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Biofilm penetration, triggered release and *in vivo* activity of inhaled liposomal amikacin in chronic *Pseudomonas aeruginosa* lung infections

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***Objectives*: Chronic infections of *Pseudomonas aeruginosa* in the lungs of cystic fibrosis patients are intractable antibiotic targets because of their biofilm mode of growth. We have investigated the biofilm penetration, mechanism of drug release and *in vivo* antimicrobial activity of a unique nanoscale liposomal formulation of amikacin designed specifically for nebulization and inhaled delivery.**

***Methods*: Penetration of fluorescently labelled liposomes into sputum or *P. aeruginosa* (PA3064) biofilms was monitored by a filter assay and by epifluorescence or confocal scanning laser microscopy. Amikacin release *in vitro* and rat lung levels after inhalation of nebulized material were measured by fluorescence polarization immunoassay. A 14 day agar bead model of chronic *Pseudomonas* lung infection in rats was used to assess the efficacy of liposomal amikacin versus free aminoglycosides in the reduction of bacterial count.**

***Results*: Fluorescent liposomes penetrated readily into biofilms and infected mucus, whereas larger (1 μm) fluorescent beads did not. Amikacin release from liposomes was mediated by sputum or *Pseudomonas* biofilm supernatants. Rhamnolipids were implicated as the major releasing factors in these supernatants, active at one rhamnolipid per several hundred lipids within the liposomes. Inhaled liposomal amikacin was released in a slow, sustained manner in normal rat lungs and was orders of magnitude more efficacious than inhaled free amikacin in infected lungs.**

***Conclusions*: Penetration of biofilm and targeted, sustained release from liposomes can explain the superior *in vivo* efficacy of inhaled liposomal amikacin versus free drug observed in a 14 day infection model. Inhaled liposomal amikacin may represent an important therapy for chronic lung infections.**

Keywords: cystic fibrosis, drug delivery, inhaled liposomes

Introduction

Cystic fibrosis is a life-threatening, inherited disorder caused by an abnormality in the cystic fibrosis transmembrane conductance regulator (CFTR) and characterized by chronic progressive lung disease. Abnormal function of CFTR (and other ion channels) leads to inspissated static mucus in the lungs and a situation in which mucociliary clearance and other antimicrobial defences are damaged. The damage is so extensive that persistent infection by a predictable set of pathogens, especially *Pseudomonas aeruginosa*, and a concomitant chronic neutrophilic inflammatory response are characteristic consequences, which are progressive and ultimately fatal.[1–3]

P. aeruginosa grows in small colonies with biofilm-like characteristics in the hypoxic environment of such stationary mucus[4] where the bacterial cells elaborate a quorum-sensing system to control gene expression specifically for growth as a

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biofilm.[5] Observation of tissue samples from cystic fibrosis patients indicates that *P. aeruginosa* is predominantly intraluminal, localized in hypoxic mucopurulent masses.[6] This environment may include a matrix composed of alginate or other exopolysaccharides from mucoid bacteria, mucins from lung epithelial cells and DNA from damaged leucocytes.[7] Alginate production by *P. aeruginosa* is actually stimulated in such hypoxic conditions,[6] converting non-mucoid cultures to mucoid.[8]

The biofilm-like mode of growth results in a difficult challenge for antibiotic therapy. For aminoglycosides, slow penetration due to electrostatic interactions with the mucus and biofilm matrices and lack of activity against cells in a slow growth phenotype are particularly important.[9] To make matters worse, subinhibitory levels of aminoglycosides help to induce biofilm formation.[10,11] One approach to increase efficacy in this situation is direct administration of antibiotics to the lungs via inhalation. However, because of the relatively small size of these drugs (e.g. inhaled tobramycin), they are rapidly removed from the lungs after inhalation,[12,13] limiting the amount of time they remain at a concentration above the effective minimum inhibitory concentration in the local vicinity of the bacteria. Additionally, because of their short residence time in the lung, these drugs require at least twice-a-day administration. There is concern that this added inconvenience may adversely impact compliance.

Based on these considerations, it is clear that an improvement in aminoglycoside therapy of cystic fibrosis lung infections may be realized if delivery of sufficient drug at sustained levels in and around the target infections could be achieved. One approach is the use of inhaled liposomes to deliver the drug in a localized and sustained manner. An intratracheally administered liposomal tobramycin formulation with a fluid membrane phase has shown a significant increase in drug retention in the lung and enhanced antimicrobial activity,[14–16] but the clinically relevant demonstration of sustained release and long-term efficacy (>24 h) after inhalation of a nebulized formulation was not demonstrated. Because cystic fibrosis patients commonly use nebulized delivery for a number of medications, a nebulized form of liposomal antibiotics could be an important contribution.

We have extended the liposomal delivery approach using an alternative design to develop an inhaled liposomal formulation of amikacin (Arikace^{TM}) with high drug loading (drug-to-lipid ratio) and stability to be administered via nebulization. The liposomal membrane of this formulation comprised the saturated lipid dipalmitoyl phosphatidylcholine (DPPC) and cholesterol, which are natural constituents of lung surfactant. These lipids are not only biocompatible but also impart a high degree of membrane stability (reduced leakage), due to factors including suppression of the gel-to-liquid crystalline phase transition, thus making nebulized delivery feasible and maintaining high localized concentrations of drug in the lung.

After initial verification of slow, sustained release of the inhaled formulation in normal uninfected rat lungs, two major factors important for the action of liposomes in cystic fibrosis were investigated in this study: (i) the ability to penetrate through mucus and into biofilms to attain access and close proximity to bacteria; and (ii) the identification of a factor triggering the release of amikacin from liposomes at the site of *P. aeruginosa* infections. Based on *in vitro* studies, we demonstrate that both of these events can occur. Specifically, the data show that the triggering of liposome release is mediated by the virulence factors, rhamnolipids, produced by biofilm-localized bacteria. The potential importance of these findings was then tested *in vivo* with a chronic *Pseudomonas* infection model which demonstrated significantly greater efficacy of amikacin inhaled as the liposomally encapsulated form versus the free drug, consistent with penetration and sustained release at the site of the infection.

Materials and methods

Liposome preparation

Fluorescently labelled liposomes. A stock of liposomes was prepared in a manner nearly identical to liposomal amikacin but containing 0.2 wt% of a fluorescent carbocyanine probe, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate [diI(3)C18, Molecular Probes, Eugene, OR, USA], in the membrane instead of encapsulated amikacin. Briefly, 2 g of lipid, 2:1 by weight of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and cholesterol, respectively, was dissolved in ethanol (90 mL) along with the fluorescent probe. The lipid solution was infused into a 0.9% saline solution (260 mL) mixed thoroughly via two intersecting streams. The resulting preparation was concentrated by diafiltration to a final volume of ~50 mL. This suspension was filtered through a 1.2 μm filter as a final step. The average size of the liposomes was measured by dynamic light scattering using a Nicomp particle size analyser in a Gaussian mode. An intensity weighted mean diameter of 294 nm was obtained for this preparation.

Liposomal amikacin. Stocks of liposomes encapsulating amikacin were prepared by a proprietary method similar to the ethanol infusion described earlier. The only difference was that the preparations were at a much larger scale and are not filtered as a final step. The final total amikacin concentration in liposomal amikacin stocks ranged from 20 to 75 mg/mL. Total administered amikacin concentrations are indicated in the text. The lipid composition was the same as described earlier. The drug-to-lipid weight ratios in these preparations were 0.7–1.5. The intensity weighted average size of these liposome preparations as analysed by dynamic light scattering was found to be ~300 nm (Gaussian distribution). Typical liposomal amikacin preparations contain 99% of liposomes below ~700 nm and 75% above 170 nm in the Gaussian fit.

Sputum penetration

Sputum was obtained by voluntary submission under an IRB protocol at the Robert Wood Johnson Hospital cystic fibrosis clinic in New Brunswick, NJ, USA. A previously frozen sample of sputum was used in the experiments. Thawed samples that have been previously frozen do not change rheological characteristics.[17] Approximately 0.1 g of the thawed sputum sample was added with a marked sterile pipette to each Transwell apparatus (8.0 μm pores, Becton–Dickinson, Franklin Lakes, NJ, USA). Phosphate-buffered saline (PBS) (400 μL) was layered on the sputum and each well was placed in a dry 24-well plate and allowed to shake gently in a 37°C incubator for ~1–2 h. The final sputum layer was in the range of 1 mm thick. Then, 400 μL of PBS was added to the plate well and 450 μL of a mixture of liposomes (equivalent in total lipid to ~20 mg/L liposomal amikacin) and 1 μm polystyrene beads labelled with a green fluorophor (Molecular Probes, #F13080) was added to the upper part of the Transwell apparatus. The particle numbers were estimated to be $\sim 5 \times 10^7$/mL for the beads and $\sim 3 \times 10^{10}$/mL for the liposomes (assume 10^5 lipids/liposome). Controls without sputum were also set up. The plates and Transwells

were allowed to incubate at 37°C with gentle shaking for 24 h. Fluorescence in the bottom compartment was measured using a Cytofluor plate reader (ex. 395 nm, em. 460 nm, gain 75, for polystyrene beads; ex. 560 nm, em. 620 nm, gain 75, for liposomes). A control well of only buffer and sputum was also used to correct for any background fluorescence.

A sputum sample was also used to observe penetration microscopically. Liposomes were prepared in a manner identical to amikacin-loaded liposomes as described earlier, but containing the hydrophobic fluorophor, diI(3)C18 (Molecular Probes), in the membrane at 0.1 mol% instead of encapsulated amikacin. The approximate lipid concentration in the liposome preparations was 40 mg/mL. A small aliquot of a mixture of diI-liposomes and green fluorescent 1 μm polystyrene beads (Molecular Probes) was added to the centre of the top of the sputum sample and incubated for 30 min at 36°C. All beads or liposomes were diluted 100-fold from the original concentrations to obtain fluorescence readings in a useful range. For the 1 μm beads, the concentration was $\sim 10^8$ beads/mL. For liposomes, an approximately calculated particle concentration assuming a 0.3 μm diameter is 4×10^{11} per mL. The biofilm material was frozen for sectioning perpendicular to the surface at the end of the experiment. Sections (10 μm) were cut with a cryostat. After the sections were made, they were transferred to a microscope slide and covered with aqueous mounting medium and a cover glass. Images of the biofilms were taken with a camera mounted on a Nikon Microscope using Nikon ACT 1 software. Photos were taken at a 200× magnification.

Biofilm penetration in a flow cell

Biofilms were grown from 24 h stock cultures of *Pseudomonas* strain PA3064 in glass capillary tubes with square cross-sections (nominal inside dimension of 900 μm and a wall thickness of 170 ± 10 μm, Friedrich & Dimmock, Millville, NJ, USA) under continuous flow conditions. The strain (provided by Dr Donald Woods) is a mucoid derivative from PA01, isolated on the basis of growth in chronic rat lung models.

The growth medium consisted of 1% strength tryptic soy broth (TSB; Difco, USA) and was delivered via peristaltic pump (Masterflex® L/S, Cole Parmer, USA) through bubble traps (Biosurface Technologies, Bozeman MT) to the capillaries at a flow rate of 10 mL/h per channel. The entire system was sealed from the environment by 0.22 μm filters.

The biofilm was grown for 4 days. Dil(3)C18 liposomes (200 μL) and 40 μL of the 1 μm polystyrene beads (F-8816, Molecular Probes) were added to 9.76 mL of 1% TSB in a 15 mL tube. A 5 mL syringe was filled with the dilution and connected to the influent tubing of the capillary. After the first image, flow of the liposome solution was initiated into the capillary at a rate of 20 mL/h using a syringe pump.

Imaging was performed by confocal scanning laser microscopy (CLSM) using a Leica TCS NT confocal scanning laser microscope, with excitation at 488 and 568 nm and with emission collected at 500–530 nm (green channel) and 585–615 nm (red channel). Transmitted light images were also collected to determine the position of the biofilm. A 10–20 μm thick colony was located growing on the luminal surface of the capillary using transmitted light mode on the CLSM. A 100× oil objective was used to image the biofilm, liposomes and beads, which provided a resolution limit of ~0.3 μm. Once a colony was selected, the imaging was initiated. The CLSM was set up to take an image every 30 s for 1 h for a total of 120 images. The photo shown in Figure 2 is one image from this series of the 120 images.

Sputum-mediated release

Expectorated sputum from a patient with cystic fibrosis was refrigerated upon collection and utilized within 1 h of collection. The sample was liquefied with bovine DNase I (Sigma, St Louis, MO, USA) and alginate lyase (Sigma). Enzymes were at a final concentration of 0.125 mg/mL. The sputum was diluted 2-fold with the combined enzymes (assuming 1 g sputum = 1 mL) and incubated at 37°C for 2 h with shaking on an Innova 4000 Incubator/Shaker (New Brunswick Scientific, Edison, NJ, USA). Liposomal amikacin or soluble amikacin (USP) at a concentration of ~1 mg/mL amikacin was mixed with liquefied sputum or a DNase/lyase only solution at a 1:1 ratio in a final volume of 6 mL. These solutions were incubated at 37°C with gentle shaking. At each time point, 100 μL aliquots of the solutions were removed and analysed for amikacin concentration by fluorescence polarization immunoassay using a TDx analyser (Abbott Diagnostics, Abbot Park, IL, USA). Intact liposomes were lysed in a separate aliquot of each sample using 1% Triton X-100 detergent (Sigma T9284) in TDx dilution buffer (Abbott Diagnostics). All samples were centrifuged in a microfuge (Abbott Diagnostics) at 9500 **g** for 5 min and the supernatants used for analysis.

Bacterial supernatant preparation and fractionation

Adherent biofilms of PA3064 were grown via an adaptation of the procedures of O'Toole and Kolter.[18] A culture of 10^9cfu were allowed to adhere to polystyrene plates for 2 h in Minimal Davis Broth followed by washing and growth in shaken culture for 96 h at 37°C. Biofilms were disrupted with sterile transfer pipettes, resuspended in the Minimal Davis Broth and sedimented to remove insoluble material. Supernatants were then incubated (typically 50 μL) for varying times at 37°C with liposomal amikacin (50 μL at a concentration of 1 mg/mL amikacin). Free amikacin was measured on ultrafiltrates of samples (Centricon 30 kDa cutoff filters, Millipore, Inc., Bedford, MA, USA) using a fluorescence polarization immunoassay (TDx analyser, Abbott Diagnostics).

Part of the biofilm supernatant was tested for activity as described earlier after various treatments or fractionations to help identify the release factors. In all cases, a 1:1 mixture of sample with 1 mg/mL liposomal amikacin (in terms of amikacin concentration) was incubated for 4 h at 37°C, after which total and free amikacin were measured as described earlier. The per cent released was used as the measure of activity and compared with the per cent released by an untreated original sample of supernatant. For solvent precipitation, samples of 200 μL were mixed with 1 mL of ethanol or 1 mL of acetone and incubated at 4°C overnight. Pellets were sedimented and solvent supernatants were collected for drying under a nitrogen stream. The samples were resuspended in 200 μL of saline and tested for release activity. For heat treatment, a sample of bacterial supernatant was heated to 60°C for 1 h and then allowed to cool before testing for activity. Another sample was frozen overnight before thawing and testing for release activity, as described. Other samples were passed through a 30 or 10 kDa cut-off centrifugal filter before testing the filtrate in the same manner.

Identification of release factors

A commercially available rhamnolipid solution (JBR-515; Jeneil Biosurfactant Company, Saukville, WI, USA) consisting primarily of α-L-rhamnopyranosyl-β-hydroxyecanoyl-β-hydroxydecanoate (mono-rhamnolipid or RLL) and 2-*O*-α-L-rhamnopyranosyl-α-L-rhamnopyranosyl-β-hydroxydecanoyl-β-hydroxydecanoate (di-rhamnolipid or RRLL) was used. This solution was separated into

its major components of mono- and di-rhamnolipid by fractionation on a reverse-phase C-8 HPLC column [Phemonex 3 μm C8(2) 100A, 75 × 4.6 mm]. A binary mobile-phase gradient was used for elution (Phase A—99.9% acetonitrile, 0.1% acetic acid; Phase B—49.9% acetonitrile, 50% H_2O, 0.1% acetic acid). Shimadzu 10 AVP system was used equipped with Sedex 55 Evaporative Light Scattering Detector. Under these conditions, RRLL retention time was 4.2 min and RLL retention time was 6.4 min. The content of each peak was collected in multiple runs, dried and reconstituted at known concentration in water for preparation of HPLC standards and for amikacin release experiments.

For release measurements, varying ratios of rhamnolipids and liposomal lipid in the form of liposomal amikacin were mixed and allowed to incubate at 37°C for 24 h. The approximate total lipid concentration was 12.5 mM for the mono-rhamnolipid experiment and 5.5 mM for the di-rhamnolipid experiment. Samples were then diluted 5-fold and centrifuged through a Centricon 30 spin-filter. The free concentration of amikacin in the filtrates was measured by TDx analyser (Abbott Diagnostics) and compared with the total concentration to obtain the per cent leakage.

Dosing of amikacin and liposomal amikacin to rats

All procedures were performed under Institutional Animal Care and Use Committee (IACUC)-approved protocols at Transave Inc. (Protocol #002T, #003T and #004T). Nebulized aerosols were administered to Sprague–Dawley rats by inhalation in a 12-port nose-only inhalation chamber (CH Technologies, Westwood, NJ, USA). A nebulizer (PARI LC Star, Monterey, CA, USA) was used to aerosolize the test items. A total of 4 h of inhalation was administered. Delivered doses were calculated as in Wolff and Dorato,[19] based on measured aerosol concentrations in this apparatus. A dose of 6 mg/kg for liposomal amikacin (assuming 10% deposition) and 6.8 mg/kg for nebulized free amikacin was estimated for this administration time using a 20 mg/mL amikacin concentration. The same calculation applies to other concentrations administered. Rats were anaesthetized and sacrificed at the indicated time points, and lungs were removed and homogenized in 0.25% Triton X-100 (1 g of tissue/10 mL of diluent), centrifuged at 1500 rpm and aliquots taken for amikacin assay via fluorescence polarization immunoassay on a TDx analyser (Abbott Diagnostics). A typical lung weight was ~1.5 g. This assay has a limit of detection of 0.8 mg/L.

Chronic Pseudomonas infection model

A chronic *Pseudomonas* infection model based on instillation of agar beads containing bacteria was used[20,21] (Transave IACUC-approved protocol #002T and #004T). Agar beads were produced by mixing a stock of 2% Noble agar maintained at 50°C with a stock of log-phase *P. aeruginosa*, strain 3064 (provided by Dr Donald Woods, Department of Microbiology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada), and then adding it to a rapidly stirred aliquot of mineral oil, also maintained at 50°C. This mixture was then rapidly chilled with continued stirring to form agar beads of ~20–100 μm containing live bacteria.

Sprague–Dawley female rats were instilled intratracheally under anaesthesia with 100 μL of beads containing in the range of 10^6 cfu per lung. Treatments were begun at day 4 after instillation of beads. Rats were administered liposomal amikacin, free amikacin or free tobramycin by inhalation in a 12-port nose-only inhalation chamber (CH Technologies). A nebulizer (PARI LC STAR) was used to aerosolize the test items.

The deposited dose was calculated as in Wolff and Dorato[19] and based on aerosol concentration measurements in the inhalation chamber and an assumption of 10% deposition. Groups of 12 animals were randomized after instillation and treated by inhalation (specific conditions described in figure legends). After treatment, lungs were homogenized with a Polytron® homogenizer (Brinkmann, Rexdale, Ontario, Canada) using the maximum speed setting. Cfu were analysed from dilutions of the homogenates into Mueller–Hinton broth, subsequently streaked onto agar plates incubated at 37°C overnight to count colonies. Because of the dilutions used, the limit of detection was 2 log units/lung. Therefore, log cfu was counted as 2 when colonies were undetectable.

Results

Sustained release in the lung

Liposomes must retain amikacin in the lung for a sufficient period of time to be available for targeted release and to minimize dosing requirements. To test the effect of liposomal delivery, normal uninfected rats were given a single equal nebulized dose of either inhaled liposomal amikacin or inhaled free tobramycin, a similar aminoglycoside antibiotic currently administered by inhalation clinically. Under the conditions of these experiments, a reproducible proportion (~65%) of the amikacin remains in the liposomes after nebulization (data not shown). No adverse reaction was observed in any of the animals that had inhaled any of the test solutions. At the initial time point (time = 0 after inhalation), the total lung concentration of amikacin was higher than tobramycin at an equal dose. This is as expected because of the rapid clearance of the free aminoglycoside (either amikacin or tobramycin) during the inhalation dosing time (60 or 80 min dosing time, ~90 min half-life).[13] In fact, assuming a 90 min exponential half-life for free drug, the zero time concentration of tobramycin should be 81% of the liposomal amikacin concentration. The measured concentration is 82% of the initial amikacin concentration (645 versus 782 μg/lung).

Within the first few hours after inhalation, most of the free tobramycin has been cleared from the lungs, although there is a small residual component with a slow clearance (Figure 1). Results for inhaled free amikacin (data not shown) were virtually identical to free tobramycin, as expected. The clearance of amikacin from lungs of animals receiving liposomal amikacin (Figure 1) is apparently biphasic (or more complex) with a rapid component (<2 h half-life) and a larger slow component (>>24 h half-life) representing more than half of the drug. The amikacin component exhibiting the slower lung clearance is consistent with being derived from that which was initially deposited in the lung in the liposomally encapsulated form (65%), while the rapid component may represent the portion of amikacin reaching the lung in a free form.

This sustained release from the lung can lead to lower drug concentrations in non-target organs. Typically, for a 6 mg/kg deposited dose, blood levels are at a peak level immediately post-dosing of <8 mg/L (data not shown). Beyond 2 h, plasma levels remain very low (e.g. <2 mg/L). Likewise, the slow release from the lung also results in lower levels in the kidneys as drug transits into the urine. The data in aggregate are consistent with the relatively long-term retention of amikacin in the lungs as a result of liposomal encapsulation.

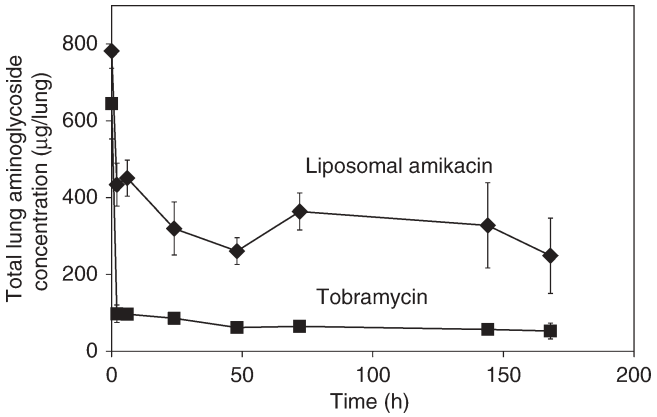

Figure 1. Clearance of aminoglycoside in rat lungs after inhalation of liposomal amikacin (75 mg/mL) versus free tobramycin (60 mg/mL). Single, equal doses were given by nebulization, 80 and 100 min for liposomal amikacin (diamonds) and free tobramycin (squares), respectively. Total lung deposited dose for both was estimated to be ~6 mg/kg from measured aerosol concentrations. Lung levels were measured at the indicated times, where time indicates hours after the end of inhalation dosing. Zero time indicates immediately after dosing, where total amikacin and tobramycin levels were 782 and 645 μg/lung, respectively. Error bars represent ± SD.

Biofilm penetration

The potential for access of this delivery system to the static mucus that surrounds the *Pseudomonas* infections in the lungs of patients is expected to be highly dependent on the size of the carrier.[22] These amikacin-containing liposomes are nanoscale delivery systems with a mean diameter of ~300 nm (smaller than bacteria) as measured by intensity-weighted dynamic light scattering (the number weighted average is even smaller). Penetration and release of the drug near the infection site could greatly enhance the efficacy of treatment. Previous measurements analysing the penetration of zwitterionic liposomes of this type into sputum or *Pseudomonas* biofilms have not been reported.

Sputum samples from cystic fibrosis patients were used to investigate the penetration of the liposomes into mucus and biofilms. Empty liposomes of the same average size and lipid composition as the liposomal amikacin formulation were prepared with a small amount (0.2 wt%) of a membrane-localized fluorescent probe [diI(3)C18, see the Materials and methods section]. Fluorescence photomicrographs of the penetration of a sputum sample are shown in Figure 2(a). These samples were frozen for sectioning after addition of a mixture of fluorescent liposomes and 1 μm fluorescent polystyrene beads to the top of the sputum layer and incubation for 30 min at 37°C. Most of the fluorescence is seen at the top of the layer, as expected. It can also be seen that a relatively higher concentration of liposomal (red) fluorescence is observed near the bottom of the sputum layer than is observed for the polystyrene beads (green), indicating a much more rapid penetration by the liposomes.

In another type of experiment, the flow of a mixture of the same liposomes and beads through a supported layer of sputum was measured (see the Materials and methods section). After 24 h, there was substantial recovery of the liposomal fluorescence on the opposite side of the layer (62% of the maximal value, if all liposomes had access to both sides—confirmed in controls without sputum), while almost no beads penetrated the

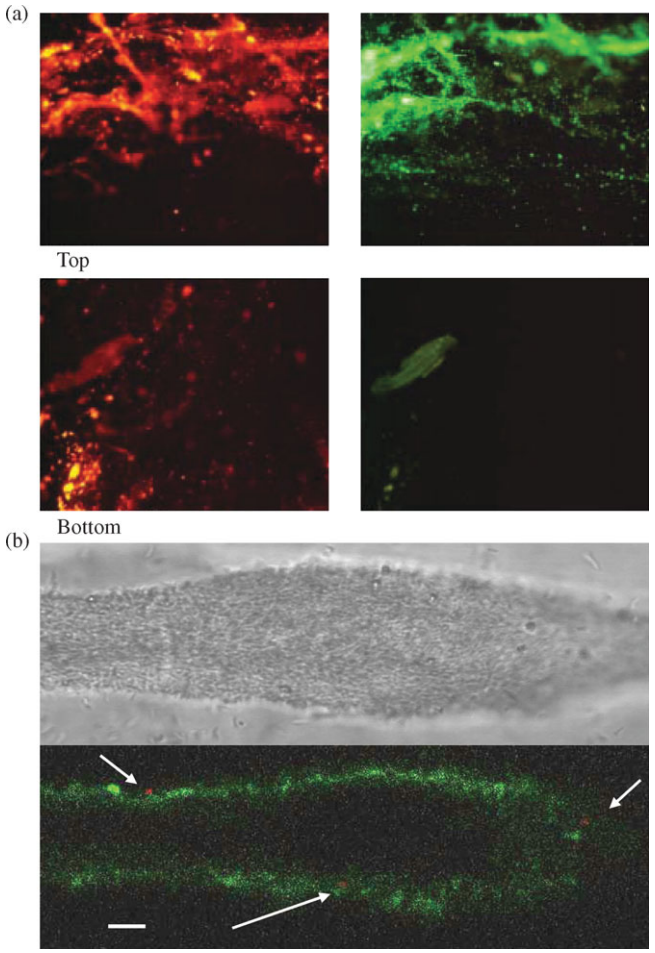

Figure 2. Penetration of liposomes into *Pseudomonas* biofilms and cystic fibrosis sputum. (a) Penetration of liposomes into a cystic fibrosis sputum sample. Fluorescent liposomes (red) and beads (green) were added to the top of a layer of sputum and incubated for 30 min. The sample was frozen and a section perpendicular to the layer was prepared in a cryo-microtome. The section was laid on a slide for observation. The images on the left and right are the same sample. The top images are the top 200–250 μm of the sample, whereas the bottom images are the bottom with a total sample thickness from top to bottom in the range of 500–1000 μm. (b) Experiments with a *P. aeruginosa* biofilm. Mucoid strain PA3064 was grown as a biofilm in an optical flow cell for observation by confocal laser scanning microscopy. Liposomes with a trace of membrane-associated carbocyanine probe (shown green) were introduced under flow along with fluorescent 1 μm polystyrene beads (shown red). The upper photo shows a light microscopic image of a section of biofilm, whereas the lower image shows the corresponding confocal fluorescence image taken ~50 min after addition of liposomes. Arrows show polystyrene beads that have bound to biofilm. Bar represents ~10 μm.

sputum (9% of maximal). It is possible in this experiment that some liposomes are remodelled as they pass through the sputum (see data below). However, smaller beads, 200 nm in diameter, were also able to penetrate the sputum under these conditions (45% of maximal), consistent with size-dependent penetration.

Because *Pseudomonas* biofilms exist as isolated colonies surrounded by mucus in the lungs of cystic fibrosis patients, penetration of pure *Pseudomonas* biofilms was also tested as a model for this environment. Biofilms grown under flow for 60 h in optical flow cells were observed with confocal laser scanning

microscopy. An optical slice within the biofilm was probed for the presence of fluorescent liposomes.

In the representative photo shown (Figure 2b), a biofilm is monitored after addition of fluorescent liposomes to the surrounding solution under flow conditions. Liposomes, which are near the resolution limit of light microscopy, can be seen as small punctate green dots (artificial colour in this image). It should be noted that the liposome concentration used in this experiment is about two orders of magnitude lower than the anticipated therapeutic dose. The fluorescently labelled liposomes can be clearly seen to penetrate the biofilm, but in addition, they appear to reach higher concentrations within the biofilm than in the bulk fluid.

The concentration of liposomes in the peripheral part of the biofilm is very high under these conditions, whereas at the centre, the concentration would appear to be about the same as in the fluid surrounding the biofilm. In contrast, a small number of larger fluorescent 1 μm polystyrene beads were included in the liposome solution. These beads, coloured red in the photo, flowed past the biofilm, or were bound to the surface (Figure 2b), but were never observed to significantly penetrate. It is clear that liposomes of the size used here readily penetrate *Pseudomonas* biofilms *in vitro*. Together with the sputum data, these results strongly suggest that zwitterionic amikacin-loaded liposomes have the ability to penetrate the site where *Pseudomonas* infections reside in the lungs of cystic fibrosis patients.

Release of antibiotic from liposomes as mediated by the infection

Because potentially lytic factors are associated with the biofilm mode of growth of *Pseudomonas*, the possibility of triggered release of amikacin mediated by such factors was investigated. Figure 3(a) shows the result of incubation of amikacin-loaded liposomes with a liquefied *Pseudomonas*-infected sputum sample from a cystic fibrosis patient. A sustained release was observed over the course of 48 h under the conditions of this experiment.

It was hypothesized that at least some of the factors responsible for release in this sample may be associated with the bacteria. Therefore, a culture of a mucoid strain of *P. aeruginosa*, PA3064, was grown as a biofilm over a 96 h period. A supernatant prepared from this biofilm was harvested and tested for the propensity to cause release of amikacin from liposomal amikacin. This supernatant was particularly active in release of amikacin as can be seen in Figure 3(b). Nearly all of the drug was released over 24 h.

To analyse the lytic components of this supernatant, standard biochemical methods were employed. A series of treatments and partial fractionations of the bacterial supernatants were performed and the resulting release activity was expressed as a percentage of the activity observed in the original samples. Results for the percentage of retained activity were as follows: heat treatment (60°C) 70%, freeze-thawing 95%, cold ethanol-soluble fraction 66%, cold acetone soluble 35%, 30 kDa filtrate 78% and 10 kDa filtrate 58%. Preliminary data also indicated that activity was retained after proteolytic treatment. A substantial portion of the activity was found to be associated with a small organic solvent soluble molecule. These results were consistent with the existence of a non-protein mediator as a major part of the observed release activity.

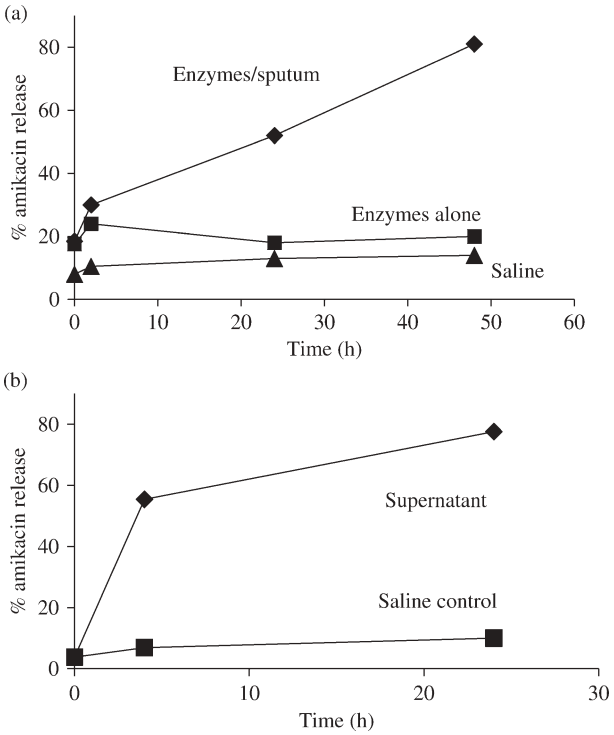

Figure 3. Release of amikacin is mediated by factors produced by biofilm-associated *Pseudomonas*. (a) Release of amikacin from liposomes in sputum. A sputum sample from a cystic fibrosis patient with a *Pseudomonas* infection (diamonds) was liquefied enzymatically and amikacin-loaded liposomes were incubated with it at 37°C. Incubation with enzymes alone in saline (squares) or with saline (triangles) alone served as controls. Release of amikacin was measured over time and is expressed as percent maximal. (b) Release of amikacin mediated by a supernatant from a mucoid *Pseudomonas* biofilm culture. Strain PA3064 was grown for 96 h as a biofilm. The culture was homogenized and clarified by sedimentation. Liposomes were incubated with the supernatant (diamonds) or saline alone (squares) at 37°C and release was monitored as shown.

Because a large portion of the activity could be extracted into organic solvent, an extract was prepared for analysis by HPLC. Three major peaks were observed on the chromatogram at retention times of 2.5 (19% peak area), 4.2 (65%) and 6.4 (16%) min. Two of the observed peaks from the supernatant extract correspond exactly to the chromatographic retention time for two major secreted biosurfactant lipids of *P. aeruginosa*, mono-rhamnolipid and di-rhamnolipid with respective retention times of 4.2 and 6.4 min. The concentration of rhamnolipids in the biofilm supernatant extract was estimated to be 43.3 and 54.5 mM for mono- and di-rhamnolipid, respectively.

These two rhamnolipid species were purified from a commercial mixture and their effect on release from the liposomes was tested. As shown in Figure 4, each of these molecules very efficiently caused release of amikacin from liposomal amikacin. As little as one rhamnolipid molecule was required for every hundred lipid molecules in the liposomes to cause release under the conditions of these experiments. Based on these results, it is clear that rhamnolipid virulence factors are particularly potent release factors and can play a major role in the targeted release of amikacin from liposomes at or near the site of infection.

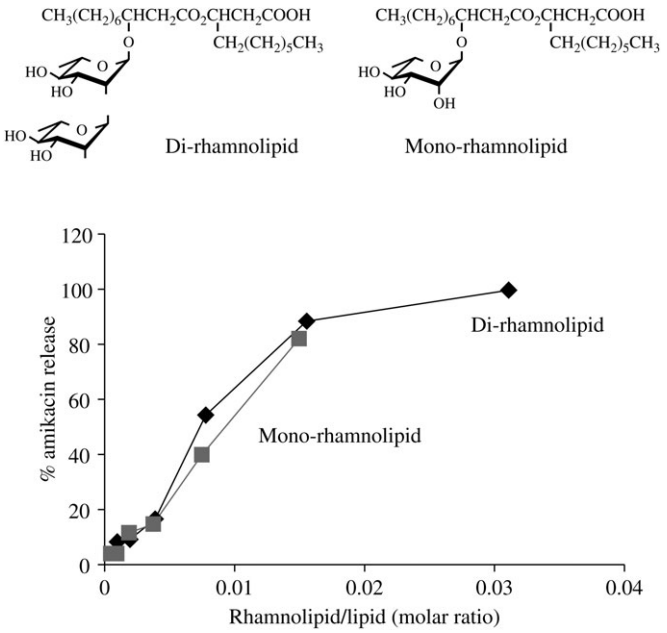

Figure 4. Identification of amikacin-releasing activity in a *Pseudomonas* biofilm extract. Effect of rhamnolipids on release of amikacin. Amikacin-loaded liposomes were incubated with the indicated amounts of rhamnolipid (di-rhamnolipid, diamonds; mono-rhamnolipid, squares) for 24 h at 37°C and the relative amount of release was measured. Top: structures of di- and mono-rhamnolipids.

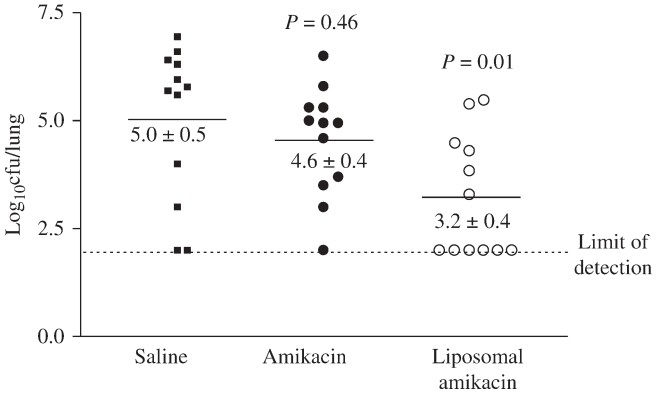

Figure 5. Efficacy of inhaled liposomal amikacin or free amikacin in a chronic lung infection model. Rats were instilled with agar beads containing live *P. aeruginosa* strain 3064. After 4 days of growth, inhaled treatments at an estimated delivered dose of 6 mg/kg were given three times per week (M,W,F) for 2 weeks (i.e. days 4, 6, 8, 11, 13 and 15). Lungs were harvested for analysis on day 16. Symbols represent $\log_{10}$ cfu value for each lung. Mean values (bars) $\pm$ SD are indicated. Because of the dilutions used, the values at 2.0 $\log_{10}$ cfu represent the lower limit of detection, indicated by the dotted line. Therefore, log cfu was counted as 2 when colonies were undetectable.

In vivo activity of inhaled liposomal amikacin

Penetration of the biofilm and the triggered release with locally high sustained concentrations of amikacin could potentially result in greater efficacy of liposomal versus free amikacin. To assess efficacy, an *in vivo* model of chronic lung infections was used.[20,21] Rats were instilled with agarose beads containing a mucoid strain of *Pseudomonas* (PA3064) developed specifically for this use. After establishment of infection, rats were treated by inhalation dosing of the nebulized drug. First, a 14 day infection model with inhalation treatment on a tri-weekly schedule (M,W,F) was performed at 6 mg/kg delivered per dose. An identical dosing of free amikacin was performed for comparison to directly assess the effect of liposomal delivery. The results, shown in Figure 5, indicated that nebulized free amikacin was relatively ineffective in the reduction of cfu under these conditions, while an equal dose of nebulized liposomal amikacin reduced cfu by two orders of magnitude in the lungs of these animals on average. In fact, bacteria were undetectable in a large proportion of the group treated with liposomal amikacin. The remaining amikacin in the liposome-treated group was determined in a parallel experiment to be 1395 ± 238 μg/lung (824 ± 135 μg/g lung tissue) at 3 days after final dosing, consistent with sustained amikacin levels in the lungs.

In a second study, the model was tested with a positive control regimen similar to the currently used inhaled aminoglycoside, i.e. twice daily dosing of free tobramycin (~3 mg/kg delivered per dose or 6 mg/kg/day total) over 14 days. This led to a reduction in $\log_{10}$ cfu from 6.3 ± 0.9 in saline control to 3.0 ± 1.4 in the treated group (Figure 6). In addition, it was verified that empty liposomes exert no effect on cfu levels when administered at the same concentration as liposomal amikacin (data not shown). It was also found that liposomal amikacin administered once daily was as effective as the twice daily treatment with tobramycin. Differences in mean $\log_{10}$ cfu values between treated animals and those receiving saline were Δ3.6 and Δ3.3 for liposomal amikacin and tobramycin, respectively. Additionally, treatment every other day with liposomal amikacin was quite effective. In fact, there was no significant difference between $\log_{10}$ cfu values for animals treated every other day with liposomal amikacin (half the cumulative dose) and those treated twice daily with tobramycin, despite the fact that

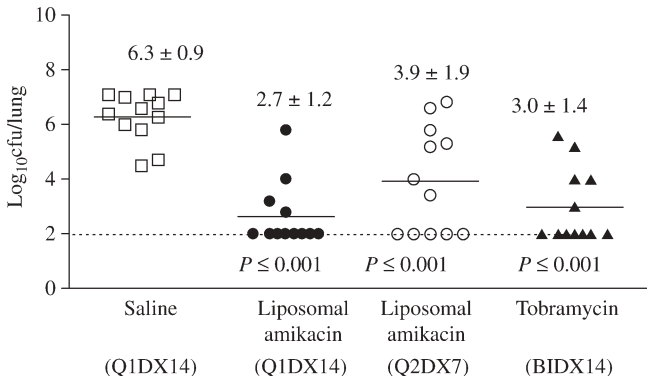

Figure 6. Efficacy of inhaled liposomal amikacin or free tobramycin in a chronic lung infection model. Symbols represent the $\log_{10}$ cfu/lungs of each rat 18 days after the instillation of beads and 3 days after the last inhalation session. Liposomal amikacin was dosed either daily (14 treatments, designated Q1D×14) or every other day (7 treatments, designated Q2D×7) at an estimated delivered dose of 6 mg/kg/day. Tobramycin was dosed twice daily (BID×14) at an estimated delivered dose of 3 mg/kg for each administration or 6 mg/kg/day total daily dose. Mean values (bars) $\pm$ SD are indicated. The dotted line represents the lower limit of detection (Figure 5 legend). Total amikacin in the lungs of animals treated daily or every other day was determined to be 1054 ± 252 and 607 ± 205 μg/lung, respectively, 3 days after the last inhalation treatment.

tobramycin has a lower MIC value than amikacin for the planktonic form of this bacterial species and strain. This may be related to the long residence time of amikacin administered in the form of liposomes, as the level was still 1054 ± 252 and 607 ± 205 μg/lung in the daily and every other day treatment groups, respectively, even though 3 days had passed since the last dose.

These results suggest that inhaled delivery of a nebulized liposomal formulation of amikacin may have significant advantages over inhalation of the free aminoglycoside. The combination of optimal liposome size, composition and loading along with locally triggered release of the drug appear to lead to a significant improvement in activity *in vivo* and may translate into clinical efficacy.

Discussion

Liposomes signify an early milestone in the history of nanoscale drug delivery systems. While most early development of liposomal technology was aimed towards their use as intravenous delivery systems, more recently the prospect of inhaled liposomes to deliver therapeutics to the lung has been explored.[23–25] The data presented here indicate that inhaled delivery of a liposomal formulation of amikacin may represent an important new approach for the treatment of bacterial infections in cystic fibrosis.

For inhalation treatment of infections, the stability of the liposomal formulation is crucial. To take advantage of the possibility of infection-targeted local release, the liposomes must be delivered to the lung predominantly intact and must not rapidly release the drug after reaching the lung. The data in Figure 1 indicate that this formulation can meet this initial requirement as evidenced by the very long-term retention of a large portion of the delivered amikacin. Although the biphasic appearance of the data is consistent with clearance of free and liposomal drug, there may be many other parameters related to the distribution, clearance and release of the drug from liposomes along with *in vivo* data variation that make a classical two exponential fit insufficient or not testable. Nonetheless, the overall picture remains consistent with remarkably stable liposomes that can mediate a sustained release in the lung.

Delivery systems specifically designed to treat cystic fibrosis infections face two major ensuing obstacles: (i) penetration into the mucus and biofilm-like colonies where much of the infection resides; and (ii) release of the drug at that site. The preceding data show that these amikacin-containing liposomes can penetrate *Pseudomonas* biofilms and may even have an enhanced concentration within biofilms.

Penetration of other types of liposomes into other types of biofilms has been previously noted. However, each type of biofilm is distinct with respect to its physicochemical characteristics as are various types of liposomes. Jones and coworkers[26,27] showed that positively and negatively charged liposomes could significantly penetrate the interior of biofilms formed by oral and skin bacteria. Several studies have also measured the penetration of positively charged liposomes into sputum or mucus.[17] In general, significant penetration is observed in the size range of hundreds of nanometers. However, positive charge appears to lead to more surface adsorption of liposomes at the expense of further penetration. In this respect, the neutral or zwitterionic lipids used to prepare the amikacin-bearing liposomes in our study preclude strong ionic interactions and may help to enhance the penetration. In fact, similar neutral liposomes appear to interact the directly with several strains of planktonic *P. aeruginosa in vitro*.[28] Therefore, these liposomes are good candidates for the delivery of antibiotic to static mucus where infections of *P. aeruginosa* reside.

The triggered release of drug mediated by bacterial virulence factors is an important aspect of the action of liposomal amikacin. Lytic enzymes and membrane-active molecules are expected to be concentrated in and near the colonies of *P. aeruginosa* growing in hypoxic biofilm-like environments, such as the situation in the static mucus in the lungs of cystic fibrosis patients. These include a bacterial haemolytic phospholipase C from *P. aeruginosa*, which is associated with the ability of this organism to form a biofilm.[29] This enzyme is found in the sputum of cystic fibrosis patients[30] and correlates with poor patient status.[31] In addition, a number of lytic factors associated with the host inflammatory response are expected to play a role in directed release, such as neutrophil phospholipase A_2 and defensins.

The experiments we have presented demonstrate the potentially important role of one of the *Pseudomonas* virulence factors, namely rhamnolipids. Rhamnolipids are associated with regulation of *Pseudomonas* biofilm architecture[32] and have been demonstrated in the sputum of cystic fibrosis patients.[33] A quorum-sensing controlled gene for a rhamnosyltransferase, *rhlA*, is induced in mature biofilms[1] and is in fact localized in the stalks of pure *Pseudomonas* biofilms grown under flow conditions.[34]

Rhamnolipids represent a remarkably potent release factor for these liposomes compared with most commonly used detergents. With release occurring at only one rhamnolipid molecule per hundreds of phospholipids, there may be a mechanism that does not involve complete dissolution of the liposomes into rhamnolipid detergent micelles.

Although it is not entirely clear how much rhamnolipid would be available *in vivo* for interaction with liposomes, a significant contribution to release of the drug is possible. Based on the recovery of ~100 μg of rhamnolipid from the 96 h culture (Figure 3), it can be estimated that each of the 10^{10} bacteria in the culture secreted enough rhamnolipid to release amikacin from ~1000 liposomes. From another perspective, rhamnolipids have been detected in the sputum of cystic fibrosis patients at levels as high as 60 mg/L, but more typically at ~1 mg/L.[33,35] This lower concentration of rhamnolipid would be sufficient to induce release of amikacin from ~0.16 mM total lipid, which is at the lower end of the range of total lipid delivered to the lungs of rats in the experiments above. Obviously, it is the local concentrations and environment that will ultimately be important, but these estimates suggest that rhamnolipid-mediated release is possible.

A sustained release of antibiotic near the infection site may be particularly effective in treating the recalcitrant *Pseudomonas* biofilms within the lungs of cystic fibrosis patients. It is well known that the highly positively charged aminoglycosides can become bound to the negatively charged polymers found in the infected mucus, such as DNA from dying cells, alginate in the biofilm matrix and mucins,[36] effectively retarding penetration of such drugs.[9] The transient nature of the inhalation dosing of small molecule drugs may preclude sufficient penetration into sites of infection before the drug is cleared away.[12,13] In

addition, the rate of uptake of aminoglycosides into *Pseudomonas* may be slower than the clearance from the lung.[37] By potentially supplying a sustained level of antibiotic near the bacteria, liposomal antibiotics may be able to overcome such a limitation.

Another major factor in the resistance of bacteria within biofilms is the establishment of a population of slowly growing or dormant cells in the oxygen-deprived interior of the biofilms. Although the access of liposomes to the interior areas of biofilms is also decreased compared with the surface, the overall density of liposomes can be quite high within the biofilm (Figure 2). A category of cells referred to as persisters has been identified in the interior of *Pseudomonas* biofilms.[38,39] Based on current knowledge, it has been calculated that a low sustained antibiotic concentration would be the best regimen to kill them.[9,40] The continued release of antibiotic from biofilm-localized liposomes can provide a sustained level of the drug near these cells. Therefore, it is possible that this subpopulation may be better addressed by liposomal antibiotics than the free drug.

The use of nebulized medication is particularly prevalent among cystic fibrosis patients. The demonstration of the effectiveness of an inhaled formulation of amikacin-containing liposomes, administered as a nebulized solution in our study, may indicate an important advance in stability, making this formulation practical for administration to patients. Ultimately, administration of amikacin in this form can lead to less frequent dosing, better efficacy through targeted delivery, lower systemic toxicity by maintaining most of the drug in the lung and potentially less resistance due to maintenance of sustained levels of antibiotic.

Conclusions

(i) Liposome stability. The particular liposomal formulation (Arikace™) of amikacin represented in this study can be administered as a nebulized solution, retaining the drug in the liposomes as demonstrated by sustained release in normal uninfected lungs.

(ii) Liposome penetration. Labelled liposomes of the same size and lipid composition as liposomal amikacin can penetrate into sputum and biofilm, suggesting the potential to reach sites of infection in the lungs of cystic fibrosis patients.

(iii) Release of drug. A possible mechanism of release of amikacin mediated by bacterial virulence factors has been identified, and rhamnolipids may play a prominent role.

(iv) Efficacy. Inhalation of nebulized liposomal amikacin in this form (Arikace™) is significantly more efficacious in the reduction of bacterial load in a chronic *Pseudomonas* infection model than an equal dose of free amikacin, possibly as a result of targeted release mediated by factors associated with the bacterial infection.

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Transparency declarations

All authors are or were employees of Transave, Inc. except those at Montana State University. Transave, Inc. is developing liposomal amikacin (Arikace™) as a commercial therapeutic and employees are issued stock options as part of compensation. The Center for Biofilm Engineering at Montana State University performed research under contract to Transave, Inc. and its employees have no other financial connection to Transave, Inc.

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