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Novel serum-tolerant lipoplexes target the folate receptor efficiently

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ABSTRACT

Gene transfer using non-viral vectors is a promising approach for the safe delivery of nucleic acid therapeutics. In this study, we investigate a lipid-based system for targeted gene delivery to malignant cells overexpressing the folate receptor (FR). Cationic liposomes were formulated with and without the targeting ligand folate conjugated to distearoylphosphatidyl ethanolamine polyethylene glycol 2000 (DSPE-PEG_{2000}), the novel cytofectin 3β[$N(N^1,N^1$-dimethlaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SGO4), which contains a 13 atom, 15 Å spacer element, and the helper lipid, dioleoylphosphatidylethanolamine (DOPE). Physicochemical parameters of the liposomes and lipoplexes were obtained by zeta sizing, zeta potential measurement and cryo-TEM. DNA-binding and protection capabilities of liposomes were confirmed by gel retardation assays, EtBr intercalation and nuclease protection assays. The complexes were assessed in an *in vitro* system for their effect on cell viability using the MTT assay, and gene transfection activity using the luciferase assay in three cell lines; HEK293 (FR-negative), HeLa (FR^+-positive), KB (FR^{++}-positive). Low cytotoxicities were observed in all cell lines, while transgene activity promoted by folate-tagged lipoplexes in FR-positive lines was tenfold greater than that by untargeted constructs and cell entry by folate complexes was demonstrably by FR mediation. These liposome formulations have the design capacity for *in vivo* application and may therefore be promising candidates for further development.

1. Introduction

Gene therapy is a treatment modality for many diseases with a genetic origin. Thus, the delivery of the appropriate, therapeutic gene (DNA) into the cells that will replace, repair or regulate the defective gene that causes the disease is a key step in gene therapy. DNA, however is a negatively charged polyanion and does not easily traverse the negatively charged and hydrophobic cell membrane. Consequently, gene delivery carriers (also called vectors or vehicles) have been developed (Ward and Georgiou, 2011). Antitumor drug delivery systems with nanometric dimensions have received much attention due to their unique accumulation behaviour at the tumour site. Various nanoparticulate carriers such as liposomes, polymer conjugates, polymer micelles, and nanoparticles are utilized for selective delivery of various anti-cancer drugs to tumours in a passive targeting manner. However, a more effective and active targeting system is needed to enhance the uptake of drugs using nanocarriers into cancerous cells at the tumour site (Kawano and Maitani, 2011). This may be achieved by ligand–receptor, antigen–antibody and other forms of molecular recognition for site-specific delivery (Steichen et al., 2013). The over-expression of receptors such as those for folate and transferrin, by tumour cells, may be exploited for this purpose (Liechty and Peppas, 2012). Non-viral vectors are generally cationic in nature. They include cationic polymers such as polyethyleneimine (PEI) and poly L-lysine (PLL), cationic peptides and cationic liposomes. Recently, a liposomal preparation LPD (liposomes–protamine–DNA) has shown transfection efficiency greater than that of conventional liposome:DNA complexes (lipoplexes) (Tros de Ilarduya et al., 2010). Physical properties such as size and zeta potential play a critical role in determining their efficiency. Selected modifications to these approaches that can produce safe, efficient and targetable gene carriers are desirable. Although non-viral vectors are less efficient than their viral counterparts, they have the advantages of safety, simplicity of preparation and high gene encapsulation capability (Nasiruddin, 2007).

Although cationic liposomal vectors mediate effective gene transfer, tissue specific *in vivo* DNA delivery is still a major challenge in gene therapy (Zhang et al., 2012; Jin et al., 2014). To date, tissue-specific targeting of cationic liposomal DNA has been accomplished by two distinct techniques. The first method involves transfection of selected tissues, such as nasal epithelium, arterial endothelium, lung or tumours by locally administering the complexes within a defined region (Reddy et al., 2002; Alton, 2007). This method has proven to be a viable option for the clinical

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treatment of several diseases, including cystic fibrosis, and cancer. A second method used to enhance the specificity of gene delivery is by the coupling of cell-binding ligands, such as folic acid (FA), transferrin or carbohydrates to liposomes for the purpose of combining the intrinsic activities of lipids with the receptor-mediated uptake properties of the attached ligand (Kamaly et al., 2012). We have been interested in the use of FA as a targeting ligand to deliver attached therapeutic and imaging agents to cancer cells that over-express the folate receptors (Kukowska-Latallo et al., 2005). Because FA-linked cargoes are efficiently bound and internalized by folate receptor-expressing cells, we have explored the possibility of using FA to enhance cationic liposomal vector delivery to FR-enriched tumours (Reddy and Low, 2000). When FA is linked via a carboxyl group to virtually any molecular construct (e.g. a drug, imaging agent, proteins, virus, liposome, etc.), folate receptor binding proceeds unhindered and folate-conjugate uptake occurs via receptor-mediated endocytosis (Lu and Low, 2012). The high affinity of folate for its receptors ($\sim 10^{-10}$ M), the small size of FA, and the compatibility of FA with a variety of solvents and solution conditions also adds to the attractiveness of the targeting ligand (Reddy and Low, 2000). It has been shown that folate-combined nanoparticles concentrated in tumour cells and liver tissue over four days longer after administering than non-targeted agents (Kukowska-Latallo et al., 2005). The role of folate receptors in the cellular transport of folate is not well understood, although a potocytosis (caveolin-coated endocytosis) model has been proposed. After binding to receptors on the cell surface, folate conjugates have been shown to traffic to endosomes (Lu and Low, 2012). Recently, it has been reported that folate mediated delivery of drug loaded nanoparticles can enable binding, promote uptake, and exhibit increased cytotoxicity to cancer cells *in vitro* and *in vivo* (Zhao et al., 2010). For *in vitro* applications, lipoplexes are usually formed with excess positive charge (cationic moiety (+) to nucleotide (−)). However for gene transfer applications *in vivo*, lipoplexes formed with excess positive or excess negative charge ratios have been used (Xu et al., 1999). It has been shown that lipoplexes adsorb a 'protein corona' in serum by low affinity and competitive binding, which may promote the formation of large aggregates of intact lipoplexes (Caracciolo et al., 2010). This, in turn may affect the mode of cellular uptake *in vitro*. The possible effects on *in vivo* applications are also far reaching as the nanoparticles perceived by cells may differ considerably from the intended formulation. However, the inclusion of polyethylene glycol (PEG) in lipoplexes creates a steric hindrance, which greatly reduces protein adsorption, opsonization and elimination by macrophages (Pozzi et al., 2014).

The aim of this study was to formulate novel serum-tolerant folate-decorated stealth lipoplexes for gene delivery to tumour cells that overexpress the folate receptor. Thus a new cholesteryl cytofectin featuring a 13 atom 15 Å spacer element separating the cationic head group and the hydrophobic cholesteryl fused ring anchor element was prepared to facilitate DNA interaction with PEGylated liposomes displaying folate on the distal end of membrane tethered polyethylene glycol 2000. Interactions were characterized by cryo transmission electron microscopy (cryo-TEM), gel retardation and ethidium displacement assays. Systems were further assessed for cytotoxicity and transfection activity in folate receptor-positive (HeLa, KB) and receptor-negative (HEK293) cell lines.

2. Materials and methods

2.1. Materials

Dioleoylphosphatidylethanolamine (DOPE), folic acid and bicinchoninic acid (BCA) assay reagents were purchased from the Sigma Chemical Company, St. Louis, USA. Distearoylphosphatidylethanolamine poly(ethylene glycol) 2000 (DSPE-PEG_{2000}) and amino distearoylphosphatidylethanolamine poly(ethylene glycol) 2000 (DSPE-$PEG_{2000}NH_2$) were purchased from Avanti Polar Lipids, Alabaster, USA. Cholesteryl chloroformate, 2-[4-(2-hydoxyethyl)-1-piperazinyl] ethane sulphonic acid (HEPES), ethidium bromide and silica gel $60F_{254}$ thin layer plates were purchased from Merck, Damstadt, Germany. Cationic lipid, 3β[*N*(N^1,N^1-dimethlaminopropylsuccinamidoethane)-carbamoyl]cholesterol (SGO4) was synthesized as described below. Ultrapure DNA grade agarose was purchased from Bio-Rad Laboratories, Richmond, USA. pCMV-luc DNA was purchased from Plasmid Factory, Bielefeld, Germany. HEK293 cells were supplied by the University of the Witwatersrand Medical School (South Africa). HeLa cells were obtained from Highveld Biologicals (Pty) Ltd. (Lyndhurst, South Africa). KB cells were provided by Professor S.T. Chen, Institute of Biological Chemistry, Academia Sinica (Taipei, Taiwan). Minimum Essential Medium (MEM) containing Earle's salts and L-glutamine, trypsin-versene and penicillin (5000 units/ml)/streptomycin (5000 μg/ml) were purchased from Lonza BioWhittaker, Walkersville, USA. The Luciferase Assay kit was purchased from the Promega Corporation, Madison, USA. All tissue culture plastic consumables were purchased from Corning Incorporated, New York, USA. All other reagents were of analytical grade. Milli-Q ultrapure 18 MΩ cm water was used throughout.

2.2. Chemistry

2.2.1. Preparation of 3β[N(2-aminoethyl)-carbamoyl]cholesterol (SGO1)

To a solution of ethylenediamine (2.25 g, 37.5 mmol) in dry dichloromethane (40 ml) was added a solution of 2.0 g of cholesterylchloroformate (4.45 mmol) in dry CH_2Cl_2 (40 ml) drop wise over 5 min. After 48 h at room temperature the reaction mixture was extracted with 3 × 150 ml H_2O. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and evaporated to a white powder. Thereafter, the product was recrystallized from cyclohexane. Next, the product was purified further by column chromatography on a silica gel 60 column (2.2 × 23 cm) equilibrated with chloroform (50 ml). Column elution was with $CHCl_3$:MeOH:conc.NH_4OH (95:4:1, v/v/v) (100 ml) followed by $CHCl_3$:MeOH:conc.NH_4OH (90:10:1, v/v/v). Product fractions were pooled and evaporated and the title compound was obtained in crystalline form from cyclohexane. Yield: 67%; Mp: 168–170 °C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.67 (s, 3H, H-18′), 0.86 (d, 6H, *J* = 7 Hz, overlapping 2 Hz, H-26′, H-27′), 0.91 (d, 3H, *J* = 6.5 Hz, H-21′), 1.00 (s, 3H, H-19′), 1.0–2.1 (m, 28H, cholesteryl), 2.35 (m, 2H, H-4′), 2.85 (m, 2H, H_2NCH_2), 3.25 (q, 2H, *J* = 5.7 Hz, $H_2NCH_2\underline{CH_2}$), 4.49 (m, 1H, H-3′), 5.37 (d, 1H, *J* = 4.9 Hz, H-6′) ppm. ^{13}C NMR (100 MHz, $CDCl_3$): 11.9 (C-18′), 18.7 (C-21′), 19.3 (C-19′), 21.0 (C-11′), 22.6 (C-26′), 22.8 (C-27′), 23.9 (C-23′), 24.3 (C-15′), 28.0, 28.2 (C-2′, C-16′, C-25′ overlapping), 31.9 (C-7′, C-8′ overlapping), 35.8 (C-20′), 36.2 (C-22′), 36.6 (C-1, C-10′), 37.0 (C-1′), 38.6 (C-24′), 39.5, 39.7 (C-4′, C-12′), 42.3 (C-13′), 50.0 (C-9′), 56.2 (C-17′), 56.7 (C-14′), 74.4 (C-3′), 122.5 (C-6′), 156.5 (NH$\underline{C}$OO), 139.8 (C-5′). HR-MS (ESI-QTOF +ve): *Anal.* Calcd. for $C_{30}H_{53}O_2N_2$: (M+H) 473.4113, Found 473.4290.

2.2.2. Preparation of 3β[N(hemisuccinamidoethane)-carbamoyl]cholesterol (SGO2)

A solution of 3β[*N*(2-aminoethane)-carbamoyl]cholesterol (237 mg, 0.5 mmol) in CH_2Cl_2 (5 ml) was added drop wise to a solution of succinic anhydride (60 mg, 0.6 mmol) in pyridine (1 ml). After 24 h a gel-like product was formed. A further aliquot of succinic anhydride (60 mg, 0.6 mmol) was added and 1 ml of DMF. A clear solution was obtained. After a further 24 h a