In Vitro Aerosol Delivery and Regional Airway Surface Liquid Concentration of a Liposomal Cationic Peptide

CARLOS F. LANGE,[1] ROBERT E. W. HANCOCK,[2] JOHN SAMUEL,[3] WARREN H. FINLAY[1]

[1]Aerosol Research Laboratory of Alberta, Department of Mechanical Engineering, University of Alberta, Edmonton, Alberta, Canada, T6G 2G8

[2]Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada, V6T 1Z3

[3]Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, Canada, T6G 2N8

Received 20 July 2000; revised 7 March 2001; accepted 25 May 2001

ABSTRACT: A liposome encapsulation was optimized for the entrapment and aerosol delivery of an α-helical cationic peptide, CM3, which had shown good antimicrobial and antiendotoxin activity *in vitro*. The encapsulation procedure and the phospholipids used were selected to maximize both the encapsulation and nebulization efficiencies, without compromising liposomal integrity during nebulization. The best compromise was found with dimyristoyl phosphatidylcholine and dimyristoyl phosphatidylglycerol (3:1 molar ratio), which allowed for peptide encapsulation levels of 730 μg/mL using 30 mM lipid concentration. The aerosol produced with the selected liposomal formulation was subsequently analyzed for determination of size distribution and nebulizer efficiencies. These quantities were used as input for a mathematical lung deposition model, which predicted local lung depositions of the liposomal peptides for three models of lung geometry and breathing patterns: an adult, an 8-year-old child, and a 4-year-old child. The deposition results were then applied to a novel model of airway surface liquid in the lung to assess the concentration of the deposited peptide. The resulting concentration estimates indicate that the minimum inhibitory levels of CM3 can be reached over most part of the tracheobronchial region in the adult model, and can be exceeded throughout the same region in both pediatric model subjects, using a valved jet nebulizer with a 2.5mL volume fill. J Pharm Sci 90:1647–1657, 2001

Keywords: cationic peptide; liposome encapsulation; nebulization efficiency; lung deposition simulation

INTRODUCTION

Cationic peptides are being developed as a new class of antibiotics.[1] They are highly effective against bacteria, such as *Pseudomonas aeruginosa*, which commonly infect cystic fibrosis (CF) patients. The antimicrobial action of these peptides is proposed to involve "self-promoted uptake" across the outer membrane of Gram-negative bacteria followed by interaction with and crossing of the cytoplasmic membrane to attack cytoplasmic targets.[2] The initial interaction of the peptides with bacteria, involving self-promoted uptake, dictates two additional features of cationic peptides. First, these peptides have antiendotoxin activity, because they bind to lipopolysaccharide (LPS), a by-product of the bacteria killing that stimulates tumor necrosis factor production and death from endotoxaemia. Indeed, their ability to neutralize endotoxin has been demonstrated both *in vitro* and *in vivo* in the galactosamine-sensitized mouse model[3,4] (in contrast, other

Correspondence to: W.H. Finlay (Telephone: 780-492-4707; Fax: 780-492-2200; E-mail: warren.finlay@ualberta.ca)

Journal of Pharmaceutical Sciences, Vol. 90, 1647–1657 (2001)

antibiotics promote the release of LPS, inducing endotoxaemia[5]). Secondly, some cationic peptides show synergy with conventional antibiotics against antibiotic-resistant strains of bacteria, acting as enhancers of those antibiotics. Scott et al.[3] compared *in vitro* levels of the three abovementioned features in several cationic peptides. Among the peptides they tested, CM3 showed good antimicrobial activity against Gram-negative bacteria, one of the highest LPS binding activities, and marginal enhancer activity. In addition, a preliminary study of the relative efficacy of diverse α-helical peptides in animal models demonstrated that CM3 was the best peptide when delivered by aerosol to the lungs of rats chronically infected with *P. aeruginosa* or by injection to the peritoneum of *Pseudomonas* infected neutropenic mice.[6] Hence, CM3 was chosen for the present investigation.

Liposome encapsulation of CM3 was developed to allow modification of the pharmacokinetics after delivery to the lung due to the issues of toxicity and stability that remain of concern for these peptides.[7] Simultaneous reduction of systemic toxicity and enhancement of drug efficacy with liposome encapsulation has been shown, for instance, by Parthasarathy et al.[8] with all-*trans*-retinoic acid. One of the major considerations in using antimicrobial peptides therapeutically is their potential to be toxic when applied systemically.[7] Unfortunately, the basis for toxicity is unknown, because to date there have been no published studies of toxicity. Some studies have noted that antimicrobial peptides are significantly hemolytic, although this is not an issue for the α-helical peptide CM3 studied here. Alternatively, we have observed that certain peptides tend to precipitate rapidly in the blood of mice at high concentrations (>40 mg/kg).[9] The only evidence of toxicity for CM3 is the observation that single injections of CM3 and related peptides give greater protection against peritoneal infections of *P. aeruginosa* than do double injections.[9] Another consideration is the lability of natural peptides to proteases, which are abundant in the host.[7] Therefore, liposome encapsulation with a final concentration of approximately 1 mg/mL is being considered, in the expectation that it will reduce CM3 toxicity and reduce proteolytic degradation in the host, while demonstrating at least a similar level of efficacy as the aqueous solution.

Nebulization of liposome encapsulated drugs has been studied for many years. Among the various aspects investigated were the stability of liposomes during nebulization,[10,11] influence of the vesicle size,[12,13] nebulizer operating conditions,[14] and lipidic concentration.[15] Several studies pointed to the damage that the liposomal vesicles might suffer during nebulization because of the high shear forces that occur in the nebulizers.[10,13] Another possible cause of vesicle disruption are the discontinuities in fluid properties that occur during droplet impact on baffles.[16] The breakup of vesicles causes them to leak the entrapped drug back into the supernatant. Waldrep and coworkers compared the liposomal aerosols generated with different phospholipids[17] and by various nebulizers,[18] estimating numerically the amount deposited in each lung region. Farr et al.[19] and more recently Vidgren et al.[20] studied *in vivo* regional deposition and clearance of nebulized liposomes labeled with 99m-technetium on healthy volunteers. Their studies demonstrated that deposition is primarily determined by the aerosol characteristics, and not by the type or size of liposome vesicles.

The main objective of the present work was to develop and select a liposome preparation for CM3 that optimized encapsulation and nebulization, minimizing leakage due to nebulization, to be subsequently used for animal testing. Another aim of the present work was to estimate the initial peptide concentration in the airway surface liquid (ASL) of human lungs[21] immediately after aerosolization, to determine if efficacious levels of the drug can be expected. Because of the topical action envisioned for the CM3 peptide in the lung, an estimate of the local concentration of the antibiotic seems more relevant than the estimate of total regional dosages, obtained in previous studies.[17,18,22] This concentration estimate required the combination of the measured aerosol characteristics with a mathematical lung deposition model and a new model for the ASL distribution in the human lung. The only other work known to the authors that used regional deposition results to numerically estimate drug concentrations in the lung, was a study on mucous concentration of nebulized dextran.[23] The present ASL model builds upon the foundation established in that study.

MATERIALS AND METHODS

Peptide Preparation

The α-helical cationic peptide CM3 is derived from a hybrid of silk moth cecropin and bee melittin

peptides, containing the first eight amino acids of cecropin followed by the first 18 amino acids of melittin with small amino acid changes. It was synthesized by Fmoc (9-fluorenylmethoxycarbonyl) chemistry at the Nucleic Acid/Protein Service Laboratory, University of British Columbia, Vancouver, BC, Canada. The amino acid sequence of CM3 is KWKKFIKSLTKSAAKTVVKTAKKPLIV, as described in Scott et al.[3]

Liposome Preparation and Vesicle Sizes

Six liposome preparations were compared regarding their encapsulation efficiency, nebulization efficiency, and leakage during nebulization. Three different phospholipids [dilauroyl phosphatidylcholine (DLPC), dimyristoyl phosphatidylcholine (DMPC), and dipalmitoyl phosphatidylcholine (DPPC)] were used in this comparison. The liposomes consisted of combinations of each of these phospholipids with dimyristoyl phosphatidylglycerol sodium salt (DMPG-Na). One set of preparations included cholesterol (Chol) (sigma grade: 99+ %), frequently used to reduce encapsulated drug leakage during storage,[24] in the form phosphatidylcholine:Chol:DMPG-Na = 3:1:2 (molar ratio), lipid concentrations 30 and 60 mM. The other set consisted of the same lipidic formulations, but without cholesterol (as in Knight et al.[25] and Parthasarathy et al.[8]), in a molar ratio of phosphatidylcholine:DMPG-Na = 3:1 and a concentration of lipids of 30 mM. Thus, the six preparations tested were: (1) DLPC:Chol:DMPG-Na = 3:1:2 molar ratio; (2) DMPC:Chol:DMPG-Na = 3:1:2 molar ratio; (3) DPPC:Chol:DMPG-Na = 3:1:2 molar ratio; (4) DLPC:DMPG-Na = 3:1 molar ratio; (5) DMPC:DMPG-Na = 3:1 molar ratio; and (6) DPPC:DMPG-Na = 3:1 molar ratio.

For simplicity, the suffix Na will be omitted in subsequent notations.

The lipids were first dissolved in chloroform HPLC grade, then placed on a rotary evaporator at 40°C for chloroform evaporation. The resulting lipid film was flushed with nitrogen and left in a vacuum oven overnight to remove any residual solvent. The desiccated lipid film was treated with phosphate buffered saline (PBS) containing the CM3 peptide (pH = 7.4). This method of hydration is known to generate liposomal multilamellar vesicles (MLVs).[26,27] The procedure was completed by the freeze–thaw method (freezing in dry ice/acetone bath, thawing in water bath at 45°C, followed by 1-min vortexing). A total of five freeze–thaw cycles were performed, shown by Mayer et al.[26] to ensure maximum trapping efficiency with MLVs. The hydrated liposomes were separated from the unencapsulated peptide by centrifuging two times at 4°C and 150,000 × *g* for 20–30 min. Finally, the remaining pellets were resuspended in PBS to a final lipid concentration of 30 mM.

The preparations were stored at 4°C and used within 14 days. They were tested for leakage before every nebulization by analysis of the presence of CM3 in the isolated supernatant (20–30 min centrifugation, 4°C, 150,000 × *g*), but no leakage occurred during storage.

The sizes of the selected liposomal MLVs were measured with a Zetasizer (Malvern Instruments Ltd., Malvern, UK).

Nebulization and Total Output

Nebulization of the liposomal preparations was done with the valved jet nebulizer Pari LC STAR (Pari, Starnberg, Germany), which has shown superior performance in previous studies.[22,23] For determination of the total output of encapsulated peptides, the nebulizer was connected directly to an Andersen cascade impactor (Andersen Mark II, Graseby Andersen, Smyrna, GA) with constant flow rate of 28.3 L/min. Nebulization took in most cases approximately 10 min, and was terminated when no aerosol was produced. Because this commonly used setup was recently shown to underestimate the particles' size distribution (cf. Ref. 28), no distinction between the impactor plates was made in this case, i.e., only the total deposition in the impactor was considered. For this measurement the impactor was used in lieu of an absolute filter.

The aerosol collected in the impactor was washed from the plates with 4–5 mL PBS, as was the residue from the nebulizers. Samples from each of the washings (2 mL) were centrifuged at 4°C and 150,000 × *g* for 30 min. After dilution with methanol, the CM3 content was assayed separately in pellets and supernatants with UV spectrophotometry ($\lambda = 280$ nm), so that the fraction of encapsulated peptide could be determined. To distinguish leakage owing to nebulization from leakage owing to simple dilution, a sample of the original unnebulized liposomal preparation was treated with PBS (4–5 mL) and submitted to the same work-up as cascade impactor plates and nebulizer washings. This sample preparation was used as standard for comparison of leakage with the washings following nebulization.

Droplets' Size Distribution and Liposome Distribution in the Droplets

To determine the aerosol size distribution, the nebulizer was run intermittently. In this way the above-mentioned underestimation of particle sizes was avoided. Five cycles of 10 min were performed, consisting of 30 s of nebulization and 9 min 30 s of pause. The short nebulization time reduced the cooling effect that compromises the measurement,[28] and the long pauses allowed for the temperature of the nebulizer fill to return to equilibrium with the ambient.

Two size distribution measurements had to be distinguished. Because of the colloidal nature of the liposome vesicles, it could not be assumed that the liposomes were evenly distributed in the droplets.[29] If the liposomes were, for instance, preferentially concentrated in larger droplets, an assay of CM3 content in the washings of the impactor plates would misrepresent the size distribution, overestimating the droplet sizes. On the other hand, besides the actual aerodynamic droplet diameters, it is important to know how the drug is distributed in the droplets to correctly estimate local deposited dosages. These two distributions were determined by two separate cascade impactor measurements.

To determine the actual droplet size distribution, methylene blue (30 μL, conc. = 2180 mg/mL, certified reagent, dye content 82%) was added to the liposomal preparation immediately before nebulization, to serve as a tracer for the aqueous phase. The mixture (2.5 mL) was then intermittently nebulized as described above, and the impactor plates were assayed for methylene blue by UV spectrophotometry. In an additional experiment, it was verified that, despite the cationic nature of methylene blue, the dye was not trapped by preformed liposomes, which are negatively charged, thus, validating its use as tracer for the aqueous phase.

To determine the distribution of liposomal peptide in the droplets, a separate set of cascade impactor measurements was performed, and this time the impactor plates were assayed for CM3 in the same way as in the above-described measurement of total output.

Calculation of Efficiencies and Losses

The parameters used for the comparison and selection of the liposomal preparations can be explained in terms of efficiencies and losses. The percentage of the initial amount of CM3 peptide (M_{initial}) that was successfully encapsulated (M_{encaps}) is called encapsulation efficiency

$$E_{\text{encaps}} = \frac{100\, M_{\text{encaps}}}{M_{\text{initial}}} \tag{1}$$

M_{encaps} was obtained with the same procedure as in the above-described assay of impactor plates washings.

After separation by centrifugation, encapsulated CM3 was submitted to nebulization. Because of retention in the walls of the nebulizer, only a fraction of the nebulizer fill ($M_{\text{neb. fill}}$) gets actually aerosolized ($M_{\text{total output}}$), resulting in the nebulization efficiency

$$E_{\text{neb}} = \frac{100\, M_{\text{total output}}}{M_{\text{neb. fill}}} \tag{2}$$

The high shear forces experienced by the liposomal suspension[10,11,30] and the effect of the droplet impaction on the baffles[16] during the nebulization process may cause rupture of the vesicles and leakage of the encapsulated drug. The amount of drug that remains encapsulated after nebulization as a percentage of the total output of the nebulizer defines the stability efficiency

$$E_{\text{stabil}} = \frac{100\, M_{\text{encaps}}}{M_{\text{total output}}} \tag{3}$$

The stability efficiency cannot be determined directly. Dilution of the liposomal suspension during the washing of impactor plates causes additional leakage due to osmotic pressure.[30] To compensate for this procedural effect, a standard unnebulized liposomal preparation was diluted by the same amount as the impactor washings. The percentage of released peptide during simple dilution (M_{free} in the standard) was then added to the measured percentage of encapsulated peptide after nebulization (M_{encaps} in the impactor washings), resulting in the stability efficiency

$$E_{\text{stabil}} = \left|\frac{100\, M_{\text{encaps}}}{(M_{\text{encaps}} + M_{\text{free}})}\right|_{\text{impactor washings}} + \left|\frac{100\, M_{\text{free}}}{(M_{\text{encaps}} + M_{\text{free}})}\right|_{\text{standard}} \tag{4}$$

Finally, the actual amount of aerosol inhaled depended also on the inhalation efficiency, defined

as the percentage of the total output of the nebulizer that is actually inhaled

$$E_{\text{inhal}} = \frac{100\, M_{\text{inhaled}}}{M_{\text{total output}}} \quad (5)$$

The losses in this case are caused by the discharge of particles during exhalation. Vented jet nebulizers, such as those used in the present study, tend to diminish these losses by reducing the aerosol production during exhalation. As a result, the inhalation efficiency varied with the breathing pattern, and was determined in a separate set of experiments that involved the connection of the nebulizer to a breath simulator, consisting of an in-house computer-controlled piston. Three different breathing patterns were tested: an adult pattern, based on literature data on breathing patterns of CF patients,[31–33] and two pediatric patterns, corresponding to normal children 8 and 4 years old, using data by Hofmann et al.[34] The corresponding values for tidal volume, breathing frequency and duty cycle are summarized in Table 1. All breathing patterns were approximated by asymmetric sine wave functions (inhalation:exhalation = 1:1.3) without breath hold. The measured inhalation efficiency determined the actual amount of drug inhaled by each model subject and was used as input for the numerical prediction.

Numerical Prediction

To predict the initial peptide concentration in the airway surface liquid (ASL), the amount of peptide deposited in each lung generation must be estimated first. For the local deposition of the inhaled droplets in the lung, a numerical lung deposition model was employed, as described in Finlay et al.[23] This deposition model has been shown to compare well with *in vivo* gamma scintigraphic measurements on normal subjects[35,36] and will be briefly described here for completeness. In this model, the aerosol particles were followed as they traveled through the airways, in what is termed a one-dimensional Lagrangian approach, or compartmental method. The lung of a healthy adult was represented by a symmetrically branching model derived from the data given in Phillips et al.[37] for the conducting airways (generations 0–14), and Haefeli–Bleuer and Weibel[38] for the alveolar region (generations 15–23). This lung model was scaled to give also pediatric lung models for healthy children of ages 4 and 8 years old using the procedures of Phalen et al.[39] (tracheobronchial region) and Hofmann et al.[34] (alveolar region). More details about the three lung models can be found in Finlay et al.[23] Amounts of aerosol depositing in each generation of these lungs were estimated by the model using the equations of Chan and Lippmann[40] for inertial impaction, Pich[41] and Heyder and Gebhart[42] for sedimentation and Gormley and Kennedy[43] for diffusion. Deposition in the mouth–throat was estimated using the equations of Rudolf et al.[44] The breathing pattern for each model subject consisted of asymmetric square waves defined by the data in Table 1. Although the model was capable of computing two-way coupled hygroscopic effects, these were not considered because of the high aerosol mass fraction produced by the jet nebulizer. The negligibility of hygroscopic effects in the present case was indicated by the high value of the parameter γ proposed by Finlay[45] to estimate the importance of such hygroscopic effects, and was confirmed by the small dosage differences ($< 3\%$) obtained with simulations that included two-way coupled hygroscopicity.

In addition to the calculated local dosages of liposomal peptide, the local volume of ASL in the three lung models required estimation to predict ASL drug concentrations. A new model of the generational distribution of ASL in the tracheobronchial region was developed for this purpose. The new model distinguishes the mucous and the periciliary liquid (PCL) layers that characterize the ASL in the upper airways,[21] treating them separately as two concentric continuous annular layers. The PCL layer is formed by a watery liquid that facilitates the beating of the cilia and keeps the thicker mucous layer afloat at an exact distance to be reached by the tip of the cilia during their forward beating.[21] Although the regulation mechanism of the PCL layer thickness in the airways is still the subject of controversy,[46]

Table 1. Description of Breathing Patterns for Each Model Subject

Model Subject	Tidal Volume (L)	Frequency (Breaths/min)	Duty Cycle
Adult (CF)	0.620	18	0.435
8-year-old (normal)	0.343	20.4	0.435
4-year-old (normal)	0.231	23.3	0.435

it is recognized that this regulated thickness is well approximated by the length of the cilia. Measurements of cilia lengths in humans by Serafini & Michaelson[47] were interpolated with an exponential function and used in all tracheobronchial generations as the average thickness of the PCL layer in the generation. The volume of PCL in each generation was calculated using the layer thickness and the morphometric dimensions from the respective lung model.

The mucous layer is essentially a gel formed by secretions from goblet cells located in the airway epithelium, and also from submucosal glands in the larger airways.[21] The thickness of the mucous layer was estimated using mass conservation and models of average mucous velocity and production rate for each generation. A reference set of mucous velocities was determined by the combination of the present lung deposition model and the *in vivo* clearance data from Stahlhofen et al.[48] The mucociliary clearance of the tracheobronchial region was treated as a series of "escalators," and the mucous velocities, assumed constant inside each generation, were estimated to match the clearance rates of those *in vivo* data, as explained in detail by Finlay et al.[23] This procedure generated a series of velocities that had a maximum at the trachea, progressively slowing down to approximately one-thousandth the tracheal velocity at the most distal tracheobronchial generation. The resulting set of velocities was scaled with prescribed values of tracheal mucous velocity to cover a meaningful range, as described below. For the distribution of the mucous production along the conducting airways there are no human data currently available.[49] As an approximation for the distribution of mucous secretion in humans, the airway surface density of total secretory material measured by Plopper et al.[50] in various lung generations of the rhesus monkey was adopted. The values were linearly interpolated and multiplied by the generational airway surface of the respective lung model, resulting in a reference cumulative production rate of mucus, starting from the terminal bronchiole and reaching the total at the trachea. Again, this reference production distribution was scaled according to prescribed tracheal values.

Because of the variability of possible mucous velocities and production rates, two combinations for each model subject were chosen, so as to result in a lower and an upper bound for the local peptide concentration in the ASL. The upper bound for peptide concentrations corresponds to low production rates and high tracheal velocities. Because Yates et al.[51] did not find mucous tracheal velocities in patients with cystic fibrosis that were higher than normal, a maximum of 15 mm/min was combined with arbitrarily low daily production rates (5 mL/day) to estimate maximum peptide concentrations in the ASL. On the other hand, several studies point to increased daily mucous productions in CF patients, for example, Oberwaldner et al.[52] observed an average of 37 mL/day and later Oberwaldner et al.[53] reported mean production values between 31 and 37 g after special physiotherapeutic treatment. Hence, a production rate of 40 mL/day together with relatively low tracheal mucous velocities (5 mm/min for the adult and 10 mm/min for the pediatric models) were chosen for the prediction of minimum concentration levels of liposomal peptide in the ASL. It is worth mentioning that this combination of high production rate and low velocity of the mucus represented airway lumens that were nearly clogged, a situation that may not be uncommon with CF patients.

Finally, the concentrations in the total ASL layer were calculated assuming uniform deposition of the liposomal peptide in each generation and homogeneous dispersion in the ASL volume (sum of PCL and mucous layers) diluted by the water content of the deposited droplets. The estimates of liposomal peptide concentrations are representative of the initial state immediately following nebulization and before significant mucociliary transport takes place.

Statistical tests were performed using ANOVA and Tukey HSD means comparisons. Results are presented as mean $\pm$ SD.

RESULTS

For comparison of the liposomal preparations, encapsulation, nebulization, and stability efficiencies, described in the previous section, were considered. All liposome formulations containing cholesterol showed low encapsulation efficiencies. High lipid concentrations (60 mM) were needed to achieve encapsulation efficiencies of 35–55% when including cholesterol. Higher lipid concentrations result in higher viscosity of the preparation and lower nebulization efficiency, as shown by Bridges and Taylor.[15] The liposomal preparations with cholesterol also showed a tendency to agglomerate during nebulization. This effect combined with the higher viscosity contributed

to reduce the nebulization efficiencies to approximately 20%. DLPC:Chol:DMPG was less viscous, but more prone to leakage due to nebulization (low stability efficiency), resulting again in low output of encapsulated peptide. Although cholesterol is generally associated with higher liposome stability,[13] Niven & Schreier[11] have shown that cholesterol might in some cases increase nebulization leakage.

In contrast, the liposomal preparations without cholesterol showed considerably higher encapsulations efficiencies, ranging between 70–90% for lipid concentrations of 30 mM. Nebulization was, in general, also more efficient in the absence of cholesterol and with lower lipid concentrations. However, DPPC:DMPG was prone to precipitation after the typical temperature drop that takes place during nebulization, resulting in low nebulization efficiencies ($E_{neb} = 10–30\%$). This effect, also found by Waldrep and coworkers,[17] can be explained by the relatively high phase transition temperature of DPPC.[24] The two other formulations, DLPC:DMPG and DMPC:DMPG, were less prone to precipitation and showed good nebulization efficiencies ($E_{neb} = 30–50\%$). Moreover, DPPC:DMPG showed high leakage during nebulization ($E_{stabil} = 35–45\%$), whereas with DLPC:DMPG and DMPC:DMPG leakage was very small ($E_{stabil} = 80–100\%$). Despite the similar overall efficiency levels of DLPC:DMPG and DMPC:DMPG, the latter was more readily centrifuged and handled.

Because of the limited availability of CM3 peptide, the above-described selection part of the study had to rely on single or double experimental runs, and the given results are to be seen as indicative only. To provide an illustration of how the combination of efficiencies results in the total output of encapsulated peptide, Figures 1 and 2 show graphically two typical cases of the process.

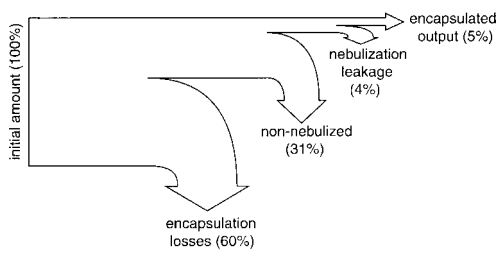

Figure 1. Example of the various losses in each step of the preparation and nebulization of DPPC:Chol:DMPG = 3:1:2 (60 mM lipid concentration) encapsulated peptide. Values are approximate percentages of the initial amount of CM3 peptide.

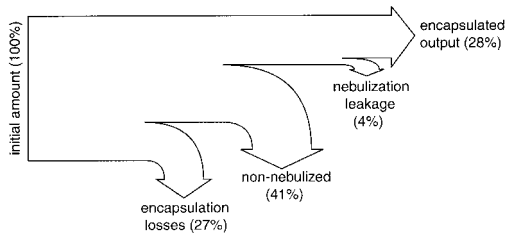

Figure 2. Average losses in each step of the preparation and nebulization of DMPC:DMPG = 3:1 (30 mM lipid concentration) encapsulated peptide. Values are approximate percentages of the initial amount of CM3 peptide.

Figure 1 shows the losses that occurred in one particular test of DPPC:Chol:DMPG, whereas Figure 2 shows average results for the case of DMPC:DMPG. In contrast to the efficiencies, defined in each step of the process, losses are shown in both figures as percentages of the initial amount of CM3 peptide. Figures 1 and 2 clearly show the superiority of DMPC:DMPG over DPPC:Chol:DMPG.

Taking into account all the above considerations, DMPC:DMPG was selected for the full characterization required as input to the mathematical lung deposition and ASL concentration models. Encapsulation efficiency of DMPC:DMPG was $E_{encaps} = 73.0 \pm 11.9\%$ ($n = 6$) and a liposomal peptide concentration of 730 μg/mL was used for nebulization. The sizes of the DMPC:DMPG = 3:1 MLVs showed a mean diameter of 262 nm with 96% of the liposomes between 190–342 nm and 4% in the range of 930–1700 nm.

The nebulization of 2.5 mL of the selected liposomal preparation with the vented jet nebulizer resulted in a total nebulization efficiency of $E_{neb} = 43.9 \pm 6.7\%$ ($n = 6$). The stability efficiency obtained was $E_{stabil} = 86.5 \pm 14.8\%$ ($n = 6$). In fact, no statistically significant differences ($p > 0.1$) between the leakage from the cascade impactor washings of nebulized DMPC:DMPG and the standard were measured. The size distribution of the aerosolized preparation, based on the aqueous phase, was lognormal with mass median aerodynamic diameter (MMAD) of 2.84 ± 0.1 μm and geometric standard deviation (GSD) of 1.97 ± 0.01 ($n = 3$). The independent assessment of the liposomal peptide distribution in the aerosol showed no statistically significant difference ($p > 0.01$) in the MMAD, indicating that the peptide is homogeneously distributed in the aerosol droplets. This result was expected in view of the small sizes of the liposomal vesicles.

Table 2. Relative Regional Deposition[a] of Liposomal Peptide for Each Model Subject

	4-Year-Old	8-Year-Old	Adult
Extrathoracic (%)	18.1	15.3	12.0
Tracheobronchial (%)	26.7	26.6	11.3
Alveolar (%)	17.0	17.2	14.0
Total Inhaled[b] (mg)	0.43	0.50	0.52

[a]Given in percentages of the total inhaled amount of CM3.
[b]Resulting from a nebulizer fill of 2.5 mL containing 1.8 mg of liposomal CM3.

Inhalation efficiencies (E_{inhal}) for the three breathing patterns described in Table 1 were 65.3, 62.7, and 54.3% for the adult, 8-year-old and 4-year-old model subjects, respectively. The inhaled amounts resulting from a 2.5 mL nebulizer volume fill (corresponding to 1.8 mg of liposomal peptide) were included in Table 2. The full characterization of the selected preparation described above is summarized in Table 3.

Using the information about the aerosol and the inhaled amounts obtained from the experiments, the deposited dosages of liposomal peptide in the three lung models were estimated. A summary of the relative deposition results grouped by region can be seen in Table 2. Lung dosages for the case of an adult are approximately half as large as the pediatric dosages.

The estimated bounds for the liposomal CM3 peptide concentration in the ASL along the tracheobronchial airways of the model subjects are shown in Figure 3. Results for the two pediatric simulations were similar and substantially higher (10 times in average) than for the adult case. Concentrations were in general higher in the proximal airways, tapering off at the more distal bronchioles.

DISCUSSION

Based on the overall performance, i.e., the combination of all efficiencies, the liposome preparation DMPC:DMPG = 3:1 molar ratio was chosen as the best formulation for the aerosol delivery of encapsulated CM3 peptide, as shown in the previous section. The encapsulation efficiencies obtained with DMPC:DMPG = 3:1 and 30 mM lipid concentration compare well with published values for cationic species.[26] Cationic peptides, in particular, have previously been encapsulated in liposomes to induce an anticancer immune response as part of the development of therapeutic cancer vaccines, as reported by Guan et al.[54] But the encapsulation levels required for stimulation of immune response were an order of magnitude smaller (approximately 100 μg/mL) than the levels targeted in the present study. The successful encapsulation of 730 μg/mL of CM3 peptide, allied to the relatively high nebulization efficiency of the formulation and the small leakage due to nebulization (Table 3), distinguished the selected liposomal preparation as well suited for the aerosolized delivery of the encapsulated antibiotic.

The numerical simulation of drug deposition in the lung showed a large difference between the pediatric and the adult cases (Table 2). This pronounced difference in predicted lung dosages can be partially explained by the morphological differences of the lung models. The narrower airways of the youngsters caused an increase in the two main deposition processes (impaction and sedimentation) in the conducting airways (tracheobronchial region). This effect was then magnified by the differences in tidal volumes of the three breathing patterns. Although the absolute values of the tidal volume increased with age, as seen in Table 1, the relative values as fractions of the model lung volume, i.e., the depths of inhalation, actually decreased. The tidal volumes

Table 3. Characterization of the Selected Liposomal Preparation

DLPC:DMPG = 3:1 Molar Ratio, 30 mM Lipid Concentration				
E_{encaps}	E_{neb}	E_{stabil}	MLV Mean Size	
73.0 ± 11.9%	43.9 ± 6.7%	86.5 ± 14.8%	262 nm	
E_{inhal} (4-Year-Old)	E_{inhal} (8-Year-Old)	E_{inhal} (Adult)	MMAD	GSD
54.3%	62.7%	65.3%	2.84 ± 0.1 μm	1.97 ± 0.01

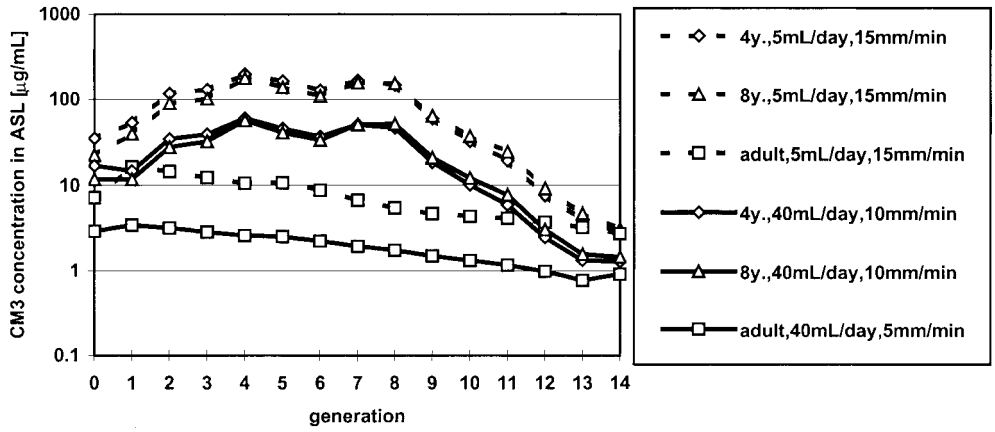

Figure 3. Estimated generational concentrations of liposomal peptide CM3 in the ASL immediately after completion of nebulization for various subjects, mucus production rates, and tracheal mucous velocities. Generation 0 corresponds to the trachea.

corresponded to 44 and 30% of the lung volume (at 50% of TLC) in the 4- and 8-year-old models, respectively, but only to 20% of the volume in the adult model. The shallower inhalation resulted in a reduction of the relative alveolar deposition (Table 2). It is worth noting that this analysis is based on lung data from healthy subjects. Although there is need for a morphologic model of diseased lungs in general, and of CF lungs in particular, the present analysis represents the closest approximation possible.

Higher tracheobronchial dosages in the pediatric models were combined with a much smaller amount of ASL (approximately 1/3 of the adult ASL volume), resulting in the prediction of 10 times higher concentrations than in the adult model in average. Although no experimental studies of pediatric ASL concentrations could be found in the literature, the present estimates for the adult model show good agreement with the measurements of Sinicropi et al.[55] Sinicropi and coworkers measured sputum DNase concentrations in 18 CF patients 15 min after inhalation of a 2.5 mg nebulizer fill of rhDNase, and found an average of 2.9 μg/mL. This compares well with the lowest bound of concentrations predicted in Figure 3 for an adult after inhalation of 1.8 mg nebulizer fill of CM3 peptide in the present study.

In both pediatric cases the model predicts minimum concentrations of CM3 peptide that are well above the minimum inhibitory concentration of 2–4 μg/mL, shown by Scott et al.[3] to be effective against several strains of *P. aeruginosa*. Despite the lower dosage received by the adult model and the dispersion of this dosage in a larger volume of ASL, most of the range of concentrations predicted still lies above the 2 μg/mL threshold. The estimated lower bound of concentrations for the more distal conducting airways falls short of this limit, but still ensures a minimum of 0.8 μg/mL. These simulation results indicate that the optimized liposome encapsulation of the new antibiotic CM3 combined with an efficient nebulization is a viable delivery option, thus fulfilling the main objectives of the present work. This finding supports future *in vivo* testing of the developed formulation.

The new model for the ASL distribution in the human lung, which to the authors' knowledge is the first of its kind, in association with the lung deposition model was revealed as a valuable tool for the pretrial evaluation of the aerosol delivery of new drugs, such as the investigated new liposomal peptide CM3.

ACKNOWLEDGMENTS

The authors are thankful to H. Orszanska (Aerosol Research Lab. of Alberta) for the liposome preparation and the laboratory analysis, and to M. King for the help on development of the ASL model. This work was supported financially by the Canadian Cystic Fibrosis Foundation's SPARX program.

REFERENCES

1. Hancock REW. 1997. Peptide antibiotics. Lancet 349:418–422.

2. Hancock REW, Chapple DS. 1999. Peptide antibiotics. Antimicrob Agents Chemother 43:1317–1323.
3. Scott MG, Yan H, Hancock REW. 1999. Biological properties of structurally related a-helical cationic antimicrobial peptides. Infect Immun 67:2005–2009.
4. Gough M, Hancock REW, Kelly NM. 1996. Anti-endotoxic potential of cationic peptide antimicrobials. Infect Immun 64:4922–4927.
5. Goto H, Nakamura S. 1980. Liberation of endotoxin from *Escherichia coli* by antibiotics. Jpn J Exp Med 50:35–43.
6. Hancock REW, Gough M, Patrykzat A, Jia X, Woods D. 2000. Anti-endotoxic, antimicrobial cationic peptides and methods of use therefore. World Intellectual Property Organization, publication WO012528A1.
7. Hancock REW. 1998. The therapeutic potential of cationic peptides. Expert Opin Invest Dis 7:167–174.
8. Parthasarathy R, Gilbert B, Mehta K. 1999. Aerosol delivery of liposomal all-*trans*-retinoic acid to the lungs. Cancer Chemoter Pharmacol 43:277–283.
9. Hancock REW. unpublished data.
10. Taylor KMG, Taylor G, Kellaway IW, Stevens J. 1990. The stability of liposomes to nebulisation. Int J Pharm 58:57–61.
11. Niven RW, Schreier H. 1990. Nebulization of liposomes. I. Effects of lipid composition. Pharm Res 7:1127–1133.
12. Niven RW, Speer M, Schreier H. 1991. Nebulization of liposomes. II. The effects of size and modeling of solute release profiles. Pharm Res 8:217–221.
13. Bridges PA, Taylor KMG. 1998. Nebulisers for the generation of liposomal aerosols. Int J Pharm 173:117–125.
14. Niven RW, Carvajal TM, Schreier H. 1992. Nebulization of liposomes. III. The effects of operating conditions and local environment. Pharm Res 9:515–520.
15. Bridges PA, Taylor KMG. 2000. An investigation of some of the factors influencing the jet nebulisation of liposomes. Int J Pharm 204:69–79.
16. Finlay WH. 2001. Chap. 8. In: The mechanics of inhaled pharmaceutical aerosols: An introduction. London: Academic Press.
17. Waldrep JC, Scherer PW, Hess GD, Black M, Knight V. 1994. Nebulized glucocorticoids in liposomes: Aerosol characteristics and human dose estimates. J Aerosol Med 7:135–145.
18. Waldrep JC, Keyhani K, Black M, Knight V. 1994. Operating characteristics of 18 different continuous-flow jet nebulizers with beclomethasone dipropionate liposome aerosol. Chest 105:106–110.
19. Farr SJ, Kellaway IW, Parry-Jones DR, Woolfrey SG. 1985. 99m-Technetium as a marker of liposomal deposition and clearance in the human lung. Int J Pharm 26:303–316.
20. Vidgren M, Waldrep JC, Arppe J, Black M, Rodarte JA, Cole W, Knight V. 1995. A study of ^{99m}technetium-labelled beclomethasone dipropionate dilauroylphosphatidylcholine liposome aerosol in normal volunteers. Int J Pharm 115:209–216.
21. Widdicombe JG. 1997. Airway surface liquid: Concepts and measurements. In: Rogers DF, Lethem MI, editors. Airway mucus: Basic mechanisms and clinical perspectives Chap. 1, Basel: Birkhauser. pp 1–17.
22. Finlay WH, Wong JP. 1998. Regional lung deposition of nebulized liposome-encapsulated ciprofloxacin. Int J Pharm 167:121–127.
23. Finlay WH, Lange CF, King M, Speert DP. 2000. Lung delivery of aerosolized dextran. Am J Respir Crit Care Med 161:91–97.
24. Kulkarni SB, Betageri GV, Singh M. 1995. Factors affecting microencapsulation of drugs in liposomes. J Microencapsul 12:229–246.
25. Knight V, Koshkina NV, Waldrep JC, Giovanella BC, Gilbert BE. 1999. Anticancer effect of 9-nitrocamptothecin liposome aerosol on human cancer xenografts in nude mice. Cancer Chemother Pharmacol 44:177–186.
26. Mayer LD, Hope MJ, Cullis PR, Janoff AS. 1985. Solute distributions and trapping efficiencies observed in freeze-thawed multilamellar vesicles. Biochim Biophys Acta 817:193–196.
27. Cullis PR, Hope MJ, Bally MB, Madden TD, Mayer LD, Janoff AS. 1987. Liposomes as pharmaceuticals. In: Ostro MJ, editor. Liposomes from biophysics to therapeutics. New York: Marcel Dekker. pp 39–72.
28. Finlay WH, Stapleton KW. 1999. Undersizing of droplets from a vented nebulizer caused by aerosol heating during transit through an Anderson impactor. J Aerosol Sci 30:105–109.
29. Finlay WH, Stapleton KW, Zuberbuhler P. 1997. Predicting regional lung dosages of a nebulized suspension: Pulmicort (budesonide). Part Sci Technol 15:243–251.
30. Schreier H. 1992. Liposome aerosols. J Liposome Res 2:145–184.
31. Cerny F, Armitage L, Hirsch JA, Bishop B. 1992. Respiratory and abdominal muscle responses to expiratory threshold loading in cystic fibrosis. J Appl Physiol 72:842–850.
32. Denyer J, Dyche A, Nikander K. 1996. Breathing patterns in adult patients. ISAM Focus Symposium on Aerosol Therapy with Small Volume Nebulizers. Sept. 4–5.
33. Diot P, Palmer L, Smaldone A, DeCelie-Germana J, Grimson R, Smaldone GC. 1997. RhDNase I aerosol deposition and related factors in cystic fibrosis. Am J Respir Crit Care Med 156:1662–1668.
34. Hofmann W, Martonen TB, Graham RC. 1989. Predicted deposition of nonhygroscopic aerosols in the human lung as a function of subject age. J Aerosol Med 2:49–68.

35. Finlay WH, Stapleton KW, Chan HK, Zuberbuhler P, Gonda I. 1996. Regional deposition of inhaled hygroscopic aerosols: In vivo SPECT compared with mathematical deposition modelling. J Appl Physiol 81:374–383.
36. Finlay WH, Hoskinson M, Stapleton KW. 1998. Can models be trusted to subdivide lung deposition into alveolar and tracheobronchial fractions? In: Respiratory drug delivery VI. Buffalo Grove: Interpharm Press. pp 235–242.
37. Phillips CG, Kaye SR, Schroter RC. 1994. A diameter-based reconstruction of the branching pattern of the human bronchial tree. Part I. Description and application. Resp Physiol 98:193–217.
38. Haefeli-Bleuer B, Weibel ER. 1988. Morphometry of the human pulmonary acinus. Anatom Rec 220:401–414.
39. Phalen RF, Oldham MJ, Beaucage CB, Crocker TT, Mortensen JD. 1985. Postnatal enlargement of human tracheobronchial airways and implications for particle deposition. Anat Rec 212:368–380.
40. Chan TL, Lippmann M. 1980. Experimental measurements and empirical modelling of the regional deposition of inhaled particles in humans. Am Ind Hyg Assoc J 41:399–409.
41. Pich J. 1972. Theory of gravitational deposition of particles from laminar flows in channels. J Aerosol Sci 3:351–361.
42. Heyder J, Gebhart J. 1977. Gravitational deposition of particles from laminar aerosol flow through inclined circular tubes. J Aerosol Sci 6:311–328.
43. Gormley PG, Kennedy K. 1949. Diffusion from a stream flowing through a cylindrical tube. Proc R Irish Soc 52A:163.
44. Rudolf G, Köbrich R, Stahlhofen W. 1990. Modelling and algebraic formulation of regional deposition in man. J Aerosol Sci 21:S403–S406.
45. Finlay WH. 1998. Estimating the type of hygroscopic behaviour exhibited by aqueous droplets. J Aerosol Med 11:221–229.
46. Matsui H, Randell SH, Peretti SW, Davis CW, Boucher RC. 1998. Coordinated clearance of periciliary liquid and mucus from airway surfaces. J Clin Invest 102:1125–1131.
47. Serafini M, Michaelson ED. 1977. Length and distribution of cilia in human and canine airways. Bull Eur Physiopath Resp 13:551–559.
48. Stahlhofen W, Gebhart J, Heyder J. 1980. Experimental determination of the regional deposition of aerosol particles in the human respiratory tract. Am Ind Hyg Assoc J 41:385–398.
49. Davis CW. 1997. Goblet cells: Physiology and pharmacology. In: Rogers DF, Lethem MI, editors. Airway mucus: Basic mechanisms and clinical perspectives Chap. 7. Basel: Birkhauser. pp 149–177.
50. Plopper CG, Heidsiek JG, Weir AJ, St. George JA, Hyde DM. 1989. Tracheobronchial epithelium in the adult rhesus monkey: A quantitative histochemical and ultrastructural study. Am J Anat 184:31–40.
51. Yeates DB, Sturgess JM, Kahi SR, Levison H, Aspin N. 1975. Mucociliary transport in trachea of patients with cystic fibrosis. Arch Dis Childhood 51:28–33.
52. Oberwaldner B, Evans JC, Zach MS. 1986. Forced expirations against a variable resistance: A new chest physiotherapy method in cystic fibrosis. Pediatr Pulmonol 2:358–367.
53. Oberwaldner B, Theißl B, Rucker A, Zach MS. 1991. Chest physiotherapy in hospitalized patients with cystic fibrosis: A study of lung function effects and sputum production. Eur Respir J 4:152–158.
54. Guan HH, Budzynski W, Koganty RR, Krantz MJ, Reddish MA, Rogers JA, Longenecker BM, Samuel J. 1998. Liposomal formulations of synthetic MUC1 peptides: Effects of encapsulation versus surface display of peptides on immune responses. Bioconjugate Chem 9:451–458.
55. Sinicropi DV, Williams M, Prince WS, Lofgren JA, Lucas M, Devault A, Baughman S, Nash M, Fuchs H, Shak S. 1994. Sputum pharmacodynamics and pharmacokinetics of recombinant human DNase I in cystic fibrosis. Am J Respir Crit Care Med 149:A671.