

Research Paper

Tat-Conjugated PAMAM Dendrimers as Delivery Agents for Antisense and siRNA Oligonucleotides

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Purpose. PAMAM G5 dendrimer (P) was conjugated to Tat peptide (T), a cell penetrating peptide, in search of an efficient cellular delivery vehicle for antisense and siRNA oligonucleotides.

Methods. PAMAM G5 dendrimer was reacted with 4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt (BODIPY) for visualization to yield the conjugate BP. Bifunctional sulfosuccinimidyl 6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT) was then used to conjugate primary amino groups of BP to cysteine derivatized Tat peptide to give the designed conjugate, BPT. This conjugate was complexed with antisense and siRNA oligonucleotides designed to inhibit MDR1 gene expression. NIH 3T3 MDR cells were used for the evaluation of biological activity of the conjugate.

Results. Both antisense and siRNA readily formed complexes with the synthesized BPT, introduced into NIH 3T3 MDR cells, and primarily accumulated in intracellular vesicles. MDR1 gene expression was partially inhibited by the antisense-BPT complex and weakly inhibited by the siRNA-BPT complex when both were tested at nontoxic levels of dendrimer. Conjugation with Tat peptide did not improve the delivery efficiency of the dendrimer.

Conclusions. Dendrimer-oligonucleotide complexes were moderately effective for delivery of antisense and only poorly effective for delivery of siRNA. Conjugation of the dendrimer with the Tat cell penetrating peptide failed to further enhance the effectiveness of the dendrimer.

KEY WORDS: antisense; cell penetrating peptide; oligonucleotide; PAMAM dendrimer; siRNA; Tat.

INTRODUCTION

There is currently a great deal of interest in the potential therapeutic applications of antisense and siRNA oligonucleotides (1,2). Although most clinical and *in vivo* studies to date have involved the use of “free” oligonucleotides (not associated with any carrier agent), there is also interest in the possible application of various delivery strategies to oligonucleotide therapeutics (3,4). One approach has been to conjugate oligonucleotides to various “cell penetrating peptides” that have been reported to promote the entry of molecules and particles into cells (5,6). This strategy has been very successful for several types of antisense oligonucleotides (7) and is also beginning to be applied to siRNA (8). Another delivery approach has been to complex oligonucleotides to various cationic carriers including polymers and lipid particles. Dendritic polymers such as PAMAM dendrimers have been extensively used as carriers for plasmid DNA (9,10) and

for antisense oligonucleotides (11,12). Cationic dendrimers may have some advantages over other types of positively charged carriers such as cationic lipids in that the dendrimer-oligonucleotide complexes are of macromolecular size (13) and dendrimers are known to have extended lifetimes *in vivo* (14), whereas cationic lipid complexes are large particles that are usually rapidly cleared from the circulation by splenic and hepatic phagocytes (15).

In the current study we examined the impact of conjugating PAMAM dendrimers with the Tat cell penetrating peptide. Our aim was to further enhance the delivery capabilities of dendrimers for oligonucleotides. We prepared PAMAM dendrimers conjugated with a fluorophore (BODIPY) and with Tat, and then examined cell uptake, intracellular distribution, and pharmacological effects of conjugated or unconjugated dendrimer complexes with antisense or siRNA oligonucleotides. The target gene used as a model was *MDR1*, which codes for P-glycoprotein, a transmembrane protein that is a drug transporter (16).

MATERIALS AND METHODS

Tat peptide with a cysteine residue at C terminus (NH₂-RKKRRQRRRPPQGGC-COOH) was synthesized at the UNC Microprotein Sequencing and Peptide Synthesis Facility or purchased from Cell Essentials (Boston, MA, USA). Phosphorothioate oligonucleotide was purchased from Mid-

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Table I. Antisense and siRNA Targeted to the MDR1 Gene and Scrambled/Mismatched Controls

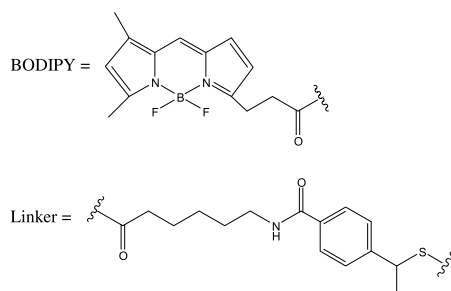
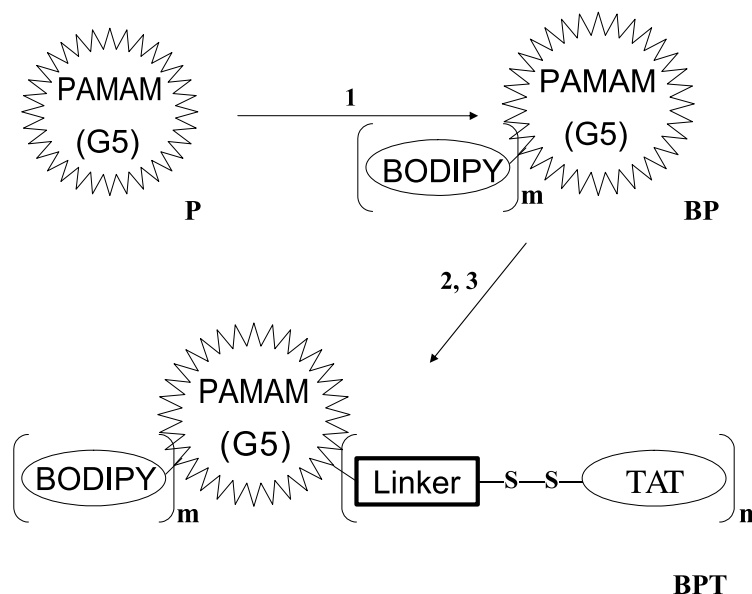
Name	Target	Sequence	Chemical property
5995	AUG start codon of MDR1	5'-CCA TCC CGA CCT CGC GCT CC-3'	Phosphorothioate antisense
Cy5-5995	AUG start codon of MDR1	5'-(Cy5)CCA TCC CGA CCT CGC GCT CC-3'	Phosphorothioate antisense
10221	Scrambled control of 5995	5'-CAC CAC CCC CCT CGC TGG TC-3'	Phosphorothioate antisense
ORF1	1545–1565 of MDR1	5'-GUAUUGACAGCUAUUCGAAAdTdT-3' 3'-dTdTCAUAACUGUCGAUAAGCUU-5'	siRNA
Cy5-ORF1	1545–1565 of MDR1	5'-(Cy5)GUAUUGACAGCUAUUCGAAAdTdT-3' 3'-dTdTCAUAACUGUCGAUAAGCUU-5'	siRNA
Nischarin	Nischarin targeting control for ORF1	5'-CCUCGUGCACCUUGACCUGdTdT-3' 3'-dTdTGGAGCACGUGGAACUGGAC-5'	siRNA

land Certified Reagents Company (Midland, TX, USA). SiRNA was purchased from Dharmacon (Lafayette, CO, USA). PAMAM G5 dendrimer was purchased from Dendritech Inc. (Midland, MI, USA). Names, sequences, and targets of the antisense and siRNA used are shown in Table I. The BODIPY fluorophore (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a-diaza-s-indacene-3-propionic acid, sulfosuccinimidyl ester, sodium salt) (see Scheme 1) and thiol and sulfide quantitation kit were purchased from Molecular Probes (Eugene, OR, USA). Bifunctional sulfosuccinimidyl 6-[α -

methyl- α -(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-SMPT) was purchased from Pierce (Rockland, IL, USA).

Preparation of PAMAM Conjugate of Tat Peptide (BPT)

PAMAM G5 dendrimer (P, 24.32 mg, 0.844 μ mol) was reacted with BODIPY (2 mg, 4.219 μ mol) in 100 mM NaHCO₃ for 12 h at 4°C. The reaction was then purified on a G-25 size exclusion column to remove remaining free BODIPY derivatives. The large molecular weight product



Tat peptide = NH₂-RKKRRQRRRPPQGGC-COOH

Scheme 1. Preparation of PAMAM G5 dendrimer conjugates of BODIPY and Tat peptide.

recovered from G-25 was dried under reduced pressure to give BP conjugate in an 82% yield. The product was then analyzed by spectrophotometry for BODIPY absorbance ($\lambda_{\max} = 502$ nm) to give an average BODIPY/PAMAM ratio of 1.8. The BODIPY-PAMAM conjugate (10 mg, 0.343 μmol) was then reacted with sulfosuccinimidyl 6-[α -methyl- α -(2-pyridyldithio)toluamido]hexanoate (4 mg, 6.63 μmol) in phosphate-buffered sulfate (PBS) for 12 h at 4°C, and purified on a G-25 column. Tat peptide (25 mg, 13.30 μmol) in PBS/50 mM EDTA (pH 7.4) was then added to the intermediate, and the reaction was maintained at room temperature for 24 h. Using a spectrophotometer, the ratio of Tat/PAMAM in the reaction product was determined as 15.9 by the release of pyridine-2-thione ($\lambda_{\max} = 343$ nm). The reaction was purified on a G-25 column with a yield of 68%. The product (BPT) was subjected to thiol and sulfide quantitation according to the manufacturer's recommendation.

Physical Characterization of BPT

Gel filtration fast protein liquid chromatography (FPLC) was conducted on an AKTA/Amersham Pharmacia Biotech system using a Superdex 75 10/300 column (Amersham Biosciences/Tricorn). Unmodified P or derivatized dendrimers BPT were eluted at 0.5 mL/min in 50 mM phosphate/150 mM NaCl buffer and monitored at 280 or 515 nm. Dendrimer (20 $\mu\text{g}/\mu\text{L}$) was dissolved in high-performance liquid chromatography (HPLC)-grade water and 50 μL was injected per run. Data analysis and peak integrations were performed using Unicorn software. To compare fractions, the height of the monomer peak was arbitrarily set to a value of 1 and the other peak heights were compared to this value. This allowed the calculation of the ratios of monomer to dimer or multimer. The multimer peaks eluted near the void volume and could not be further resolved.

Complex Formation of PT with Antisense and siRNA

P (128 free amino groups per molecule), or its Tat peptide derivative BPT (110 free amino groups per molecule) (150 nM–1.2 μM), was complexed with 20 bp antisense or 19 bp siRNA (100 nM) in Opti-MEM I Reduced Serum Medium (20 μL). Positive/negative charge ratios for P complexes were 10.1–80.9 for antisense and 5.6–45.1 for siRNA. Positive/negative charge ratios for BPT complexes were 8.7–69.7 for antisense and 4.9–38.9 for siRNA. After 20 min, the mixture was analyzed by 20% nondenaturing polyacrylamide gel electrophoresis with ethidium bromide. The complex was then visualized under ultraviolet light.

RNase Stability Assay

One microliter of siRNA (100 μM) was diluted with 9 μL Opti-MEM I Reduced Serum Medium and/or complexed to PAMAM as above contained in 0.6-ml Eppendorf tubes; 3.5 μg of pancreatic RNase was added to each tube in ascending order and the tubes were incubated at 37–38°C for 20, 40, 60, or 90 min, after which they were placed on ice briefly and 3 μL heparin (10 mg/mL in RNase free water)

was added to displace the bound siRNA from PAMAM. 3% agarose/ethidium bromide stained gels were loaded with bromophenol blue (10 mg/mL in RNase free water) and visualized under ultraviolet light.

Cells

NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum. NIH 3T3 cells stably transfected with a plasmid containing the human MDR1 gene (NIH 3T3 MDR cells) were a gift from M.M. Gottesman (17). These cells were grown in DMEM containing 10% fetal bovine serum (FBS) and 60 ng/mL colchicine. Cells were maintained in a humidified atmosphere of 95% air/5% CO₂ at 37°C. Cells were seeded onto 6-well plates at 3×10^5 /well in DMEM medium containing 10% FBS 24 h prior to transfection.

PAMAM Derivative Uptake by Cells

Fluorescently labeled PAMAM derivatives (BP or BPT, 1–27 $\mu\text{g}/\text{mL}$) were incubated with NIH 3T3 MDR cells in DMEM/10% FBS for 4 h. The cells were washed twice with DMEM/10% FBS, twice with PBS, detached by trypsinization, and centrifuged at 1100 rpm for 5 min. PBS was added to the pellet for normalization of cell number on an Elzone particle cell counter (Micromeritics, Norcross, GA). PAMAM derivative uptake was studied by measuring the relative fluorescence intensity of the transfected cells by flow cytometry.

Oligonucleotide Treatment

Dendritic compounds (P, BP, or BPT) or Lipofectamine 2000 was complexed to antisense or siRNA in Opti-MEM I Reduced Serum Medium 20 min before cell treatment. At

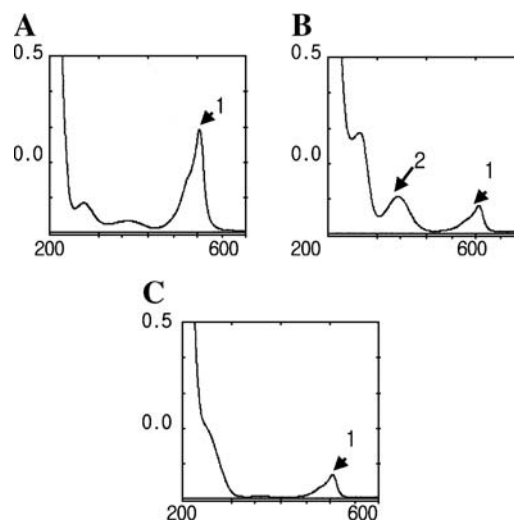


Fig. 1. UV-Vis spectra of PAMAM derivatives. (A) BODIPY-PAMAM G5 dendrimer conjugate (BP). (B) Reaction mixture of BP-SMPT and Tat peptide. (C) BODIPY-PAMAM-Tat conjugate (BPT). Peak 1 is from BODIPY and peak 2 is from pyridine-2-thione. X-axes represent wavelengths (nm). Y-axes represent absorbance.

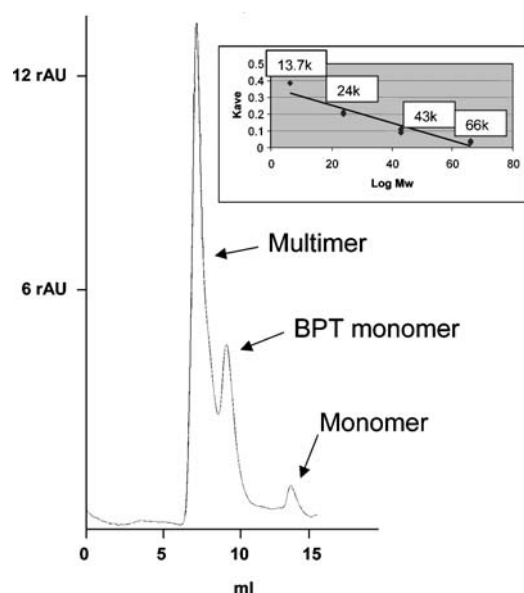


Fig. 2. Resolution of PAMAM monomer, BPT, and multimer species by gel filtration. This represents the elution profile of PAMAM doubly derivatized with BODIPY and TAT peptide, with monomer and multimer peaks detected. X-axis: elution volume. Y-axis: relative absorbance. A calibration profile for the gel filtration column is shown in the inset.

24 h after cell seeding onto 6-well plates at 3×10^5 /well in DMEM medium (2 mL) containing 10% FBS, cells were treated with the freshly prepared antisense or siRNA complexes with P, BP, BPT, or Lipofectamine 2000 in Opti-MEM I Reduced Serum Medium (2 mL) for 4 h at 37°C. The cells were then washed twice with 10% FBS/DMEM and incubated in the same medium at 37°C. After 1 h, cells were washed again with DMEM/10% FBS and further incubated in DMEM/2% FBS for 64 h.

Confocal Microscopy

NIH 3T3 MDR cells were seeded onto Glass Bottom Microwell Dishes (35 mm, MatTek) at 3×10^4 /well. After 24 h, cells were treated with Cy5-labeled antisense (100 nM, Cy5-5995) or siRNA (100 nM, Cy5-ORF1) complexed with BP (20 µg/mL) or its Tat derivatized reagent, BPT (20 µg/mL), as described. After 4 h of transfection in Opti-MEM I Reduced Serum Medium, cells were washed with DMEM/10% FBS twice and incubated in DMEM/10% FBS for 1 h. Cells were then washed with DMEM/10% FBS and further incubated in DMEM/2% FBS for 24 h. Cells were placed in DMEM without phenol red that was supplemented with 25 mM HEPES for pH control during live cell confocal microscopy. Images were taken using an Olympus confocal microscope with a 60× objective lens and processed using Fluoview software.

Cytotoxicity

NIH 3T3 MDR cells were treated with P or BPT complexes of antisense or siRNA, washed and further incubated for 64 h as described above. Cells were then washed twice with PBS, detached by trypsin, and centrifuged

1,100 rpm for 5 min at 4°C. PBS was added to dilute the pellet and the viable cells were counted on an Elzone particle counter (Micomeritics) that electronically monitors the movement of intact viable cells through an orifice by detecting changes in current as the intact cell blocks the orifice.

Immunostaining of P-glycoprotein

The inhibition of P-glycoprotein expression on cell surfaces of viable cells was studied by immunostaining using a flow cytometry assay. After treating NIH 3T3 MDR cells with antisense or siRNA and further incubating them for 64 h as described above, cells were trypsinized, washed twice with PBS, counted for normalization, and incubated with R-phycoerythrin conjugated mouse anti-human P-glycoprotein (20 µL; BD Biosciences, San Diego, CA, USA) in 50 µL PBS at 4°C. After 45 min, cells were washed once with PBS/10% FBS and twice with PBS. The levels of R-phycoerythrin immunostaining on viable cells (identified by light scattering)

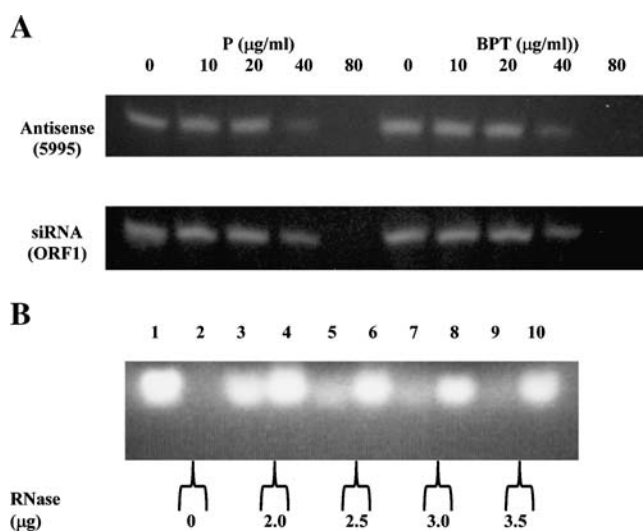


Fig. 3. (A) Complex formation of PAMAM derivatives (P or BPT) with antisense (5995) or siRNA (ORF1). PAMAM G5 dendrimer (P) or its Tat peptide derivative, BPT (10–80 µg/mL), was complexed with antisense or siRNA (100 nM) in Opti-MEM. After 20 min, the mixture was analyzed by 20% nondenaturing polyacrylamide gel electrophoresis with ethidium bromide. The residual free antisense or siRNA was then visualized under ultraviolet light. Loss of bands is indicative of complex formation. (B) Stability of siRNA to pancreatic RNase. Free ORF1 siRNA or siRNA complexed to PAMAM dendrimer was exposed to pancreatic RNase at various doses and for increasing amounts of time. After treatment the siRNA was displaced from the PAMAM complex with heparin. Degradation of the siRNA is indicated by loss of intensity at the migration position of the intact free (or heparin displaced) siRNA. Lane 1: free siRNA untreated; lane 2: PAMAM complex bound siRNA (not displaced with heparin); lanes 3, 4: free and complexed siRNA (complex treated with heparin) after 20 min incubation with 2.0 µg RNase; lanes 5, 6: free and complexed siRNA (complex treated with heparin) after 40 min incubation with 2.5 µg RNase; lanes 7, 8: free and complexed siRNA (complex treated with heparin) after 60 min incubation with 3.0 µg RNase; lanes 9, 10: free and complexed siRNA (complex treated with heparin) after 90 min incubation with 3.5 µg RNase.

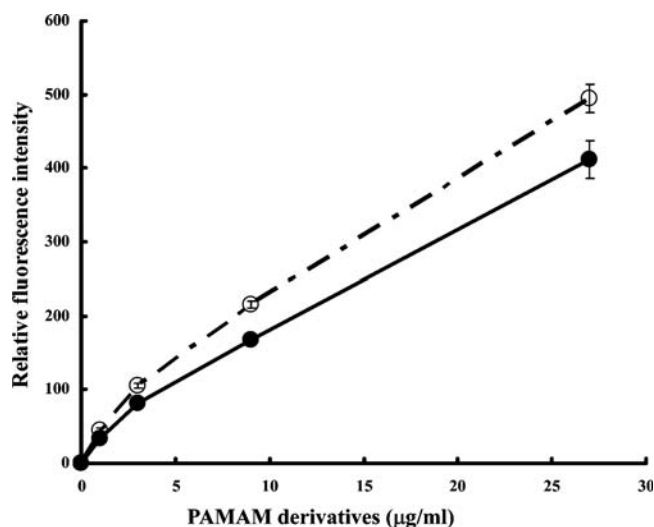


Fig. 4. PAMAM derivative uptake by NIH 3T3 MDR cells. BP or BPT was incubated with NIH 3T3 cells as described. The BODIPY fluorophore associated with cells was then analyzed by flow cytometry. (●) BODIPY-PAMAM conjugate (BP), (○) BODIPY-PAMAM-Tat conjugate (BPT). X-axis: concentration of PAMAM derivatives. Y-axis: fluorescence intensity.

were then quantified on a Becton Dickinson flow cytometer with Cicero software (Cytomation, Fort Collins, CO, USA).

RESULTS AND DISCUSSION

Preparation of PAMAM Conjugate of Tat Peptide

The synthesis of PAMAM conjugates used in this study is summarized in Scheme 1. BODIPY fluorophore was conjugated to PAMAM G5 dendrimer for visualization of the dendrimer to yield BP. A dual linker, sulfo-LC-SMPT, was then used to conjugate primary amino groups of PAMAM G5 dendrimer and the thiol group of the cysteine residue of Tat peptide (18). As P and its conjugates, BP and BPT, have relatively large molecular weights (estimated based on composition as 28,825–67,077), any unreacted materials were easily removed by G25 size exclusion chromatography. This was demonstrated by spectroscopy of the reactions taken before and after G25 column purifications. BODIPY conjugation to PAMAM G5 dendrimer was verified by the presence of the BODIPY peak at 507 nm in the G25 purified dendrimer of reaction 1 (peak 1, Fig. 1A).

The conjugation of the SMPT derivative linker to BP was verified by performing G25 column purification of both reaction 2 and free sulfo-LC-SMPT. When these materials

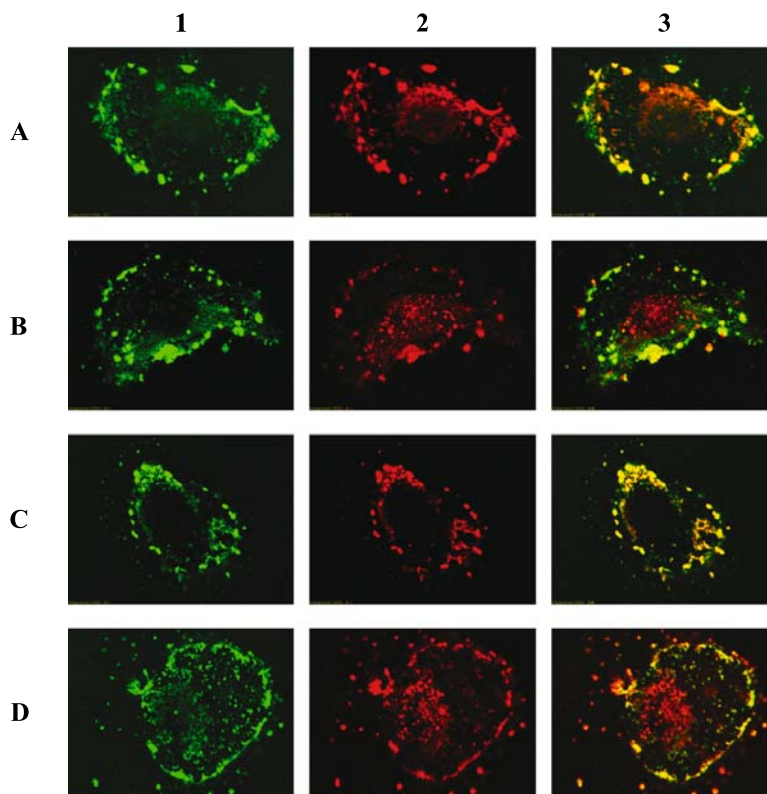


Fig. 5. Confocal microscope images of dendrimers and oligonucleotides associated with NIH 3T3 MDR cells. Cy5 antisense (5995, 100 nM) or Cy5-siRNA (ORF1, 100 nM) complexed with BP (20 µg/mL) or BPT (20 µg/mL) was incubated with NIH 3T3 MDR cells as described. The BODIPY and Cy5 fluorophores were then analyzed by confocal microscopy. (1) BODIPY-dendrimer-Tat conjugate; (2) antisense (Cy5-5995) or siRNA (Cy5-ORF1); (3) merged images of 1 and 2; (A) BP complexed to Cy5-5995; (B) BP complexed to Cy5-ORF1; (C) BPT complexed to Cy5-5995; (D) BPT complexed to Cy5-ORF1.

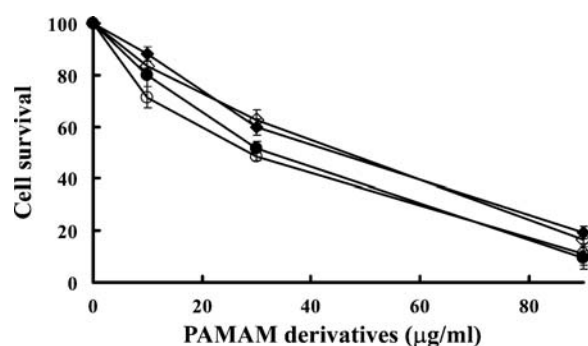


Fig. 6. Cytotoxicity of PAMAM derivatives (P or BPT) complexed to antisense (5995) or siRNA (ORF1). The cytotoxicities of free PAMAM (P) and BODIPY and Tat derivatized PAMAM (BPT) complexed with antisense (5995, 100 nM) or siRNA (ORF1, 100 nM) on NIH 3T3 MDR cells were determined after incubation for 4 h, washing for 1 h, and further incubation for 64 h as described. (\diamond) P complexed to antisense (5995), (\blacklozenge) P complexed to siRNA (ORF1), (\circ) BPT complexed to antisense (5995), (\bullet) BPT complexed to siRNA (ORF1). Values represent cell population in percent with 100% taken for untreated NIH 3T3 MDR cells. Means and standard errors of three determinations. X-axis: concentration of PAMAM derivatives. Y-axis: % of control cells.

were treated with excess cysteine, only the purified materials from reaction 2, not free SMPT derivative, generated the pyridine-2-thione leaving group, which was observed at 343 nm. This suggests the incorporation of SMPT linker into the dendrimer. The release of pyridine-2-thione was again observed during the reaction of G25 purified material from reaction 2 and a cysteine derivatized Tat peptide (peak 2, Fig. 1B). The leaving

group was removed by G25 chromatography (Fig. 1C). The removal of the unreacted Tat peptide by G25 column was verified by thiol quantitation showing that no free thiol group was present after G25 column purification of the BPT product. The conjugate has 1.8 BODIPY and 15.9 Tat peptides per PAMAM G5 dendrimer on average.

Antisense and siRNA Oligonucleotides Studied

The antisense and siRNA oligonucleotides used in this study are shown in Table I. Phosphorothioate 5995 is an antisense targeting the AUG start codon of the MDR1 gene. Phosphorothioate 10221 is a scrambled control for 5995. ORF1 is a siRNA duplex designed to target the coding region at nt 1545–1565 of MDR1 mRNA. A siRNA targeting an “irrelevant” message (Nischarin) was used as a control. The antisense activity of 5995 and the RNAi effect of ORF1 have been demonstrated previously (19,20).

Physical Characterization of the PAMAM Conjugates of Tat Peptide

P, BP, and BPT were subjected to gel filtration FPLC. As shown in Fig. 2, three peaks were detected, which based on extrapolation from the elution of standard proteins (see figure inset), represented monomer, dimer, or multimer species of dendrimer. Evidence for these species was seen in several different runs via UV absorbance, fluorescence (546 nm), and light scatter coupled to refractive index detection methods (data not shown). The multimer/dimer/monomer ratio was 5:4:1 for PAMAM, 46:8:1 for BP, and

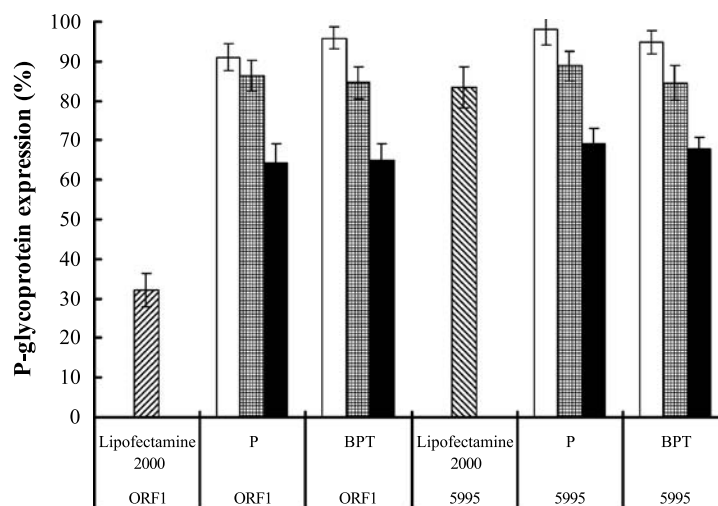


Fig. 7. PAMAM concentration-dependent effects on P-glycoprotein expression. NIH 3T3 MDR cells were treated with antisense (5995, 100 nM) or siRNA (ORF1, 100 nM) complexed to various concentrations of delivery reagents. Delivery reagents used were: (\square) 10 μ g/mL of P or BPT, (▨) 20 μ g/mL of P or BPT, (\blacksquare) 30 μ g/mL of P or BPT, (▩) 2 μ g/mL of Lipofectamine 2000. P-glycoprotein cell surface expression in the viable cells was evaluated as described in Materials and Methods. Values are percentage of P-glycoprotein expression with 100% taken for NIH 3T3 MDR cells treated with corresponding mismatched or scrambled sequence. The percentage was calculated on the basis of the fraction of the cell population shifted to greater than 1 standard deviation below the mean of the untreated controls in terms of P-glycoprotein expression. Means and standard errors of three determinations.

31:10:1 for BPT. In some cases, elution of dimer and Tat derivatized species may overlap. These data suggest that chemical modification of the dendrimer surface with hydrophobic moieties may cause some degree of aggregation.

Complex Formation of PAMAM Derivatives with Antisense and siRNA

Both free P and BPT formed complexes with either antisense or siRNA as evidenced by oligonucleotide bands disappearing from polyacrylamide gels (Fig. 3A). At 100 nM, both antisense and siRNA were completely complexed with 40–80 $\mu\text{g/mL}$ of free PAMAM or Tat derivatized PAMAM. There was no significant difference in complexation of antisense or siRNA with free or Tat conjugated dendrimers.

RNase Stability Assay

PAMAM complexation of the RNAi protects it from degradation by RNase. As seen in Fig. 3B after 90 min of incubation, RNAi complexed to PAMAM was completely intact, whereas free uncomplexed RNAi was approximately 90% degraded at this time point.

Cellular Uptake and Distribution

Cellular uptake of PAMAM G5 dendrimer derivatives, BP and BPT, were studied in NIH 3T3 MDR cells. After transfection and washing cells as described, BODIPY fluorescence associated with cells was measured by flow cytometry. Tat derivatization enhanced the cellular uptake of the dendrimer by 20–30% at the tested concentrations (Fig. 4). Confocal microscopic analyses taken 24 h after transfection indicated that both antisense and siRNA were readily delivered into cells by both free P and Tat derivatized BPT (Fig. 5). Much of the material seemed to be associated with intracellular vesicles. However, only a relatively small portion of the siRNA and antisense were released from the dendrimer complexes, as indicated by the separation of BODIPY and Cy-5 fluorescence and the presence of Cy-5 fluorescence in the cytoplasm or nucleus. This is in contrast with the use of Lipofectamine 2000, where a substantial amount of the fluor associated with the antisense oligonucleotide was located in the nucleus (data not shown).

Cytotoxicity of PAMAM Conjugates of Antisense and siRNA

Cytotoxic effects of free PAMAM G5 dendrimer, P, and Tat derivatized dendrimer, BPT, complexes of antisense or siRNA on NIH 3T3 MDR cells, were studied by measuring live cell numbers after 4 h transfection and 64 h additional incubation (Fig. 6). Little difference was observed between the cytotoxicity of antisense vs. siRNA complexes. Tat conjugates (BPT) were slightly more toxic than their unmodified dendrimer counterpart (P). Tat conjugate (BPT) displayed about 50% cytotoxicity and free PAMAM was about 40% toxic when 30 $\mu\text{g/mL}$ dendrimer was used to complex 100 nM oligonucleotides. As free antisense or siRNA are in excess of PAMAM derivatives at 0–40 $\mu\text{g/mL}$ dendrimer concentrations (Fig. 3), the observed cytotoxicity is thought to be attribut-

able to the complexes of PAMAM/oligonucleotides, and not to free dendrimer, in the tested concentration range. Lipofectamine 2000 was 50% cytotoxic at 6 $\mu\text{g/mL}$ but displayed only approximately 10% cytotoxicity at the concentration (2 $\mu\text{g/mL}$) used in functional studies (data not shown).

Inhibition of P-glycoprotein Expression

The effects of antisense or siRNA delivered either by unmodified PAMAM G5 dendrimer or Tat conjugated dendrimer on cell surface P-glycoprotein expression in viable NIH 3T3 MDR cells were evaluated using immunofluorescence and flow cytometry, and compared to the inhibitory effects of antisense and siRNA delivered by Lipofectamine 2000. In Fig. 7, the effects of various concentrations of dendrimer are examined. At a low concentration (10 $\mu\text{g/mL}$), dendrimers had little toxicity (10–20%) but were also poorly effective compared to Lipofectamine 2000-mediated delivery. At higher concentrations of P or PBT dendrimer (20–30 $\mu\text{g/mL}$), the effects on P-glycoprotein inhibition were greater; however, as shown in Fig. 6, the toxicity level was also substantially greater. The limited biological activity of antisense and siRNA delivered by either free or Tat derivatized PAMAM may be attributable to the incomplete release of antisense and siRNA delivered into the cells (Fig. 5).

CONCLUSION

Dendritic polymers have been used quite extensively to deliver both plasmid DNA and antisense oligonucleotides (10–12,21), but there are no reports in the literature on delivery of siRNA with dendrimers. Likewise, the Tat cell penetrating peptide has been widely used in the preparation of peptide–antisense conjugates and has been moderately effective in enhancing intracellular delivery in that context (7,8,22,23). In this study we sought to bring these two approaches together via the use of Tat conjugated PAMAM dendrimers. Somewhat surprisingly, however, adding the Tat peptide to the dendrimers failed to enhance the ability of the dendrimers to deliver oligonucleotides in a functionally effective manner. The reason for this is not clear. Cell penetrating peptides such as Tat are highly cationic and their abundant positively charged residues contribute to their delivery ability. As the PAMAM dendrimers are also highly positively charged at physiological pH, perhaps the charged moieties on the dendrimer serve the same delivery function as the charged residues on the Tat peptide, thus making the peptide essentially redundant. If this supposition is correct, it may be of interest to examine the effect of Tat or other cell penetrating peptides when conjugated to other forms of dendrimers that have neutral or anionic surface charges (12,24). As mentioned in the Introduction, the fact that dendrimer–oligonucleotide conjugates are substantially smaller than cationic lipid complexes may offer some advantages in the *in vivo* context.

Another observation is that PAMAM dendrimers or their conjugates seem more effective in delivering antisense oligonucleotides than siRNA. Thus, when compared to Lipofectamine 2000 as a positive control, the dendrimers were comparably effective in reducing P-glycoprotein expression when conveying a phosphorothioate antisense

oligonucleotide. However, when a siRNA was used the cationic lipid was much more effective than the dendrimers in promoting reduction of P-glycoprotein expression. The basis for this is not obvious; both types of oligonucleotides form strong complexes with both types of carriers. In addition, the fluorescence microscopy images did not reveal any major differences in the subcellular distribution of antisense or siRNA delivered via dendrimers; in both cases the oligonucleotides were primarily associated with vesicles with some diffuse distribution in the cytosol and nucleus. However, apparently subtle differences in the delivery process differentially affect the distinct mechanisms of message degradation employed by siRNA vs. antisense. In summary, this study confirms previous results that PAMAM dendrimers are moderately effective agents for delivery of antisense oligonucleotides. However, they are relatively less effective for delivery of siRNA. Furthermore, conjugation with Tat peptide failed to enhance delivery efficiency.

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