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Calcium condensed cell penetrating peptide complexes offer highly efficient, low toxicity gene silencing

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ABSTRACT

The development of short-interfering RNA (siRNA) offers new strategies for manipulating specific genes responsible for pathological disorders. Myriad cationic polymer and lipid formulations have been explored, but an effective, non-toxic carrier remains a major barrier to clinical translation. Among the emerging candidates for siRNA carriers are cell penetrating peptides (CPPs), which can traverse the plasma membrane and facilitate the intracellular delivery of siRNA. Previously, a highly efficient and non-cytotoxic means of gene delivery was designed by complexing plasmid DNA with CPPs, then condensing with calcium. Here, the CPP TAT and a longer, 'double' TAT (dTAT) were investigated as potential carriers for siRNA. Various N/P ratios and calcium concentrations were used to optimize siRNA complexes *in vitro*. Upon addition of calcium, 'loose' siRNA/CPP complexes were condensed into small nanoparticles. Knockdown of luciferase expression in the human epithelial lung cell line A549-luc-C8 was high (up to 93%) with no evidence of cytotoxicity. Selected formulations of the dTAT complexes were dosed intravenously up to 1000 mg/kg with minimal toxicity. Biodistribution studies revealed high levels of gene knockdown in the lung and muscle tissue suggesting these simple vectors may offer a translatable approach to siRNA delivery.

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1. Introduction

The discovery and development of small interfering RNA (siRNA) has provided an elegant strategy for disabling specific genes responsible for pathological disorders (Aagaard and Rossi, 2007; Fire et al., 1998). Since its discovery, siRNA has been developed as a screening tool for cancer studies (Fuchs and Borkhardt, 2006; Luo et al., 2007; Sachse and Echeverri, 2004), and has been evaluated as a potential therapeutic agent for a variety diseases such as HIV (Chakraborty, 2007), hepatitis C (Wilson and Richardson, 2006) and cancer (Cejka et al., 2006; Storvold et al., 2006). Double stranded siRNA is able to silence genes effectively in a sequence-specific manner (McManus and Sharp, 2002; Pirollo et al., 2007). The major limitation to clinical application of siRNA, like most nucleic acid based therapeutics, is poor cellular uptake of these relatively large, negatively charged molecules (Lu et al., 2005; Urban-Klein et al., 2004). Also, enzymatic lability, rapid renal clearance, low transfection efficiency, and immune stimulation by siRNA have further delayed therapeutic applications (Bartlett et al., 2007; Behlke, 2006; Kircheis et al., 1997; Wang et al., 2009). Several siRNA delivery systems have been proposed, such as direct chemical modification of siRNA or 'carrier' formulations including lipids, cationic polymers, antibody-protamines, or cell penetrating peptides (CPPs) (Corey, 2007; De Fougerolles et al., 2007; Eguchi and Dowdy, 2009; Jeong et al., 2008; Juliano and Alam, 2008; Lorenz et al., 2004; Whitehead et al., 2009; Wolfrum et al., 2007). In most cases, dose-limiting toxicity of the delivery system and intracellular delivery at target tissues remain key challenges (Akhtar and Benter, 2007; Grayson et al., 2006; Medina-Kauwe et al., 2005; Sepp-Lorenzino and Ruddy, 2008).

In general, acute toxicity of carriers may be reduced by conjugation with biocompatible, hydrophilic polymers such as poly(ethylene glycol) (PEG) or hydroxypropyl methacrylamide (HPMA), or by removing excess (uncomplexed) cationic polymers (Greenwald, 2001; Putnam and Kope ek, 1995; Rihova et al., 1989; Vicent and Duncan, 2006). The long-term toxicity of such approaches remains somewhat unclear. Biodegradable polymers that exhibit low toxicity are attractive candidates for further enhancing the safety of siRNA polyplexes (Luten et al., 2008; Park et al., 2005). Commonly, 'safe' vectors sacrifice efficacy.

After circulation and entry into the cell, the siRNA vector or released siRNA must escape to the cytosol prior to degradation in the late endolysomal compartment. In order to circumvent this

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problem, many strategies have been employed to enhance endosomal escape. Lipid or lipid-like molecules responsive to pH and viral fusogenic proteins and peptides have promoted endosomal escape via membrane destabilization, which can result from pH-dependent changes in conformation (Akinc et al., 2008; Hughson, 1995; Morrissey et al., 2005; Pal et al., 2005; Palliser et al., 2005; Ren et al., 1999; Skehel and Wiley, 2000; Sørensen et al., 2003; Zhang et al., 2006; Zimmermann et al., 2006). Ideally, such mediators of cellular entry, and any potential toxicity, would be masked within a complex or via 'shielding' prior to arrival at the target tissue (Sawant et al., 2006).

Cell penetrating peptides (CPPs) have the earmarks of promising delivery vehicles for intracellular delivery of siRNA (Chiu et al., 2004; Jones et al., 2005; Kim et al., 2006; Meade and Dowdy, 2007; Muratovska and Eccles, 2004; Turner et al., 2007). Two main strategies have been investigated; chemical linkage of CPPs with siRNA (Davidson et al., 2004; Moschos et al., 2007; Muratovska and Eccles, 2004) and carrier formulations (e.g. liposomes or polyelectrolyte complexes). Liposomes or polyelectrolyte complexes, in general, suggest some potential for this strategy (Crombez et al., 2008; Eguchi and Dowdy, 2009; Meade and Dowdy, 2007). Various CPPs including TAT, 3-methyladenine-DNA glycosylase (MPG) proteins from HIV-1 (Morris et al., 1997; Schwarze et al., 1999; Torchilin et al., 2001; Vivès et al., 1997), polyarginine, penetratin or TP10 (Derossi et al., 1994; Futaki et al., 2001; Pooga et al., 1998) have been reported to improve siRNA delivery into various cell lines. The interaction between the positive charges of CPPs and the negative charges of proteoglycans and/or phospholipids was expected to be crucial for CPP-mediated uptake (Gonçalves et al., 2005). Due to the negative charge of siRNA and the cationic nature of CPPs, it is very cumbersome to directly conjugate the two and the CPPs can be effectively neutralized (Jiang et al., 2004). In some cases, both covalent conjugation and electrostatic complexes of siRNA with CPPs were found to be trapped in endosomes even after efficiently entering cells (Endoh et al., 2008; Lundberg et al., 2007; Mäe et al., 2009; Meade and Dowdy, 2008) and required additional molecules to facilitate cytosolic release of siRNA (Cheng et al., 2006; Michiue et al., 2005; Turner et al., 2005; Wadia et al., 2004).

Previous studies demonstrated that adding calcium to CPP complexes with plasmid DNA can reduce particle size and maximize transfection efficiency *in vitro* (Baoum et al., 2009; Baoum and Berkland, 2011). This simple formulation scheme was found to display high transfection efficiency compared to controls with extremely low cytotoxicity. Here, a similar scheme was explored for siRNA delivery by employing the CPPs TAT and a 'double' TAT (dTAT; a longer form of TAT). The cell penetration enabled by TAT is well known and dTAT was selected due to the slightly larger molecular weight, which may improve siRNA complexation. In addition, preliminary *in vivo* studies were conducted to establish the toxicity and biodistribution of calcium-condensed CPP/siRNA complexes.

2. Materials and methods

2.1. Materials

Anti-luciferase siRNA-1 (Mw 13,358 g/mol) and siRNA control (non-targeting) were supplied by Thermo Scientific Dharmacon® (Chicago, IL). TAT peptide (RKKRRQRRR; Mw = 1338.6 Da) and dTAT (RKKRRQRRRHRRKKR; Mw = 2201.7 Da) peptide were purchased from Biomatik Corporation (Cambridge, Ontario, Canada). Branched polyethylenimine (PEI, 25 kDa) was obtained from Aldrich (Milwaukee, WI). Calcium chloride ($CaCl_2 \cdot 2H_2O$), Nuclease-free water and BCA™ Protein Assay were purchased from Fisher Scientific (Pittsburgh, PA). Glucose was acquired from Sigma. A549-luc-C8 Bioware® cell line was obtained from Caliper LifeSciences (Hopkinton, MA). The cell culture medium (RPMI-1640) and (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). Heat inactivated fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Penicillin-streptomycin was purchased from MB Biomedical, LLC (Solon, OH). Trypsin-EDTA was purchased through Gibco (Carlsbad, CA). MTS reagent [tetrazolium compound; 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] and Luciferase Assay System were purchased from Promega (Madison, WI).

2.2. Preparation of complexes

TAT, dTAT and PEI complexes were prepared as described previously with minor modification as described below (Baoum et al., 2009; Baoum and Berkland, 2011). Briefly, various amounts of polycations and siRNA were first dissolved in known volume of nuclease-free water (NFW). Ten microliters (e.g., 10 nM) of siRNA solution was added rapidly to 15 μL polycation (TAT, dTAT or PEI) solution while pipetting. To this solution, 15 μL (e.g., 23.1 mM) $CaCl_2$ (or NFW in the case of PEI) was added and mixed by vigorous pipetting. This resulted in different N/P ratios of polycation/siRNA complexes. The complexes then were allowed to form during 20 min incubation at 4 °C prior to use. Complexes were freshly prepared before each individual analysis.

2.3. Size and zeta potential measurement

The effective hydrodynamic diameter of the complexes was analyzed using a dynamic light scattering (DLS) system (Brookhaven Instrument, Holtsville, NY) equipped with a 50 mW HeNe laser operating at 532 nm. The complexes were prepared at a constant pDNA concentration of 100 μg/mL whereas the N/P ratios of the complexes were varied. The scattered light was monitored at 90° to the incident beam. For each sample, the data was collected continuously for three 1-min intervals. The diameter of the complexes was obtained from the diffusion coefficient by the Stokes–Einstein equation using the method of cumulants. Zeta potential measurements were obtained by phase analysis light scattering using a Brookhaven Zeta PALS instrument. The electrophoretic mobility of the samples was determined from the average of 10 cycles of an applied electric field. The zeta potential was determined from the electrophoretic mobility from the Smoluchowski approximation.

2.4. Cell culture

Culturing of human epithelial lung cell line A549-luc-C8, stably expressing luciferase, was performed according to the protocol provided by Caliper LifeSciences. A549-luc-C8 cells were grown in RPMI-1640 supplemented with 10% v/v heat inactivated FBS and 1% v/v penicillin/streptomycin at 37 °C in a humidified air atmosphere containing 5% CO_2.

2.5. In vitro luciferase gene knockdown studies

A549-luc-C8 cells were trypsinized, counted and diluted to a concentration of approximately 100,000 cells/mL. Then, 0.1 mL of that dilution was added to each well of a 96-well plate and the cells were incubated in a humidified atmosphere at 5% CO_2 and 37 °C. Twenty-four hours before transfection, the cells were washed once with PBS and 100 μL of sample at 10, 25 or 50 nM siRNA concentration (20% of complex to 80% of serum-free cell culture medium (Ham's F-12 Nutrient Mixture, Kaighn's modified with L-glutamine)) was added to each well. Cells were incubated with

the complexes for 5 h. The media was then removed by aspiration and 100 μL of fresh serum medium (RPMI-1640) was added followed by further incubation (48 h). In addition to the anti-luc siRNA, a non-silencing siRNA sequence was used to ensure that the decrease in luciferase expression was due to the anti-luc siRNA and not to cytotoxicity effects of the vector. The Luciferase Assay System from Promega was used to determine luciferase gene silencing following the manufacturer's recommended protocol. The light units were normalized against protein concentration in the cells extracts, which were measured using the BCA™ Protein Assay. The data were expressed as a percentage of control (non-specific siRNA control).

2.6. Assessment of cytotoxicity (MTS assay)

The cytotoxicity of polymers was determined by the CellTiter 96® Aqueous Cell Proliferation Assay (Promega). A549-luc-C8 cells were grown as described in the transfection experiments. Cells were treated with the TAT, dTAT or PEI for ~24 h. The media were then removed and replaced with a mixture of 100 μL fresh culture media (RPMI-1640) and 20 μL MTS reagent solution. The cells were incubated for 3 h at 37 °C in the 5% CO_2 incubator. The absorbance of each well was then measured at 490 nm using a microtiter plate reader (SpectraMax, M25, Molecular Devices Corp., CA) to determine cell viability.

2.7. Assessment of toxicity in vivo

Stock solutions of 0.3 M $CaCl_2$, 10% glucose, and 174 mg/mL dTAT were prepared. Non-silencing control siRNA (Dharmacon; sense: UGUACUGCUUACGAUUCGGtt, antisense: CCGAAUCGUAAGCAGUACAtt) was dissolved in nuclease-free water (61.5 nM). The dTAT/siRNA complexes were prepared in 1.5 mL microcentrifuge tubes by mixing stock solutions (at concentrations indicated above). Formulation 1 used 15 μL of dTAT, 10 μL siRNA, 25 μL glucose, and 5 μL $CaCl_2$. Each ingredient was added to the previous component(s) and mixed by pipetting between each addition. Four of these formulas were individually prepared and kept at 4 °C for 20 min, then kept at room temperature for 5 min prior to injection. Remaining formulations (2–10) were prepared by a serial 1:2 dilution of stock siRNA solution and stock dTAT solution following the same experimental protocol as described above. The dTAT/siRNA complexes (volume = 260 μL) were prepared for each individual animal and each 0.2 mL injection volume contained approximately the following amounts of dTAT peptide (mg/mL): 40, 20, 10, 5, 2.5, 1.25, 0.64, 0.32, 0.16, 0. Each formulation was administered via 200 μL injection into the tail vein of 12 week old male Balb/C mice (10 animals total).

2.8. Assessment of knockdown and biodistribution

Tissue biodistribution and functional siRNA delivery efficiency of dTAT/siRNA complexes were tested *in vivo*. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) siRNA is a sequence that is not expressed in mice, which allows quantitative measurement of siRNA accumulation. Quantitation of siRNA-induced gene silencing in tissues was performed by administration of GAPDH siRNA and a scrambled sequence (negative control) siRNA. GAPDH mRNA expression levels were determined from selected tissues.

The dTAT/siRNA complexes were prepared similarly to the toxicity study except that a 94.6 mg/mL stock solution of the dTAT peptide and a 500 μM stock solution of siRNA were used. Nuclease free water was also added to dilute the 500 μM stock solution of siRNA to attain the lower siRNA dose. The final dose of siRNA was 77 or 38.5 μM. Each formulation was administered via 200 μL injection into the tail vein of 8–12 week old male Balb/C mice (12 animals total; 3 per group). Forty-eight hours after administration, animals were sacrificed and tissues extracted (brain, liver, kidney, lung, muscle, stomach).

Tissues were homogenized and processed using a miRvana isolation kit that allows isolation of both total protein fraction and total RNA fraction (qRT-PCR analysis) from the same tissue. A TaqMan qRT-PCR assay was performed on all isolated mouse samples (duplicate RT step followed by a single PCR step). The geometric mean of two genes, 18S and CyclophilinA, were used to normalize the raw GAPDH mRNA data. A qRT-PCR method using sequence-specific primers was also employed to quantify GAPDH siRNA delivered to these tissue samples. The amount of GAPDH siRNA was normalized to the appropriate miR-24 value and then calculated as percent accumulation relative to the tissue samples from animals given non-silencing siRNA.

2.9. Statistical analysis

Statistical evaluation of data was performed using an analysis of variance (one-way ANOVA). Newman–Keuls was used as a post hoc test to assess the significance of differences. To compare the significance of the difference between the means of two groups, a

Table 1
The diameter of (A) TAT and (B) dTAT complexes as a function of N/P ratio and $CaCl_2$ concentration. Results are presented as mean ± SD (n = 3).

A			B		
TAT - N/P (Ca mM)	Diameter (nm)	Polydispersity	dTAT - N/P (Ca mM)	Diameter (nm)	Polydispersity
7 (0.00)	1012.0 ± 23.4	0.265	6 (0.00)	1170.4 ± 90.4	0.199
7 (23.1)	139.7 ± 3.9	0.108	6 (23.1)	124.3 ± 9.4	0.143
7 (34.6)	113.7 ± 3.7	0.211	6 (34.6)	101.7 ± 15.2	0.179
7 (69.2)	116.6 ± 11.3	0.125	6 (69.2)	98.5 ± 6.8	0.201
18 (0.00)	1411.3 ± 54.1	0.195	17 (0.00)	2256.5 ± 45.1	0.253
18 (23.1)	112.0 ± 6.9	0.230	17 (23.1)	189.3 ± 5.1	0.230
18 (34.6)	99.9 ± 10.8	0.134	17 (34.6)	203.4 ± 9.4	0.177
18 (69.2)	60.6 ± 7.5	0.139	17 (69.2)	152.0 ± 13.6	0.215
25 (0.00)	2105.0 ± 87.9	0.263	23 (0.00)	1978.2 ± 68.2	0.223
25 (23.1)	144.2 ± 2.1	0.222	23 (23.1)	155.7 ± 4.2	0.242
25 (34.6)	117.8 ± 9.7	0.178	23 (34.6)	113.4 ± 7.9	0.196
25 (69.2)	59.3 ± 6.4	0.203	23 (69.2)	89.7 ± 5.1	0.103
33 (0.00)	2210.0 ± 76.9	0.271	31 (0.00)	2190.5 ± 39.6	0.247
33 (23.1)	152.9 ± 8.1	0.169	31 (23.1)	197.4 ± 8.3	0.122
33 (34.6)	122.8 ± 4.5	0.225	31 (34.6)	145.2 ± 1.9	0.108
33 (69.2)	58.5 ± 7.3	0.189	31 (69.2)	90.1 ± 3.3	0.127

t-test was performed; in all cases, a value of $p < 0.05$ was accepted as significant.

3. Results

3.1. Particle sizes and zeta potentials of TAT, dTAT and PEI complexes

TAT, dTAT and PEI complexes were prepared by mixing siRNA with each polycation at various N/P ratios. These complexes were thoroughly mixed by pipetting and $CaCl_2$ was added (final concentration 23.1–69.2 mM). The size of the complexes prepared with 25 nM of siRNA was determined by DLS (Table 1A and B). In general, the added $CaCl_2$ produced small TAT and dTAT complexes at all N/P ratios (58.5–201.3 nm) with polydispersity values below 0.24. The size of the TAT and dTAT complexes generally decreased with increasing N/P ratios and increasing concentrations of calcium. The zeta potential of TAT and dTAT complexes was ~15 mV. The charge did not substantially change with the N/P ratio. In comparison, PEI complexes showed a small particle size (90 nm) with a higher zeta potential of ~20 mV. Calcium was not used to prepare the PEI complexes.

3.2. Cytotoxicity of TAT, dTAT and PEI complexes

Efficient delivery together with low cytotoxicity is extremely desirable to translate RNAi therapeutic vectors. To evaluate the cytotoxicity of free TAT, dTAT, and PEI, an MTS assay was performed by incubating A549-luc-C8 cells with up to 5 mg/mL of TAT, dTAT or PEI for 24 h (Fig. 1). TAT peptide revealed almost no evidence of cytotoxicity and cells maintained high viability, while dTAT showed modest cytotoxicity (IC_{50} ~ 4000 μg/mL as estimated from interpolating the data trend). The branched PEI induced substantial cytotoxicity (IC_{50} of 22 μg/mL) as expected.

3.3. In vitro luciferase gene knockdown by TAT, dTAT and PEI complexes

The silencing efficiency of TAT and dTAT complexes was investigated using the human lung carcinoma cell line A549-luc-C8. This cell line stably expresses firefly luciferase. Luciferase knockdown was evaluated 48 h after treatment with the TAT, dTAT or PEI polyplexes. The data were normalized to the luciferase protein levels of cells treated with control siRNA complexes. Complexes prepared at five different N/P ratios of TAT/siRNA or dTAT/siRNA were condensed by adding different concentrations of $CaCl_2$ (23.1, 34.6, or 69.2 mM) after complex formation. Different siRNA doses (10, 25 or 50 nM) were studied and compared to PEI polyplexes (N/P 10).

In general, several of the TAT and dTAT complexes showed a significantly higher degree of luciferase knockdown for the various N/P ratios and $CaCl_2$ concentrations when compared to PEI, which showed excellent knockdown in the absence of $CaCl_2$ (Figs. 2 and 3). For TAT complexes, the lowest average luciferase expression for each dose was always at the N/P ratio 25 (69.2 mM $CaCl_2$). TAT complexes showed a minimum luciferase expression of 8.3% for this particular formulation at the highest dose (50 nM). Complexes using dTAT followed a similar trend with the highest calcium concentration and N/P ratios of 23 knocking luciferase expression down to just 8.0% for the 50 nM dose. It should be noted that, these

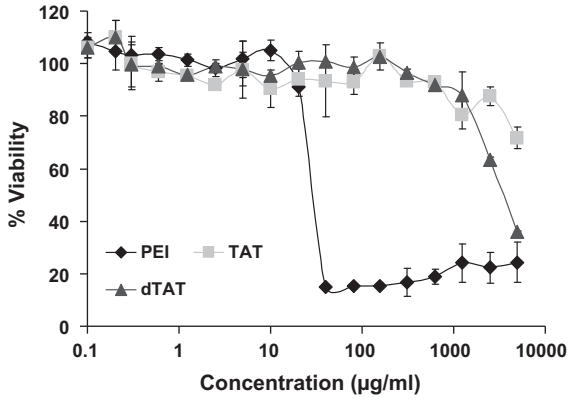

Fig. 1. Cytotoxicity profiles of PEI, TAT and dTAT for A549-luc-C8 cells. Viability is expressed as a function of polymer concentration. Results are presented as mean ± SD ($n = 3$).

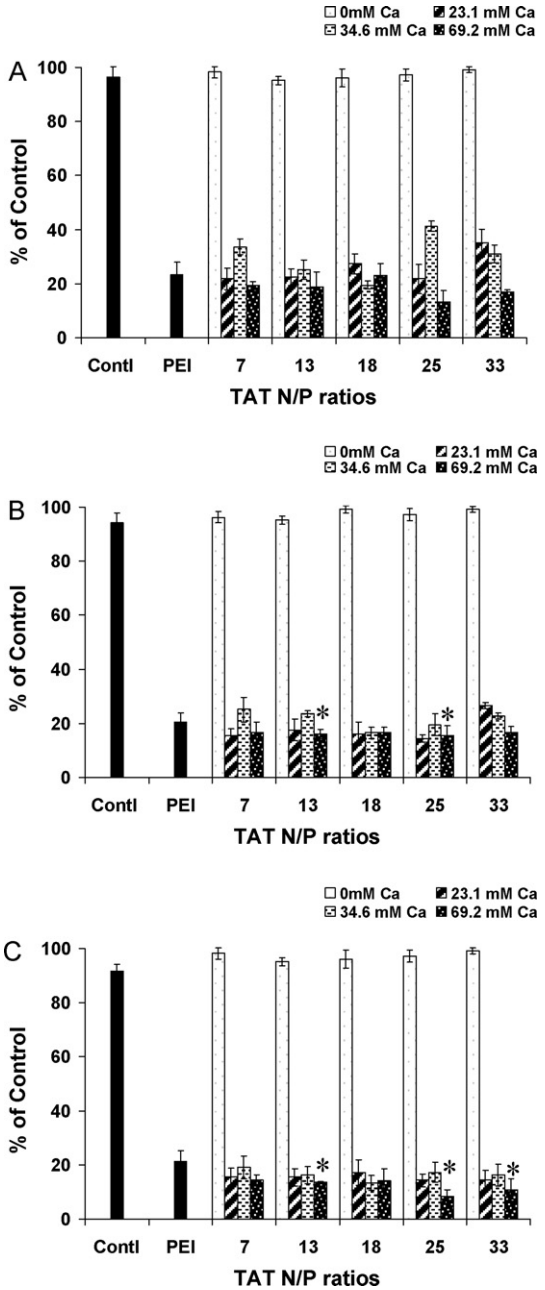

Fig. 2. The luciferase knockdown was assessed in A549-luc-C8 cells using TAT complexes prepared across a range of N/P ratios and condensed with different concentrations of added $CaCl_2$. Doses studied were (A) 10, (B) 25 and (C) 50 nM of siRNA. Results are presented as mean ± SD ($n = 3$), $^*p < 0.05$ when compared to the PEI formulation (PEI N/P = 10).

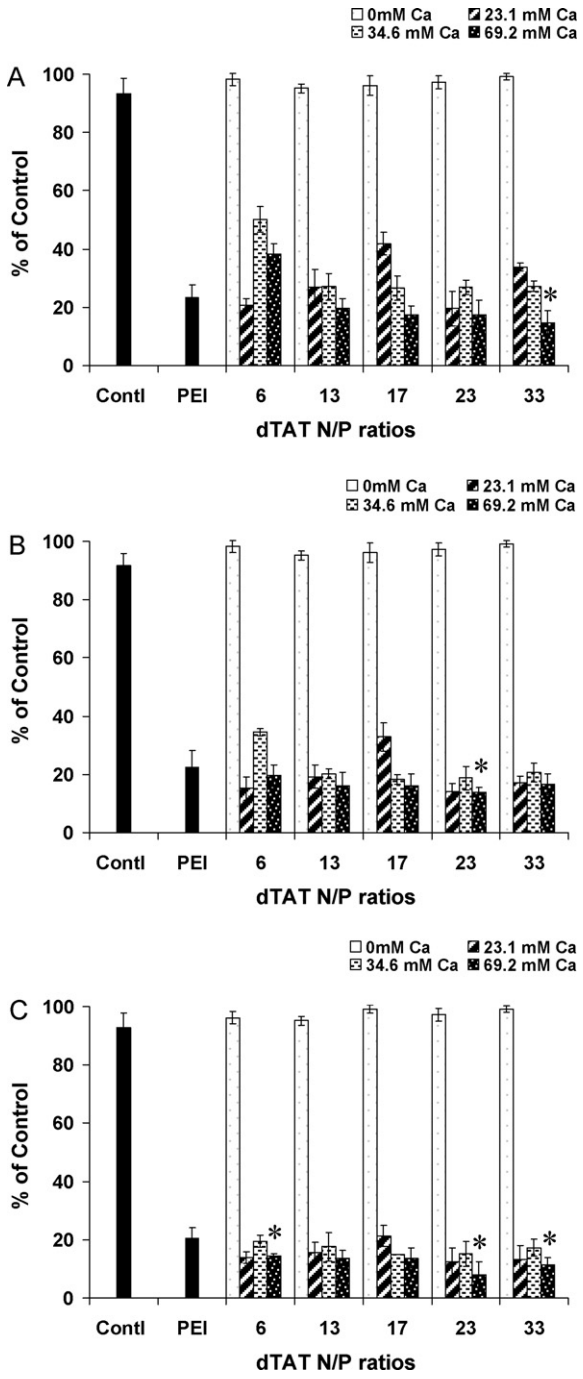

Fig. 3. The luciferase knockdown was assessed in A549-luc-C8 cells using dTAT complexes prepared across a range of N/P ratios and condensed with different concentrations of added $CaCl_2$. Doses studied were (A) 10, (B) 25 and (C) 50 nM of siRNA. Results are presented as mean ± SD ($n = 3$), $^*p < 0.05$ when compared to the PEI formulation (PEI N/P = 10).

minima were not significantly different from the 3–5 next best performing formulations; however, both TAT and dTAT formulations tended towards the greatest gene silencing at high calcium concentration (69.2 mM) and moderately high N/P ratios (N/P ratios of 25 and 23, respectively). The luciferase knockdown of PEI complexes was found to be independent of the siRNA dose (no statistical differences). Strikingly, no luciferase knockdown was observed for TAT and dTAT complexes without $CaCl_2$. It is important to note that the TAT and dTAT siRNA control complexes including 69.2 mM $CaCl_2$ did not affect the overall luciferase expression levels, which further supported the premise that these vectors did not influence the viability of A549-luc-C8 cells.

3.4. In vivo dose escalating toxicity study

The CPP dTAT was selected for further studies *in vivo*. A dose escalating study was conducted in an effort to establish a lethal dose (LD_{50}) for the dTAT peptide. For these studies, only a minimal amount of siRNA was included so that animal responses would be more indicative of the toxicity of dTAT. The inclusion of a small amount of siRNA was necessary to form complexes, since the colloidal properties of these CPP complexes were expected to alter biodistribution as compared to dTAT alone. Animals were administered the formulations via tail vein injection (200 μL) at doses up to 1000 mg/kg of dTAT (Table 2). The highest dose yielded a transient behavioral response, with the animal fully recovering.

3.5. In vivo gene knockdown and biodistribution of dTAT complexes

The dTAT complexes were also investigated for gene silencing *in vivo*. The siRNA-induced gene silencing was determined by tail vein injection of dTAT complexed with GAPDH siRNA. Animals were given a 200 μL injection of either GAPDH siRNA (38.5 μM or 77 μM), control siRNA (77 μM), or PBS (Table 3). The increased siRNA loading levels for this study yielded particles of 251 nm (polydispersity of 0.117) and 483 nm (polydispersity of 0.241) for the 38.5 μM or 77 μM doses, respectively. These particle sizes were larger than those studied *in vitro* due to the substantially higher siRNA loading and overall concentration. In this case, the siRNA loading was maximized to improve detection *in vivo*, however, the smaller (~50–60 nm) particles are of great interest and will be explored in future studies. Tissues were analyzed 48 h after administration.

To assess the performance of GAPDH siRNA, the amount of GAPDH mRNA was determined in brain, liver, kidney, lung, muscle, and stomach. The relative knockdown was then determined by normalizing these data to the GAPDH mRNA expression in tissue samples from animals given PBS (Fig. 4). As expected, animals treated with control siRNA did not show GAPDH mRNA knockdown. At the higher GAPDH siRNA dose (77 μM), knockdown was most

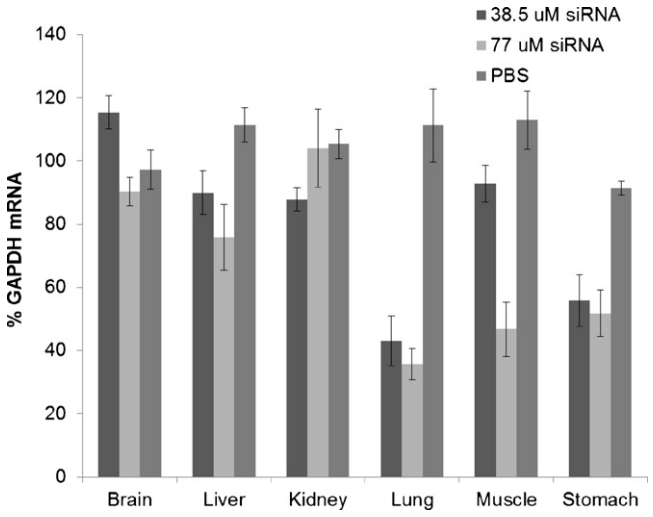

Fig. 4. GAPDH knockdown was determined for dTAT/siRNA complexes condensed with calcium (siRNA dose noted in the legend). The percentage of GAPDH mRNA expression in each tissue was quantified relative to the negative control siRNA/dTAT complexes ($n = 3$). When comparing GAPDH siRNA/dTAT treatments to PBS placebo, differences were significant ($p < 0.05$) for both doses in lung, muscle, stomach and liver ($t = 48$ h).

Table 2
Dose escalation toxicity study indicated negligible toxicity of dTAT. The RNA dose was minimized to focus on animal response to dTAT.

Animal ID	dTAT (mg/mL)	Total dose (mg/kg)	Final siRNA concentration (200 μL injection volume) (nM)	Toxicity/behavioral effects
1	40	1000	9.46	Behavioral phenotype[a]
2	20	500	4.73	Normal
3	10	250	2.36	Normal
4	5	125	1.18	Normal
5	2.5	62.5	0.59	Normal
6	1.25	31.25	0.29	Normal
7	0.64	15.63	0.14	Normal
8	0.32	7.81	0.07	Normal
9	0.16	3.90	0.03	Normal
10	0.00	0.00	0.00	Normal

[a]Mouse was less active after injection, laid down for 1 h, dragged back legs. During 8 h post-injection animal was less alert and moving compared to other animals. Resumed full activity after 8–12 h post-administration.

pronounced in lung, muscle, and stomach tissue. The lower siRNA dose (38.5 μM) also showed significant knockdown of GAPDH mRNA in lung tissue. Very little knockdown was noted in liver and kidney tissue (Fig. 5).

The amount of GAPDH siRNA in these tissues was also quantified, which provided more direct evidence of the biodistribution of complexes. The amount of siRNA found in brain, liver, kidney, lung, muscle, and stomach was normalized to the amount of GAPDH siRNA in the corresponding tissues of animals receiving PBS. This GAPDH siRNA is not expressed in mice, so all measured siRNA was due to delivery. GAPDH siRNA was detected at relatively high levels in lung tissue at both doses. The amount of siRNA in the lung tissue nearly doubled when the dose was doubled. The muscle and stomach tissue also showed significant siRNA accumulation at the higher dose (77 μM). Results were more variable in the liver tissue; however, some accumulation of GAPDH siRNA was evident at the higher dose.

4. Discussion

The strikepoint of dose limiting toxicity versus efficacy has been a common issue plaguing translation of all types of gene and RNAi formulations. Previous studies have shown that delivery of siRNA by liposomes may provide efficient knockdown *in vitro*, but such systems can be inefficient when applied *in vivo* (Griesenbach et al., 2006). Current approaches often aim to improve the safety of the best transfection reagents (e.g. PEI or cationic liposomes). For example, derivatives of PEI such as degradable oligo-ethylenimine, PEGylated PEI and low molecular weight PEI have been used as siRNA carriers (Mao et al., 2006; Tarcha et al., 2007; Werth et al., 2006). Small chemical changes in PEI or other highly effective transfection reagents (e.g. cationic lipids) commonly improved safety at the expense of efficacy, thus the problem of dose-limiting toxicity remains. Furthermore, current trends may also suggest that these systems may have limited efficiency in siRNA delivery compared to pDNA delivery and that toxicity is still be a concern (Hassani et al., 2005; Richards Grayson et al., 2006).

Highly efficient intracellular delivery mediated by CPPs has been confirmed in a variety of cell lines with minimal toxicity, overcoming challenges often faced with other delivery methods. Previously, such CPP polyelectrolyte complexes were demonstrated as an efficient and non-cytotoxic means of gene delivery (Baoum et al., 2009; Baoum and Berkland, 2011). The use of calcium to condense CPP/pDNA complexes resulted in small particles leading to gene expression levels that were even higher than optimized PEI polyplexes. These complexes were stable, maintaining particle size in the absence and presence of 10% of FBS. Moreover, the transfection efficiency was not significantly affected by the presence of serum. The CPPs also showed negligible cytotoxicity compared with PEI, which was very cytotoxic. Thus, previous reports using calcium condensation of CPP/pDNA complexes offered a potential strategy to improve safety while maintaining transfection efficiency.

In this study, the effect of calcium on TAT and dTAT complexes was investigated to determine whether this formulation could also effectively deliver siRNA while enhancing safety when compared to current vectors. Various N/P ratios of TAT and dTAT with different calcium concentrations and different doses of siRNA were used to optimize particle size and knockdown *in vitro*. Calcium was found to form compact TAT and dTAT complexes (58.5–201.3 nm) leading to high knockdown efficiencies in A549-luc-C8 lung epithelial cells. TAT and dTAT showed the smallest particle sizes at high calcium concentration (69.2 mM) across various N/P ratios. Small differences in the size of these complexes did not appear to directly affect gene silencing. A small siRNA dose of 10 nM was enough to knockdown luciferase expression by up to 87%. Also, it is important

Table 3
Animal groups and formulations for biodistribution study (200 μL injection volume).

Group number	dTAT (mg/mL)	siRNA (μM)	Glucose (%)	$CaCl_2$ (mM)
1	40	38.5	2	70
2	40	77	2	70
3	40	77	2	70
4	(PBS control)	–	–	–

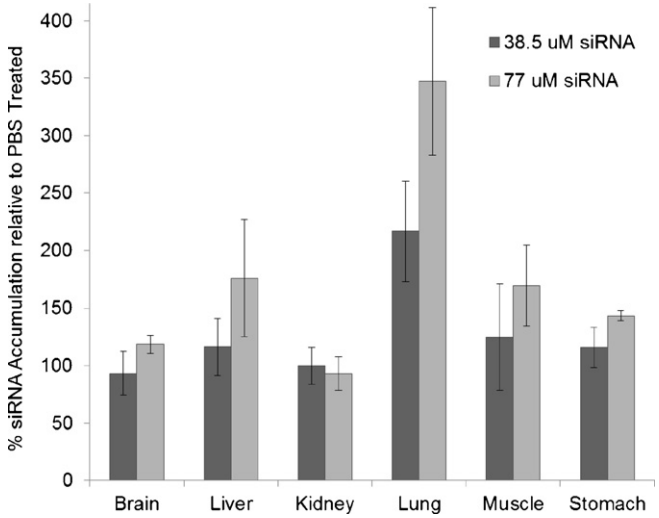

Fig. 5. The distribution of siRNA was determined for dTAT/siRNA complexes condensed with calcium (siRNA dose noted in the legend). The accumulation of GAPDH siRNA per tissue was quantified relative to mice given PBS placebo ($n = 3$). When comparing GAPDH siRNA to PBS control, differences were significant ($p < 0.05$) for the high dose in muscle, stomach and liver, and for both doses in lung ($t = 48$ h).

to note that absolutely no gene knockdown was observed for TAT and dTAT complexes that were not condensed with calcium. This supports previous studies on CPP complexes with pDNA that point to calcium condensation as an essential part of the formulation (Baoum et al., 2009; Baoum and Berkland, 2011).

The toxicity of TAT and dTAT was also systematically evaluated *in vitro* and *in vivo*. Low molecular weight CPPs were selected due to the potential for safe administration of these biodegradable polycations. Lung carcinoma cells studied here were highly tolerant of CPPs ($IC_{50} \geq 4$ mg/mL) and previous studies suggest that multiple other CPPs perform similarly (Baoum et al., 2009; Baoum and Berkland, 2011). The dose escalation of calcium condensed dTAT complexes administered via tail vein injection confirmed the safety of this CPP. Even at exceptionally high doses of 1000 mg/kg of dTAT, only a reversible behavioral phenotype was noted. In contrast, the LD_{50} of PEI (25 kDa) has been reported to be ~1-5 mg/kg in mice. β-Cyclodextrin-based polymers showed an LD_{40} of 200 mg/kg with some compromise in gene delivery efficacy (Hwang et al., 2001). Results for the CPP complexes reported here suggested that dose-limiting toxicity may not be a bottleneck in the translation of this siRNA delivery system.

Knockdown of a target gene requires successful delivery of the siRNA to the tissue of interest; therefore, efficacy and biodistribution studies were undertaken. Studies confirmed both the delivery of GAPDH siRNA and the knockdown of the target in several tissues. The relative quantity of siRNA delivered to the tissue samples correlated moderately well to the knockdown in GAPDH mRNA. The knockdown was noticeable in multiple tissues despite the fact that siRNA doses were modest (~19 and 38 μg/kg) in comparison to other reports. For example, doses of 150–250 μg/kg are common when using liposomal delivery systems (Landen et al., 2005). Researchers have dosed siRNA up to very high values, but are commonly limited by the toxicity of the carrier, when one is employed. In the case of CPP complexes reported here, the siRNA doses could be further increased, even without additional optimization of the current formulation approach. Thus, calcium condensed CPP complexes were effective RNAi vectors *in vivo* and dose may be escalated to further improve performance.

Another difficulty with current siRNA delivery strategies is the accumulation of these colloids in organs of the reticuloendothelial system. Attempts to increase the dose of therapeutic to target tissue can be confounded by unwanted accrual in liver, kidney, spleen, or other organs. In this study, the low levels of siRNA in liver and kidney tissues of mice may provide an important advantage over existing delivery systems. One hypothesis for the preferential targeting of lung over liver tissue observed here is the evidence of small particle size for CPP complexes. CPP complexes condensed with calcium were previously shown to maintain a very small size when challenged with serum, which may have facilitated delivery to highly vascularized tissue such as the lung and muscle. In addition, formulation parameters are easily adjusted to produce larger or smaller particles (~50 to >500 nm) that preferentially accrue in target tissue.

5. Conclusion

A myriad of design strategies for siRNA vectors in the last several years has led to a broad spectrum of various liposomal and cationic polymer formulations. Recently, there have been several advances in the use of complexes employing cell penetrating peptides, but simple, safe, and effective formulation approaches are still needed (Johnson et al., 2007; Kumar et al., 2007, 2008; Moschos et al., 2007; Schaffert and Wagner, 2008). Calcium condensation of TAT or dTAT complexed with siRNA induced a substantial decrease in the particle size and enabled knockdown. The gene silencing of these CPP/siRNA complexes was significantly higher than PEI complexes in A549-luc-C8 lung epithelial cells. Furthermore, the TAT and dTAT peptides showed negligible cytotoxicity up to 5 mg/mL. *In vivo* data demonstrated that up to 1000 mg/kg dose of dTAT in complexes was tolerated by animals. Also, appreciable knockdown was favored in lung, muscle and stomach tissue over traditional sites (e.g. liver and kidneys). Much higher doses and/or local administration may still be explored to amplify the silencing effect observed from calcium condensed CPP complexes.

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