the selectivity factor α. When plotting ln α against 1/T, the so called isoelution temperature T_{iso} could be assessed on the basis of experimentally determined retention data. Knowing T_{iso} of a certain analyte under given experimental conditions might be helpful in the determination of optical purities, as the elution order will be reversed and other selectivities should be observed when increasing the temperature above T_{iso}. The low selectivities observed for some analytes in these studies appear to be due to the fact that some temperatures chosen were close to this isoelution temperature. The conformationally restricted *cis*-α-methyl analogue of 8-methoxy-2-amidotetralin was best resolved on the Whelk-O stationary phase, whereas the respective *trans*-isomer showed only low selectivites. Over the temperature range investigated, a decline of the column efficiency with decreasing temperature was observed for all compounds. At lower temperatures the kinetics of mass transfer are lower, which will result in peak broadening.

3. Experimental

3.1. Apparatus and column

A Hewlett Packard G1205A supercritical fluid chromatography system (Hewlett-Packard, Little Falls, Wilmington DE, USA), consisting of dual pumps, pressure controller, oven module and autosampler, was used.
The analytes were monitored in the UV range by means of an HP 1050 multiwavelength detector. Chromatograms were generated and evaluated with the HP SFC 2D ChemStation software package, version A.01.02, operated under Windows 3.1. on a Compaq Deskpro 590 personal computer. Low-temperature experiments were carried out by thermostating the column externally in a water-ethylene glycol bath which was cooled to the desired temperature using the LAUDA compact low-temperature thermostat RM 6B (Lauda Dr. R. Wobser GmbH & co. KG, Lauda-Königshofen, Germany). The (3*R*, 4*S*)-Whelk-O 1 column (25 cm × 0.46 cm I.D.) was supplied by Regis (Morton Grove, IL, USA) via Chrompack (Deventer, The Netherlands).

3.2. Materials and sample preparation

Methanol, gradient grade, was purchased from E. Merck (Darmstadt, Germany). Spectroscopic ethanol, supplied by Kemetyl (Stockholm, Sweden), was used for dissolving the analytes. SFC-grade carbon dioxide (99.9%) was supplied by AGA (Lidingö, Sweden).
The racemic 2-amidotetralins were synthesized at the Department of Medicinal Chemistry, University Centre for Pharmacy, Groningen, The Netherlands [7, 8]. About 1 mg of each compound, either as HCl-salts or free bases, was dissolved in 1.5 ml ethanol.

3.3. Chromatographic conditions

The SFC apparatus was operated in the downstream mode at five different temperatures: 50, 30, 15, 0, and −15 °C. The outlet pressure of the supercritical carbon dioxide was set to 200 bar and 20% methanol was added to increase the polarity of the mobile phase. Per run, 5 µl of the prepared solutions were injected. The total flow-rate was set to 2 ml/min. The analytes were monitored by UV-detection at 220 and 272 nm, respectively.
Chromatographic data were calculated as follows: $\alpha = k'_2/k'_1$, $k'_i = (t_i - t_0)/t_0$, whereas the peak of the solvent front was considered to be equal to the dead time t_0 and was taken from each particular run. The resolution was determined according to: $R_S = (t_2 - t_1)/[(w_{0.5})_1 + (w_{0.5})_2]$, where $(w_{0.5})_i$ stands for the peak width at half height of the individual peaks. This value was provided by the ChemStation software. The column efficiency was calculated for the second eluted enantiomer according to $N_2 = 5.54\,(t_2/(w_{0.5})_2)^2$.

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A spermine-deoxycholic acid conjugate based lipid as a transfecting agent

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Deoxycholic acid-spermine conjugate (DAS), which is composed of natural components (deoxycholic acid and spermine), was incorporated in liposomes and evaluated for its interaction with plasmid DNA (pDNA) and *in vitro* transfection efficiency. Electromicrographs demonstrated that DAS-pDNA complexes are spherical, compact and electronically dense compared to the toroidal shapes formed by the monovalent lipid 1,2-dioleoyl-3-trimethylammonium propane (DOTAP) and pDNA. In comparison to the singly charged, non-cholesterol based lipid (DOTAP), the multivalent lipid DAS had similar transfection efficiency in two cell lines. The monovalent sterol, deoxycholic acid propyldiamine conjugate (DAP) was not effective as a transfecting agent. This suggests that multivalent facial amphiphiles such as DAS may serve as excellent candidates for non-viral gene transfer and warrant further study.

1. Introduction

Gene therapy has the potential to be beneficial in the treatment of a number of disorders [1, 2] including cystic fibrosis [3, 4] and stimulation of the immune system for cancer therapy [5]. Non-viral gene delivery [6–11] is an approach which utilizes various delivery systems such as injection of naked plasmid DNA (pDNA) and ligands conjugated to polycationic carriers including polylysine [12] and liposomes [13–15]. The non-viral approach is generally considered to be safer than viral delivery due to the lessened chance of eliciting an immune response. However, when compared to viral vectors, non-viral vectors are

not as effective due to problems in clearance resulting from their low stability in biological fluids and lower transfection efficiency.

Cationic liposomes are a promising class of non-viral vectors, which form complexes with pDNA mainly through electrostatic attractions. pDNA is protected from nuclease degradation, and the interaction of pDNA with the cellular membrane is enhanced. However, the structure and characteristics of the pDNA/liposome complex has not been thoroughly investigated. Both a tight complex and the coating of the pDNA into a lipid tubule have been reported in the literature [16, 17].

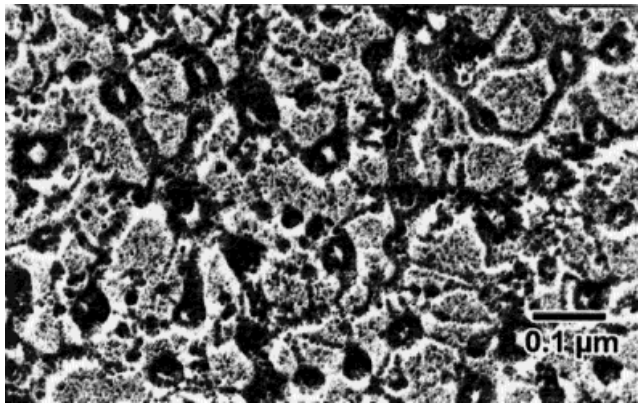
In this report, a novel multivalent serol based lipid composed of natural products, deoxycholic acid-spermine (DAS) was evaluated in liposomes as a non-viral vector. The binding characteristics of DAS liposomes as well as their ability to transfect cultured cells were determined and compared to the performance of two monovalent lipids, deoxycholic acid propyldiamine conjugate (DAP) and 1,2-dioleoyl-3-trimethylammonium propane (DOTAP).

2. Investigations, results and discussion

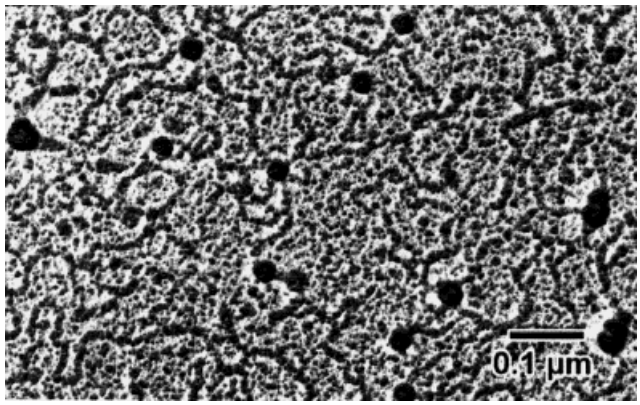
Endogenous compounds may offer unique advantages in the production of new cationic molecules (e.g. lower toxicity) and may have superior properties in transfection studies. It has been shown in the literature that facial ampiphiles can be efficient transfection agents [18].

Electromicrographs have been used to visualize the particle size and morphology of pDNA lipid complexes [16]. The complex formed between a lipid particle made of a singly charged lipid and pDNA has been reported to be toroid in shape with a diameter of approximately 70 nm [19]. Fig. 1 shows the electromicrographs of pDNA bound to the two-liposome preparations (one containing the multivalent lipid DAS and the other the monovalent lipid DOTAP) used in the transfection and binding studies. The DOTAP/DOPE (1 : 1, w/w) complex appears to be of a toroid shape, while the final pDNA/lipid complex with DAS/DOPE (2 : 1, w/w) liposomes has a more spherical shape which may facilitate inter and intra cellular movement. The differences in the structure of the liposome-pDNA complexes may also be related to the differences in the backbone of the lipid structure.

Studies were conducted to evaluate the extent of pDNA/liposome binding interaction. These experiments demonstrated that the molar amount of lipid incorporated into a liposome required to successfully bind the pDNA, was less for the multiply charged lipid DAS compared to the singly charged DAP liposomes. SYBR, a commonly used intercalating agent, is a fluorescent probe which fluoresces



A)

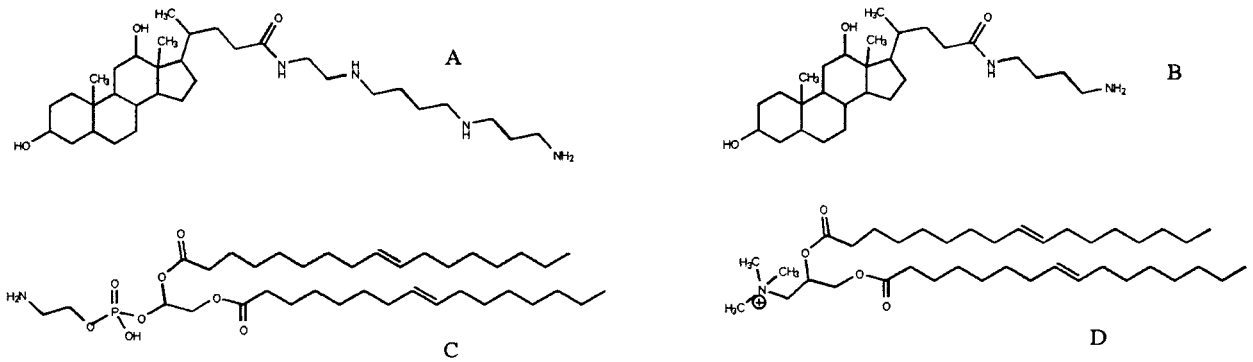


B)

Fig. 1: Electromicrographs of (A) DOTAP/DOPE (1 : 1, w/w) and (B) DAS/DOPE (2 : 1, w/w). Both complexed with pDNA in a 1 : 3 w/w ratio of pDNA/liposome

brightly when complexed with pDNA and offers greater sensitivity and less toxicity [20–22] than ethidium bromide. Fig. 2 shows that DAS/DOPE (2 : 1, w/w) liposomes had a greater tendency to quench fluorescence and thus interact with pDNA compared to the singly charged DAP/DOPE (1 : 1, w/w). It is possible that the structural features of this interaction are facilitated by the proximal charges on the spermine portion of DAS. This effect was not a function of the backbone, since the monovalent sterol DAP did not interact with pDNA to a significant extent.

Using an *in vitro* cell culture system, we evaluated the liposome formulations as transfecting agents in the SKnSH and CHO cell lines. The 2 : 1 (w/w) DAS/DOPE liposomes had a higher transfection activity compared to



(A) Deoxycholic acid spermine conjugate (DAS); (B) deoxycholic acid propyldiamine conjugate (DAP); (C) dioleoyl phosphatidylethanolamine (DOPE); (D) 1,2-dioleoyl-3-trimethylammonium propane (DOTAP)

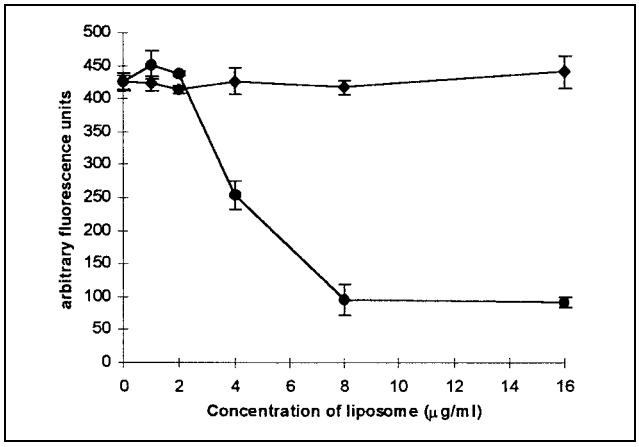


Fig. 2: SYBR Green Binding Assay. (◆) DAP/DOPE (1 : 1, w/w); (●) DAS/DOPE (2 : 1, w/w), 1 μg pDNA/ml, n = 3

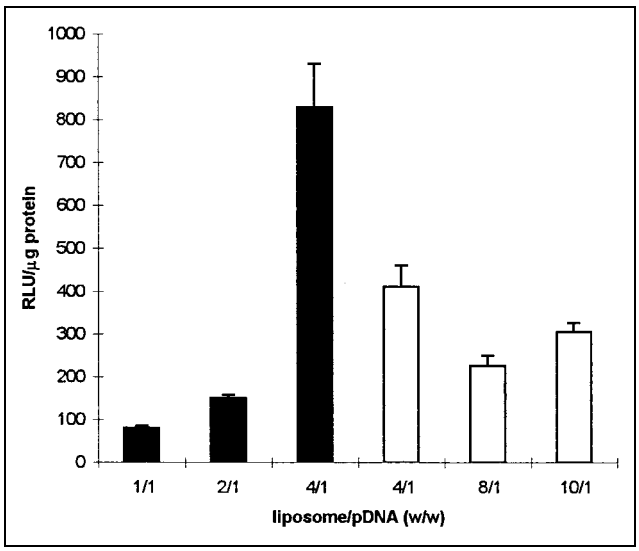


Fig. 3: Effect of the different cationic liposomes on transfection efficiency in CHO cells. (filled bars) DAS/DOPE (2 : 1, w/w); (empty bars) DOTAP/DOPE (1 : 1, w/w), 1 μg pDNA/well, n = 4

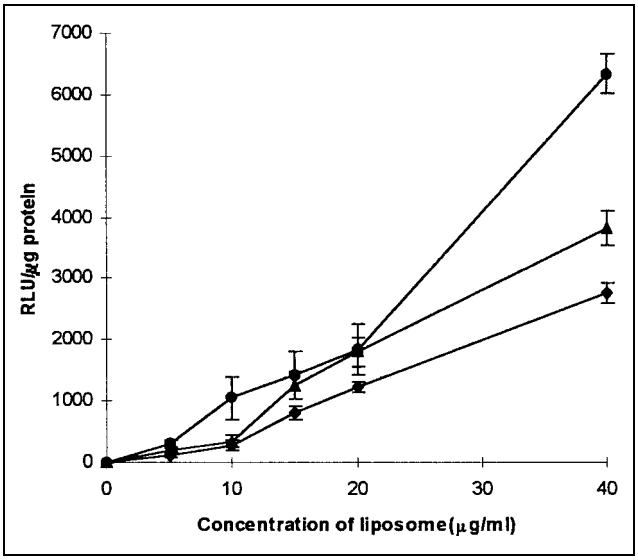


Fig. 4: Effect of the different cationic liposomes on transfection efficiency in SKnSH cells. (●) DAS/DOPE (2 : 1, w/w); (▲) DOTAP/DOPE (1 : 1, w/w); (◆) DAS/DOPE (1 : 1, w/w); 1 μg pDNA/well, n = 4

the DOTAP/DOPE (1 : 1, w/w) in the CHO cell line (Fig. 3). DAS without DOPE was also evaluated (data not shown), but no production of transgene was noted. This is probably due to the fact that DOPE has the ability to enhance fusogenic events and facilitate penetration of cell membranes in *in vitro* models. The two liposomal formulations were also evaluated in SKnSH cells (Fig. 4). The DAS/DOPE (2 : 1 w/w) liposomes showed higher transfection activity with respect to the monovalent DOTAP/DOPE (1 : 1 w/w) liposomes. The monovalent sterol DAP was not an effective transfecting agent in our studies. The optimal ratio of DAS to DOPE was not determined in this investigation but the 2 : 1 w/w ratio out-performed the 1 : 1 w/w. The transfection efficiency could be enhanced by incorporating DOPE/egg lecithin (1 : 1, w/w) liposomes into the transfection media (data not shown). This finding suggests that the DAS/DOPE ratio was not optimal, and also that the transfection agents do not have to be in the same molecular package to elicit gene transfection.

The introduction of pDNA's into cells is a challenging step in gene therapeutics. Cationic lipids are frequently used to facilitate anionic gene delivery. The effective control of complexation of the pDNA to cationic lipids is essential for obtaining optimal pDNA expression. The complexation event may influence the final particle size and net charge associated with the particle. The resulting complex, created by the electrostatic interaction between the pDNA and cationic liposomes requires characterization to ensure a reproducible system. The optimal lipid/DNA ratio and particle size would also depend on the transfected cell and other variables. The diverse structure of the pDNA/liposome complex has already been reported in the literature [16]. Electron microscopy studies have revealed both a tight spherical complex and a coating of the pDNA into a lipid tubule. Both events protect the pDNA from metabolic degradation and facilitate gene transfection, yet they are likely to have different transport features.

The rate determining step in non-viral gene delivery has yet to be elucidated. This step may be different for particular delivery systems and target cells. In this report, multivalent DAS/DOPE (2 : 1, w/w) liposomal formulation was a more effective transfecting agent compared to the monovalent DOTAP/DOPE (1 : 1, w/w) and DAP/DOPE (1 : 1, w/w) liposomal preparations. The DOTAP/DOPE (1 : 1, w/w) and the facial amphiphile/DOPE (2 : 1, w/w) ratios have been reported in literature and were used in this study. The reason for the enhanced performance of DAS/DOPE (2 : 1, w/w) liposomes is not clear. The differences in transfection efficiency could arise from the different lipid characteristics, such as the ability to protect pDNA from degradation, efficient cellular uptake of the lipid/pDNA complexes, rate of escape of the particles from the cellular endosomes, or the rate at which the complex dissociates within the cell. In addition, an MTT toxicity assay [23] showed an IC_{50} value of 0.8 mM for DAS that is much higher than the concentrations used in our studies. It should be noted that comparison between various liposomal formulations for transgene delivery is complex as the comparisons should be made under optimal conditions for each preparation, and not based on a mass or charge ratio. In this study, the DAS formulation resulted in a greater production of the transgene, but only one time point was addressed. The result may have differed with different cell lines, or with changes in the transfection protocol.

In this preliminary report, we have not differentiated the mechanisms that enhanced the transgene activity, but have

addressed one of the physicochemical parameters of the pDNA/lipid complex. A striking difference in the two systems is the shape of the final complex. It is not known what influence the size or shape of the particle has on the uptake efficiency. The transport of the pDNA/lipid complex is likely to be dependent on the cell type. The attractiveness of the current approach is its use of natural products in the formation of new cationic gene delivery vehicles; overall toxicity of the system is most likely reduced, thus improving transgene expression. In this preliminary report, we addressed the issue of pDNA-lipid interactions and whether multivalent lipid vectors could facilitate gene transfer. The results indicate that facial amphiphiles such as DAS may serve as excellent candidates for non-viral gene transfer and warrant further study.

3. Experimental

3.1 Materials

DAS and DAP were synthesized as previously reported [24]. DOTAP and DOPE (dioleoyl phosphatidylethanolamine) were purchased from Avanti Polar Lipids (Alabaster, AL) and stored at −20 °C in chloroform. The structures of the cationic lipids (A–D) used in this study are illustrated above. All supplies for the plasmid purification were purchased from Promega (Madison, WI). RPMI-1640 and DMEM media for cell culture work were obtained from Life Technologies (Grand Island, NY). Fetal Bovine Serum (FBS) and penicillin/streptomycin were also obtained from Life Technologies. All other chemicals used in the study were of analytical grade or better.

3.2. Liposome preparation

The liposomes were prepared by mixing the lipids (DAS/DOPE 2 : 1 w/w, DAP/DOPE 1 : 1 w/w and DOTAP/DOPE 1 : 1 w/w) in chloroform and drying the mixture under nitrogen at 60 °C in a Büchi Rotavapor. The dried lipid film was reconstituted in sterile water and shaken in a waterbath at 60 °C for 15 min.

The liposomes were sized by extrusion through a 0.2 μm polycarbonate membrane (Poretics Corporation, Livermore, CA). The particle size of the liposomes was measured in a Nicomp 370 Submicron Particle Sizer (Particle Sizing Systems, Santa Barbara, CA) using a volume weighted parameter. The average diameter of liposomes was 200 ± 80 nm.

3.3. Plasmid DNA

pDNA was extracted from Escherichia Coli (strain JM-109; pGL3 luciferase gene with SV-40 promoter and enhancer sequences obtained from Promega, Madison, WI) according to a Wizard DNA Purification Kit. The concentration and purity of the pDNA were determined spectrophotometrically.

3.4. Cell culture and transfection

SKnSH (neuroblastoma) and Chinese hamster ovary (CHO) cell lines were obtained from American Type Culture Collection (Rockville, MD). The cells were cultured in RPMI-1640 (SKnSH cells) or DMEM (CHO cells) media supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were grown at 37 °C in a humidified 5% CO_2 atmosphere. The confluent cells were seeded at 2×10^5 cells/well in 24-well plates. Cells were transfected at 60–80% confluency, approximately 18–24 h after seeding. A fixed amount of p-DNA was complexed with varying concentrations of liposomes in serum free media and incubated at room temperature for 30 min. The cells were washed once with 500 μl of 1X PBS, and the treatments were added to the cells in serum-free media. The cells were incubated for 12 h at 37 °C, and the serum free media was replaced with serum containing media and the cells were allowed to grow for an additional 48 h.

3.5. Transfection activity

The cells were washed with phosphate buffered saline (PBS) and lysed in 100 μl of luciferase lysis buffer (0.1 M potassium phosphate buffer pH 7.8, 2 mM EDTA, 1% Triton X-100, 1 mM DTT) at room temperature on a shaker. The luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA) by adding 100 μl luciferase assay buffer (30 mM Tricine, 3 mM ATP, 15 mM $MgSO_4$, 10 mM DDT, pH 7.8) to 20 μl cell lysate and injecting 100 μl of 1 mM D-luciferin, pH 6.3. The emitted light was measured for 10 s and expressed in terms of relative light units (RLU) and corrected for total cellular protein concentration which was determined by a BCA protein assay reagent kit (Pierce, Rockford, IL) using bovine serum albumin as a standard.

3.6. Electron microscopy

Samples were prepared by complexing pDNA (3 μg) and liposomes (15 μg) for 30 min at room temperature and visualized by electron microscopy. Carbon films, made by evaporation onto freshly cleaved mica, were placed on 400 mesh nickel grids. Suspensions of pDNA/lipid complexes were adsorbed to the surface of the films and the grids were exposed to 1% OsO_4 for 1 h, washed with water and dried. Specimens were rotary shadowed with carbon/platinum by electron beam evaporation at a 10^0 angle and observed at 75 kV on a Hitachi H-7000 transmission electron microscope.

3.7. SYBR binding study

A constant amount of pDNA (1 μg) was complexed with increasing concentrations of liposomes for 30 min at room temperature in 500 μl of sterile water. 500 μl of 2X SYBR Green (Molecular Probes, Eugene, OR) was added and the mixture was incubated for 30 min. The fluorescence was measured on a Perkin Elmer LS-50B Spectrophotometer (Norwalk, CT) at an excitation wavelength of 497 nm and an emission wavelength of 520 nm.

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