The Lower-Generation Polypropylenimine Dendrimers Are Effective Gene-Transfer Agents

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Objective. To evaluate polypropylenimine dendrimers (generations 1–5: DAB 4, DAB 8, DAB 16, DAB 32, and DAB 64) as gene delivery systems.

Methods. DNA binding was evaluated by measuring the reduced fluorescence of ethidium bromide, and molecular modelling of dendrimer-DNA complexes also was performed. Cell cytotoxicity was evaluated against the A431 cell line using the MTT assay. *In vitro* transfection was evaluated against the A431 cell line using the β-galactosidase reporter gene and *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethylammonium methylsulphate (DOTAP) served as a positive control.

Results. Molecular modeling and experimental data revealed that DNA binding increased with dendrimer generation. Cell cytotoxicity was largely generation dependent, and cytotoxicity followed the trend DAB 64 > DAB 32 > DAB 16 > DOTAP > DAB 4 > DAB 8, whereas transfection efficacy followed the trend DAB 8 = DOTAP = DAB 16 > DAB 4 > DAB 32 = DAB 64.

Conclusion. The generation 2 polypropylenimine dendrimer combines a sufficient level of DNA binding with a low level of cell cytotoxicity to give it optimum *in vitro* gene transfer activity.

KEY WORDS: polypropylenimine dendrimers; gene delivery; Astramol; cytotoxicity; molecular modeling.

INTRODUCTION

The possibility of using genes as medicines to correct genetic disorders or treat cancers is hampered by the inability to efficiently deliver genetic material to diseased sites (1). A variety of viral and non-viral systems are being used experimentally, each with distinct advantages and disadvantages. Viral systems (2) have been studied extensively and include a wide variety of viral types such as retroviruses, adenoviruses, adeno-associated viruses, herpes simplex virus, and the HIV-based lentivirus. All have various inherent disadvantages, such as safety concerns and scale-up difficulties (3). Non-viral systems, such as cationic liposomes (4,5), cationic polymers (6,7), cationic polymeric vesicles (8), and both polyamidoamine (9,10) and phosphorus containing (11) dendrimers, have thus been studied as gene delivery agents in an effort to circumvent some of the safety and production problems associated with viruses.

Polypropylenimine dendrimers contain 100% protonable nitrogens (12) , making them ideally suited as DNA binding and possibly DNA transporting agents (Fig. 1). Polypropylenimine dendrimers also contain up to 64 terminal amino groups (generation 5, DAB 64; Reference 13) (Table I). However, the use of dendrimers as gene delivery agents has largely focused on the high generation polyamidoamine (9,10,14–16) and phosphorous-containing compounds (11) with a mixture of amine/ amide or N-P(O_2)S as the conjugating units, respectively, with no work being reported on the use of the lower generation polypropylenimine dendrimers for gene delivery. However, polypropylenimine dendrimers have been studied as pH-sensitive controlled release systems for drug delivery (17) and their cytotoxicity documented (18). Data on polypropylenimine dendrimers and DNA that could be found in the literature were limited to the interaction of polypropylenimine dendrimers with DNA (19) and a publication describing the poor transfection efficacy of DAB 64 (20). In actual fact, Malik and others concluded that cationic dendrimers, including the polypropylenimine dendrimers, as opposed to the anionic dendrimers are too toxic for parenteral use without further derivatisation with biocompatible groups, such as polyethylene glycol units (18), and Gebhart and Kabanov have reported that DAB 64 is far too toxic above a dendrimer, with a DNA weight ratio of 0.62: 1 (20), for use as a gene transfer agent.

This current work details the gene transfer and cytotoxicity of the polypropylenimine dendrimers, and although the higher-generation dendrimers are indeed comparatively toxic, favorable qualities were found in the lower-generation polypropylenimine dendrimers. The cytotoxicity of gene transfer agents must be taken into account when developing such agents, and we propose that the lower-generation polypropylenimine dendrimers may find a use as non-toxic gene delivery agents.

METHODS

Materials

Dendrimers (DAB 4, DAB 8, DAB 16, DAB 32, and DAB 64) were obtained from Sigma-Aldrich, Co, United Kingdom and were subjected to elemental analysis before use. Elemental analysis data were consistent with the theoretical dendrimer structure. Ethidium bromide, phosphate-buffered saline tablets, and dextrose were obtained from Sigma-Aldrich, Co. United Kingdom, whereas *N*-[1-(2,3-dioleoyloxy)propyl]-*N*,*N*,*N*-trimethyl-ammonium methylsulphate (DOTAP) was obtained from Avanti Polar Lipids (Alabaster, Alabama). Plasmid (pCMVsport β-galactosidase) and all tissue culture media and reagents were obtained from Life Technologies, United Kingdom.

DNA Dendrimer Formulations

Dendrimer-DNA (pCMVSport β-galactosidase) formulations were made by mixing DNA and dendrimers in a 5% dextrose solution and allowing to stand for no longer than 5 min before use. The resulting colloidal dispersion was sized by photon correlation spectroscopy (Malvern Instruments, United Kingdom). Control DNA formulations were produced by diluting DNA stock solutions in 5% w/v dextrose, and control DOTAP formulations were prepared by mixing DNA and a probe sonicated dispersion of DOTAP in a 5% w/v dextrose solution and allowing to stand for no longer than 5 min before use.

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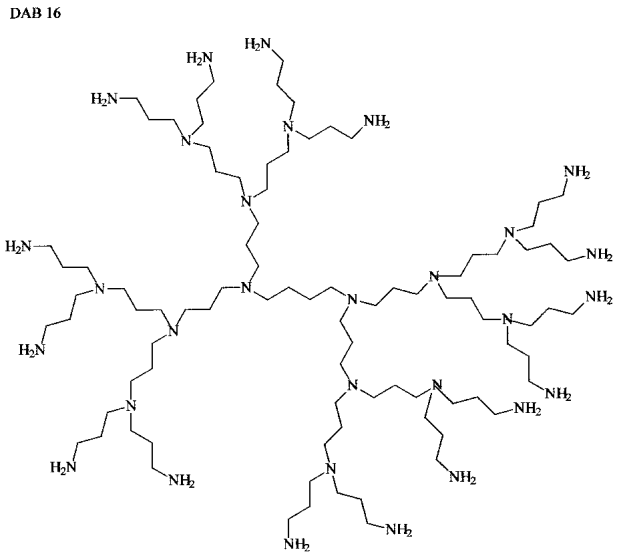

Fig. 1. DAB 16 generation 3 polypropylenimine dendrimer, DAB 64 would contain 2 more generations of propylenimines attached to this molecule.

DNA Condensation

Plasmids were grown in *Escherichia coli* and plasmid purification performed using a QIAGEN Endo-toxin free Giga Plasmid Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Purity was confirmed by agarose gel electrophoresis (21). The reduced fluorescence of ethidium bromide was used to probe for DNA condensation by the dendrimers. Ethidium bromide fluorescence increases significantly (factor 40 compared to unbound ethidium bromide) on intercalation with double stranded DNA (22). The electrostatic interaction between the anionic DNA and cationic groups of the carrier on formation of the DNA-dendrimer complex condenses the DNA and reduces the number of ethidium bromide binding sites, ultimately reducing the fluorescence intensity of the ethidium bromide solution. Complexes of the dendrimers with DNA were prepared at various polymer, DNA weight ratios, and at various time points the fluorescence intensity ($\lambda_{excitation}$ = 526 nm, $\lambda_{emission}$. = 592 nm) of the complexes was determined in the presence of ethidium bromide (0.4 $\mu g\ mL^{-1}$). The DNA concentration in the cuvette was kept constant (10 $\mu g{\cdot}mL^{-1}$), and the polymer solutions in 5% w/v dextrose and a solution of DNA in 5% w/v dextrose served as controls. The reduced fluorescence (F_t/F_0) was determined for each of the samples, where F_t = the fluorescence of the DNA, polymer complexes and F_0 = the fluorescence of DNA alone.

TABLE I. Percent Condensation of DNA by DAB Dendrimers

Dendrimer	Dendrimer molecular weight	Number of surface amine groups	Dendrimer, DNA weight ratio	Percent DNA condensation after 30 min
DAB 4	317	4	30	0
			20	4
			10	14
			5	12
			1	2
DAB 8	773	8	20	81
			15	62
			10	72
			5	59
			1	11
DAB 16	1684	16	5	90
			2	94
			1	96
			0.5	96
			0.25	94
			0.13	43
DAB 32	3508[a]	32	5	94
			2	97
			1	97
			0.50	96
			0.25	87
			0.13	22
DAB 64	7156[a]	64	5	93
			2	95
			1	93
			0.50	96
			0.25	88
			0.13	26

[a] Molecular weight estimated from a perfect dendrimer structure.

DNA-Dendrimer Sizes and Zeta Potential

DNA-dendrimer complexes were sized by photon correlation spectroscopy (Malvern Zetasizer 3000, Malvern Instruments, United Kingdom) and their zeta potential measured (Malvern Zetasizer 3000, Malvern Instruments, United Kingdom).

Molecular Modeling

Molecular modeling was performed using the InsightII (Release 2000)/ Discover (Version 95.0/3.00) software (Accelrys, California) mounted on a Silicon Graphics Octane R12000 workstation. All molecules were constructed using predefined residue libraries in the Biopolymer application of InsightII and parameters and charges assigned using the cff91 force field. All calculations were performed *in vacuo* using a distance dependent dielectric = 1.

Each dendrimer structure initially was minimized using the conjugate gradient algorithm to an energy tolerance value of 0.1 kcal $mol^{-1}Å^{-1}$. To obtain a global minimum, simulated annealing was performed on each dendrimer following the method described by Bhaglet and Roberts (23). A 20-base pair random sequence of double helical B-DNA incorporating two helical turns was constructed and minimized using the conjugate gradient algorithm with the terminal bases restrained using a force constant of 10 kcal $mol^{-1}Å^{-1}$ to an energy tolerance value of 0.1 kcal $mol^{-1}Å^{-1}$. No counterions were included. Each annealed dendrimer was docked with the minimized DNA structure until a minimum intermolecular energy between the two species (summed non bonded contributions from the electrostatic and van der Waals terms from the force field functional form) was achieved. Each complex was then minimized with the terminal bases restrained using the same convergence criteria described above. All studies used a distant-dependent dielectric of 4.0. The intermolecular energies for each complex were then recalculated.

In Vitro Cytotoxicity Assay

A human epidermoid carcinoma cell line (A431, ATCC CRL-1555) was maintained in Dulbecco's minimum essential

medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine at 10% CO_2 and 37°C. Dendrimer cytotoxicity was assessed by measuring the IC_{50} in a modified MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide thiazolyl-blue indicator dye) assay (24). Briefly, 96-well microtiter plates were seeded with 5000 cells per well and incubated for 24 h. Dilutions of the dendrimer, DOTAP, and dendrimer-DNA formulations (100 μL) in tissue culture medium (Opti-Mem devoid of serum) were incubated with the cells for 4 h. The samples were then replaced with fresh DMEM, supplemented with 10% FCS, and glutamine (2 mM) daily and incubated for 72 h. After this period, the indicator dye (50 μL, 50 mg mL^{-1}) was added to each well and incubated with the cells for 4 h in the dark. The medium and indicator dye were then removed and the cells lysed with dimethylsulphoxide (200 μL). After addition of Sorensen's glycine buffer (25 μL), the absorption was measured at 570 nm. Values were expressed as a percentage of the control to which no dendrimers had been added.

In Vitro Transfection

A431 cells (human epidermoid carcinoma cell line, ATCC, CRL-1555), maintained in DMEM supplemented with FCS and L-glutamine (2 mM) were seeded at a density of 10^4 cells per well in 96-well flat-bottomed plates. Cells were incubated for 24 h at 37°C in 10% CO_2. Dendrimer-DNA complexes of varying concentration and volume and serum-free medium (100 μL, OPTIMEM, Life technologies, United Kingdom) were incubated with the cells for 4 h at 37°C in 10% CO_2. Naked DNA served as the negative control, whereas a formulation comprising DOTAP, DNA (5:1) served as the positive control. The negative control was dosed at a level of 20 μg DNA per well, whereas the level of DNA dosed with the dendrimers and DOTAP varied as indicated. At the end of the incubation period, the incubation medium was replaced with DMEM culture media, supplemented with 10% FCS, and 2 mM glutamine and also containing both penicillin (100 U·mL^{-1}) and streptomycin (0.1 mg·mL^{-1}). Cells were once again incubated at 37°C in 10% CO_2 for 48 h. The cells were then washed in phosphate-buffered saline (200 μL) and lysed with 1X passive lysis buffer (80 μL, Promega, UK) for 30 min. The cell lysates were subsequently analyzed for β-galactosidase expression as described below.

To the assay buffer [50 μL (sodium phosphate buffer = 200 mM, pH = 7.3, magnesium chloride = 2 mM, mercaptoethanol = 100 mM, *o*-nitrophenol-β-galactopyranoside = 1.33 mg·mL^{-1})] was added an equal volume of the cell lysate from above within 96-well flat-bottomed plates. Samples were incubated for 1 h, and the visible absorbance read at 405 nm on an automatic plate reader.

RESULTS

DNA Condensation

DNA binds electrostatically with the nitrogen rich dendrimers DAB 8, DAB 16, DAB 32, and DAB 64 (Table I), and this binding prevents the intercalation of ethidium bromide, an indication of DNA condensation. DNA condensation is further confirmed by the size data below. DNA is condensed by DAB 16, 32, and 64 polypropylenimine dendrimers at a minimum nitrogen to phosphate ratio of 1.47, 1.49, and 1.45, respectively (Table I) and a surface nitrogen to phosphate ratio of 0.78, 0.77, and 0.74 respectively. DNA is not completely condensed by DAB 8 within the half hour time period even at high nitrogen to phosphate ratios of 123:1 (Table I). Presumably DAB 4 binds to DNA as a result of the opposing charge carried by this molecule; however, DNA condensation with the resultant exclusion of ethidium bromide from the DNA double helix does not occur with DAB 4.

DNA-DAB Dendrimer Sizes and Zeta Potential

On interaction of DAB 8, 16, 32, and 64 dendrimers with DNA a colloidal suspension is formed with a mean particle size ranging from 163–518 nm (Table II). The lack of condensation of DNA by the DAB 4 dendrimer gives rise to a clear solution and no colloidal dispersion is formed with DAB 4. However, a reduction of the negativity shown by the zeta potential of DNA when mixed with DAB 4 (zeta potential negativity decreases from −36 mV to −13 mV) indicates that an electrostatic interaction occurs between DAB 4 and DNA. The relatively neutral zeta potential (and resulting comparatively large particle size −518nm) shown by DAB 8-DNA complexes (+2 mV) further confirms the incomplete DNA condensation achieved with this molecule. Within the DAB4 and DAB 8 complexes, there is an excess of free phosphate groups, which essentially neutralize the positive charges on the polypropylenimine dendrimer. Charge screening by counterions and resultant complex aggregation, is minimized because all DNA binding experiments are performed in 5% w/v dextrose.

The zeta potential of the dendrimer-DNA complex becomes more positive with dendrimer generation (up to DAB 32, where it plateaus; Table II).

Molecular Modeling Studies

Preliminary molecular modeling studies show that the number of dendrimer-DNA contact points per molecule increases with an increase in dendrimer generation (Fig. 2) as mirrored by the increase in simulated intermolecular energies (Table III). DAB 4 is able to bind across the major groove and the less favorable intermolecular energy of this interaction may be sufficient for the exchange of bound and bulk DAB 4. It should be noted that the intermolecular energies quoted should be viewed as a means to rank the affinities of the dendrimer-DNA complexes and are not representations of the binding enthalpies or free binding energies. DAB 16,

TABLE II. Sizes and Zetasizer Potential of DNA–DAB Formulations in a Continuous Phase of 5% w/v Dextrose

Formulation in 5% dextrose	Mean size (nm) (mean of three measurements)	Zeta potential (mV)[a] (Mean of six measurements)
DAB4, DNA (5:1 g · g^{-1})	Clear solution	−13
DAB8, DNA (5:1 g · g^{-1})	518	+2
DAB16, DNA (5:1 g · g^{-1})	180	+12
DAB32, DNA (3:1 g · g^{-1})	163	+34.1
DAB64, DNA (3:1 g · g^{-1})	n.d.	+30.5

[a] Zeta potential recorded on a solution of DNA in 5% dextrose = −35.8 mV.

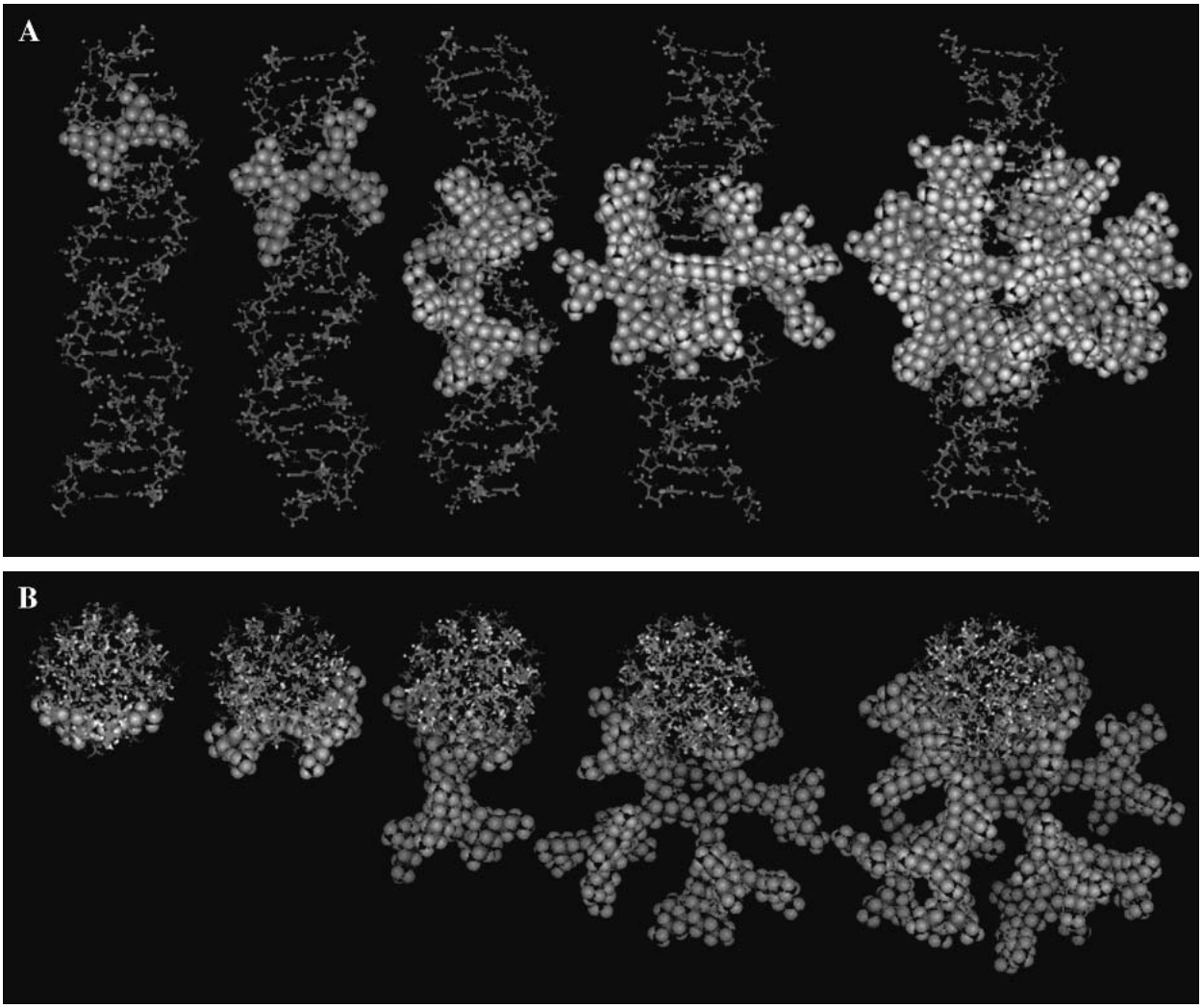

Fig. 2. (A) Molecular models of the dendrimer-DNA binding (side view). From left to right: generation 1–5. (B) Molecular models of the dendrimer-DNA binding (top view). From left to right: generation 1–5.

however, is able to bind an entire helical turn of the DNA molecule, and we conclude that this level of potential contact points is the minimum requirement for the total prevention of ethidium bromide intercalation. The nature of the DAB 16, DNA interaction, by spanning both the major and minor grooves, would prevent base pair separation, which must occur before intercalation. Even allowing for similar weights of dendrimer associating with the DNA, it must be borne in mind that a limited number of dendrimer molecules can bind to a single piece of DNA because of the charge repulsion between closely spaced polycationic molecules and, hence, DNA charge neutralization increases with dendrimer size as evidenced by the zeta potential measurements (Table II).

TABLE III. Comparative Dendrimer–DNA Intermolecular Energies

Dendrimer	Binding energy (Kcal mol^{-1})
DAB 4	–3,932
DAB 8	–9,619
DAB 16	–17,569
DAB 32	–31,325
DAB 64	–61,701

Also the extension into space beyond the DNA double helix of the DAB 16, DAB 32, and DAB 64 dendrimers (Fig. 3b) would hinder the approach of ethidium bromide through charge repulsion. Charge repulsion between dendrimers binding in close proximity to the same DNA molecule or charge repulsion between a bound dendrimer molecule and an approaching ethidium bromide species will be influenced by water. Water has not been represented in these studies other than by a dielectric constant. Simulations, which include an explicit representation of water, are currently in progress in our laboratories. It is also assumed that the dendrimers themselves will replace the phosphate counterions.

The higher generation dendrimers, such as DAB 32 and DAB 64, show no constraint to DNA wrapping around these molecules. DNA is also believed to wrap around the higher generation polyamidoamine dendrimers (25).

In Vitro Cytotoxicity

The cell cytotoxicity of the DAB dendrimers generally increases as the generation increases (Table IV), following the trend DAB 8 < DAB 4 < DAB 16 < DAB 32 < DAB 64. These data support the hypothesis that the cytotoxic activity

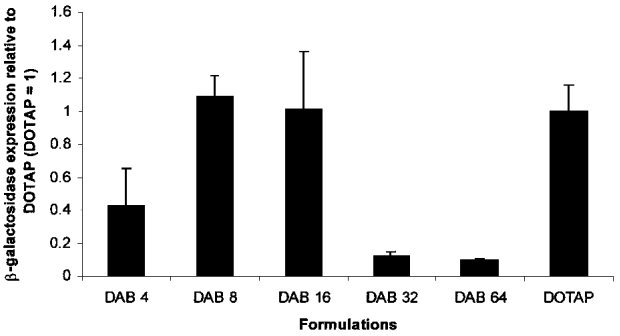

Fig. 3. Transfection efficacy of polypropylenimine dendrimers relative to DOTAP in the A431 cell line studied in 96-well plates. DAB 4 and DAB 8 were dosed at a dendrimer, DNA ratio of 5:1, and a DNA dose of 20 μg per well was used. DAB 16 was also dosed at a dendrimer, DNA ratio of 5:1, but a DNA dose of 5 μg per well DNA was used; DAB 32 and DAB 64 were dosed at a dendrimer DNA ratio of 3:1 and a DNA dose of 20 μg per well. DOTAP was dosed at a DOTAP, DNA ratio of 5:1, and a DNA dose of 20 μg per well. Data represented as the mean ± SD of at least 3 replicates.

of macromolecular cations is in some way dependent on multiple interactions with cellular anions (26). It is worth noting that both DAB 4 and DAB 8 are less cytotoxic than the cationic liposome formulation DOTAP.

The formation of the dendrimer-DNA formulations has a profound effect on the toxicity of the lower generation DAB dendrimers (DAB 4 and DAB 8, Table IV) but not on the higher generation dendrimers. The reduced cytotoxicity of DAB 4 when in the presence of DNA indicates that DAB 4 does bind electrostatically to DNA and that this binding modulates the cytotoxicity of DAB 4. The reduced cytotoxicity of both DAB 4 and DAB 8 on binding to DNA also gives evidence to the hypothesis that the binding of the polypropylenimine dendrimers to essential cellular anions must be responsible for the cytotoxicity observed, just as with other polycations (26) because a reduction in the anion binding sites on the dendrimers by the binding of the lower generation dendrimers with DNA has a positive effect on cell cytotoxicity. These lower generation dendrimers contain a limited number of potential anion binding sites. In the case of the larger dendrimers a number of anion binding sites are still available even after DNA binding is complete. The cytotoxicity of polypropylenimine dendrimer-DNA complexes follows the trend DAB 64 > DAB 32 > DAB 16 > DAB 8 > DAB 4 and is purely generation dependent (Table IV).

TABLE IV. *In vitro* Cytotoxicity against the A431 Cell Line

Formulation	IC50 ($\mu g \cdot mL^{-1}$)
DAB 4	220
DAB 4, DNA (5:1 $g \cdot g^{-1}$)	800
DAB 8	352
DAB 8, DNA (5:1 $g \cdot g^{-1}$)	669
DAB 16	39
DAB 16, DNA (5:1 $g \cdot g^{-1}$)	36
DAB 32	5.7
DAB 32, DNA (3:1 $g \cdot g^{-1}$)	5.8
DAB 64	<5
DOTAP	62

In Vitro Transfection

In vitro transfection efficacy with the polypropylenimine dendrimers reveals that the polypropylenimine dendrimer transfection activity decreased according to the trend DAB 8 > DAB 16 > DAB 4 > DAB 32 > DAB 64 with the DAB 8 and DAB 16 dendrimers being as active as DOTAP at the highest and the lowest DNA doses respectively (Fig. 3, Table V). The improved activity of DAB 16 when only a low dose of DNA is administered (Table V) is due to the reduced cytotoxicity of DAB 16 at the lower dose level. At the lower dose level; 5 μg of DNA and 25 μg of DAB 16 are dosed to the cells, compared to 20 μg of DNA and 100 μg of DAB 16, which is dosed at the higher dose level. The IC_{50} of DAB 16 when complexed to DNA is 36 μg mL^{-1} (Table IV) and a dose of 100 μg DAB 16 per well would have to be administered to the cells at a concentration of 500 μg mL^{-1} (Table V, a dose that is 1 order of magnitude in excess of its IC_{50}! Although a dose of 5 μg DNA would be administered with DAB 16 at a concentration of 200 $\mu g \cdot mL^{-1}$, a less toxic dose but still a dose at a level that exceeds the IC_{50} of this formulation by 8-fold.

It is clear that the toxicity of DAB 16 formulations limits their gene transfer ability. Comparing this with the dosing of DAB 8-DNA complexes, which have an IC_{50} of 669 $\mu g \cdot mL^{-1}$ (Table IV), it can be seen that a dose of 20 μg of DNA per well would require a concentration of DAB 8 of 500 μg mL^{-1} (for a dendrimer, DNA ratio of 5:1, Table V), a concentration below its IC_{50}. As the dose of DNA decreases with the DAB 8 compound, the level of gene expression decreases, indicating that the toxicity of DAB 8 is not interfering with gene expression at this DAB 8, DNA dose ratio. Also at a higher DAB 8, DNA ratio of 10:1 (Table V), transfection efficacy at the higher DNA doses is decreased slightly as a result of the toxicity of DAB 8 becoming apparent with this high concentration of DAB 8 (1 $mg \cdot mL^{-1}$). It should be noted that twice as many cells were used in the transfection experiments when compared to the MTT experiments for cell cytotoxicity.

A comparison of the transfection efficacies of both DAB 8 and DAB 16 at the lowest DNA dose level of 5 μg reveals that the DAB 16 molecule is a more efficient gene transfer agent than DAB 8 as the two compounds give transfection levels relative to DOTAP of 100.8 and 59.2% respectively (Table V).

The toxicity of the higher generation dendrimers may be used to explain the poor transfection efficacy shown by the DAB 32 and DAB 64 dendrimers which have IC_{50}s of 5.8 $\mu g \cdot mL^{-1}$ and less than 5 $\mu g \cdot mL^{-1}$, respectively. Administering DNA at the lowest dose of 5 μg would require that a dose of DAB 32 or DAB 64 of at least 15 μg (for a DAB, DNA ratio of 3:1) be administered. This would have to be administered at a DAB concentration of 120 $\mu g \cdot mL^{-1}$ (Table V), a concentration of approximately 20-fold the IC_{50} of these high generation polypropylenimine dendrimers.

DISCUSSION

The use of dendrimers as gene delivery agents has been largely focused on the use of the polyamidoamine molecules (9,10) and although polypropylenimine dendrimers contain 100% protonable nitrogens (12) (Fig. 1) and bind DNA (19), they have been relatively poorly studied as gene transfer

TABLE V. The *in Vitro* Transfection of DAB Dendrimers in the A431 Cell Line

Formulation	Final concentration of DNA added to well ($\mu g \cdot mL^{-1}$)	Final concentration of dendrimer/DOTAP added to well ($\mu g \cdot mL^{-1}$)	DNA dose per well (μg)	β-galactosidase expression (mean of 3 measurements ± SD)[a]
DAB 4, DNA ($3:1\ g \cdot g^{-1}$)	100	300	20	25.7 ± 2.6
	86	257	15	21.9 ± 0.2
	67	200	10	23.0 ± 2.9
	40	120	5	21.0 ± 0.4
DAB 4, DNA ($5:1\ g \cdot g^{-1}$)[b]	100	500	20	42.6 ± 22.7
	86	429	15	22.4 ± 1.1
	67	334	10	25.9 ± 5.7
	40	200	5	44.0 ± 8.5
DAB 4, DNA ($10:1\ g \cdot g^{-1}$)	100	1000	20	42.0 ± 13.4
	86	857	15	30.1 ± 6.5
	67	667	10	25.8 ± 3
	40	400	5	22.5 ± 2.3
DAB 8, DNA ($3:1\ g \cdot g^{-1}$)	100	300	20	101.3 ± 39
	86	257	15	102.6 ± 9.5
	67	200	10	80.1 ± 18.6
	40	120	5	45.1 ± 14.2
DAB 8, DNA ($5:1\ g \cdot g^{-1}$)[b]	100	500	20	108.7 ± 13
	86	429	15	96.5 ± 28.6
	67	334	10	73.1 ± 18.6
	40	200	5	59.2 ± 8.9
DAB 8, DNA ($10:1\ g \cdot g^{-1}$)	100	1000	20	82.0 ± 57.1
	86	857	15	76.4 ± 25.8
	67	667	10	78.6 ± 22.5
	40	400	5	71.7 ± 10.7
DAB 16, DNA ($1:1\ g \cdot g^{-1}$)	100	100	20	54.8 ± 8
	86	86	15	59.2 ± 13.4
	67	67	10	53.3 ± 16.6
	40	40	5	44.7 ± 10.8
DAB 16, DNA ($3:1\ g \cdot g^{-1}$)	100	300	20	23.9 ± 11.1
	86	257	15	40.7 ± 18.9
	67	200	10	57.7 ± 11.4
	40	120	5	56.5 ± 29.1
DAB 16, DNA ($5:1\ g \cdot g^{-1}$)[b]	100	500	20	18.3 ± 1.6
	86	429	15	24.1 ± 2.8
	67	334	10	88.4 ± 54.9
	40	200	5	100.8 ± 35.5
DAB 32, DNA ($3:1\ g \cdot g^{-1}$)	100	300	20	12.2 ± 2.2
	86	257	15	16.3 ± 3.5
	67	200	10	17.6 ± 2.3
	40	120	5	16.6 ± 7.9
DAB 64, DNA ($3:1\ g \cdot g^{-1}$)	100	300	20	10.1 ± 0.8
DOTAP, DNA ($5:1\ g \cdot g^{-1}$)	100	500	20	100.0 ± 15.5
	86	429	15	89.2 ± 13
	67	334	10	84.7 ± 15
	40	200	5	90.5 ± 15.1
DNA	100	0	20	25.2 ± 1.3

[a] Percent expression relative to expression obtained for optimum DOTAP (DOTAP, β-galactosidase reporter DNA ratio = 5.1) formulation on dosing cells with 20 μg DNA.
[b] Most effective dendrimer, DNA ratio.

agents. DNA binding is an initial prerequisite for transport of this macromolecule, and evidence is provided to show that as the size of the dendrimer increased, dendrimer-DNA binding was more effective (Fig. 2, Tables I–III) with little binding by DAB 4 and partial binding by DAB 8. The modeling data indicate that with DAB 16, it is possible that the dendrimer binds with DNA across a complete helical turn (Fig. 2a). DAB 16-bound DNA resists intercalation by ethidium bromide (Table I), and thus the level of contact shown by DAB 16 as modeled in this work (Fig. 2) appears to be the minimum requirement for ethidium bromide exclusion with these dendrimers. The modeling data also indicates that DAB 32 and DAB 64 have the ability to bind DNA on the opposite face (Fig. 2b), thus causing DNA to wrap around the higher generation dendrimers.

The complete exclusion of ethidium bromide by DABs 16, 32, and 64 occurs at a minimum surface nitrogen to phosphate ratio of 0.7:1 or a total nitrogen to phosphate ratio of 1.5:1. It appears that with the larger dendrimers, involvement of at least the second shell of nitrogens in the binding may occur. Our findings are contrary to earlier reports, which suggest that DNA only binds to the outer shell of primary amines in polypropylenimine dendrimers (19). We are assuming perfect or near-perfect dendrimers were used in our work, although it is known that imperfections exist with the higher generation dendrimers (27).

The toxicity of the dendrimers increased with molecular size, implying that the availability of multiple contact points between the dendrimer molecules and essential cellular anions may be implicated in the toxicity of these molecules. A similar hypothesis has been put forward for other polycations (26). The decreased toxicity of the lower generation molecules (Table IV) leads to improvements in the observed level of gene transfer (Fig. 3, Table V). The removal of some of the available anion binding sites by binding of the dendrimers to DNA reduces the toxicity of DAB 4 and DAB 8 (Table IV). This is a further advantage for gene transfer. With the higher generations (DAB 16 and DAB 32), the toxicity of these agents is not modulated (Table IV), which reveals that a sufficient number of available anion binding sites are still present after the binding of DAB 16 and DAB 32 to DNA.

In the absence of detailed structural data from NMR or crystallographic studies, the modeled structures are to be viewed as suggestions for the binding complexes. However, these studies can confidently predict that the third and higher generation dendrimers will have significant polycation protrusions that are not neutralised when bound to a single, polyanionic DNA sequence.

DAB 8 is 5-fold less toxic than DOTAP (Table IV) and hence has a good biocompatibility profile when assessed in the light of its gene transfer activity.

The gene transfer activity of DAB 16 is superior to DAB 8 at the 5 μg DNA dose level, where the toxicity of DAB 16 is reduced (Table V). This suggests that this molecule, which shows good DNA binding, would be the most efficient specie if it did not have an excess of anion binding sites. Molecules that combine the effects of DNA binding and a lack of excess cationic amine groups may be better gene transfer systems. We are in the process of constructing such molecules. The lack of extra anion binding sites does not prevent transfection however as both DAB 4 and DAB 8 dendrimers are internalized by the cell and gene expression is seen (Fig. 3, Table V), which is 40% of that obtained with the positive control cationic lipid formulation DOTAP, in the case of DAB 4, and equivalent to DOTAP in the case of DAB 8. DAB 8 is the most effective of the dendrimers. Although the DNA-DAB 8

dendrimer complex possesses a near neutral charge, it is assumed to contain areas of both high positive and negative charge density, with cellular entry occurring via the areas of high positive charge density. Our data present clear evidence that complete DNA condensation is not a strict necessity for efficient gene transfer. Previous work with polyamidoamine dendrimers had reported that the tight binding of DNA to the G7 polyamidoamine dendrimer is responsible for the greater gene transfer activity of this molecule (28) when compared to the lower generation polyamidoamine compounds (25). However, our experience suggests that the tight binding of DNA to polypropylenimine dendrimers is not necessary for optimum activity and factors that modulate toxicity may be more important.

For the DAB 16 formulation, transfection appears to be optimum when using DAB 16 complexes with DNA at a nitrogen-to-phosphate ratio of 30:1, forming complexes of 150 nm in size. Transfection with DAB 8 is also optimal at a nitrogen-to-phosphate ratio of 30:1, and complexes are 518 nm in size.

Although previous polypropylenimine dendrimer gene delivery communications report low gene transfer activity and toxicity with DAB 64 (18,20), this communication reveals that reducing the anion binding sites per molecule by simply reducing the molecular weight of the dendrimer may be an alternative strategy to reduce toxicity and ultimately improve gene transfer activity. Hence, for the first time relatively efficient gene transfer has been found to be affected by a relatively small molecular weight non-amphiphilic compound (DAB 8, Mw = 773). Optimum gene transfer is obtained with the polyamidoamine (Starburst dendrimers) with a molecular weight in excess of 20 kDa (10). We thus argue that these lower generation cationic polypropylenimine dendrimers warrant further testing as gene transfer systems. We are not the only ones to report an effect of molecular size/weight on gene transfer activity and an optimum molecular weight has been found for both polyethylenimine (29) and chitosan (30).

CONCLUSIONS

In summary, the lower generation polypropylenimine dendrimer (DAB 8) shows improved biocompatibility when compared to DOTAP and *in vitro* transfection activity, which is equivalent to the cationic liposome formulation DOTAP; whereas the toxicity of the higher generation dendrimers preclude their use as efficient gene-transfer agents.

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