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Factors mediating lipofection potency of a series of cationic phosphonolipids in human cell lines

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Abstract

A series of cationic liposomes known as cationic phosphonolipids (CPs) were evaluated as vehicles for in vitro gene transfer in K562 erythroleukemia cells and 5637 epithelial carcinoma cells. For each CP and target cell type examined, detailed analyses were performed to determine optimal transfection conditions (lipid/ DNA (+/−) charge ratio, amount of complexed episomal DNA, liposomal and lipoplex size, complexation medium and duration of complex-cell exposure time). Lipofection conditions were determined to be both cell- and lipid-type specific. Complexation medium critically affected transfection competence. The initial size of the liposome was not always predictive of lipofection potency. The lipid chemical composition had a strong impact upon lipofection efficiency; DOPE inclusion in the liposome formulations was found to affect the levels of transgene expression in a cell-dependent way. Notably, effective transgene expression was characterized by prominent plasmid nuclear incorporation. Human Aγ- and ε-globin transgene nuclear incorporation and expression in 5637 cells post GLB.391-mediated lipofection lends credence to its use as a vehicle of therapeutic transgene delivery.

Keywords: Cationic phosphonolipid; Lipofection condition; Gene transfer; Transgene expression; Nuclear DNA incorporation

1. Introduction

Since the publication of the DEAE-Dextran technique in 1965 [1], various methods have been introduced for the transfer of DNA into mammalian cells, including viral and non-viral-mediated delivery [2–6]. The search for efficient non-viral techniques for gene delivery is warranted because of immune anti-viral reactions and safety issues that arise when viruses are used as vectors [7,8]. Liposome-mediated gene transfer (lipofection) is known to exhibit a number of desirable features, such as decreased immunogenicity and interference with cell survival, lack of mutagenesis, significant transgene expression, successful generation of stable transfectants, internalization of episomal DNA irrespective of size, reproducibility and ease of use [6,9–15]. To improve lipofection efficacy, a series of cationic lipids, called cationic phosphonolipids (CPs), have been formulated and successfully tested both in vitro and in vivo gene transfer protocols [16–20].

In this study, we examined a group of cationic phosphonolipids formulated with or without the neutral lipid DOPE, for their ability to serve as vehicles for efficient gene transfer in K562 human erythroleukemia cells and 5637 epithelial carcinoma cells. Several reports indicate the need for a better understanding of the biochemical and biophysical factors that affect lipoplex carrier properties. In this context, we studied in detail the effect of several parameters [e.g., impact of lipid to DNA (+/−) charge ratio (lipoplex ratio), amount of plasmid DNA, liposome size, complexation

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Table 1
Molecular formula of cationic phosphonolipids

Cationic phosphonolipid	R	R′	R″	Z	*n*	X^-	Molecular formula
GLB.73	$C_{14}H_{29}$	CH_3	CH_3	N	1	I	$C_{32}H_{69}INO_3P$
GLB.43	$C_{18}H_{35}$	CH_3	CH_3	N	1	I	$C_{40}H_{81}INO_3P$
GLB.391	$C_{14}H_{29}$	CH_3	C_2H_5	N	1	I	$C_{33}H_{71}INO_3P$

medium, duration of cell-complexes exposure and lipoplex removal] on lipid transgene delivery performance. Our dual approach, based on quantification of transgene nuclear incorporation as well as evaluation of the expression of both reporter and functional genes, indicates that lipofection parameters critically affect the delivery properties of CPs.

2. Materials and methods

2.1. Cell lines

K562 human erythroleukemia suspension cells (#CCL-243 ATCC, Rockville, MD, USA) [21] and 5637 human epithelial carcinoma bladder cells (#HTB-9 ATCC, Rockville, MD, USA) were cultured under standard conditions. Cell viability was quantified by trypan blue exclusion staining.

2.2. Expression vectors

The plasmid vectors used in this study were pCMVβ-gal, encoding β-galactosidase under the control of CMV promoter (Clontech Laboratories, Inc.), pGL3bLuc$^+$ which encodes for the luciferase gene and the plasmids pTkneoAγ (3.3 kb *Hin*dIII human Aγ-globin gene) and pTkneoε (3.7 kb *Eco*RI human ε-globin gene) (a generous gift from N. Anagnou).

2.3. Preparation of cationic phosphonolipids

CPs synthesized by the Laboratoire de Chimie Organique, UBO, UMR, Brest Cedex, France (Table 1, Fig. 1) were tested for their gene transfer properties. CPs were used alone or in combination with the neutral co-lipid dioleylphosphatidylethanolamine (DOPE) (Sigma, USA). The commercially available cationic lipid Lipofectamine^{2000} was purchased from GIBCO-BRL, Life Technologies. Unilamellar liposomes were prepared by the extrusion method employing a laboratory extruder (LiposoFast-Pneumatic, Avestin Inc.). In a typical experiment for preparing 1 ml dispersion of liposomes, 1 mg of CP was dissolved in chloroform/methanol solution (2:1, v/v) and the solvents were evaporated under reduced pressure. The resulting lipid film was hydrated with 1 ml sterile pyrogen-free water and the sample was vortexed for 1 min at room temperature to produce large multilamellar liposomes (MLVs). The obtained suspension was extruded through two-stacked polycarbonate filters of 100 or 50 nm pore size. Twenty-five cycles were applied at temperature 5 °C above the corresponding T_m of lipid. All types of liposomal dispersions were stable at + 4 °C for more than 1 week.

2.4. Dynamic light scattering studies

The size of the liposomes and generated lipoplexes was measured by a Light Scattering Apparatus (AXIOS-150/EX, Triton Hellas), with a 30 mW laser source and an Avalanche photodiode detector at an angle of 90°. Ten measurements were obtained for each dispersion and the results were averaged.

2.5. Preparation of lipoplexes

Lipoplex solutions were prepared by sequentially adding varying amounts of plasmid DNA (1–8 μg) and CP reagent at 0.5:1 to 3.5:1 lipid/DNA charge ratios (+/−) into polystyrene tubes to a final volume of 500 μl of either of the following varying composition complex medium [OPTI-MEM I, RPMI-1640, 150 mM NaCl, 300 mM NaCl, 500 mM NaCl, 1 M HEPES, 5 mM $CaCl_2$, 10 mM $CaCl_2$, 15 mM $CaCl_2$, ddH_2O]. The tube was vortex-mixed and incubated at room temperature for 30 min.

2.6. Lipofection protocols

K562 suspension cells were seeded in OPTI-MEM I medium into 6-well plates (Greiner Labortechnik GmbH) at a density of 5×10^5 cells/well and to a total volume of 2 ml 1 h before transfection. 5637 adherent cells were seeded into 6-well plates at a density of 2.5×10^5 cells/well and to a total volume of 1 ml 1 day prior to lipofection. Following exposure of cells to the lipoplexes for varying time-periods (1–6 h), 2 ml of complete growth medium (RPMI supplemented with 10% FBS) were added to each well and the cells were further cultured until a luciferase assay or X-Gal staining was performed (3–20 days post-transfection).

2.7. Assay for luciferase and β-galactosidase activity

Luciferase assays were performed using a luciferase assay kit (Promega) on a Lumat LB953 luminometer (EG and G, Berthold, Evry, France). Each experiment was performed in triplicate and reported as relative light units (RLU)/μg total cellular protein as determined with the Bradford protein assay (Sigma).

β-galactosidase activity was measured spectrophotometrically (420 nm) by an ONPG assay.

2.8. In situ X-gal cell staining

One to 20 days post-transfection, cells were stained with X-gal solution (Sigma). Briefly, cells were washed twice with PBS 1× buffer, fixed with 0.25% (v/v) glutaraldehyde solution in PBS for 15 min, washed three times with 1x PBS and incubated with 1 mg/ml X-Gal solution in 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6 \cdot 3H_2O$, 5 mM $K_3Fe(CN)_6$ at + 37 °C for 3 to 16 h. Transfection frequency was determined by scoring the number of β-gal expressing cells over the total number of seeded cells.

2.9. Dot-blot transfer and hybridization

Nuclear DNA was extracted from cell pellets by standard methods. 3 μg of denatured nuclear DNA samples and standard amounts of the appropriate DNA probes (50–800 ng) were applied on to a nylon membrane (Zeta-Probe GT, Bio-Rad). The probes were prepared after digestion of the plasmids pTkneoAγ (*Hin*dIII, 3.3 kb), pTkneoε (*Eco*RI, 3.7 kb) and pGL3bLuc$^+$ (*Hin*dIII/*Bam*HI, 2.2 kb) with the appropriate enzymes. Labeling of the probes was performed by random priming (RadPrime DNA Labeling System, BRL). Quantification of transgene nuclear incorporation was performed by a radioanalytic scanner (Molecular Imager FX, BIO-RAD) according to the standard curve derived from standard amounts of DNA probe (10–1000 ng).

2.10. RNA preparation and cDNA analysis

RNA was extracted from 5637 cells by the RNAzol method. Aγ- and ε- globin transcripts were RT-PCR amplified as previously described [22].

3. Results

3.1. Optimal lipoplex ratio

The degree of lipofection efficiency as a function of the lipid to DNA charge ratio differed with the lipid formulation and size

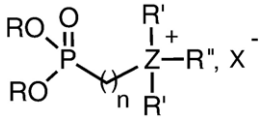

Fig. 1. General structure of cationic phosphonolipids used in this study.