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Enhanced gene transfection efficiency by polyamidoamine (PAMAM) dendrimers modified with ornithine residues

Ajay Kumar[a], Venkata K. Yellepeddi[a], Gareth E. Davies[b], Kevin B. Strychar[c], Srinath Palakurthi[a,*]

[a] Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, Texas A&M Health Science Center, Kingsville, TX, 78363, United States
[b] Department of Pharmaceutical Sciences, College of Pharmacy, South Dakota State University, Brookings, SD, 57007, United States
[c] Department of Life Sciences, Texas A&M University-Corpus Christi, Corpus Christi, TX, 78412, United States

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ABSTRACT

Aim of the study was to prepare and to evaluate gene transfection efficiency and cytotoxicity of the ornithine-conjugated PAMAMG4 dendrimers. Ornithine-conjugated PAMAMG4 dendrimers were prepared by Fmoc synthesis. A comparative gene transfection study between PAMAMG4 dendrimers and the surface modified dendrimers was conducted in HEK 293T, GM7373 and NCI H157G cell lines. Effect of excess of ornithine (100 μM) on transfection efficiency of the ornithine-conjugated PAMAMG4 dendrimers was investigated in separate experiment. Cytotoxicity of the dendriplexes was tested in HEK 293T cells by MTT assay. ^{1}H NMR and MALDI-TOF spectral analysis showed that about 60 molecules of ornithine (PAMAMG4-ORN60) were conjugated to a PAMAMG4 dendrimer. Preliminary studies indicated that dendriplexes at charge ratio (N/P 10) show higher transfection efficiency and presence of serum does not affect the transfection efficiency of the dendriplexes. Transfection efficiency of PAMAMG4-ORN60 dendriplexes was slightly higher in cancer cells (NCI H157G) as compared to HEK 293T cells. Transfection efficiency of the PAMAMG4-ORN60 dendrimers decreased in presence of excess of ornithine while there was no effect on the parent PAMAMG4 dendrimers. Cytotoxicity assay has shown that PAMAMG4-ORN60 dendriplexes at N/P 10 were safe at concentrations ≤50 μg/mL. It may be concluded that the ornithine-conjugated dendrimers possess the potential to be novel gene carrier.

1. Introduction

Polyamidoamine (PAMAM) dendrimers are attractive gene carriers because of their well defined structure and ease of surface modification, safety and lack of immunogenicity (Manunta et al., 2004; Lee et al., 2003; Wang et al., 2001; Chen et al., 2000; Kukowska-Latallo et al., 1996; Dufes et al., 2005; Fischer et al., 2003). Because of their net cationic charge, PAMAM dendrimers interact with phosphate groups of DNA electrostatically, condensing DNA into compact complexes called dendriplexes. The compaction of DNA by the dendrimers protects the DNA from degradation by nucleases and enhances cellular uptake of these compact particles via adsorptive endocytosis or phagocytosis (Tang and Szoka, 1997; Belinska et al., 1996; Belinska et al., 1997). Dendriplexes bind to the cell surface by electrostatic interaction with heparan sulfate proteoglycans (HSPG) and integrins ($\alpha_v\beta_3$) at the cell surface and are internalized by endocytosis (Mislick and Baldeschwieler, 1996; Mounkes et al., 1998). Once in the acidic environment of endosome, dendrimer acts as a 'proton sponge' triggering osmotic effect (Tang et al., 1996). The osmotic drag of counter ions and water leads to vesicle rupture and release of DNA complexes. Cationic dendrimers are considered advantageous over other positively charged carriers (e.g. cationic lipids) because of their extended lifetimes *in vivo* (Tang et al., 1996), whereas cationic lipid complexes are usually rapidly cleared from the circulation by the reticuloendothelial system (Kobayashi and Brechibel, 2004). Although dendrimers can carry high gene load, their major limitations for *in vivo* application are: (i) low transfection efficiency, (ii) unspecific sequestration by non-target tissues or lack of target specificity, and (iii) limited transport into the nucleus of the target cells (Zhang et al., 2005; Cassidy and Schatzlein, 2004; Schatzlein, 2003; Audouy et al., 2002; Ogris and Wagner, 2002).

Polyamines are ubiquitous in living systems and are essential for various biological processes such as cellular proliferation including tumor growth, neoplastic transformation and carcinogenesis (Pegg, 1988). Ornithine decarboxylase (ODC) is a key enzyme responsible for decarboxylating ornithine to putrescine, the rate-limiting step in polyamine biosynthesis. ODC has very rapid turnover rate (only few minutes) and is considered as a biomarker for can-

* Corresponding author at: Department of Pharmaceutical Sciences, Irma Lerma Rangel College of Pharmacy, 1010 West Avenue B, MSC 131, Texas A&M Health Science Center, Kingsville, TX 78363, USA. Tel.: +1 361 593 2566; fax: +1 361 593 4303.
E-mail address: palakurthi@pharmacy.tamhsc.edu (S. Palakurthi).

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cer (Criss, 2003). It is present both in the cytoplasm and in the nucleus (Marton and Pegg, 1995) providing the driving force for ornithine uptake by polyamine transporter system (PAT) and transportation into the nucleus as well. Many tumor types have been shown to contain elevated polyamine levels and an activated PAT for importing exogenous polyamines (Gardner et al., 2004; Cullis et al., 1999). Based on the enhanced cellular need for these amine growth factors and existence of an activated transport system for their import, we hypothesized that polyamines and their analogs can be used as biomarkers of cancer. In accordance with the hypothesis, we and other research groups have demonstrated high uptake of polyamines in various cancer cell lines, suggesting particularly ornithine and putrescine as potential biomarkers for cancer (Gardner et al., 2004; Cullis et al., 1999; Palakurthi et al., 2002; Wang et al., 2003; Bergeron et al., 1997; Kramer et al., 1993). A recent biodistribution study in AT3B-1 rat prostate tumor model by our research group has also demonstrated the potential of putrescine and ornithine as positron emission tomography (PET) imaging agents for early detection of cancer (Palakurthi et al., 2007).

Polyamines and their analogs are well known for condensing and packaging DNA into compact forms such as rods, toroids, and spheroids, which are structurally similar to phage DNA in the capsid of viruses (D'Agostino et al., 2006; Ahmed et al., 2006; Vijayanathan et al., 2001; Golan et al., 1999; Fang and Hoh, 1998; Bloomfield, 1997). Polyamines interact with DNA reversibly and the resulting DNA complexes do not withstand dilution or binding to polyanions, hence cannot be used as DNA vectors (Clamme, 2000; Remy et al., 1994). To explore the potential of polyamine-based vectors, lipopolyamines with varied unsaturation in N^4,N^9-dioctadecanoyl spermines were synthesized and their transfection efficiency and DNA condensation ability were found to be better than the Transfectam® [dioctadecylamidoglycyl spermine] formulation (Ahmed et al., 2006). Similarly, Ewert et al. (2006); have synthesized a dendritic lipid, MVLBG2 (starting from ornithine methyl ester), terminated by carboxyspermine moieties. These cationic lipids upon interaction with DNA, form hexagonally ordered cylindrical micelles embedded in a DNA honeycomb lattice. The resultant lipoplexes were found to be significantly more transfectant than commercially available, optimized DOTAP-based complexes.

Above-mentioned studies have propelled us to hypothesize that conjugation of the polyamines to PAMAM dendrimers may increase their cellular uptake by the actively growing cells such as cancer cells resulting in high transfection efficiency and cancer cell specificity of the dendriplexes. As a first step to realize the above goal, ornithine-conjugated dendrimers were prepared and their transfection efficiency was tested in various cell lines using plasmid DNA encoding for green fluorescent protein (pmaxGFP) as the reporter gene. Comparative cytotoxicity of these surface modified dendrimers and parent PAMAM dendrimers was assessed by MTT (methylthiazoletetrazolium) assay.

2. Materials and methods

2.1. Materials

PAMAM dendrimers of different generations were obtained from Dendritic Nanotechnologies Inc. (Mount pleasant, MI). 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), piperidine, *N*,*N*-dimethylformamide (DMF), *N*,*N*-diisopropylethylamine (DIPEA), diethyl ether, D_2O, 2,5-dihydrobenzoic acid (DHB), tetramethyl silane (TMS) and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), ornithine hydrochloride, paraquat and DNase I were purchased from Sigma-Aldrich (Saint-Louis, MO). Trifluoroacetic acid (TFA), ethylene diamine tetra acetic acid (EDTA), phosphate buffered saline (PBS, pH 7.4) and cell culture materials were purchased from Fisher Scientific (Chicago, IL). *N*-hydroxybenzotriazole (HOBt), and 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium (HBTU) were purchased from Anaspec (San Jose, CA). Fmoc-ornithine(Boc)-OH was purchased from Novabiochem (San Diego, CA). One shot® Top10 chemically competent cells were from Invitrogen (Carlsbad, CA). Hank's balanced salt solution (HBSS) with calcium and magnesium (without phenol red) was purchased from Cellgro (Herndon, VA). Ethidium bromide (EtBr) was obtained from Fisher Bio Reagents (Fair Lawn, NJ).

2.2. Methods

2.2.1. Synthesis of ornithine-conjugated PAMAM dendrimers (PAMAM-ORN)

Ornithine-conjugated PAMAM dendrimers (generation 4) were prepared using Fmoc synthesis as reported by our research group earlier (Choi et al., 2004; Pisal et al., 2008). Briefly, to 1.0 mmol of PAMAMG4-NH_2 dendrimer in 3.0 mL of DMF, required amount of each of HOBt, HBTU, Fmoc-ornithine(Boc)-OH and DIPEA were added at a molar ratio 1:60. The reaction mixture was allowed to stir for 4 h at room temperature. The product was precipitated in about 5 mL of diethyl ether and washed with excess of diethyl ether. Fmoc groups of Fmoc-ornithine(Boc)-OH-coupled dendrimer were removed by adding 2.0 mL of 30% piperidine in DMF (v/v). After 1 h of deprotection reaction, the mixture was precipitated in diethyl ether and washed with excess of diethyl ether. Deprotection of the BOC group was achieved by 90% TFA for 1 h at room temperature and the final product was precipitated in diethyl ether and washed with excess of diethyl ether. The product was then solubilized in deionized water, dialyzed against deionized water at 4 °C overnight and then purified by Sephacryl S-300 column chromatography with acetonitrile:Tris buffer (70:30) as the elution buffer. The elution fractions corresponding to the dendrimer size were collected, dialyzed against deionized water at 4 °C, lyophilized and stored at 4 °C for further studies. The yield was always more than 95%.

2.2.2. NMR and Mass spectral analysis

^{1}H NMR spectra (chemical shift in ppm with respect to TMS set at zero) of ornithine-conjugated PAMAMG4 dendrimers (PAMAMG4-ORN60) were recorded on a Bruker AMX-400 (400 MHz) Spectrometer using D_2O as the solvent (with 0.05%, v/v TMS).

To confirm the molecular weight of surface modified dendrimers, mass spectral analysis of the dendrimers was performed on a Bruker MALDI-TOF (matrix assisted laser desorption/ionization-time of flight) using DHB as the matrix.

2.2.3. Plasmid preparation

A 3.4 kbp plasmid encoding for green fluorescent protein (pmaxGFP), with molecular weight of about 2.3 MDa (given an average of 330 Da per nucleotide, 660 Da per base pair (Felgner et al., 1997), carrying 6972 negative charges) was used as a reporter gene to monitor the results of gene transfection. DNA plasmid pmaxGFP purchased from Amaxa Inc. (Gaithersburg, MD) was transformed into One shot® Top10 cells and highly purified covalently closed circular plasmid DNA was isolated by plasmid purification mini and maxi kits from Qiagen (Valencia, CA), according to the manufacturer's instructions. Plasmid concentration and purity, $A_{260}/A_{280} > 1.9$, was assessed using Nanodrop ND-1000 Spectrophotometer (Wilmington, DE). Plasmid integrity was confirmed by 1% agarose gel electrophoresis and stored at −20 °C until further use.

2.2.4. Preparation of dendriplexes

Dendriplexes were prepared at different charge ratios (N/P). Charge ratios (N/P) were calculated based on the number of terminal amine groups on the PAMAMG4 dendrimer (64) and the number of phosphate groups in pmaxGFP (6972). PAMAMG4-ORN60 has 124 surface amino groups. PAMAMG4-pmaxGFP and PAMAMG4-ORN60-pmaxGFP dendriplexes were prepared by adding known amount of pmaxGFP to the required amount of dendrimers while vortexing in PBS. The dendriplexes were incubated for 1 h at 37 °C prior to analysis.

2.2.5. DNA condensation

DNA condensation was monitored by ethidium bromide (EtBr) fluorescence quenching assay (Chen et al., 2000; Ahmed et al., 2006; Geall and Blagbrough, 2000). Briefly, DNA (pmaxGFP, 2 μg) was diluted to 100 μL with buffer (20 mM NaCl, 2 mM HEPES, 10 μM EDTA, pH 7.4) in a glass cuvette. Aliquots of different dendrimers were added to a final charge ratios (N/P) ranging from 0.1 to 40 and allowed to equilibrate for 10 min. EtBr solution (1 μL, 0.5 mg/mL) was added to the stirring solution. The fluorescence was measured, after equilibration with EtBr for 1 min, using SpectraMax M2 Multi-detection Reader (Molecular Devices, Sunnyvale, CA) with excitation and emission at 260 and 600 nm, respectively. Fluorescence was expressed as the percentage of the maximum fluorescence when EtBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EtBr in solution.

2.2.6. Gel retardation assay

Dendriplexes were prepared at different N/P ratios (0.5–20) between the dendrimer and the plasmid DNA by incubating in HEPES buffer (25 mM, 10 mM $MgCl_2$, pH 7.4) at room temperature for 30 min (Kukowska-Latallo et al., 1996; Bergeron et al., 1997). Each sample was analyzed by electrophoresis on a 1% agarose containing ethidium bromide (0.5 μg/mL) at 75 V for 1 h.

2.2.7. Zeta potential and particle size measurements

Zeta potential and size of the dendriplexes prepared at different N/P ratios were measured by using NICOMP 380 ZLS Particle Sizing System (Santa Barbara, CA). All measurements were carried out on the dendriplexes with 5 μg/mL plasmid DNA in HEPES buffer at pH 7.4.

2.2.8. Dendriplex stability

Dendriplexes were prepared at different N/P ratios (1, 5, 10 and 20) and each dendriplex solution was incubated with DNase I at a final DNase I concentration of 5 U/μg plasmid DNA at 37 °C for 45 min. 3 μL of EDTA (500 mM) solution was added to stop the DNA degradation (Huang et al., 2007). Naked DNA, with and without DNase I treatment was used as the control. All the samples were analyzed by 1% agarose gel electrophoresis to evaluate the integrity of DNA in the dendriplexes.

2.3. Transfection studies

2.3.1. Determination of optimal N/P ratio:

HEK 293T (human embryonic kidney) cells were plated at a density of 7.5×10^4 cells/well in a 12 well plate, grown for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity using RPMI 1640 Medium (pH 7.4) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin solution. Dendriplexes were prepared by complexing 1 μg of pmaxGFP with the dendrimers (PAMAMG4 and ornithine-conjugated PAMAMG4 dendrimers) at various N/P from 1 to 20, and were added to the cells and incubated for 7 h at 37 °C. The medium was removed, washed with PBS pH 7.4 and the cells were incubated with growth medium for another 48 h. Levels of GFP in the transfected cells were detected and corrected for background fluorescence of the control cells by FITC (emission 530/30 bandpass) filter using fluorescence-activated cell sorting (FACS) machine (BD FACS Calibur system, argon ion laser 488 nm). Histograms were generated and analyzed using CellQuestPro software program (BD Biosciences). Transfection efficiency was calculated based on the percentage of the cells that expressed pmaxGFP (positive cells) in the total number of cells.

2.3.2. Effect of serum on transfection

To determine the effect of serum on transfection efficiency, upon incubation of HEK 293T cells in growth medium for 24 h, the medium was removed and 1 mL of either of the following, a) dendriplex solution in RPMI, b) dendriplex solution in RPMI with 10% FBS were added. Following incubation for 7 h the medium was removed, washed with PBS, and incubated in 10% FBS-containing RPMI for another 48 h. Transfection efficiency was determined by flow cytometry.

2.3.3. Transfection efficiency in various cell lines

A comparative transfection study was performed in HEK 293T, GM7373 (bovine aorta endothelial cell line) and NCI H157G (human non-small cell lung carcinoma) cell lines. NCI H157G cell line was a kind gift from Dr. Robert A. Casero Jr., School of Medicine, Johns Hopkins University, Baltimore, MD and GM7373 cell line was provided by Dr. Daniel Cervantes, Department of Chemistry and Biochemistry, South Dakota State University, Brookings, SD. Cells were plated at a density of 7.5×10^4 cells/well in a 12 well plate, grown for 24 h at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity using RPMI 1640 Medium (pH 7.4) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin solution. Dendriplexes were prepared by complexing 1 μg of pmaxGFP with the dendrimers (PAMAMG4, PAMAMG4-ORN60) in RPMI 1640 Medium at N/P ratio 10 and were added to the cells and incubated for 7 h at 37 °C. Cells were washed with PBS pH 7.4 and incubated with growth medium for 48 h and qualitative assessment of pmaxGFP expressed in transfected cells was done by observing the cells under a fluorescent microscope (Olympus IX70) and the transfection efficiency was determined by flow cytometry as described above. The geometric mean fluorescence intensity (GMFI) obtained by statistical analysis (CellQuestPro software) was quantified in relative fluorescence units (RFU) and all the values were normalized to the control.

2.3.4. Transfection in presence of excess of ornithine

HEK 293T cells were seeded at a density of 7.5×10^4 cells/well in a 12 well plate and were grown at 37 °C for 24 h in an atmosphere of 5% CO_2 and 95% relative humidity using RPMI 1640 Medium (pH 7.4) supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin solution. Cells were treated with or without excess of ornithine (100 μM) 90 min prior to the addition of the dendriplexes and incubated for 7 h at 37 °C. The medium was removed, washed with PBS pH 7.4 and the cells were incubated in 10% FBS-containing RPMI 1640 for another 48 h. pmaxGFP expression was analyzed by flow cytometry as described above. A concentration of 100 μM of ornithine was selected in the experiments as many previous studies have used this concentration in the polyamine transport studies and ornithine at this concentration does not interfere with the viability of the cells. Transfection experiment in the absence of ornithine served as the positive control while cells transfected with naked DNA served as negative control.

2.4. In vitro cytotoxicity

In vitro cytotoxicity of the dendriplexes was evaluated by MTT (methylthiazoletetrazolium) assay (Sgouras and Duncan, 1990).

HEK 293T cells were seeded at a density of 1×10^4 cells/well in a 96 well plate. Following overnight incubation, cells were treated with various concentrations (10–80 μg/mL) of dendriplexes prepared at N/P ratio 10. After 24 h incubation, 50 μL of MTT (5 mg/mL) was added and the cells were incubated for approximately 6 h. The growth medium was removed and 150 μL of dimethyl sulfoxide (DMSO) was added to dissolve the MTT crystals and the optical density was read using a SpectraMax M2 microplate reader (Molecular devices, Sunnyvale, CA) with 590 nm as excitation wavelength and 650 nm as the background. Viability of cells exposed to dendriplexes was expressed as a percentage of the viability of cells grown in the absence of dendriplexes.

2.5. Statistical analysis

Statistical analysis was performed using two-way ANOVA with general linear model (Graph Pad InStat 3.05 version software (San Diego, CA) followed by multiple comparison. $p < 0.05$ was considered statistically significant and $p < 0.01$ was considered very significant.

3. Results

Present study aims at developing a potent transfection agent by grafting polyamine molecules onto the surface of the PAMAM (generation 4) dendrimers. PAMAM Dendrimers are commercially available at a relatively low cost, are reasonably small in molecular weight, and contain a reasonable number of terminal amines that are believed to contribute to the endosome-buffering effect. It was assumed that synthesis of the dendrimer that has surplus of spatially oriented polyamine molecules after complex formation with DNA might contribute to enhanced uptake by cells, resulting in high transfection efficiency.

It has already been demonstrated that transfection efficiency of the dendrimers increases with the generation size with PAMAM (generation 6) being the most efficient transfecting agent in Rat2 cells (Dufes et al., 2005). However, dendrimer cytotoxicity also increases with generation (Geall and Blagbrough, 2000; Duncan and Izzo, 2005; Brazeau et al., 1998). It is, therefore, advantageous to select a lower generation dendrimer with high cell penetration efficacy and this is exactly the basis for the selection of PAMAM (generation 4) dendrimers in the present study.

3.1. Synthesis of ornithine-conjugated PAMAM dendrimers

^{1}H NMR spectra of the surface modified dendrimers have shown the protons between δ 1 and 2 ppm unlike the PAMAMG4 dendrimers where protons were seen only after δ 2 ppm (Fig. 1a). The protons between δ 1–2 ppm are the characteristic protons derived from four protons on the β- and γ-carbons of ornithine ($COCHN_2CH_2CH_2CH_2NH$). Additionally, the proton attached to the α-carbon of the amino acid ornithine is the most downfielded based on its structure (b, 1H, NHCH CO) and it was not seen in the spectrum of the PAMAMG4 dendrimer. The ratio of the integration observed for the protons at α-carbon and protons at β- and γ-carbons is 1:4, which is consistent with the structure of ornithine. Chemical shift of the protons at the δ-carbon were mixed with those of the protons present in the dendrimer. Overall, the NMR spectrum confirms the conjugation of ornithine to the dendrimer. MALDI-TOF spectra (Fig. 1b) have revealed that out of 64 surface amine groups of PAMAMG4 dendrimer, 60 molecules of ornithine were attached (PAMAMG4-ORN60).

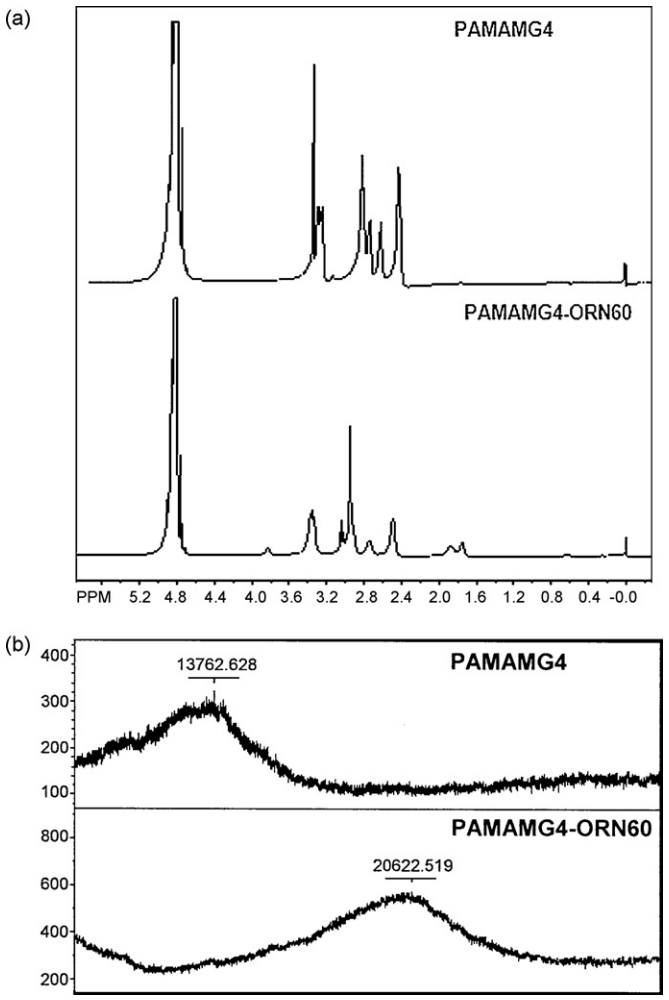

Fig. 1. (a) ^{1}H NMR (400 MHz) spectra of PAMAMG4 and ornithine-conjugated PAMAMG4 dendrimers. (b) MALDI-TOF of PAMAMG4 and ornithine-conjugated PAMAMG4 dendrimers.

^{1}H NMR (400 MHz, D_2O) spectra of the dendrimers are as follows:

PAMAMG4: δ (ppm) 3.5–3.2 (m, $CONCH_2$) 3.0–2.6 (m, NH_2CH_2–$CH_2NCH_2CH_2CO$); 2.75–2.4 (m, CH_2CO).
PAMAMG4-ORN60: δ (ppm) 3.83–3.78 (b, NHCHCO); 3.5–3.3 (m, $CONCH_2CH_2CO$) and (m, $CONCH_2CH_2NH$); 3.10–2.62 (m, $CONCH_2CH_2NCH_2CH_2CO$); 2.0–1.8 (m, $COCNH_2CH_2$ CH_2CH_2NH).

3.2. DNA condensation

Ethidium bromide is a cationic dye. When the phenanthridium moiety of this molecule intercalates DNA, a large increase in its fluorescence is observed making it a useful probe to measure polymer–DNA interactions. Interactions between PAMAMG4 dendrimers (and the modified dendrimers) and the plasmid DNA (pmaxGFP) were evaluated from the ability of the ethidium bromide to displace dendrimers from the DNA. As shown in Fig. 2, a gradual decrease in the percent displacement by ethidium bromide with increase in N/P ratio (from 0.2 and 40) was observed with both PAMAMG4 as well as PAMAMG4-ORN60. No significant difference in percent displacement was seen between PAMAMG4 and PAMAMG4-ORN60 at the N/P ratios tested. Lowest % displacement of PAMAMG4 (23.19 ± 6.9%) and PAMAMG4-ORN60 (16.42 ± 5.12%) was at N/P ratio 40.

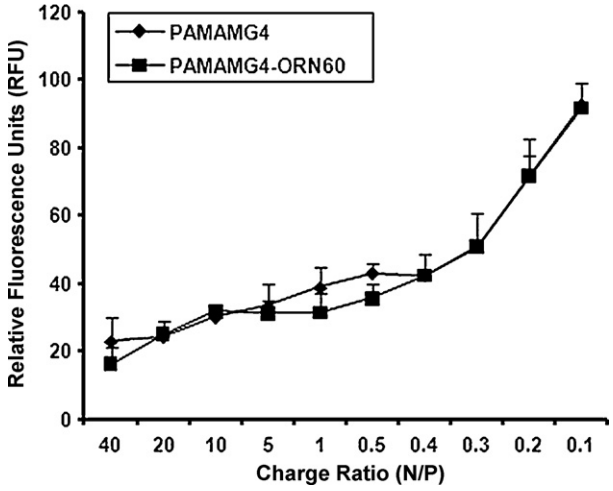

Fig. 2. DNA condensation assay of dendrimers (PAMAMG4, PAMAMG4-ORN60) at different charge ratios (0.1 to 40). pmaxGFP was mixed with aliquots of different dendrimers to a final N/P ratio ranging from 0.1 to 40 for 10 min followed by EtBr for 1 min. Fluorescence was expressed as the percentage of the maximum fluorescence when EtBr was bound to the DNA in the absence of competition for binding and was corrected for background fluorescence of free EtBr in solution ($n = 3$, $p < 0.05$).

3.3. Gel retardation assay

Formation of dendrimer–DNA complexes was assessed by 1% agarose gel electrophoresis at different N/P ratios as shown in Fig. 3. Complete retardation of DNA was seen at N/P ratio 1 and above with the PAMAMG4 and PAMAMG4-ORN60 dendrimers. At N/P ratio ≥ 1 where the dendrimer was in excess, the dendriplexes appeared to have net positive charge as the DNA migrated towards the cathode.

3.4. Size and zeta potential measurement of the dendriplexes

It is well known that transfection efficiency of the dendriplexes depend on their charge, stability and size (Belinska et al., 1997; Mislick and Baldeschwieler, 1996; Wiethoff et al., 2003; Braun et al., 2005; Hung et al., 2005). Agarose gel electrophoresis has revealed that both PAMAMG4 as well as PAMAMG4-ORN60 dendrimers have formed relatively compact complexes at N/P ≥ 1. Therefore, size of the dendriplexes prepared at N/P ratio 1, 5 and 10 was measured

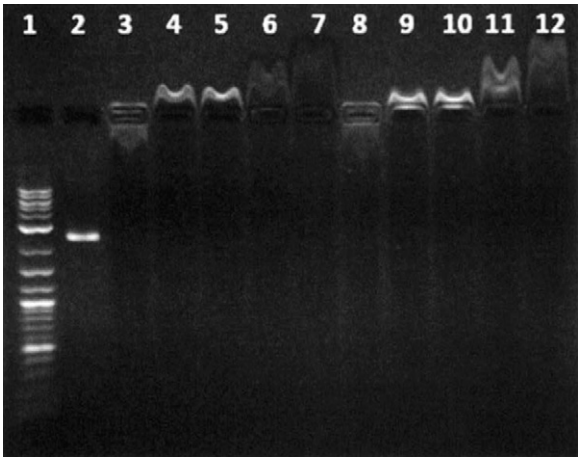

Fig. 3. Agarose gel electrophoretic analysis of dendriplex formation of ornithine-conjugated PAMAMG4 dendrimers. Dendrimers and pmaxGFP were mixed at varying charge ratios (N/P). Values in the brackets represent N/P ratio. Lane 1: DNA marker; Lane 2: pmaxGFP; Lane 3–7: PAMAMG4 dendriplexes (0.5. 1, 5, 10 and 20 N/P); Lane 8–12: PAMAMG4-ORN60 dendriplexes (0.5. 1, 5, 10 and 20).

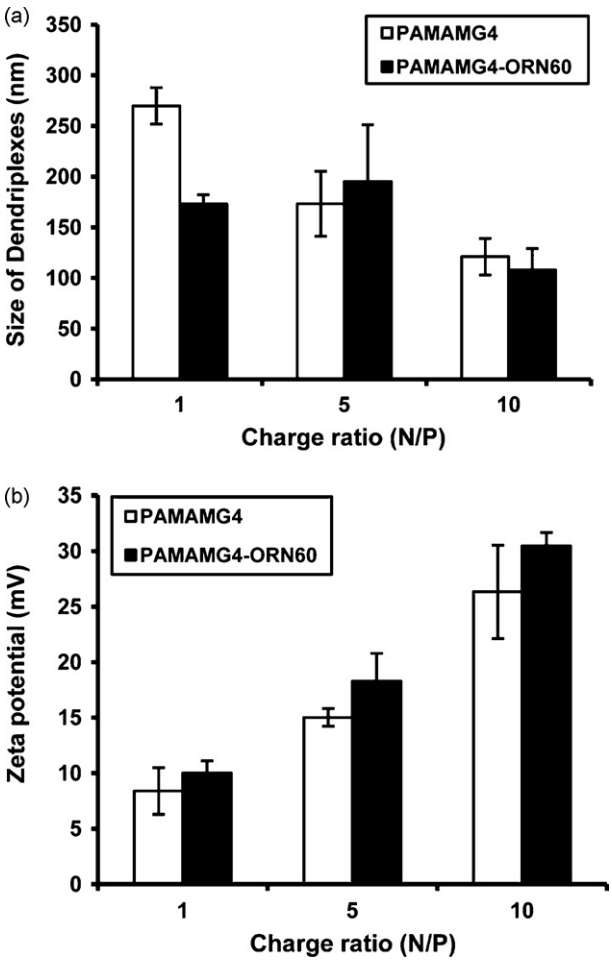

Fig. 4. (a) Size analysis of the dendriplexes. Dendriplexes were prepared with ornithine-conjugated PAMAMG4 and parent PAMAMG4 dendrimers with pmaxGFP at N/P ratios (1, 5 and 10) and their sizes were determined. Results are expressed as mean ± standard deviation ($n = 3$). Size of PAMAMG4-ORN60 dendriplexes was significantly lower than PAMAMG4 at N/P = 1 ($p < 0.05$), but no significant difference was seen at higher N/P (5 and 10). (b) Zeta potential of the dendriplexes. Dendriplexes were prepared with ornithine-conjugated PAMAMG4 and parent PAMAMG4 dendrimers with pmaxGFP at N/P ratios (1, 5 and 10) and their zeta potential were determined. Results are expressed as mean ± standard deviation ($n = 3$). No significant difference in zeta potential was observed between PAMAMG4 and PAMAMG4-ORN60 dendriplexes at all the N/P ratios tested ($p < 0.05$).

by a NICOMP 380 ZLS Particle Sizing System. As shown in Fig. 4a, size of the dendriplexes decreased gradually with increase in N/P ratio. At higher N/P ratios (5 and 10), there was no significant difference in the size between the PAMAMG4 and the PAMAMG4-ORN60 dendriplexes, however at N/P ratio 1, size of PAMAMG4-ORN60 dendriplexes was significantly lower than PAMAMG4 dendriplexes ($p < 0.05$). At N/P ratio 10, size of the dendriplexes with PAMAMG4 and PAMAMG4-ORN60 dendrimers was 121 ± 18 and 108 ± 21 nm, respectively.

Zeta potential measurement helps to predict the stability of the dendriplexes as well as their ability to interact with cell membranes (Wiethoff et al., 2003; Braun et al., 2005; Hung et al., 2005). Dendriplexes are considered stable when they have pronounced ζ-potential values, either positive or negative, but the tendency to aggregate is higher when the ζ-potential is close to zero (Ahmed et al., 2006). A gradual increase in the zeta potential of the dendriplexes was observed as the N/P ratio increased from 1 to 10. At N/P ratio 10, PAMAMG4 dendriplexes have shown a zeta potential of 26.3 ± 4.2 mV, whereas PAMAMG4-ORN60 dendriplexes have shown a zeta potential of 30.5 ± 1.2 mV (Fig. 4b).

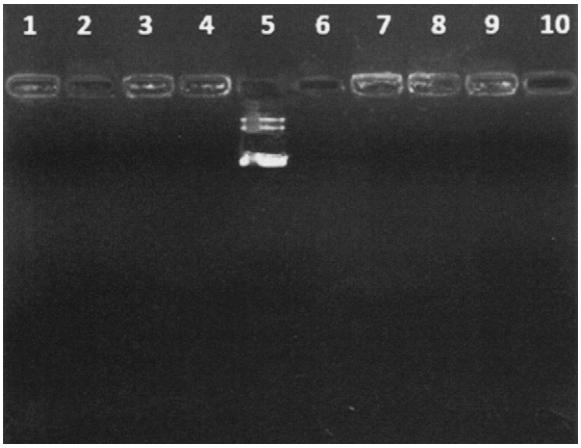

Fig. 5. Stability of dendriplexes against DNase I digestion. Agarose gel electrophoresis was performed following incubation of the dendriplexes with 5U of DNase I/μg of DNA at 37 °C for 45 min. Values in the parentheses represent the N/P ratios. Left to right: Lane 1–4: PAMAMG4 (1, 5, 10 and 20); Lane 5: pmaxGFP; Lane 6: digested pmaxGFP; Lane 7–10: PAMAMG4-ORN60 (20, 10, 5 and 1).

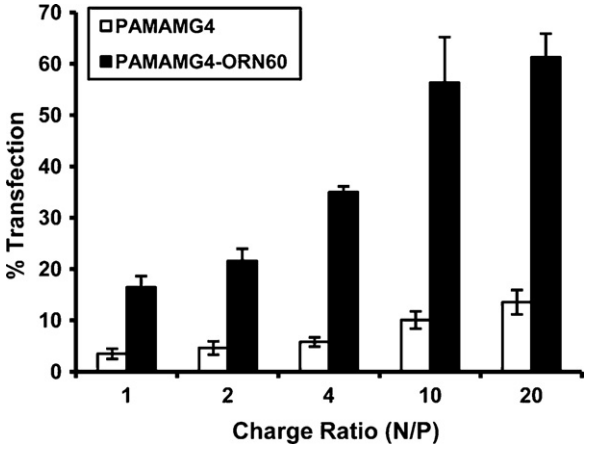

Fig. 6. Effect of charge ratio (N/P) on gene transfection efficiency of the PAMAMG4, PAMAMG4-ORN60 in HEK 293T cells. Cells were transfected pmaxGFP complexed with PAMAMG4, PAMAMG4-ORN60 dendrimers at increasing N/P from 1 to 20. The pmaxGFP expression was analyzed by flow cytometry. Each data point represents the mean ± standard deviation ($n = 3$). Transfection efficiency was significantly higher with PAMAMG4-ORN60 as compared to PAMAMG4 at all the N/P ratios tested ($p < 0.01$).

3.5. Dendriplex stability

The plasmid DNA protection in dendrimer-pmaxGFP complexes was studied against DNase I. Dendriplexes prepared at various charge ratios (N/P) were incubated with DNase I and their stability was analyzed by 1% agarose gel electrophoresis (Fig. 5). Intact pmaxGFP served as the positive control (lane 5). While plasmid pmaxGFP was completely digested with DNase I, PAMAMG4-ORN60 and PAMAMG4 dendriplexes (at N/P ≥5) were found to be stable and appear to move towards cathode because of their high positive charge.

3.6. Transfection studies

Preliminary gene transfection experiments were performed with PAMAMG4 as well as PAMAMG4-ORN60 dendrimers in HEK 293T cells. HEK 293T cells were chosen for the transfection experiments as they are usually vulnerable to non-viral transfection agents.

3.6.1. Determination of optimal N/P ratio

Initially, optimal charge ratio (N/P) of dendrimer-pmaxGFP complexes for effective transfection efficiency was determined by incubating the HEK 293T cells with dendriplexes at different charge ratios (N/P) from 1 to 20. Naked (uncomplexed circular) pmaxGFP DNA, a negative control, typically gave a 0.4–1% transfection. As shown in Fig. 6, transfection efficiency increased with increase in charge ratio (N/P) for both PAMAMG4 as well as PAMAMG4-ORN60 dendriplexes. However, transfection efficiency of PAMAMG4-ORN60 dendriplexes was significantly higher than the parent PAMAMG4 dendriplexes ($p < 0.01$). No significant difference in transfection efficiency of PAMAMG4-ORN60 dendriplexes was seen between N/P ratios 10 (56.4 ± 8.8) and 20 (61.3 ± 4.6%). Therefore, N/P ratio 10 was chosen for further transfection studies.

3.6.2. Effect of serum on transfection

Many cationic non-viral vectors are known to be sensitive to serum. To study the effect of serum, transfection experiments were conducted in the presence and absence of serum containing growth media. While transfection efficiency of PAMAMG4 dendriplexes was not affected by the serum, transfection efficiency of PAMAMG4-ORN60 dendriplexes was insignificantly increased from 48.2 ± 2.1% to 52.8 ± 4.6% when incubated with and without serum (10% fetal bovine serum) containing media, respectively (Fig. 7). It seems that PAMAMG4 as well as ornithine-conjugated PAMAMG4 dendriplexes not only tolerate the presence of other negatively charged macromolecules such as serum proteins but also protect the plasmid DNA from degradation by nucleases.

3.6.3. Transfection efficiency in various cell lines

As shown in Fig. 8a, transfection efficiency with PAMAMG4-ORN60 dendriplexes was significantly higher than the PAMAMG4 dendriplexes in all the three cell lines tested. In spite of significantly longer doubling time (and therefore presumably less PAT activity) of NCI H157G cells (26 h) as compared to HEK 293T cells (16 h), transfection efficiency of PAMAMG4-ORN60 dendriplexes in NCI H157G cells (65.8 ± 4.8%) was slightly higher as compared to HEK 293T cells (49.6 ± 5.6) (Fig. 8b). Interestingly even in GM7373, a bovine endothelial cell line known hard to be transfected, PAMAMG4-ORN60 dendriplexes have shown a transfection efficiency of 17.5 ± 1.4%, whereas, it was only 6.4 ± 0.62% with parent PAMAMG4 dendrimers ($p < 0.05$). Similarly, as shown in Fig. 8b, the levels of GFP expression (represented as RFU) were signifi-

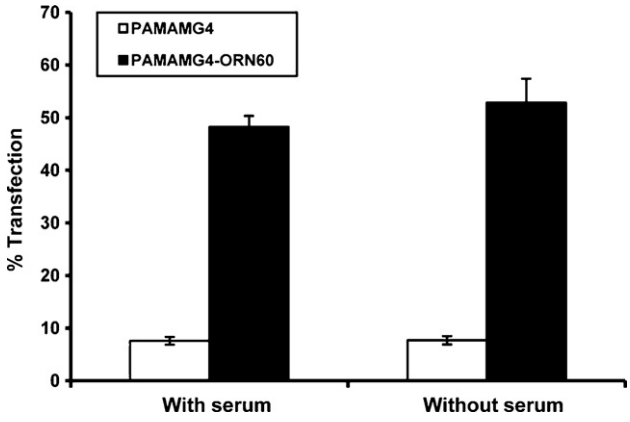

Fig. 7. Effect of serum on transfection efficiency of PAMAMG4 and Ornithine-conjugated PAMAMG4 dendrimers. Following incubation of PAMAMG4-ORN60 and PAMAMG4 dendriplexes in HEK 293T cells, for 7 h in with or without serum containing RPMI, cells were washed and incubated in 10% FBS-containing RPMI for 48 h. Transfection efficiency was determined by flow cytometry.

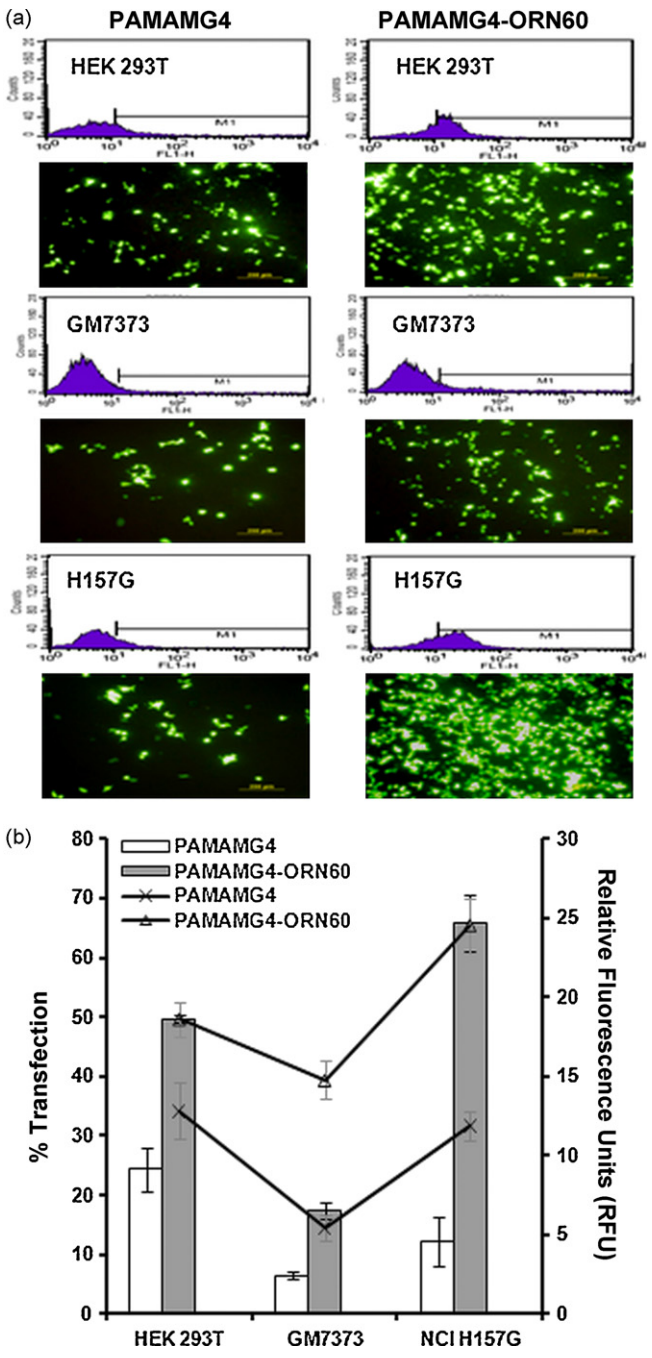

Fig. 8. (a) Gene transfection in HEK 293T, GM7373 and NCI H157G cells. Cells were transfected with pmaxGFP complexed in PAMAMG4, PAMAMG4-ORN60 dendrimers at N/P 10. Histograms were generated using CellQuestPro software and corresponding fluorescent micrographs obtained by fluorescent microscopy of expressed GFP in the above mentioned cell lines. (b) Comparative flow cytometry analysis of PAMAMG4 and PAMAMG4-ORN60 dendriplexes in various cell lines. Cells were transfected with pmaxGFP complexed in PAMAMG4, PAMAMG4-ORN60 dendrimers at N/P 10. Transfection efficiency was determined using CellQuestPro software. Bars represent number of GFP positive cells (%) while line exhibits amount of GFP expressed per cell. Each data point in line represents the mean ± standard deviation ($n = 3$). Percent transfection and RFU with PAMAMG4-ORN60 was significantly higher than PAMAMG4 dendrimers in all three cell lines tested ($n = 3$, $p < 0.05$).

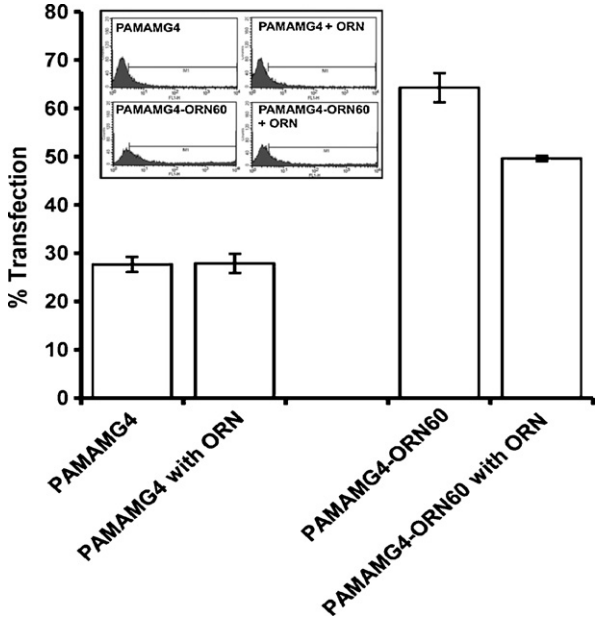

Fig. 9. Presence of ornithine reduces the transfection efficiency of PAMAMG4-ORN60 dendriplexes. HEK 293T cells were transfected in absence or presence of ornithine (ORN) 100 μM. A comparative transfection was performed with PAMAMG4, PAMAMG4-ORN60. All transfection experiments were performed in triplicate wells and the GFP expression was analyzed by flow cytometry. Results are expressed as the mean ± standard deviation ($n = 3$). Transfection efficiency of PAMAMG4-ORN60 significantly decreased in presence of free ornithine ($p < 0.05$), unlike parent PAMAMG4 dendrimers.

cantly higher with PAMAMG4-ORN60 (18.61 ± 1.05, 14.76 ± 1.21, 24.51 ± 1.75) as compared to PAMAMG4 (12.83 ± 1.76, 5.47 ± 0.83, 11.87 ± 0.95) in HEK 293T, GM7373 and NCI H157G cells, respectively.

3.6.4. Transfection in presence of excess of ornithine

A control experiment with 100 μM ornithine did not show any change in the viability of the HEK 293T cells. As shown in Fig. 9, transfection efficiency of PAMAMG4 dendrimers was not affected in presence of excess of ornithine (100 μM) but PAMAMG4-ORN60 dendriplexes have shown a decrease in transfection efficiency from 64.3 ± 3.02% to 49.6 ± 0.53% indicating that both free ornithine and ornithine-conjugated dendrimers may share the same transporter system (PAT).

3.7. In vitro cytotoxicity

Cytotoxicity of the cationic polymers is generally attributed to the interaction of polymers with the cell membrane and efficiency of cellular uptake (Fischer et al., 2003; Duncan and Izzo, 2005; Brazeau et al., 1998). Dendriplexes prepared at N/P ratio 10 were tested for their toxicity at different concentrations from 10 to 80 μg/mL based on the dendrimer concentration. No significant difference in cell viability between the PAMAMG4 and PAMAMG4-ORN60 dendriplexes was seen up to 50 μg/mL concentration (Fig. 10). At 80 μg/mL, PAMAMG4-ORN60 was more toxic (53.5 ± 4.3% cell viability) than PAMAMG4 (98.4 ± 6.8% cell viability) dendriplexes. Toxicity of PAMAMG4-ORN60 dendriplexes may be attributed to their high internalization capacity as compared to parent PAMAMG4 dendrimers. Typically 70% cell viability has been reported to be acceptable for a safe DNA delivery (Ahmed et al., 2006). Results above show that the PAMAMG4-ORN60 dendriplexes are relatively safe at concentrations ≤50 μg/mL at N/P ratio 10, while they are toxic above this concentration.

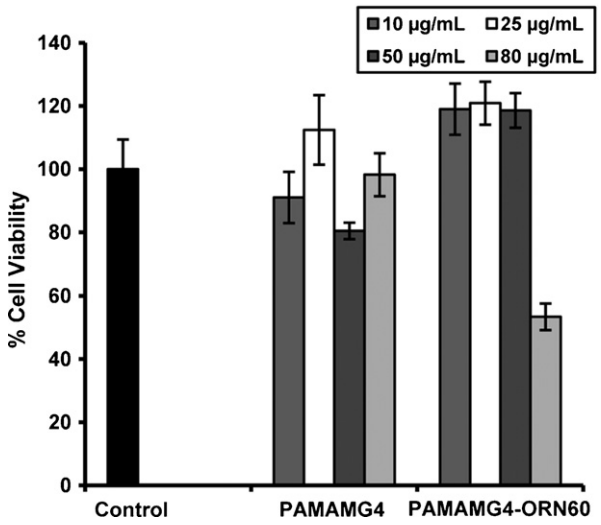

Fig. 10. Effect of dendriplexes (10–80 µg/mL) on HEK-293T cell viability after 24 h of incubation at 37 °C. Cells were incubated with the dendriplexes prepared at N/P ratio 10, with final dendrimer concentration ranging from 10 to 80 µg/mL. Results are reported as percent cell viability as compared to control ($n = 4$, $p < 0.05$).

4. Discussion

Dendrimers are considered as one of the most efficient vectors for gene transfection. In spite of their relatively high transfection efficiency, there is an urgent need to develop dendrimers with target specificity and low toxicity. Exploring the various membrane transporter proteins to improve the cellular internalization of these vectors in cancer cells is an attractive direction to design novel cancer cell-specific transfection agents. As it is well known that cancer cells show activated PAT protein, we investigated the potential of ornithine, a precursor of polyamine, to improve the transfection efficiency and specificity of PAMAM dendrimers.

Initially, ornithine was conjugated to PAMAMG4 dendrimers by EDC coupling reaction. But, because of low conjugation efficiency and the formation dendrimers–ornithine–dendrimers conjugates, Fmoc synthesis protocol was used as an alternative. MALDI-TOF of the conjugate shows that that out of 64 surface amine groups of PAMAMG4 dendrimer, 60 amine groups were conjugated with ornithine (PAMAMG4-ORN60).

Ethidium bromide assay demonstrated that at charge ratio (N/P) >1, the dendrimers–DNA complexes were compact and stable and ethidium bromide could not displace the DNA from the complexes, indicating the higher affinity between the dendrimers and the DNA as compared to DNA and ethidium bromide. Therefore, a decreased fluorescence signal was observed in presence of dendrimers–DNA complexes as compared to when there was no dendrimers. An inverse relationship was observed between the charge (N/P) ratio and the percent displacement with both the PAMAMG4 and PAMAMG4-ORN60 dendrimers. No significant difference in DNA condensation was observed between PAMAMG4 and PAMAMG4-ORN60 dendrimers indicating that both of these dendrimers could form very compact complexes with DNA. Similar results were observed with agarose gel retardation assay. Gel retardation assay demonstrated that the dendrimers could completely retard DNA migration at charge (N/P) ratio >1, indicating that the dendrimers–DNA complexes were stable, and they have net positive charge as the complexes migrated towards cathode. PAMAMG4 dendrimers have 64 amine groups on the surface, whereas, PAMAMG4-ORN60 dendrimers have 124 amine groups. At the physiological pH only about 45% of these amine groups are ionized. Therefore, at lower charge ratios (N/P $\leq$1), PAMAMG4-ORN60 could condense the DNA more effectively as compared to simple PAMAMG4 dendrimers. However, at higher charge ratios (N/P of 5 and 10), because of the availability of optimal number of charged surface amine groups, no significant difference in dendriplex size and zeta potential was observed between these dendrimers.

Transfection efficiency of the dendriplexes increased with increase in charge ratio (N/P) for PAMAMG4 as well as PAMAMG4-ORN60 dendrimers. As mentioned previously, because of presence of relatively higher positive charge on the surface, transfection efficiency of PAMAMG4-ORN60 dendriplexes is expected to be greater than the simple PAMAMG4 dendriplexes. With PAMAMG4-ORN60 dendriplexes, transfection efficiency increased up to charge ratio of 10, and further increase in charge ratio could not increase the transfection efficiency significantly. It is possible that at charge ratio of 10, the dendrimers–DNA complexes were stable with optimal positive charge for interaction with the cell membrane. At charge ratio of 20, cellular internalization and endosomal escape may have been increased, but the more compact PAMAMG4-ORN60 complexes might not have released as many DNA molecules as expected resulting in insignificant increase transfection efficiency. It is possible that further increase in charge ratio may result in significant decrease in transfection efficiency of these dendrimers as the release of the DNA from the compact dendriplexes may be difficult.

Simple plasmid DNA rapidly undergoes degradation by the nucleases present in the serum or cytoplasm in the cells. Complexation of the plasmid DNA with cationic vector results in compact particles conferring protection against nucleases. However, serum proteins may block the interaction between the dendriplexes and the cell membrane thus retarding the transfection efficiency. To assess the ability of the dendrimers to protect the DNA against serum nucleases, the effect of serum on the transfection efficiency was determined. In the present study, presence of serum did not affect the transfection efficiency of PAMAMG4 as well as PAMAMG4-ORN60 dendrimers. Based on the physicochemical parameters of the dendrimers–DNA complexes (such as gel retardation, size and zeta potential, and stability against DNase I), it appears that the dendrimers could efficiently condense DNA because of the excessive positive charge on their surface, which in turn helps in charge-mediated interaction between the cell membrane and the dendriplexes.

It is well known that transfection efficiency of the synthetic vectors depends on the nature of the cell line. This may be because of the difference in cellular binding, internalization mechanism and the properties of the cell line such as doubling time, rate of uptake of nutrients and related biochemical processes (Douglas et al., 2008; Mo and Lim, 2004; Panyam et al., 2002; Qaddoumi et al., 2003). Percent transfection efficiency and GFP expression levels with PAMAMG4-ORN60 dendriplexes were significantly higher than simple PAMAMG4 in all the cell lines tested. Similarly, GFP expression was also greater in NCI H157G cells than HEK 293T and GM 7373 cells. Fluorescent microscopy and flow cytometry data presented in Fig. 8a and b demonstrate that cellular internalization of pmaxGFP and protein (GFP) expression are significantly higher with PAMAMG4-ORN60 as compared to PAMAMG4 dendriplexes. Results clearly indicate that PAMAMG4-ORN60 is a better transfecting agent as compared to simple PAMAMG4 dendrimers. However it is not clear if the higher transfection efficiency of the PAMAMG4-ORN60 dendriplexes is due the combined effect of rapid internalization via PAT mediated uptake and charge-mediated endocytosis or is just charge-mediated endocytosis alone.

To determine the role of PAT in the internalization of PAMAMG4-ORN60 dendriplexes, a preliminary transfection experiment was performed in presence of free ornithine. As difference in size and zeta potential between PAMAMG4 and PAMAMG4-ORN60 dendriplexes were moderate, higher transfection efficiency with

PAMAMG4-ORN60 demonstrates the involvement of cellular internalization mechanisms other than charge-mediated endocytosis. In the present study, presence of 100 μM of ornithine significantly reduced the transfection efficiency of ornithine-conjugated dendrimers and the viability of the cells was not affected. However, though 200 μM of ornithine also significantly decreased the transfection efficiency of these dendrimers, it was toxic to the cells as several dead cells were seen (data not shown). 50 μM of ornithine reduced the transfection of the dendrimers to a lesser extent as compared to 100 μM of ornithine. As the presence of ornithine decreased the transfection efficiency of PAMAMG4-ORN60 unlike simple PAMAMG4, the role of PAT in their uptake may be considered significant. However, further studies are warranted to confirm this hypothesis.

5. Conclusion

In conclusion, PAMAMG4-ORN60 dendrimers have shown high transfection efficiency as compared to parent PAMAMG4 dendrimers. Conjugation of ornithine to PAMAMG4 dendrimers increased their molecular weight and the number of surface amine groups resulting in higher zeta potential of PAMAMG4-ORN60 dendriplexes as compared to PAMAMG4 dendriplexes. Nevertheless, no significant difference in size was observed between the PAMAMG4 and PAMAMG4-ORN60 dendriplexes. PAMAMG4-ORN60 dendrimers have also shown high transfection efficiency as compared to parent PAMAMG4 dendrimers in all the cell lines tested in the present study. It has been suggested that polyamines are transported by a plasma membrane carrier PAT and then sequestered into pre-existing polyamine sequestering vesicles (PSVs) and/or by polyamine receptor mediated endocytosis (Soulet et al., 2004; Casero and Marton, 2007). Several polyamine conjugates with diverse chemical structures were shown to be transported by PAT indicating that PAT is a non-selective transporter (Gardner et al., 2004; Kaur et al., 2008; Tsen et al., 2008). Though the exact basis for the high transfection efficiency of PAMAMG4-ORN60 dendrimers is not known at present, one possibility is that conjugation with ornithine increases the surface charge density of the dendrimer resulting in enhanced adosrptive endocytosis. Additionally, results above suggest that uptake PAMAMG4-ORN60 dendrimers was inhibited by ornithine indicating the role of PAT in their uptake. Further studies to understand the mechanism of PAMAMG4-ORN60 dendrimers uptake are in progress which may help in developing new polyamine-based vectors with higher cancer cell specificty.

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