articles

molecular pharmaceutics

Increased Intracellular Targeting to Airway Cells Using Octaarginine-Coated Liposomes: In Vitro Assessment of Their Suitability for Inhalation

Sally-Ann Cryan,*,† Marc Devocelle,‡ Padraig J. Moran,† Anthony J. Hickey,§ and John G. Kelly†

Advanced Drug Delivery Research Centre, School of Pharmacy, Royal College of Surgeons in Ireland, Dublin 2, Ireland, Centre for Synthesis and Chemical Biology, Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland, Dublin 2, Ireland, and Division of Drug Delivery and Disposition, School of Pharmacy, University of North Carolina, Chapel Hill, North Carolina 27599-7360

Received August 30, 2005

Abstract: Delivery of macromolecular drugs to airway cells after inhalation can be limited by rapid clearance, in vivo degradation, and poor intracellular targeting. Liposome carriers offer an effective method of improving drug stability, but conventional liposomes have limited intracellular targeting capacity and are cleared rapidly by the lungs. Further modification is required to improve liposome−cell interaction and intracellular targeting. Therefore, we proposed conjugating three arginine-rich membrane translocating peptides, namely, HIV-TAT, Antennapedia, and octaarginine, to neutral liposomes as a biocompatible alternative to cationic lipids for intracellular delivery of macromolecules to airway cells. Conjugation did not significantly affect liposome stability, and each system was nebulized to produce aerosols of mean aerodynamic diameter $<1.5\ \mu$m. The peptides caused a significant ($p < 0.05$) increase in liposome−airway cell association compared to untagged liposomes and to DOTAP liposomes. Up to 30% of the peptide-conjugated liposomes added were bound and internalized (via a temperature-dependent, endocytic process) after just 2 h. The novel carriers all delivered encapsulated dextrans rapidly and efficiently to the cytoplasm of Calu-3 cells. Once internalized by the cells, the modified carriers localize for the most part in the cytoplasm with only a small amount of nuclear localization. These peptide-conjugated liposomes were significantly ($p < 0.05$) less toxic than DOTAP liposomes with octaarginine-coated liposomes the least toxic. These systems, particularly octaarginine-coated liposomes, offer many advantages for drug delivery to airway epithelial cells including increased stability, improved cell binding, and cell uptake with an improved toxicity profile.

Keywords: Liposomes; protein transduction domains; airway cells; inhalation

Introduction

There is a growing need to develop methods of delivery of drugs, particularly macromolecular drugs, intracellularly to airway cells. Examples include antimicrobial peptides, antiinflammatory proteins, antioxidants such as catalase and dismutase, and gene therapies. Some of the fundamental obstacles to intracellular macromolecule delivery to the lungs have been inefficient vectors.[1]

Liposomes have been used as carriers for delivery of small molecule drugs and in more recent years as delivery vectors

* Corresponding author. Mailing address: School of Pharmacy, Royal College of Surgeons in Ireland, York House, York Street, Dublin 2, Ireland. Phone: 353-1-4022741. Fax: 353-1-4022765. E-mail: scryan@rcsi.ie.

† School of Pharmacy, Royal College of Surgeons in Ireland.

‡ Department of Pharmaceutical & Medicinal Chemistry, Royal College of Surgeons in Ireland.

§ University of North Carolina.

(1) Klink, D. T.; Chao, S.; Glick, M C.; Scanlin, T. F. Nuclear translocation of lactosylated poly-L-lysine/cDNA complex in cystic fibrosis airway epithelial cells. *Mol. Ther.* **2001**, *3* (6), 831−841.

10.1021/mp050070i CCC: $33.50
Published on Web 12/08/2005

for macromolecules, such as DNA, RNA, and proteins, to the lungs.[2] Liposome aerosols have a number of advantages including sustained release, prevention of local irritation, reduced toxicity, improved stability in the large aqueous core, and the possibility to manipulate release and targeting by altering the bilayer constituents and changing the preparation technique.[3] Liposomes may be prepared for inhalation in liquid[4] or dry powder form.[5] Conventional liposomes offer only limited cell binding and intracellular delivery, and until now cationic lipids, such as dioleoyloxy-3-(trimethylammonio) propane (DOTAP), have been used. These lipids are toxic at high doses, have been shown to cause immunogenic and inflammatory responses in vivo,[6] and can be inactivated by pulmonary surfactants.[7]

Recent studies have shown that, by modifying carriers to allow multiple molecular interactions with the cell membrane, carrier systems can be used to both prolong exposure and trigger cell transport processes in the epithelium.[8] A number of small regions of proteins called protein transduction domains (PTD) have been identified which are rapidly and efficiently bound to and internalized by cells.[9] These peptides represent nature's very own highly evolved macromolecular delivery system and include the *Drosophilia* homeotic transcription protein Antennapedia (ANTP) and the human immunodeficiency virus I transcriptional activator (HIV-TAT). The use of these peptides for drug delivery is receiving increased interest.[10] The structural features of these two peptides were extensively studied, and the guanidinium headgroups of the arginine residues appeared to be the critical structural feature involved in transduction.[11] Comparing a series of homopolymers of arginine, the polymer with eight arginine (R8) residues was found to produce the optimum membrane transduction. This simple homopolymer was found to be even more effective at entering cells than HIV-TAT in some cases.[11,12] Compared with polyarginine the toxicity associated with this short chain R8 peptide is much lower.[13]

Recently, several groups have successfully improved the cellular uptake of neutral liposomes by attaching the arginine-rich peptides, HIV-TAT and ANTP peptides, to liposomes.[14,15] To date, no attempt has been made to determine the ability of R8 to improve the intracellular delivery of liposomes. We have synthesized R8-conjugated liposomes, as another biodegradable alternative to cationic lipids, in order to determine its effect on intracellular delivery of liposomes to airway cells and compare this with the effects of HIV-TAT and ANTP. These systems offer many potential advantages for macromolecular delivery including improved stability, cell binding, and uptake with an improved toxicity profile. The added advantage of these liposomal carriers is that the drug molecule does not need to be conjugated directly to the PTD but rather can be encapsulated within the targeted liposome. Given the ubiquitous nature of their uptake into a range of cell types, it appears that local delivery may offer the best approach to harness these carriers for site-specific targeting. When delivered locally to the lungs they can improve targeting to the airway epithelium.

Here we compare the size, aerodynamic properties, membrane translocating efficiency, and toxicity of the PTD-conjugated liposomes (LIP-TAT, LIP-ANTP, LIP-R8) in airway cells. We also present results demonstrating the improved biocompatibility and intracellular delivery achieved using R8-conjugated liposomes compared to DOTAP liposomes.

Experimental Section

Materials. Dioleoylphosphatidylcholine (DOPC), cholesterol (Chol), rhodamine-phosphatidylethanolamine (Rho-PE),

(2) Niven, R. Modulated Drug Therapy with Inhalation Aerosols. In *Pharmaceutical Inhalation Aerosols Technology*; Hickey, A., Ed.; Marcel Dekker: New York, 2003; pp 551−570.
(3) Cryan, S. A. Carrier-based Strategies for Targeting Protein and Peptide Drugs to the Lungs. *AAPS J.* **2005**, *07* (01), E20−E41.
(4) Niven, R.; Schreier, H. Nebulisation of liposomes. I Effects of lipid composition. *Pharm. Res.* **1990**, *7*, 1127−1133.
(5) Schreier, H.; Mobley, W. C.; Concessio, N.; Hickey, A. J.; Niven, R. W. Formulation and in vitro performance of liposome powder aerosols. *STP Pharma Sci.* **1994**, *4*, 38−44.
(6) Zelphati, O.; Szoka, F. C. Intracellular Distribution and Mechanism of Delivery of Oligonucleotides Mediated by Cationic Lipids. *Pharm. Res.* **1996**, 1367−1373.
(7) Duncan, J. E.; Whitsett, J. A.; Horowitz, A. D. Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro. *Hum. Gene Ther.* **1997**, *8* (4), 431−438.
(8) Abu-Dahab, R.; Schafer, U. F.; Lehr, C. M. Lectin-functionalized liposomes for pulmonary drug delivery: effect of nebulization on stability and bioadhesion. *Eur. J. Pharm. Sci.* **2001**, *14* (1), 37−46.
(9) Suzuki, T.; Futaki, S.; Niwa, M.; Tanaka, S.; Ueda, K.; Sugiura, Y. Possible existence of common internalization mechanisms among arginine-rich peptides. *J. Biol. Chem.* **2002**, *277* (4), 2437−2443.
(10) Plank, C.; Zauner, W.; Wagner, E. Application of membrane-active peptides for drug and gene delivery across cellular membranes. *Adv. Drug Delivery Rev.* **1998**, *34* (1), 21−35.
(11) Mitchell, D. J.; Kim, D. T.; Steinman, L.; Fathman, C. G.; Rothbard, J. B. Polyarginine enters cells more efficiently than other polycationic homopolymers. *J. Pept. Res.* **2000**, *56* (5), 318−325.
(12) Wender, P. A.; Mitchell, D. J.; Pattabiraman, K.; Pelkey, E. T.; Steinman, L.; Rothbard, J. B. The design, synthesis, and evaluation of molecules that enable or enhance cellular uptake: peptoid molecular transporters. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97* (24), 13003−13008.
(13) Futaki, S. Membrane-permeable arginine-rich peptides and the translocation mechanisms. *Adv. Drug Delivery Rev.* **2005**, *57* (4), 547−558.
(14) Tseng, Y. L.; Liu, J. J.; Hong, R. L. Translocation of liposomes into cancer cells by cell-penetrating peptides penetratin and tat: a kinetic and efficacy study. *Mol. Pharmacol.* **2002**, *62* (4), 864−872.
(15) Torchilin, V. P.; Rammohan, R.; Weissig, V.; Levchenko, T. S. TAT peptide on the surface of liposomes affords their efficient intracellular delivery even at low temperature and in the presence of metabolic inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98* (15), 8786−8791.

dioleoyl-*sn*-glycero-3-phosphoethanolamine-*N*-[4-(maleimidophenyl) butyramide] (N-MPB-PE), and dioleoyltrimethylammonium propane (DOTAP) were purchased from Avanti Polar Lipids. Spin-OUT columns were purchased from Geno Technology Inc. and fluorescein isothiocyanate (FITC)-dextran, Ellman's reagent, MTT, and buffer components from Sigma. Cell culture media were sourced from Gibco Invitrogen Corporation. Transwell Clear inserts (25.4 mm in diameter and pore size 0.4 μm) (Co-star) were used.

Peptide Synthesis. Octaarginine (RRRRRRRR) (R8), human immunodeficiency virus transactivating factor (AYGRKKRRQRRR) (TAT), and Antennapedia (ARQIKIWFQNRRMKWKK) (ANTP) peptides were prepared with cysteine residues: R8-Cys, TAT-Cys, ANTP-Cys. The peptides were prepared by standard solid phase peptide synthesis[16,17] according to the Fmoc-*t*Bu strategy[18−20] with HBTU/HOBt/DIEA coupling chemistry, in *N*-methylpyrrolidone (NMP) solvent. Single coupling cycles using a 10-fold excess of Fmoc amino acid derivatives to resin-bound peptide were employed. The side chain protecting groups were *t*Bu for Tyr; Trt for Asn, Gln, and Cys; Boc for Lys and Trp; Pbf for Arg. Syntheses were carried out on a 1.0×10^{-4} mol scale. Assembly of the amino acid sequence, starting from a Rink Amide MBHA resin, and attachment of an N-terminal cysteine were carried out on an automated peptide synthesizer (Applied Biosystems 433A). Peptides were deprotected and cleaved from the synthesis resin using a mixture of 82.5% trifluoroacetic acid, 5% water, 5% triisopropylsilane, 5% thioanisole, 2.5% ethanedithiol (EDT), at room temperature for 2.5 h. The peptides were precipitated and washed three times with 10 mL portions of diethyl ether. They were then dried, dissolved in distilled water, and lyophilized.

Chromatographic analysis and purification were performed on a BioCAD SPRINT perfusion chromatography workstation (*PerSeptive Biosystems*) using POROS 20R2 reversed phase perfusion chromatography packing (column: 4.6 mm diameter × 100 mm length, self-packed), or a Jupiter column (5μ, C5, 4.6 mm diameter/250 mm length, Phenomenex) for analysis and a Jupiter column (15μ, C5, 100 mm diameter/250 mm length, Phenomenex) for purification, when required. Mobile phase A: 0.1% TFA in water. Mobile phase B: 0.1% TFA in acetonitrile. Gradient: 5% to 65% B in 18 column volumes (Poros-analytical) or 6 column volumes (Jupiter-analytical) and 15 column volumes (Jupiter-semi-preparative). Flow rate: 4 mL/min (Poros-analytical) or 1 mL/min (Jupiter-analytical) and 5 mL/min (Jupiter-semi-preparative); single wavelength detection at 214 nm. The peptides were characterized by matrix assisted laser desorption ionization time-of-flight mass spectrometry (α-cyano-4-hydroxy-cinnamic acid matrix).

Liposome−Peptide Conjugation. Liposomes composed of DOPC:Chol:N-MPB-PE:Rho-PE (6:3:1:0.1) were formed by dehydration−rehydration. Controls (unmodified liposomes) were prepared by the same method without the MPB-PE. In brief, a lipid film was prepared by rotary evaporation of the lipid mix (detailed above) in chloroform. The lipids were then left under vacuum for 1 h. The film was rehydrated in phosphate-buffered saline (PBS), pH 7.4, to produce a concentration of 10 μmol of lipid/mL. When required, liposomes were reconstituted with FITC-dextran (45 mg/ml). Size reduction was carried out using a thermobarrel extruder (Northern Lipids) with a 0.2 μm filter (each batch passed through the extruder 10 times). When FITC-dextran was encapsulated, the unencapsulated dextran was removed using a Sephadex PD-10 column (Sigma-Aldrich) prior to conjugation with peptides.

The coupling of the peptides to liposomes was performed according to the strategy of Martin and Papahadjopoulos.[21] Liposomes were reacted with the thiolated peptides at a molar ratio of 10:0.66. Stocks (10 mg/mL) of each of the thiolated peptides (HIV-TAT-Cys, ANTP-Cys, R8-Cys) were made using PBS immediately prior to reaction; 0.66 μmol of each peptide was added per milliliter of liposomes (10 μmol/mL) and reacted for 2 h at 25 °C. Reaction progress was monitored by determination of residual thiols using Ellman's reagent.[22] One hundred microliter aliquots were mixed with 1 mL of Ellman's reagent [2 mM DTNB, 1 mM ethylenediaminetetraacetic acid (EDTA) in 0.2 M sodium phosphate buffer pH 7.27].[23] The unreacted peptide was removed using a Spin-OUT column with a molecular weight cutoff of 6000. The lipid (fluorescence) and peptide content (Bio-rad DC protein assay kit) was determined for each batch.

DOTAP liposomes (DOPC:Chol:DOTAP:Rho-PE (6:3:1:0.1)) were similarly prepared. This was also prepared at 10μmol/mL final concentration.

Characterization of the Peptide-Tagged Liposomes. To determine the effect of conjugation on liposome size and stability, the particle size of the PTD-conjugated liposomes was determined using dynamic light scattering and electron microscopy (EM). Particle size was measured by dynamic light scattering using a high performance particle sizer (Malvern). For EM studies 400-mesh pioloform-coated

(16) Merrifield, R Solid phase peptide synthesis I. Synthesis of a terapeptide. *J. Am. Chem. Soc*. **1963**, *85*, 2149−2154.
(17) Merrifield, R. Solid Phase Synthesis. *Science* **1986**, *232*, 341−347.
(18) Carpino, L.; Han, G. J. The 9-fluorenylmethoxycarbonyl amino protecting group. *J. Org. Chem.* **1972**, *37*, 3404−3409.
(19) Atherton, E.; Cameron, L.; Sheppard, R. Peptide synthesis: Part 10. Use of pentafluorophenyl esters of fluorenylmethoxycarbonylamino acids in solid phase peptide synthesis. *Tetrahedron* **1988**, *44*, 843−857.
(20) Sheppard, R. The Fluorenylmethoxycarbonyl Group in Solid Phase Synthesis. *J. Pept. Sci*. **2003**, *9*, 545−552.
(21) Martin, F. J.; Papahadjopoulos, D. Irreversible coupling of immunoglobulin fragments to preformed vesicles. *J. Biol. Chem.* **1982**, *257*, 286−288.
(22) Riddles, P.; Blakeley, R. L.; Zerner, B. Ellman's reagent: 5,5′-dithiobis(2-nitrobenzoic acid) a reexamination. *Anal. Biochem.* **1979**, *94*, 75−81.
(23) Frisch, B.; Boeckler, C.; Schuber, F. Synthesis of Short Polyoxyethylene-Based Heterobifunctional Cross-Linking Reagents. Application to the Coupling of Peptides to Liposomes. *Bioconjugate Chem.* **1996**, *7*, 180−186.

copper grids were glow-discharged for several minutes and used within 30 min. A drop of the liposome sample (10-fold dilution of sample in water) was placed on the grid for 2 min followed by washing with deionized water. The grid was negatively stained with a drop of uranyl acetate (2% w/v) for 1 min and again blotted. The grids were stored at 4 °C and viewed the same day using a Hitachi H7000 transmission electron microscope (TEM).

Cell Culture. Calu-3 cells were obtained from American Collection of Animal Cell Cultures (ATCC, Rockville, MD). Calu-3 cells are a human bronchial submucosal adenocarcinoma cell line. Calu-3 cells of passage 30−50 were used throughout. The cell line was cultured using modified Eagle's medium (MEM) supplemented with fetal calf serum (10% v/v), sodium pyruvate (1 mM), nonessential amino acids (NEAA) (0.1 mM), 100 μg/mL streptomycin and 100 units/mL penicillin. Cells were maintained at 37 °C in a 5% CO_2 humidified atmosphere.

Cellular Binding and Uptake Assays. Calu-3 cells were seeded at a density of 1×10^5 cells/well in 24-well plates (Nunc). The cells were grown for 2 days, and prior to treatment the medium was removed and replaced with fresh medium. PTD-conjugated liposomes (rhodamine-labeled) were added to each well and incubated in medium at 37 °C under 5% CO_2. After incubation the cells were washed three times with phosphate-buffered saline (PBS) and incubated with 1 mL of Triton-X 100 (1% w/v) for 30 min at 37 °C. Cell-associated fluorescence was determined at excitation and emission wavelengths of 550 and 590 nm, respectively, on a spectrofluorimeter (Perkin-Elmer) and expressed as fluorescence/mg of protein (Bio-rad protein assay kit). In order to determine the mechanism of liposome−cell interaction, incubations were carried out at 4 °C and in the presence of endocytosis inhibitors (sodium azide (0.1% w/v), sodium fluoride (10 mM), and antimycin A (1 μg/mL)).

Cell Viability. Calu-3 cells were seeded in 96-well tissue culture plates and grown to 90% confluency (24−48 h). The cell medium was removed and replaced with fresh medium. The PTD-conjugated liposomes were added over a range of concentrations (10−200 μM) for 4 h at 37 °C. The cell viability at the end of the incubation period was determined using the Thiazolyl Blue (MTT) assay.[24]

Confocal Laser Scanning Microscopy (CLSM). For intracellular distribution studies Calu-3 cells were seeded on coverslips at a density of 1×10^4 cells/well in 24-well plates. At 50% confluency cells were incubated with rhodamine-tagged liposomes in medium at 37 °C under 5% CO_2. Some samples were fixed using paraformaldehyde. The medium was removed, and the cells were washed with sterile phosphate-buffered saline (PBS), pH 7.4. Coverslips were mounted cell-side down with fluorescence-free glycerol-based mounting medium and viewed using an LSM510 Axioplan 2 upright confocal microscope (Zeiss, Germany). In order to prevent artifacts of fixation, live cells and fixed cells were imaged.

Transport Studies. In order to assess the degree to which the PTD-conjugated liposomes are transported across the airway, epithelial transport studies were carried out. Calu-3 cells were seeded on 24 mm diameter, 0.4 μm pore size, polycarbonate filters, Transwells, at a density of 10^5 cells/cm^2.[25] Cell culture medium was added to the apical (1.5 mL) and basolateral (2.6 mL) chambers. The apical medium was removed after day 1, and cells were fed daily with 1.5 mL of fresh basolateral medium to produce an air-interfaced culture (AIC).[25] The cultures were used 7−8 days post-seeding.

Transport studies were carried out in Hanks balanced salt solution (HBSS), pH 7.4. Rhodamine-labeled liposomes were added to the apical chamber of $n = 3$ wells. The initial and final concentrations of liposomes in the apical chamber were measured by taking a 20 μL sample, and the basolateral chamber was sampled at 15, 30, 60, 90, and 120 min. Transepithelial electrical resistance was measured at the beginning and the end of the experiment.

The apparent permeability coefficient (P_{app}) for each system was calculated using the following equation:

$$J = P_{app}A(C_{donor} - C_{receptor})$$

The cumulative amount of each liposome transported across the cell layer was plotted versus time over 2 h (0, 30, 60, 120 min), and the slope of the plot was found using linear regression (J). C_{donor} is the concentration in the donor compartment (apical side), $C_{receptor}$ is the concentration in the receptor compartment (basolateral side), and A is the surface area of the filter used in this study. The percentage cell association at the end of the experiment was also determined by rinsing the monolayers three times with PBS followed by incubation with Triton-X 100 (1% w/v) in PBS at 37 °C for 30 min. The cells were removed from the filter by gentle pipetting, and the fluorescence was read on a spectrofluorimeter.

Nebulization. A Pari-LC nebulizer (PARI-Werk GmbH, Germany) was used to generate the liposome aerosol. The nebulizer was connected to an Andersen cascade impactor. Five milliliters (10 μmol/mL) of each system was placed in the nebulizer. After aerosolization, each stage was rinsed with 2 mL of phosphate buffer and the fractions were analyzed using a spectrofluorimeter. Using a log−probability plot, we calculated the mass median aerodynamic diameter (MMAD) and geometric standard deviation (GSD).

Statistical Analysis. Unpaired Student's t test (two-tailed) was used to test the significance of the difference between two means.

(24) Mossman, T. J. Rapid colourimetric asay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.

(25) Fiegel, J.; Ehrhardt, C.; Schaefer, U. F.; Lehr, C. M.; Hanes, J. Large porous particle impingement on lung epithelial cell monolayers—toward improved particle characterization in the lung. *Pharm. Res.* **2003**, *20* (5), 788−796.

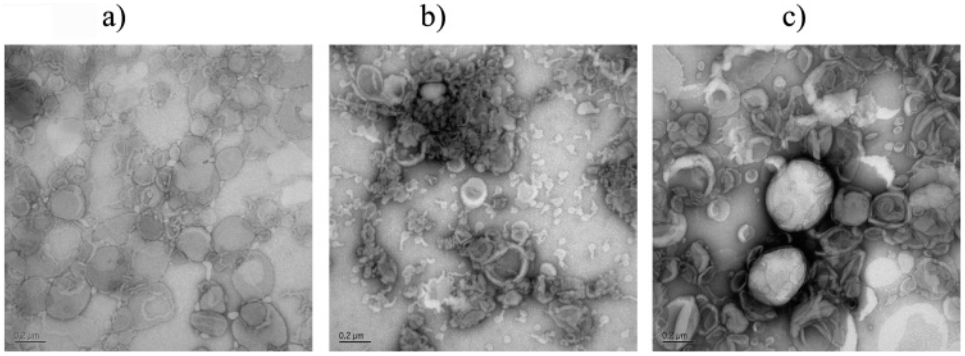

Figure 1. Electron micrographs of PTD-conjugated liposomes: (a) LIP-R8, (b) LIP-ANTP, (c) LIP-TAT.

Results

Conjugation and Characterization of the Peptide-Tagged Liposomes. No liposomes conjugated to R8 have yet been assessed for delivery, so in this study we conjugated R8 to DOPC:Chol liposomes and compared its ability to translocate into airway epithelial cells with HIV-TAT and ANTP conjugated liposomes[14,15,26] and DOTAP liposomes. The purity of each batch of peptide used for conjugation was >90%. The three peptides were conjugated to the liposomes as described. Preformed liposomes that contain MPB-PE react readily with the cysteine residue at the end of each peptide to give a stable thioether bond.

Greater than 70% of the peptide added was attached for each of the peptide systems as determined by protein assays. This indicates that, for each 1 μmol of lipid, 0.04 μmol of peptide is present. The unconjugated liposomes (LIP) alone were 200 nm in diameter, and the mean size increased when peptides were added to 213.4 nm ± 44.3 nm for LIP-R8, 221.0 nm ± 44.3 nm for LIP-TAT, and 275.2 nm ± 110.2 nm for LIP-ANTP. The increase in the mean size upon addition of the peptides is not statistically significant, however, given the polydispersity of the liposomes. The increase in polydispersity upon addition of peptides would be expected particularly given that the peptides are conjugated after size reduction. The electron micrographs indicated that the liposomes retained their structural integrity after conjugation though an increase in liposome size polydispersity can be seen, especially for LIP-TAT and LIP-ANTP (Figure 1).

Effect of Peptides on Liposome−Airway Cell Association and Transport. The effect of time and concentration on liposome−cell interaction was assessed in Calu-3 cells (a well-characterized airway cell culture model) grown in culture wells.[27] The level of cell association of the PTD-conjugated liposomes was significantly ($p < 0.01$) greater than for unmodified liposomes. It should be noted that results detailed in Figures 2, 3, 5, and 6 relate to total cell association, i.e., the level of liposome both bound to and internalized by the Calu-3 cells. The degree of association was time- (Figure 2) and concentration-dependent. The relative increase in association depended on the peptide used with HIV-TAT producing a 12-fold increase, ANTP producing a 17-fold increase, and R8 producing a 12-fold increase

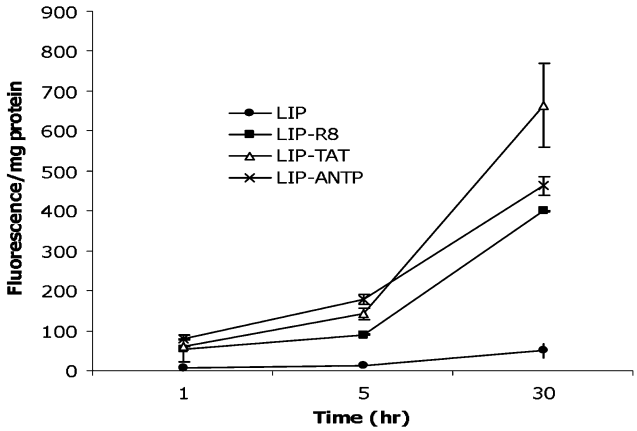

Figure 2. Association of DOPC:Chol (7:3) liposomes (LIP) or PTD-conjugated liposomes octaarginine (LIP-R8), HIV-TAT (LIP-TAT), or Antennapedia (LIP-ANTP) with Calu-3 cells after treatment with 100 μM liposomes for 1, 5, or 30 h at 37 °C ($n = 3$, mean ± SD).

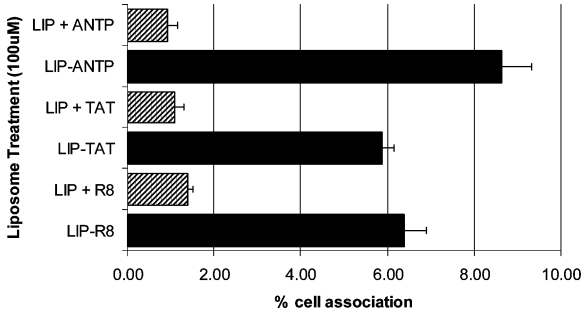

Figure 3. Percentage cell association (of total added) of PTD-conjugated liposomes octaarginine (LIP-R8), HIV-TAT (LIP-TAT), and Antennapedia (LIP-ANTP) liposomes (full columns) and liposomes mixed with the peptides (striped columns) after treatment of Calu-3 cells for 1 h at 37 °C ($n = 3$, mean ± SD).

(26) Marty, C.; Meylan, C.; Schott, H.; Ballmer-Hofer, K.; Schwendener, R. A. Enhanced heparan sulfate proteoglycan-mediated uptake of cell-penetrating peptide-modified liposomes. *Cell. Mol. Life Sci.* **2004**, *61* (14), 1785−1794.

(27) Shen, B. Q.; Finkbeiner, W. E.; Wine, J. J.; Mrsny, R. J.; Widdicombe, J. H. Calu-3: a human airway epithelial cell line that shows cAMP-dependent Cl− secretion. *Am. J. Physiol.* **1994**, *266* (5:1), L493−501.

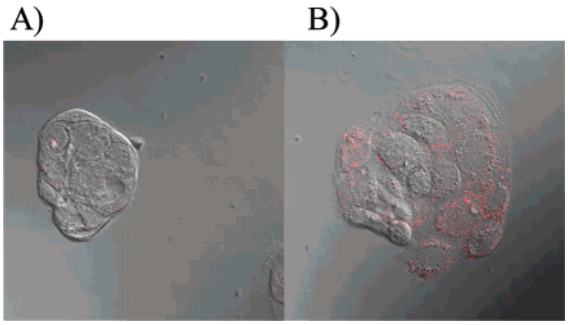

Figure 4. Confocal laser scanning images of the uptake of (A) rhodamine-tagged DOPC:Chol (LIP) and (B) DOPC:Chol: MBP-PE:Arg-8 (LIP-R8) liposomes into Calu-3 cells after treatment with 100 μM liposomes for 2 h.

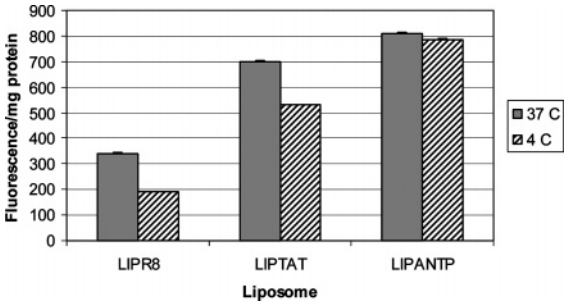

Figure 5. The effect of incubation at 4 °C on the association of LIP-R8, LIP-TAT, and LIP-ANTP with Calu-3 cells. Cells were treated with 100 μM liposomes for 4 h at 37 °C or 4 °C ($n = 3 \pm$ SD).

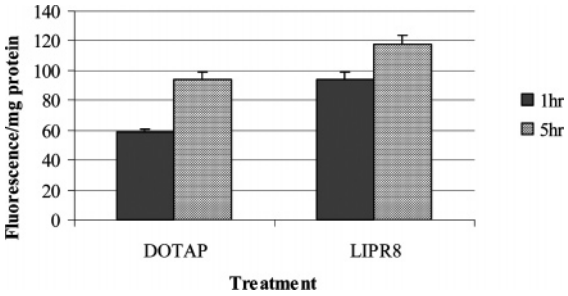

Figure 6. Association of DOPC:Chol:DOTAP (7:3:1) liposomes (DOTAP) and octaarginine tagged liposomes (LIP-R8) with Calu-3 cells after treatment with 100 μM liposomes for 1 or 5 h at 37 °C ($n = 3$, mean $\pm$ SD).

over liposomes alone (0.5% cell association) after 1 h (Figure 3). This improvement continued over time with ANTP producing the most significant increase after long-term exposure (30 h).

In order to determine if this effect was dependent on conjugation, free peptides (HIV-TAT, ANTP, and R8 without Cys amino acids) were mixed with rhodamine-labeled DOPC:Chol liposomes to give the same ratio and concentration of peptide as the conjugated liposomes. Results indicated that arginine-rich peptides in contact with the cells required conjugation to liposomes to carry their cargo efficiently into the cell. The presence of free peptide caused a small significant ($p < 0.05$) increase in liposome cell association (Figure 3) but was far less effective than the conjugated systems. The levels of cell association of each of the PTD-conjugated liposomes were also significantly greater ($p < 0.05$) than that of DOPC:Chol:DOTAP (6:3:1) liposomes.

Calu-3 cells can form tight, polarized and well-differentiated monolayers with apical microvilli when cultured appropriately on filters.[27] Limited studies of the interaction of ANTP with CaCo-2 intestinal epithelial cell monolayers[28] and HIV-TAT with Calu-3 cell monolayers[29] have been carried out. To our knowledge, no studies investigating the transport of PTD-conjugated liposomes across an airway cell monolayer have been carried out to date, so preliminary studies were carried out to determine the degree of transport across the monolayer. After the cells were cultured as described, the Calu-3 cell monolayers were treated with one of the PTD-conjugated liposomes or control, unmodified liposomes ($n = 3$). Initial studies indicate that after 2 h <5% of the peptide-modified liposomes added have crossed the monolayer, giving each an apparent permeability (P_{app}) of approximately 1×10^{-7} cm/s. The percentage fluorescence associated with the monolayer (as a % of that added) was only 3% for untagged LIP with a statistically significant ($p < 0.05$) increase seen for each of the tagged liposomes; 30% for LIP-R8, 43% for LIP-ANTP, and over 50% for LIP-TAT. The integrity of the tight junction barrier of the Calu-3 cell monolayer remained intact. Transepithelial electrical resistance was monitored before and after treatment with the PTD-conjugated liposomes. A small decrease in transepithelial electrical resistance was seen after treatment of the cells with the liposomes.

Toxicity. In order to ascertain if the increase in cell association was related to or concurrent with cell toxicity, we carried out MTT assays. Toxicity studies indicated that there was a significant difference in the level of toxicity between systems. LIP-ANTP proved the most toxic, with a significant decrease in cell viability to 80% even at low concentrations (10 μM) with further decreases to only 60% at 100 μM. LIP-TAT led to a decrease in cell viability to 75% at 100 μM while LIP-R8 remained nontoxic even at 100 μM with almost 100% cell viability. One of the major disadvantages associated with DOTAP liposomes is toxicity. Cell viability after treatment with 100 μM DOTAP liposomes was 50% compared with 100% for LIP-R8, 75% for LIP-TAT, and 60% for LIP-ANTP.

Intracellular Localization and Mechanism of Uptake. The initial cellular assays looked at cell association, and in order to determine whether this was a membrane-binding phenomenon and/or involved cell uptake, we carried out confocal studies. Confocal studies of LIP-R8 uptake into Calu-3 cells (Figure 4) clearly indicated that the modified

(28) Lindgren, M. E.; Hallbrink, M. M.; Elmquist, A. M.; Langel, U. Passage of cell-penetrating peptides across a human epithelial cell layer in vitro. *Biochem. J.* **2004**, *377* (Part 1), 69−76.

(29) Trehin, R.; Krauss, U.; Beck-Sickinger, A. G.; Merkle, H. P.; Nielsen, H. M. Cellular uptake but low permeation of human calcitonin-derived cell penetrating peptides and Tat (47−57) through well-differentiated epithelial models. *Pharm. Res*. **2004**, *21* (7), 1248−1256.

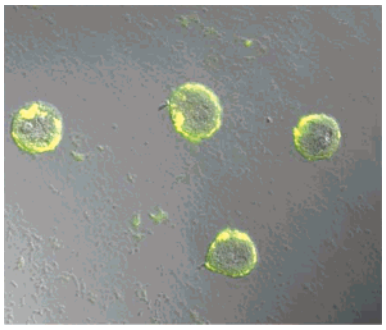

Figure 7. Confocal laser scanning images of the uptake of rhodamine-tagged DOPC:Chol:MBP-PE:Arg-8 liposomes (LIP-R8) (red) containing FITC-dextran (green) into Calu-3 cells after treatment with 100 μM liposomes for 2 h.

liposomes once internalized by the cell localize for the most part in the cytoplasm with only a small amount of nuclear localization. Similar intracellular distribution was seen for each of the modified liposomes.

In these experiments we also examined the effect of temperature on liposome−cell association. Cells were treated with 100 μM liposomes for 4 h. The cell association of the PTD-conjugated liposomes was inhibited when incubation was carried out at 4 °C but to different degrees depending on the peptide. At 4 °C the level of liposome association for LIP-R8, LIP-TAT, and LIP-ANTP was reduced by 44%, 25%, and 2% respectively (Figure 5).

The level of liposome cell association was significantly ($p < 0.05$) decreased by greater than 50% for all the conjugated liposomes in the presence of endocytosis inhibitors. The degree of inhibition also depended on the peptide attached, with a more significant decrease in liposome−cell association seen for LIP-TAT and LIP-ANTP than for LIP-R8.

Association and Uptake of LIP-R8 by Calu-3 Cells. The cell association of LIP-R8 and DOTAP liposomes with Calu-3 cells was compared, and the results are shown in Figure 6. At both time points shown, the cell association of LIP-R8 was significantly greater ($p < 0.05$) than for DOTAP liposomes. In order to determine if LIP-R8 liposomes were capable of delivering a macromolecular cargo to the airway cells, FITC-dextran 9 kDa was encapsulated within the liposomes prior to extrusion and R8 conjugation and was added to Calu-3 cells grown on coverslips. The encapsulation efficiency of the dextran within the liposomes was 87%. Free FITC-dextran showed only minimal intracellular accumulation in Calu-3 cells. The dextran encapsulated in the LIP-R8 could be seen in large quantities colocalized with liposomes (yellow) and free (green) (Figure 7).

Aerosolization. Five milliliters of the conjugated liposomes (10 μmol/mL) was nebulized using a Pari-LC nebulizer into an Andersen cascade impactor (Copley Scientific). The modified liposomes once aerosolized produced a high respirable fraction and good mass median aerodynamic diameters (MMADs) of $\leq$1.5 μm for each of the modified liposomes. The geometric standard deviations (GSDs) of the log-normal distribution of the aerosols were high, indicating a degree of polydispersity in the aerosol particle size generated. Overall the modification of the liposomes with the three peptides had no statistically significant effect on the MMAD compared to unmodified liposomes.

Discussion

The differences in size seen for each of the PTD-conjugated liposomes may be attributable to the different size and spatial conformation of each peptide. To assess the usefulness of these systems for drug delivery to the airways, the nature and efficiency of the interaction between airway cells and the PTD-conjugated liposomes was determined.

Previous studies have shown that HIV-TAT can translocate into polarized airway cells,[30] and we have found in these studies that conjugation of arginine-rich peptides to liposomes can enhance their uptake into airway cells. From our results (Figure 3) it is clear that conjugation of the cargo is required to enhance intracellular delivery of the liposomes, which corroborates previous findings by other investigators.[31] The dramatic increases in modified liposome−cell association with time seen in these experiments resembled those of other studies looking at the effect of HIV-TAT on liposome−cell interactions[15] in other cell types. The kinetic information is important both in elucidating the mechanism of uptake and in determining potential applications of the technology. A general trend in PTD cell uptake kinetics is that at 37 °C the maximal concentration of internalized peptide is achieved after less than 1 h.[32] The delayed uptake maximum seen in Figure 2 may be due to the large size of the cargo (liposomes) impeding the usually rapid internalization of the peptides. The rate of uptake of LIP-TAT and LIP-ANTP resembles that seen in previous studies[14,15,33] in which liposome uptake was also found to be time- and concentration-dependent.[26] Marty et al. found that the uptake of HIV-TAT and ANTP modified liposomes into a panel of cell types was 15−25-fold greater in comparison to that of unmodified liposomes with LIP-ANTP producing the greatest increase,[26] a finding that is confirmed by our studies in airway cells.

While the effect of HIV-TAT and ANTP appeared to be superior to that of R8, it was felt that a comparison of their relative toxicities was necessary before deciding on the usefulness of the systems in vivo. PTDs are generally regarded as inert with few side effects.[32] Toxicity and undesirable effects have not generally been detected in most in vivo applications of PTDs to date.[34−36] LIP-R8 was the

(30) Mi, Z.; Mai, J.; Lu, X.; Robbins, P. D. Characterization of a class of cationic peptides able to facilitate efficient protein transduction in vitro and in vivo. *Mol. Ther*. **2000**, *2* (4), 339−347.

(31) Morris, M. C.; Depollier, J.; Mery, J.; Heitz, F.; Divita, G. A. peptide carrier for the delivery of biologically active proteins into mammalian cells. *Nat. Biotechnol.* **2001**, *19* (12), 1173−1176.

(32) Zorko, M.; Langel, U. Cell-penetrating peptides: mechanism and kinetics of cargo delivery. *Adv. Drug Delivery Rev*. **2005**, *57* (4), 529−545.

(33) Torchilin, V. P.; Levchenko, T. S. TAT-liposomes: a novel intracellular drug carrier. *Curr. Protein Pept. Sci*. **2003**, *4* (2), 133−140.

least toxic of the three PTD-conjugated liposomes and may therefore offer the best alternative to cationic lipids, such as DOTAP in vivo. From our studies it would appear that LIP-R8 enhances cell association (Figure 6) to a greater extent than the cationic lipid DOTAP with decreased cellular toxicity. Despite the greater positive charge conferred on the LIP-R8 liposomes (8 positively charged groups associated with each charged lipid) than the DOTAP liposomes (1 positively charged group associated with each charged lipid), LIP-R8 appears biocompatible at the concentrations required for intracellular delivery. The more rapid uptake seen for LIP-R8 compared to DOTAP (Figure 5) would also decrease the likelihood of premature drug release prior to cell membrane permeation, e.g., due to destabilization.

The system with the greatest impact on cell viability was LIP-ANTP. A study looking at the effect of the HIV-TAT and ANTP peptides alone on Calu-3 cell viability similarly found that ANTP was more toxic than HIV-TAT.[29] The difference in toxicity may be related to the different mechanisms of internalization with each peptide interacting and/or disrupting the cell membrane to a lesser or greater extent. The high positive charge of most PTDs leads to strong interaction with polyanionic cell membranes; for example, ANTP has been found to interact with heparin, polysialic acid, and nucleic acids and in one study was found to cause cell death after intrastriatal injection,[37] and some apoptotic effects have also been seen with Tat-derived PTDs.[38] An in vivo study using TATp-liposomes to deliver DNA found that the modified liposomes were capable of efficient delivery with lower cytotoxicity that the commonly used cationic lipids.[39]

In vivo, after delivery via inhalation, these carriers will not encounter isolated airway cells but a polarized epithelial cell layer. The results of the transport studies would indicate that these novel PTD-conjugated liposome carriers are most suitable for local delivery of drugs to the airway epithelium. Once the liposomes have been delivered to the cell cytoplasm, they tend to remain there rather than being further translocated across the basolateral membrane into the receiver chamber. The permeability coefficient for HIV-TAT alone across a confluent Calu-3 cell monolayer was found to be approximately 7×10^{-8} cm/s,[29] which closely matches the P_{app} for LIP-TAT (6×10^{-8} cm/s) across the Calu-3 monolayer found in these studies. The transport of ANTP alone (i.e., with no cargo) was studied in CaCo-2 cell monolayers,[28] and it was found that only 0.6% of the peptide added was transported across the monolayer after 120 min. The reason for the increased transport of Antennapedia (4%) seen in this study may be attributed in part to the more permeable ("leakier") nature of Calu-3 cell monolayers compared to CaCo-2 cell monolayers. The slight decrease in transepithelial electrical resistance (TEER) values seen after 2 h of treatment did not appear to significantly enhance transport of the liposomes across the cell monolayer. The high levels of PTD-conjugated liposomes associated with the polarized monolayer is a promising result. Many viral and nonviral carriers demonstrate decreased binding to the apical side of polarized cells compared to nonconfluent cells due to changes in the structure of the apical membrane (e.g., decreased endocytosis) and in the density of receptors (e.g., integrin receptors) present, but the ability of the peptide-modified liposomes to bind to and be internalized by airway cells did not appear to be significantly affected by polarization and differentiation.

While these systems appear to be easily aerosolized and have a significant effect on delivery in cell culture, their ability to improve intracellular delivery in vivo will be influenced by a number of factors including the effect of lung surfactant on the stability of the systems and their rate of clearance. The fate of liposomes in the lungs has been studied, and their residence time within the lung was found to be dependent on the degree of ventilation and their composition.[40−42] The high GSD values would indicate that the liposome aerosols produced by the Pari-LC nebulizer are polydisperse, which could limit pulmonary deposition. For further in vivo studies alternative devices would need to be investigated.

A great deal of debate still continues about the exact mechanism of transduction of PTDs, as well as the intracellular fate of their cargoes. A diversity of mechanisms has

(34) Schwarze, S. R.; Ho, A.; Vocero-Akbani, A.; Dowdy, S. F. In vivo protein transduction: delivery of a biologically active protein into the mouse. *Science* **1999**, *285* (5433), 1569−1572.

(35) Pooga, M.; Soomets, U.; Hallbrink, M.; Valkna, A.; Saar, K.; Rezaei, K.; Kahl, U.; Hao, J. X.; Xu, X. J.; Wiesenfeld-Hallin, Z.; Hokfelt, T.; Bartfai, T.; Langel, U. Cell penetrating PNA constructs regulate galanin receptor levels and modify pain transmission in vivo. *Nat. Biotechnol.* **1998**, *16* (9), 857−861.

(36) Rothbard, J. B.; Garlington, S.; Lin, Q.; Kirschberg, T.; Kreider, E.; McGrane, P. L.; Wender, P. A.; Khavari, P. A. Conjugation of arginine oligomers to cyclosporin A facilitates topical delivery and inhibition of inflammation. *Nat. Med.* **2000**, *6* (11), 1253−1257.

(37) Bolton, S.; Jones, D.; Darker, J.; Eggleston, D.; Hunter, F.; Walsh, F. Cellular uptake and spread of the cell-permeable peptide penetratin in adult rat brain. *Eur. J. Neurosci.* **2000**, *12*, 2847−2855.

(38) Jia, H.; Lohr, M.; Jezequel, S.; Davis, D.; Shaikh, S.; Selwood, D.; Zachary, I. Cysteine-rich and basic domain HIV-1 Tat peptides inhibit angiogenesis and induce endothelial cell apoptosis. *Biochem. Biophys. Res. Commun.* **2001**, *283* (2), 469−479.

(39) Torchilin, V. P.; Levchenko, T. S.; Rammohan, R.; Volodina, N.; Papahadjopoulos-Sternberg, B.; D'Souza, G. G. Cell transfection in vitro and in vivo with nontoxic TAT peptide-liposome-DNA complexes. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100* (4), 1972−1977.

(40) Morimoto, Y.; Adachi, Y. Pulmonary uptake of liposomal phosphatidylcholine upon intratracheal administration to rats. *Chem. Pharm. Bull. (Tokyo)* **1982**, *30* (6), 2248−2251.

(41) Oyarzun, M. J.; Clements, J. A.; Baritussio, A. Ventilation enhances pulmonary alveolar clearance of radioactive dipalmitoyl phosphatidylcholine in liposomes. *Am. Rev. Respir. Dis.* **1980**, *121* (4), 709−721.

(42) Papahadjopoulos, D.; Poste, G.; Schaeffer, B. E. Fusion of mammalian cells by unilamellar lipid vesicles: inflluence of lipid surface charge, fluidity and cholesterol. *Biochim. Biophys. Acta* **1973**, *323* (1), 23−42.

been proposed for PTDs, and it has been proposed that the mechanism is also dependent on the attached cargo and the cell type.[43] This emphasizes the need to study the effect of these peptides on delivery to specific cell types depending on the route of administration and cargo proposed. Initial studies conducted on these cell permeable peptides indicated a high rate of nuclear localization, but it has recently been suggested that some of these effects on cell localization may have been artifacts of fixation.[44] Our studies indicate that PTD-conjugated liposomes are internalized into vesicular structures within the cytoplasm but have limited access to the nucleus. Studies have shown that TATp-liposomes remained intact inside the cell cytoplasm within 1 h of translocation[39] as seen for LIP-R8 in Figure 4. This is an important result when possible applications of these systems are considered. It is interesting to note that several studies have indicated that the addition of HIV-TAT and Antennapedia to a transfection system can enhance transfection using plasmid DNA.[33,45] Given that the plasmid DNA requires access to the nucleus, this would appear to contradict our findings. On the other hand the enhanced cytoplasmic delivery and trafficking evidenced in Figure 4 may be sufficient for transfection enhancement.

Inhibition of uptake at 4 °C together with the vesicular pattern seen in the confocal images would indicate that the PTDs are triggering an active uptake into the airway cells, though the specific mechanism would appear to be peptide-dependent and cargo-dependent. This corroborates data which indicated that TAT-mediated intracellular delivery of large molecules and nanoparticles proceeds via an energy-dependent macropinocytosis with subsequent enhanced escape from the cell cytoplasm.[46,47] There are several proposed mechanisms for translocation of the different arginine-rich peptides including endocytosis,[46] the induction of inverted micelles,[48,49] and the involvement of hydrogen bonding and membrane potential in the translocation process. More recent data assumes more than one mechanism of intracellular delivery depending on the cargo.[50]

In conclusion, liposomes modified with PTDs, HIV-TAT, ANTP, and R8 were bound to and internalized by airway epithelial cells in culture to an even greater extent than cationic lipids, such as DOTAP. These carriers are capable of delivering macromolecules, e.g., dextrans, rapidly and efficiently to the cytoplasm of airway cells. While all three systems have a high affinity for airway cells, LIP-R8 is the least toxic and may therefore be the most useful carrier in vivo. These systems offer an innovative method for improving the cell binding and uptake of liposomes on the airway surface and intracellular delivery of therapeutic molecules including macromolecules to airway cell cytoplasm after local delivery.

Acknowledgment. We wish to acknowledge funding from the Programme for Research in Third Level Institutes (PRTLI) administered by Higher Education Authority of Ireland, Science Foundation Ireland (SC/2004/B0419, S.-A.C.), Enterprise Ireland, and the Fulbright Commission of Ireland.

MP050070I

(43) Prochiantz, A. Protein transduction: from physiology to technology and vice versa. *Adv. Drug Delivery Rev.* **2005**, *57* (4), 491−493.

(44) Richard, J. P.; Melikov, K.; Vives, E.; Ramos, C.; Verbeure, B.; Gait, M. J.; Chernomordik, L. V.; Lebleu, B. Cell-penetrating peptides. A reevaluation of the mechanism of cellular uptake. *J. Biol. Chem.* **2003**, *278* (1), 585−590.

(45) Ou, J.; Geiger, T.; Ou, Z.; Ackerman, A. W.; Oldham, K. T.; Pritchard, K. A., Jr. AP-4F, antennapedia peptide linked to an amphipathic alpha helical peptide, increases the efficiency of Lipofectamine-mediated gene transfection in endothelial cells. *Biochem. Biophys. Res. Commun.* **2003**, *305* (3), 605−610.

(46) Snyder, E. L.; Dowdy, S F. Cell penetrating peptides in drug delivery. *Pharm. Res.* **2004**, *21* (3), 389−393.

(47) Wadia, J. S.; Stan, R. V.; Dowdy, S. F. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. *Nat. Med.* **2004**, *10* (3), 310−315.

(48) Berlose, J. P.; Convert, O.; Derossi, D.; Brunissen, A.; Chassaing, G. Conformational and associative behaviours of the third helix of antennapedia homeodomain in membrane-mimetic environments. *Eur. J. Biochem.* **1996**, *242* (2), 372−386.

(49) Derossi, D.; Calvet, S.; Trembleau, A.; Brunissen, A.; Chassaing, G.; Prochiantz, A. Cell internalization of the third helix of the Antennapedia homeodomain is receptor-independent. *J. Biol. Chem.* **1996**, *271* (30), 18188−18193.

(50) Gupta, B.; Levchenko, T. S.; Torchilin, V. P. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. *Adv. Drug Delivery Rev.* **2005**, *57* (4), 637−651.