles



Compound	R_1	R_2	Y	Molar ratio	β-Galactosidase activity
Lipofectin	—	—	—	7	100
Spermine	—	—	—	66	0
Pentamine	—	—	—	17	6
Hexamine	—	—	—	8	16
1b	OH	OH	Spermine	17	—*
1c	OH	OH	Pentamine	28	684
1d	OH	OH	Hexamine	12	778
2b	OH	H	Spermine	6	233
2c	OH	H	Pentamine	4	57
2d	OH	H	Hexamine	4	38
3d	H	H	Hexamine	28	64
4a′†	α-Glucoside	α-Glucoside	OCH_3	20	12
4b	α-Glucoside	α-Glucoside	Spermine	64	313
4c	α-Glucoside	α-Glucoside	Pentamine	126	128
4d	α-Glucoside	α-Glucoside	Hexamine	19	1053

Molar ratio is the ratio of compound to DNA base pairs. The concentration of DNA base pairs is 1.5 μM. β-Galactosidase activity is expressed as a percentage of the activity in Lipofectin-treated cells.

*Cholic acid–spermine conjugate is insoluble in water.

†**4a′** has the same structure as **4a** except that the benzyl protecting groups have been removed as reported (22).

were transfected with the most active compounds (**1c**, **1d**, and **4d**). In contrast, fewer than 10% of the cells were transfected with Lipofectin. Hence, our first efforts to design transfection agents have led to a number of compounds that are several times more effective than Lipofectin for transfecting cultured cells.

The results in Table 1 merit further comment. We have found that neither the bile acids nor the polyamines alone facilitate DNA uptake, even when used as admixtures; transfection activity requires a covalent linkage between the cationic side chain and the bile acid nucleus. Moreover, there are significant differences between the efficacy of the different bile acids. Although most other designed transfection agents contain cationic head groups attached to hydrophobic tails, our results show that the hydrophilic bile acid conjugates are generally more active than the hydrophobic conjugates (compare, for example, **1d** and **4d** to **2d** and **3d**). Hydrophilicity is not necessarily the critical feature, however. In the introduction we noted that facially amphiphilic cationic peptides have also been shown to mediate transfection of cultured cells. The high activity of transfecting particles containing amphiphilic peptides may be related to an increased fusogenic potential that makes entry into the cell more likely (17). Our hydrophilic bile acid conjugates contain steroids that are facially amphiphilic like amphiphilic peptides. In fact, the bisglycosylated steroid **4a** was specifically designed as an amphiphilic peptide mimic (20). Our results show that nonpeptidic facial amphiphiles can promote DNA uptake like peptidic facial amphiphiles. Nonpeptidic facial amphiphiles have clear advantages over peptidic facial amphiphiles in terms of both expense and chemical stability. Moreover, our cationic facial amphiphiles have much lower toxicity than many membrane-active peptides (17). A standard MTT toxicity assay shows that the IC_{50} values for DOPE formulations of the best compounds (**1c**, **1d**, and **4d**) range from 0.1 to 0.3 mM, much higher than the concentrations used in transfection. For comparison, the toxicity of Lipofectin in this assay is 0.2 mM.

Finally, we point out that very high transfection activities were achieved with some of the polyethylenediamine conjugates, particularly the hexamine conjugates. Spermine, a biogenic polyamine known to bind to DNA, has been used in the design of several other cationic lipids (14, 16). Polyethylenediamine chains have not been used in synthetic transfection agents (28) and yet our results show that they function better than the spermine side chain in a number of cases even though they have a lower intrinsic affinity for DNA (see below). Further studies will be necessary to establish whether the increased activity is related to different spacing between charged amines, to the presence of additional amines that can be protonated in the endosome (see below), or to other factors; however, it is evident from this work that there is still much to be learned about the optimum cationic head group structure.

The Role of the Lipid. Many cationic transfection formulations require a neutral phospholipid for optimal activity (12, 13, 15, 17, 29). The neutral phospholipid is usually a phosphatidylethanolamine analogue such as DOPE. DOPE forms unstable bilayers and may enhance transfection activity because it facilitates fusion of the DNA–lipid complex with the plasma membrane or the endosomal membrane (30). Lipids that form bilayers that are refractory to fusion (e.g., DOPC) generally inhibit transfection activity.

We examined the requirement for a neutral phospholipid in our formulations by carrying out transfections with some of the best bile acid–polyamine conjugates in the presence and absence of both DOPE and DOPC. None of the cationic bile acids tested facilitated transfection in the absence of DOPE. Moreover, formulations of these cationic bile acids with DOPC were also inactive. Transfection experiments carried out with compounds **1d** and **4d** and different amounts of DOPE showed that the optimum ratio for transfection in both cases was approximately 1:1 DOPE/cationic bile acid.

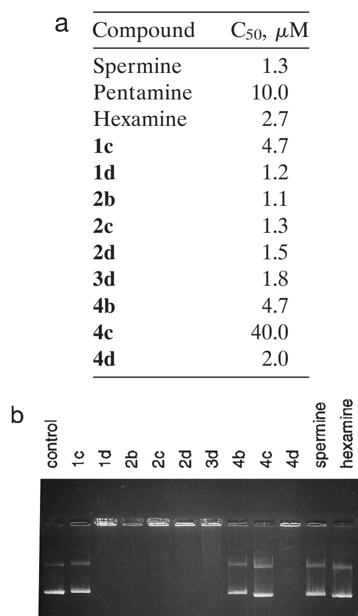
The results show that active transfecting particles must contain a phospholipid in addition to the cationic bile acids. The comparison between DOPE and DOPC indicates that the headgroup of the phospholipid is critical. The headgroups of DOPE and DOPC are known to influence the way in which these lipids organize. DOPC forms very stable bilayers. In contrast, DOPE, which contains a smaller headgroup, can form other types of structures. DOPE-containing membranes readily undergo fusion. It is possible that the cationic facial amphiphiles can facilitate this process.

Effect of Chloroquine on Transfection Activity. Cationic transfection complexes can enter the cytoplasm by direct fusion with the plasma membrane or by endocytosis followed by release of the DNA from the endocytic vesicle (16, 17, 29). Some complexes appear to be taken up efficiently by endocytosis, but they are unable to escape from the endosome before it fuses with the lysosomal compartment where macromolecular degradation occurs. Chloroquine is a weak base that inhibits fusion of the endosome with the lysosome by buffering the lysosome interior (13, 15, 29). Because complexes have more time to escape from the endosome, chloroquine often increases transfection activity. We included chloroquine during transfections with **1c**, **1d**, and **4d** but saw no significant increase in β-galactosidase activity. In contrast, chloroquine improved the activity of Lipofectin–DNA complexes by a factor of 2.5. We have concluded that if any of our complexes enter cells by an endosomal route, they are able to escape more efficiently than Lipofectin complexes. It has been suggested that transfection agents containing amines that can be protonated buffer the endosome and facilitate escape into the cytoplasm (28).

Particle Size. The complexes formed between the cationic bile acid formulations and DNA were measured by dynamic light scattering. The most active transfection formulations (i.e., **1c**, **1d**, and **4d**) formed complexes approximately 1 μm in diameter at the optimal molar ratios used for transfection. The corresponding DOPC complexes, which were found to be inactive in the transfection assay, formed significantly smaller complexes ($\approx$0.7 μm). The least active cationic bile acids formed relatively small complexes even with DOPE (e.g., $<$0.4 μm for both **2c** and **3d**).

Affinity for DNA. We have evaluated the relative affinities of the different cationic bile acids for DNA to determine whether transfection efficiency correlates with DNA binding. Relative affinities were assessed with two assays, an ethidium displacement assay and a gel retardation assay (Fig. 4). The ethidium displacement assay has been used previously to evaluate the relative DNA binding affinities of various polyamines (26, 29, 31) as well as some other bile acid–polyamine conjugates (25). This assay shows that the C_{50} values for most of our cationic bile acid compounds fall within a narrow range, between approximately 1 and 5 μM (Fig. 4*a*). One notable exception is compound **4c**, with a C_{50} value of 40 μM. Compound **4c** is comparable to Lipofectin in its ability to transfect COS-7 cells. The closely related hexamine conjugate **4d** binds about 10 times more tightly to DNA and is about 10 times more active. This comparison would seem to suggest that binding affinity and transfection activity are correlated. However, several compounds that have relatively high affinities for DNA according to the ethidium displacement assay do not show significant activity (e.g., **2c** and **2d**).

The gel retardation assay has been used by others to monitor formation of complexes between DNA and various agents used for transfecting cells (17). With a few exceptions, we found the results of the gel retardation assay to be consistent with the results of the ethidium displacement assay (Fig. 4). The most dramatic exceptions are spermine and pentaethylenehexamine, which have low C_{50} values but do not retard DNA even at



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Compound	C_{50}, μM
Spermine	1.3
Pentamine	10.0
Hexamine	2.7
1c	4.7
1d	1.2
2b	1.1
2c	1.3
2d	1.5
3d	1.8
4b	4.7
4c	40.0
4d	2.0

FIG. 4. DNA binding assays of the cationic bile acid conjugates. (*a*) C_{50} values from the ethidium displacement assay. (*b*) Agarose gel of mixtures of plasmid DNA with various compounds used in transfections. The compounds are indicated above the lanes. All compounds were used at a 5:1 charge ratio. No DOPE was included in the above experiment. The presence of DOPE slightly lowers the amount of compound required to fully retard the DNA (data not shown).

ammonium/phosphate charge ratios of 25:1 (data not shown). In contrast, all of the bile acid–polyamine conjugates that have low C_{50} values (1–2 μM) fully retard the DNA in the wells at a 5:1 charge ratio (Fig. 4*b*); the other conjugates can be fully retarded at higher charge ratios (data not shown). Hence, the polyamines themselves do not form stable complexes with DNA but the conjugates do. These results suggest that the bile acid portions of the conjugates interact favorably with one another and help to stabilize the complexes with DNA. The stabilizing interactions are not reflected in significantly decreased C_{50} values for the conjugates relative to the polyamines, but perhaps this is because the bile acids do not play a direct role in displacing ethidium (e.g., by contacting the DNA).

The binding assays indicate that the ability to form a stable complex with DNA is necessary for good transfection activity. The compounds that do not form stable complexes with DNA cannot facilitate transfection (see, e.g., the polyamines themselves). In some cases, it is possible to improve transfection activity by improving complex stability (compare **4c** and **4d**). However, the ability to form a stable complex with DNA is clearly not sufficient for high transfection activity. For example, the more hydrophobic bile acid conjugates form stable complexes with DNA at lower charge ratios than the hydrophilic bile acid conjugates (presumably because of favorable hydrophobic interactions between the steroids), and yet they tend to be far less active in transfection (compare, for example, the gel retardation and transfection results of **2c** and **2d** to **4c** and **4d**). The reason for the increased activity of the DNA complexes formed by the hydrophilic conjugates is not clear; however, the structural differences between the hydrophobic and hydrophilic bile acid conjugates influence the size of the transfecting particles that form with DNA (see above) and undoubtedly affect how the particles interact with membranes. We speculate that the active transfecting particles have an increased ability to fuse with membranes.

CONCLUSION

We have designed a class of transfection agents that function better than a commercially available cationic lipid for transfecting a standard cultured cell line. These transfection agents are very different from standard cationic lipids. On first view they might appear to resemble the cationic cholesterol transfection agents that have been tested because they contain both a steroidal portion and an amine chain (16, 29). However, the cholesterol-based transfection agents contain a nonpolar steroid tail whereas our best compounds contain a highly polar steroid tail. There is no precedence in the cationic lipid literature that increasing the polarity of the tail would improve transfection efficiency. In fact, the best precedence that these kinds of compounds might work comes from the studies of Legendre and Szoka (17), who showed that some amphiphilic peptides promote DNA uptake. Although a cursory look would suggest that there are no structural similarities between amphiphilic peptides and polyhydroxylated bile acid derivatives, further consideration reveals that both types of molecules have the unusual distribution of hydrophilic and hydrophobic domains that we call facial amphiphilicity (Fig. 2). Facially amphiphilic molecules have interesting physicochemical properties (20, 32). Some facially amphiphilic peptides are known to permeabilize membranes and promote membrane fusion (17). The bile acids and some of their glycosylated derivatives are also known to permeabilize membranes (19, 33). Because functionally active transfecting particles must penetrate cell membranes—and because Szoka (17) had shown that membrane-destabilizing amphiphilic peptides could facilitate DNA uptake—we thought that amphiphilic bile acid derivatives might make better components of gene delivery vehicles than the hydrocarbon chains used in synthetic cationic lipids.

The results reported above support the idea that incorporating facial amphiphiles into synthetic DNA delivery agents can lead to high transfection levels. The most active compounds tested have the highest degree of facial amphiphilicity. In fact, the best compound contains a glycosylated bile acid derivative that was originally designed as a minimalist analogue of a facially amphiphilic peptide (20). The differences in activity between the different cationic bile acid derivatives are clearly not related to differences in the stability of the resulting DNA complexes: the more hydrophobic compounds have a higher affinity for DNA and form stable complexes at lower charge ratios. We designed the compounds based on the idea that facially amphiphilic components known to destabilize membranes (19, 33) might increase the "fusogenic potential" of the transfecting particles and thereby enhance DNA uptake. Although the success of the compounds does not say anything about the mechanism of DNA uptake or whether efficacy correlates with the membrane destabilizing potential of the compounds, we find it interesting that we were able to design a class of compounds that work significantly better than standard cationic lipid-based formulations after observing some abstract similarities between certain amphiphilic peptides that promote DNA uptake and polyhydroxylated steroids.

We note that although the initial experiments were carried out on a single cell line to facilitate comparisons between a large number of compounds, we have since used the most active compounds to transfect other cell lines, including primary human fibroblasts, human epithelial cell lines, and human breast and colon carcinoma cell lines. Although high transfection activity *in vitro* does not necessarily imply successful gene transfer *in vivo*, we are hopeful that some of these compounds will also prove useful for *in vivo* gene delivery. In any event, the success of these unusual cationic facial amphiphiles may inspire ways of thinking about the optimal structural motifs for synthetic gene delivery agents that may lead to the development of more efficient chemical methods for DNA delivery.

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