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Efficacy of Liposomal Bismuth-Ethanedithiol-Loaded Tobramycin after Intratracheal Administration in Rats with Pulmonary *Pseudomonas aeruginosa* Infection

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We sought to investigate alterations in quorum-sensing signal molecule *N*-acyl homoserine lactone secretion and in the release of *Pseudomonas aeruginosa* virulence factors, as well as the *in vivo* antimicrobial activity of bismuth-ethanedithiol incorporated into a liposome-loaded tobramycin formulation (LipoBiEDT-TOB) administered to rats chronically infected with *P. aeruginosa*. The quorum-sensing signal molecule *N*-acyl homoserine lactone was monitored by using a biosensor organism. *P. aeruginosa* virulence factors were assessed spectrophotometrically. An agar beads model of chronic *Pseudomonas* lung infection in rats was used to evaluate the efficacy of the liposomal formulation in the reduction of bacterial count. The levels of active tobramycin in the lungs and the kidneys were evaluated by microbiological assay. LipoBiEDT-TOB was effective in disrupting both quorum-sensing signal molecules *N*-3-oxo-dodecanoylhomoserine lactone and *N*-butanoylhomoserine lactone, as well as significantly ($P < 0.05$) reducing lipase, chitinase, and protease production. At 24 h after 3 treatments, the CFU counts in lungs of animals treated with LipoBiEDT-TOB were of $3 \log_{10}$ CFU/lung, compared to 7.4 and $4.7 \log_{10}$ CFU/lung, respectively, in untreated lungs and in lungs treated with free antibiotic. The antibiotic concentration after the last dose of LipoBiEDT-TOB was $25.1 \mu\text{g/lung}$, while no tobramycin was detected in the kidneys. As for the free antibiotic, we found $6.5 \mu\text{g/kidney}$ but could not detect any tobramycin in the lungs. Taken together, LipoBiEDT-TOB reduced the production of quorum-sensing molecules and virulence factors and could highly improve the management of chronic pulmonary infection in cystic fibrosis patients.

Cystic fibrosis (CF) is an autosomal recessive genetic disease caused by mutation in a cystic fibrosis transmembrane regulator (CFTR) gene that affects organs and systems, including the lungs, the pancreas, the endocrine system, and the gastrointestinal tract (1). Pulmonary injury is the most challenging medical problem and is responsible for the majority of morbidity and mortality in the CF population (2). There are more than 1,500 mutations in CFTR genes, causing different degrees of disease severity. The mutation in CFTR caused by deletion of phenylalanine at position 508, known as ΔF508 , is more common and causes severe disease due to nonfunctional chloride ion channels (3). Normal lung epithelial cells keep the epithelial lining fluid of the airways hydrated to ensure appropriate mucociliary clearance of allergens or microbes from the airways (4). Hydration of the mucosal surface of epithelial cells is linked osmotically to sodium transport and chloride secretion. The mutations in CFTR lead to dysfunctional or compromised chloride ion channels as well as hyperabsorption of sodium through sodium channels (ENaC). The resultant thick sticky mucus (5, 6) provides a suitable growth environment for bacteria, such as *Staphylococcus aureus*, *Haemophilus influenzae*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* (7–9). Recurrent *P. aeruginosa*-induced pulmonary infection and inflammation is more common and is associated with reduced lung function and disease exacerbation (10, 11).

P. aeruginosa is a Gram-negative opportunist human pathogen found in various environments, such as fresh water, plants, sinks, hand soaps, and hospitals (12, 13). *P. aeruginosa* cells interact with specific host cell receptors through appendices, such as type IV pili, which recognize the overexpressed asialoganglioside (GM1) in CF epithelial cells, and its monotrichous flagellum binds specifically to secreted respiratory mucins (14–16). *P. aeruginosa* utilizes mucus as a shield against the host immune system and regu-

lates its cell density, virulence factor production, and biofilm formation through quorum-sensing (QS) signaling (17–20). The pathogen carries two homologues that control the QS system: *lasI/lasR* and *rhlI/rhlR* (21). The autoinducer proteins are responsible for synthesizing specific signal molecules. LasI and RhlI synthesize *N*-3-oxo-dodecanoylhomoserine lactone (3O-C₁₂-HSL) and *N*-butanoylhomoserine lactone (C₄-HSL), respectively, whereas, LasR and RhlR function as transcriptional activator proteins (22–24). Bacteria release of 3O-C₁₂-HSL occurs at a certain cell density into the external environment, where it binds to LasR, forming a complex that binds promoters to induce a wide variety of virulence factors, including lipase, chitinase, and proteases (25–29). Activation of airway epithelial cell signaling pathways in response to *P. aeruginosa* pulmonary infection results in gene expression and secretion of several cytokines and chemokines, including interleukin-8 (IL-8), a potent chemoattractant of neutrophils (30). While neutrophils eradicate bacteria, their toxic products, such as elastase and reactive oxygen radicals in the airway, damage the lungs tissue as well (31).

Aggressive chemotherapy, through various routes, has been utilized to decrease the persistence of *P. aeruginosa* in lungs (32, 33). Administration of aminoglycosides, such as tobramycin, along with β -lactams, is usually prescribed against *P. aeruginosa* to

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reduce infection (34, 35). Tobramycin at a subinhibitory concentration reduces production of *P. aeruginosa* virulence factors at the translation level by inhibiting the release of C₄-HSL and 3O-C₁₂-HSL levels (36, 37). However, since a high dosage and prolonged use of tobramycin are required to eradicate bacteria, a high risk of ototoxicity and nephrotoxicity exists (34). Furthermore, the presence of mucus, overexpression of multidrug efflux pumps, and a bacterial transition to the biofilm form result in a poor prognosis (38–41).

Bismuth subsalicylate and bismuth subcitrate have been used for years to treat gastrointestinal disorders associated with *Helicobacter pylori* (42). A combination of bismuth and thiol agents increases the bismuth solubility, lipophilicity, and its antimicrobial activity against Gram-positive and Gram-negative bacteria (43). Huang and Stewart showed that bismuth dimercaprol was able to reduce biofilm formation by *P. aeruginosa* (44). Bismuth ethanedithiol (BiEDT) along with tobramycin have a synergistic effect against *P. aeruginosa* and *Burkholderia cepacia* *in vitro* (45, 46). The cytotoxic effects of bismuth, however, limit its utility. BiEDT at concentrations of 10 and 20 μ M render human lung cells in culture nonviable (47). Microcarriers, such as liposomes, are used to overcome toxicities of the drugs, to sustain the release of drugs at the target site, and to prolong their residence time (48, 49).

Liposomes are small lipid vesicles with sizes ranging from nanometers to micrometers. They are generally a safe delivery system, since liposomes are biocompatible and biodegradable. They consist of phospholipid bilayers with an aqueous core. Hydrophilic drugs can be encapsulated in the aqueous core, whereas, lipophilic drugs can be incorporated into the bilayers. Recently, more research has focused on utilizing liposomes to deliver therapeutic molecules to target sites, including the lungs (50). Liposomes are preferred for antibiotic delivery because they provide a sustained release of the drugs and reduce side effects, as well as increasing the bioavailability of insoluble hydrophobic drugs (50, 51). Previous studies in our laboratory showed that coencapsulation of BiEDT with tobramycin in liposomes resulted in elimination of the BiEDT toxic effect on human lung cells while increasing its antibacterial efficacies against *P. aeruginosa* and *B. cepacia* (52, 53).

The current study was performed to test whether liposomal BiEDT-loaded tobramycin (LipoBiEDT-TOB) at subinhibitory concentrations is able to reduce production of virulence factors and QS signal molecules by *P. aeruginosa* *in vitro* and to enhance the antimicrobial efficacy as well as to examine anti-inflammatory effect of LipoBiEDT-TOB in an animal model of chronic pulmonary infection with the aforementioned bacteria.

MATERIALS AND METHODS

Chemicals and media. 1,2-Distearoyl-*sn*-glycero-3-phosphocholine (DSPC) was purchased from Northern Lipids (Vancouver, BC, Canada). Cholesterol, bismuth nitrate [Bi (NO₃)₃ · 5H₂O], EDT, propylene glycol (PG), heavy mineral oil, xylazine, saline, chitin azure, β -D-galactopyranoside, and Triton X-100 were obtained from Sigma-Aldrich (Oakville, ON, Canada). Sodium hydroxide (NaOH), sodium deoxycholic acid (C₂₄H₃₉O₄Na), tobramycin, chloroform, and methanol were purchased from Fisher Scientific (Ottawa, ON, Canada). Tryptic soy agar, tryptic soy broth, Luria-Bertani (LB) broth, Luria-Bertani agar, and Mueller-Hinton agar were purchased from Becton, Dickinson Microbiology Systems (Oakville, ON, Canada). Ketamine was obtained from Animal Health Inc. (Cambridge, ON, Canada).

Bacterial strains. PA-489122 strains of *P. aeruginosa* were used throughout the experiment and had been isolated from CF patients at Sudbury Regional Hospital (Sudbury, Ontario, Canada). *Staphylococcus aureus* ATCC 29213 was used as an indicator of tobramycin activity, as recommended by the Clinical and Laboratory Standards Institute (CLSI). All strains were stored in Mueller-Hinton broth at –80°C supplemented with 10% glycerol. All strains were grown for 18 h in ABt medium [27 mM (NH₄)₂SO₄, 30 mM Na₂HPO₄ · 2H₂O, 20 mM KH₂PO₄, 47 mM NaCl, 1 mM MgCl₂, 0.1 mM CaCl₂, 0.01 mM FeCl₂, 0.5% (wt/vol) glucose, 0.5% (wt/vol) Casamino Acids, and 0.00025% (wt/vol) thiamine] broth prior to the MIC, QS, and virulence factor experiments. *Agrobacterium tumefaciens* strain A136(pCF218)(pCF372) (Ti[–]) was used as the biosensor for the detection of *N*-acyl-homoserine lactone (AHL) and cultured in LB broth at 30°C.

LipoBiEDT-TOB preparation. A dehydration-rehydration method was used to prepare liposomal bismuth tobramycin. To prepare BiEDT, Bi(NO₃)₃ and 600 mM NaOH were dissolved first in 25 ml of methanol. One milliliter of EDT was then added to the mixture. To prepare the liposome vesicles, DSPC and cholesterol (2:1 molar ratio) were transferred into a round flask and dissolved in 19 ml of chloroform-methanol (2:1 molar ratio). One milliliter of ethanedithiol bismuth was then added to the round flask. The organic solvents were removed by using rotary vapor (Buchi Rotavapor R205 and Buchi vacuum controller V-800; Brinkman, Toronto, Ontario, Canada) under vacuum at 55°C, which resulted in a thin layer of lipid. The lipid film was rehydrated by adding 12 ml of phosphate-buffered saline (PBS) with hand shaking for 5 to 7 min in a water bath at 55°C until the mixture became a suspension. The suspension lipids were then sonicated at an amplitude of 50% (Sonic Dismembrator model 500; Fisher Scientific, Ottawa, ON, Canada) for 10 min (40 s on and 5 s off). Tobramycin (8 mg/ml) and PG were added to the sonicated suspension. The solution was then sonicated again for 10 min (40 s on and 5 s off). The sonicated liposomes were transferred to 15-ml tubes and frozen for 15 min at –70°C and then freeze-dried overnight (Freeze Dry system model 77540; Labconco Corporation, Kansas City, MO). The powdered liposomes were stored at 0°C. To rehydrate the powder formulation, sterile distilled water was added at 10% of the volume before lyophilization, vortexed, and then the mixture was incubated for 30 min at 45°C; then PBS was added to restore the original volume. The solution was centrifuged (Beckman L8-M ultracentrifuge) for 20 min at 100,000 × *g* and 4°C, and the supernatant was removed. This step was repeated with PBS as described previously (52). The sizes of liposomes were determined by using a submicron particle sizer (model 270; Nicomp, Santa Barbara, CA).

Tobramycin encapsulation efficiency of the LipoBiEDT-TOB formulation. The concentrations of tobramycin incorporated into LipoBiEDT were measured in an agar diffusion assay using a laboratory strain of *S. aureus* (ATCC 29213) as an indicator organism for tobramycin. We used an overnight culture of the organism in cation-adjusted Mueller-Hinton broth (CAMH) to prepare a bacterial solution equivalent to 0.5 McFarland (1.5 × 10⁸ bacteria/ml). The bacterial suspension in warm (45°C) Muller-Hinton agar was then poured onto a sterile glass plate (460 mm by 360 mm) and left to solidify at room temperature. Wells of 5-mm diameter were made with a well puncher. Standard curves of diluted tobramycin as well as samples of LipoBiEDT-TOB were prepared. Triplicate samples (25 μ l) were transferred into the agar plate holes. The plate was covered and incubated for 18 h at 37°C. We then measured the inhibition zones, and the averages of triplicate measures were used in data analysis. The standard curve was utilized to calculate concentrations of the entrapped tobramycin that were released from the liposomes by 0.2% (vol/vol) Triton X-100 (with PBS). The sensitivity of the assay was 0.75 μ g/ml. The quantifiable limit for tobramycin was 0.75 μ g/ml. At concentrations between 0.75 and 12.5 μ g/ml, the coefficients of variation ranged between 1.2 and 2.9%. Over the same concentrations, the intraday coefficients of variation ranged between 2.2 and 3.5%. For 10 samples of spiked tobramycin, the standard curve linearity extended over the range 0.75 to 12.5

µg/ml and gave a correlation coefficient greater than 0.999. Concentration measurements were the means of at least three independent experiments, with each experiment measured in triplicate.

(i) Encapsulation efficiency. The drug encapsulation efficiency (expressed as a percentage) was calculated by dividing the concentration of LipoBiEDT-TOB (determined by the microbiological assay as described in the previous paragraph) by the concentration of free tobramycin used in the original preparation of these liposomes.

Determinations of MICs. The broth microdilution method was used to determine the MICs for tobramycin. Briefly, the reference strain *S. aureus* or clinical isolates of *P. aeruginosa* PA-489122 were exposed to different dilutions of LipoBiEDT-TOB or a combination of tobramycin with BiEDT. The contribution of bismuth ethanedithiol to the MICs was assessed by exposing the aforementioned bacterial strains to different concentrations of BiEDT-TOB and LipoBiEDT-TOB, with a starting concentration of 128 mg/liter for tobramycin as well as 128 µM for BiEDT in the LipoBiEDT-TOB and free BiEDT-TOB as reported previously (52). Drug-free bacterial cultures and an ABt broth medium alone were used as positive and negative controls, respectively.

Quantification of bismuth in liposomal formulations. The bismuth content within the LipoBiEDT-TOB formulation was measured by graphite furnace atomic absorption spectroscopy (GFAAS) as described previously, with some modifications (52). To simplify, samples were lyophilized, weighed, and then transferred into Teflon digestion vessels. Total volumes of 1 ml H₂O₂ (30%, wt/wt) and 4 ml HNO₃ were added, and the samples were digested overnight at 25°C. Samples were then subjected to hot plate digestion in a glycerol bath at 135 to 140°C for 3 h and left overnight. Next, the volumes were adjusted to 25 ml with double-distilled water. A 1.25-ml aliquot from each digested sample was then subjected to 20-fold dilution with 2% HNO₃. Samples were then analyzed by GFAAS (AAAnalyst 600; PerkinElmer Precisely, Woodbridge, ON, Canada).

Evaluation of QS and virulence factor production and activity. PA-489122 was grown in ABt medium for 18 h at 37°C; then, the bacterial solution was adjusted to 0.5 McFarland standard (optical density at 600 nm [OD₆₀₀], 0.13) in a 100-ml flask and incubated for 1 h at 37°C for experiments involving QS signal molecules, lipase, chitinase, and protease. When the bacterial concentration doubled to an OD₆₀₀ of 0.26, the solutions were exposed to an equal volume of free or liposomal BiEDT-TOB (1/16 to 1/2 the MIC). Untreated *P. aeruginosa* PA-489122 served as a control. After 24 h, bacterial cultures were measured, then centrifuged at 16,000 × g for 15 min at 4°C, and filter sterilized (0.22 µm). To test that there were no killing effects of the antibiotic on bacteria at concentrations below the MICs, free or liposomal BiEDT-TOB (1/4 and 1/2 the MIC) was introduced to a PA-489122 culture that had been adjusted to 0.5 McFarland standard in 100-ml flasks and incubated at 37°C with agitation (250 rpm). The growth was monitored (OD₆₀₀) for 8 h.

Bioassay for AHL production. Supernatant samples were screened for AHL production as described previously with some modifications (54). *A. tumefaciens* strain A136(pCF218)(pCF372) (Ti⁻) cells equal to a density of 10⁶ CFU/ml with β-D-galactopyranoside (20 mg/ml in dimethylformamide) and LB agar at 45°C were poured into petri dishes. Wells of 5-mm diameter were made with a well puncher, and aliquots (80 µl) from control or treated supernatant samples were transferred to the wells. The petri dishes were incubated for 48 h at 30°C. AHL production levels were confirmed based on blue pigmentation around the wells.

β-Galactosidase activity assay. The level of AHL production from *P. aeruginosa* exposed to free or LipoBiEDT-TOB at subinhibitory concentrations was examined by measuring the ability of *P. aeruginosa* AHL signaling molecules released into the supernatants to activate the production of β-galactosidase in the reporter strain *A. tumefaciens* (A136) as described previously (55). Briefly, bioassay tubes containing 4 ml of reporter strain and 1 ml of supernatant were incubated at 30°C in a water bath for 5 h with rotation at 100 rpm. Next, the bacterial cell density was measured (as the OD₆₀₀) before centrifugation. The supernatants were removed, and the pellets were suspended in an equal volume of Z buffer

(0.06 M Na₂HPO₄ · 7H₂O, 0.04 M NaH₂PO₄ · H₂O, 0.01 M KCl, 0.001 M MgSO₄ · 7H₂O, 0.05 M β-mercaptoethanol; pH 7.0). The cells were then permeabilized by a solution of 200 µl of chloroform and 100 µl of 0.1% sodium dodecyl sulfate prior to the addition of 0.4 ml of *o*-nitrophenol-β-D-galactopyranoside (4 mg/ml in PBS). After the development of yellow color, 1 ml of 1 M Na₂CO₃ was added to stop the reaction. The optical densities of the reaction samples were measured at 420 and 550 nm. Miller units of β-galactosidase activity were calculated as [(1,000 × A₄₂₀) - (1.75 × A₅₅₀)]/(time × volume × A₆₀₀), as described previously (56).

Virulence factor assays. Lipase activity was evaluated using Tween 20 as the substrate. Briefly, the reaction mixture consisted of 0.6 ml of 10% Tween 20 in Tris buffer, 0.1 ml of 1 M CaCl₂, 0.6 ml of filtered supernatant, and 1.6 ml of double-distilled water. Samples were incubated at 37°C for 24 h with agitation (200 rpm). In the presence of lipase, Tween 20 is broken down to a fatty acid and alcohol. The fatty acid binds calcium to form a precipitate that was measured as the OD₄₀₀. For chitinase, 1 ml of filtered supernatant was mixed with 1 ml of PBS and 5 mg of insoluble chitin azure. The reaction mixture was incubated at 37°C for 24 h with agitation (200 rpm). The cleaving of chitin azure in the presence of chitinase results in the release of a blue dye that can be measured spectrophotometrically as the OD₂₉₀. The lipase and chitinase experiments were repeated three times with three replicates, and the results were normalized by dividing the optical density by cell density (OD₆₀₀). For the protease assay, 100 µl of filtered supernatant was transferred into the wells of ABt medium containing 2% agar and 2% skim milk. Plates were incubated for 48 h at 37°C. Zones of clearance due to the proteolytic activity of protease could be easily perceived and were measured (in millimeters) by using digital calipers. The experiments were repeated three times with three replicates.

Preparation of agar beads. Agar beads were prepared as described previously with some modifications (57). The *P. aeruginosa* PA-489122 strain was grown overnight at 37°C in tryptic soy broth. The bacteria were then embedded into agar beads by mixing 2% (vol/vol) of the aforementioned strain with tryptic soy agar and mineral oil (1:3 volume ratio) at 45°C. The mixture was then vortexed vigorously and cooled down by placing crushed ice around the vessel while stirring continuously for 5 min. Next, the mineral oil was removed by centrifugation at slow speed for 5 min, 500 × g, at 4°C. Agar beads were washed once with 0.5% sodium deoxycholic acid, once with 0.25% sodium deoxycholic acid, and three times in PBS for 20 min, 1,000 × g, at 4°C. The number of bacteria was determined after homogenizing the bacteria-impregnated bead suspension. The bacterial count was ascertained by working with 10-fold serial dilutions in PBS on Mueller-Hinton agar plates as described previously (58).

Experimental infections and LipoBiEDT-TOB treatment. Fifteen Sprague-Dawley rats weighing 201 to 225 g (Charles River, Saint Constant, Quebec, Canada) were used in this study. The animals were housed (Nalgene cages) in groups of three for 1 week before any experiment was undertaken and allowed free access to food and water. Animals were kept at room temperature and were exposed to alternate cycles of 12 h of light and darkness. Animals used in this study were treated and cared for in accord with the guidelines recommended by the Canadian Council on Animal Care and the Association for Assessment and Accreditation of Laboratory Animal Care. The experimental protocol was approved by the Institutional Animal Care and Use Committee.

To mimic the chronic respiratory tract infection caused by *P. aeruginosa*, PA-489122 was incorporated into agar beads. Animals were anesthetized with a mixture of 70 mg/kg of body weight of ketamine hydrochloride and 7 mg/kg of xylazine by intraperitoneal injection before infection and placed in the supine position, and the upper jaw was attached to the operating table with a rubber band brought over the incisor teeth. Using a laryngoscope, the tongue was moved aside and the mouth was opened. The larynx was identified and distinguished by its opening and closing as the rat breathed. A catheter was inserted between the vocal cords and pushed gently forward into the trachea. The catheter's insertion

was confirmed by the formation of water condensation on a cold mirror with each breath of the rat. The rats were then inoculated with 50 μ l of agar beads containing 10^6 CFU of *P. aeruginosa* at the bifurcation of the trachea with a 1-ml tuberculin syringe, followed by a bolus of air to ensure complete delivery.

Four days after the inoculation with agar beads, the rats were anesthetized using the same procedure as above to be treated with antibiotics. The rats were sorted into three groups, and each group was administered either saline, BiEDT-TOB, or LipoBiEDT-TOB for 3 days. Infected animals received a dose (same method as described for infection) of either 300 mg/liter of LipoBiEDT-TOB per kg or 300 mg/liter of BiEDT-TOB per kg. The concentration of tobramycin in free or LipoBiEDT-TOB was 300 mg/liter, and the BiEDT concentration in liposomal preparations as well as the combination with tobramycin was 300 μ M. Saline (90 μ l) was administered to the infected control animals. At 24 h after the last treatment, the animals were euthanized by using CO₂. The kidneys and the lungs were removed aseptically and homogenized in cold sterile PBS (33% [wt/vol]) for 40 s with a Polytron homogenizer. The homogenizer was rinsed, immersed in 95% ethanol, flamed, and then cooled with cold saline between samples. Lung bacterial counts were performed after homogenizing the lungs. Serial 10-fold dilutions of the homogenates in cold PBS were made, and 0.1 ml of each dilution was pipetted and spread onto Mueller-Hinton agar. The experiment was done in triplicate, and the bacterial counts for each animal were done in triplicate. CFU were counted after 24-h incubations at 37°C, and counts are expressed in log₁₀ CFU per pair of lungs. To measure the quantity of active tobramycin in tissues, the tissue samples were concentrated as follows: 1-ml samples of homogenized lungs or kidneys were lyophilized (Freeze Dry system model 77540; Labconco Corporation, Kansas City, MO) and rehydrated with 100 μ l of sterile PBS. The presence of active tobramycin was detected by an agar diffusion assay as described above.

IL-8 assay. Supernatants from sera and the lung homogenate samples were used to quantify secreted IL-8 protein. A 96-well plate was precoated with IL-8 capture antibody (primary antibody) overnight. A wash buffer, consisting of 1× PBS and Debecos buffered salts, was used between each step to rinse excess reagents from the treatment plate according to the manufacturer's protocol (BioLegend, San Diego, CA). Next, the protein blocking agent was added to each well of the 96-well plate. The blocking agent was allowed to sit in the wells for 1 h while on a shaker (200 rpm) at room temperature. The assay diluent was removed from plates with wash buffer. A standard curve was made with a 1:2 serial dilution of known IL-8 antigen. Supernatants were spun in a microcentrifuge for 10 min, 106 × g, 22°C. The supernatants were added to the wells for 2 h while shaking. The plates were washed before adding the detection antibody for 1 h on a shaker. Detection antibody was washed, and avidin-horseradish peroxidase (HRP) was added to the wells for 30 min while shaking in the dark. The Avidin-HRP was washed with wash buffer, and tetramethylbenzidine substrate solution C was added for 15 min in the dark without shaking. A 2 N H₂SO₄ solution was added to the mixture to stop the reaction. The plate was read with a Beckman Coulter AD 340 microplate reader (Beckman, Brea, CA). Data were normalized, and IL-8 concentrations are reported in pg/ml.

Data analyses. The data are presented as means $\pm$ standard errors of the means (SEM) of three independent experiments. Comparisons of groups were made by one-way analysis of variance (ANOVA) using InStat 3 from GraphPad Software (version 5.0) followed by a post-*t* test. Probability (*P*) values of <0.05, <0.01, and <0.001 are reported as statistically significant.

RESULTS

LipoBiEDT-TOB characterization. The average size of the LipoBiEDT-TOB formulation was 907.3 ± 40.1 nm, and the encapsulated tobramycin in the LipoBiEDT formulation was 1.0 ± 0.2 mg/ml. The percentage of tobramycin that was encapsulated into liposomes was $14.40 \pm 0.001\%$. Atomic absorption analysis

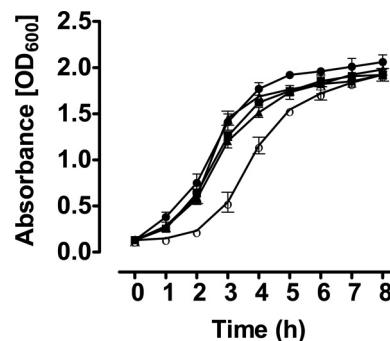


FIG 1 Effects of subinhibitory concentrations of BiEDT-TOB on growth of PA-489122. The graph shows growth curves of *P. aeruginosa* without antibiotics (filled circles), in the presence of subinhibitory concentrations of 1/2 the MIC of LipoBiEDT-TOB (0.5 mg/liter of TOB and 0.5 μ M BiEDT; open circles), 1/4 the MIC of LipoBiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT; filled triangles), 1/2 the MIC of free BiEDT-TOB (2 mg/liter of TOB and 2 μ M BiEDT; filled squares), or 1/4 the MIC of free BiEDT-TOB (1 mg/liter of TOB and 1 μ M BiEDT; open triangles).

showed that the concentration of bismuth incorporated into the LipoBiEDT-TOB formulation was 1.0 ± 0.3 mM.

Antimicrobial activities of free and LipoBiEDT-TOB. The MIC of the LipoBiEDT-TOB formulation against the *P. aeruginosa* strain used in this study was 16-fold lower than tobramycin alone and 4-fold lower than tobramycin in combination with BiEDT. For example, the MIC of tobramycin alone was 16 mg/liter, whereas for BiEDT-TOB the MIC was 4 mg/liter for tobramycin and 4 μ M for BiEDT in free BiEDT-TOB, compared to 1 mg/liter for tobramycin and 1 μ M for BiEDT in LipoBiEDT-TOB.

Effects of subinhibitory concentrations of free or LipoBiEDT-TOB. The effects of concentrations (1/4 to 1/2 the MICs) of free BiEDT or LipoBiEDT-TOB on bacterial growth are shown in Fig. 1. The rate of growth of cells treated with 1/2 the MIC of the liposomal formulation was inhibited; therefore, the formulation at that concentration was not considered subinhibitory in further investigations. Thus, all the experiments that involved QS and virulence factors were done using concentrations of 1/16 to 1/4 the MICs of free or LipoBiEDT-TOB.

QS molecules reductions. *P. aeruginosa* PA-489122 was grown in AbT medium for 24 h at 37°C with or without free or liposomal BiEDT-TOB at 1/16 to 1/4 the MICs. Both formulas had reduced AHL production up to 1/16 the MIC compared to the control, but they did not prevent AHL production completely (Fig. 2). However, LipoBiEDT-TOB reduced AHL production at subinhibitory concentrations that were 4 times lower than for free BiEDT-TOB. At 1/4 the MICs of free BiEDT-TOB, the production of the blue pigment ring around the edge was darker and more clear than with 1/4 the MIC of LipoBiEDT-TOB.

AHL quantification. The levels of β -galactosidase activity in response to AHL indicated decreasing levels of AHL signaling molecules released from *P. aeruginosa* cells exposed to LipoBiEDT-TOB (Fig. 3a and b). For instance, free BiEDT-TOB at 1/8 the MIC did not reduce the level of AHL significantly, whereas LipoBiEDT-TOB significantly reduced the level of AHL, based on β -galactosidase activity, at 1/8 the MIC ($P < 0.01$) compared to the control. LipoBiEDT-TOB was significantly more active in reducing AHL production than free BiEDT-TOB at 1/4 the MIC ($P < 0.001$).

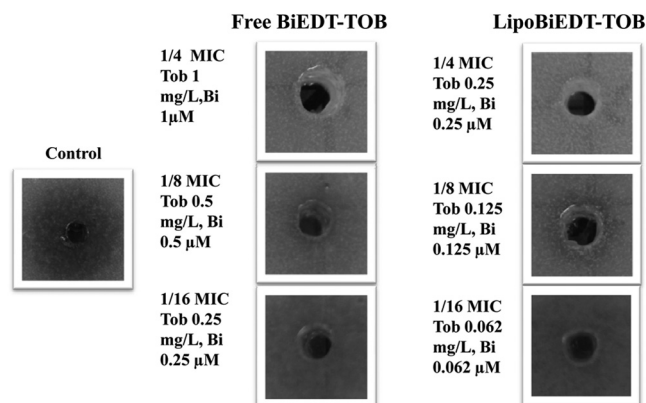


FIG 2 Effects on QS of subinhibitory concentrations of free or LipoBiEDT-TOB (1/16 to 1/4 the MIC). LB agar containing *Agrobacterium tumefaciens* and β -D-galactopyranoside was poured into petri dishes. Holes were made in the agar by using a vacuum device, and 80- μ L aliquots from control or treated supernatant samples were transferred to the wells. The plates were incubated for 48 h at 30°C.

Reduction of virulence factors by BiEDT-TOB. We compared the effects of free and liposomal BiEDT-TOB at 1/16 to 1/4 the MICs on production of the virulence factors lipase, chitinase, and protease by PA-489122. For the lipase assay, free BiEDT-TOB at 1/4 the MIC did not reduce the production of lipase significantly compared to the control (Fig. 4a). LipoBiEDT-TOB at 1/4 the MIC attenuated lipase production significantly compared to the

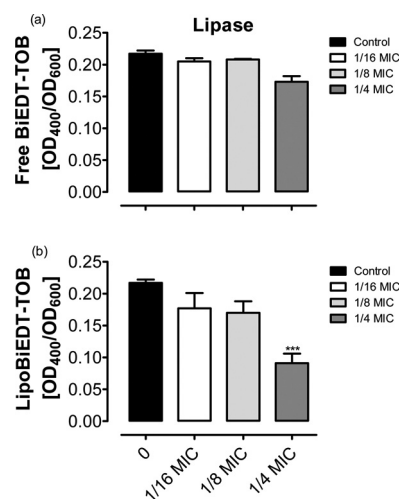


FIG 4 Lipase activities in supernatants from PA-489122 cultures. Cultures grown either without antibiotics, as a control, or in the presence of 1/4 the MIC of free BiEDT-TOB (1 mg/liter of TOB and 1 μ M BiEDT), 1/8 the MIC of free BiEDT-TOB (0.5 mg/liter of TOB and 0.5 μ M BiEDT), or 1/16 the MIC of free BiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT) (a), or in the presence of 1/4 the MIC of LipoBiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT), 1/8 the MIC of LipoBiEDT-TOB (0.125 mg/liter of TOB and 0.125 μ M BiEDT), 1/16 the MIC of LipoBiEDT-TOB (0.062 mg/liter of TOB and 0.062 μ M BiEDT) (b). Each bar represents the mean $\pm$ SEM of three independent experiments. ***, $P < 0.001$ compared with control.

control ($P < 0.001$) (Fig. 4b). Chitinase production in the supernatants was evaluated by quantifying the breakdown of chitin azure. As shown in Fig. 5a and b, the liposomal formulation was able to reduce chitinase at a concentration 8 times lower than with

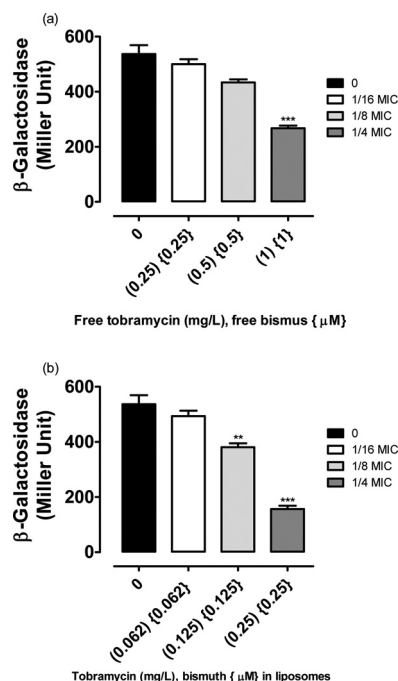


FIG 3 Production of QS molecules, as measured by β -galactosidase activity either in the presence of free BiEDT-TOB (a) or in the presence of LipoBiEDT-TOB (b) at 1/16 to 1/4 the MICs. *P. aeruginosa* was exposed to free or LipoBiEDT-TOB, and then the supernatants were collected and incubated with *A. tumefaciens* (A136). β -Galactosidase activities were measured in Miller units. Each bar represents the mean $\pm$ SEM of three independent experiments. P values were considered significant compared with the control and between groups, as follows: ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$.

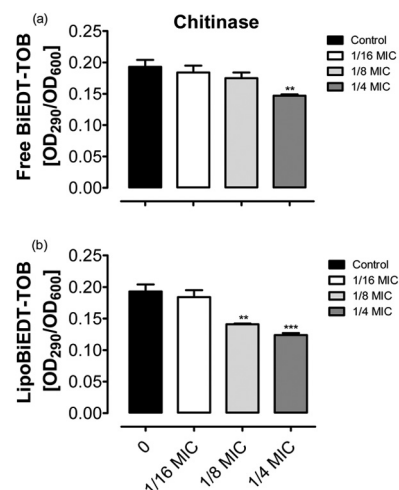


FIG 5 Chitinase activities in supernatants from PA-489122 cultures. Cultures grown either without antibiotics, as a control, or in the presence of 1/4 the MIC of free BiEDT-TOB (1 mg/liter of TOB and 1 μ M BiEDT), 1/8 the MIC of free BiEDT-TOB (0.5 mg/liter of TOB and 0.5 μ M BiEDT), or 1/16 the MIC of free BiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT) (a) or in the presence of 1/4 the MIC of LipoBiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT), 1/8 the MIC of LipoBiEDT-TOB (0.125 mg/liter of TOB and 0.125 μ M BiEDT), 1/16 the MIC of LipoBiEDT-TOB (0.062 mg/liter of TOB and 0.062 μ M BiEDT) (b). Each bar represents the mean $\pm$ SEM of three independent experiments. ***, $P < 0.001$; **, $P < 0.01$ compared with the control and between groups.

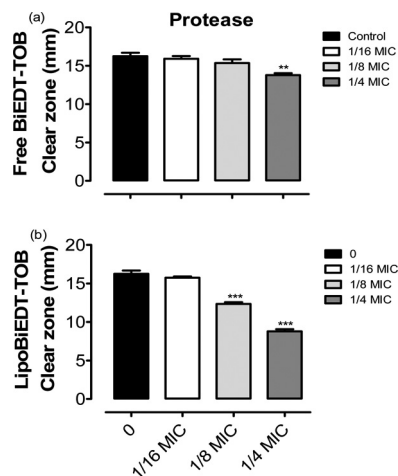


FIG 6 Protease activities in supernatants from PA-489122 cultures. Cultures were grown either without antibiotics, as a control, or in the presence of 1/4 the MIC of free BiEDT-TOB (1 mg/liter of TOB and 1 μ M BiEDT), 1/8 the MIC of free BiEDT-TOB (0.5 mg/liter of TOB and 0.5 μ M BiEDT), or 1/16 the MIC of free BiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT) (a) or in the presence of 1/4 the MIC of LipoBiEDT-TOB (0.25 mg/liter of TOB and 0.25 μ M BiEDT), 1/8 the MIC of LipoBiEDT-TOB (0.125 mg/liter of TOB and 0.125 μ M BiEDT), 1/16 the MIC of LipoBiEDT-TOB (0.062 mg/liter of TOB and 0.062 μ M BiEDT) (b). Each bar represents the mean $\pm$ SEM of three independent experiments. ***, $P < 0.001$; **, $P < 0.01$ for comparisons with the control and between groups.

free BiEDT-TOB (1/8 versus 1/4) and more effectively ($P < 0.01$) than the free formulations. The activity of extracellular protease LasA in filtered sterilized supernatants was measured in agar plates containing 2% skim milk. Free BiEDT-TOB reduced the protease level at 1/4 the MIC ($P < 0.01$) compared to the control, whereas LipoBiEDT-TOB attenuated activity significantly at 1/4 the MIC ($P < 0.001$). Furthermore, protease activity was reduced by LipoBiEDT-TOB at a concentration 8-fold lower than the free formulation (1/8 versus 1/4) ($P < 0.001$), as indicated in Fig. 6a and b.

LipoBiEDT-TOB or BiEDT-TOB activity against infected rat lungs. The number of bacteria loaded on agar beads was $8.38 \pm 0.09 \log_{10}$ CFU/ml. We instilled a total of 10^6 CFU in a 50- μ l volume in the lungs of each rat. The number of CFU enumerated following 24 h of the last treatment with saline was $7.36 \pm 0.17 \log_{10}$ CFU/lung. The bacterial load in the lungs of the rats after three doses of 300 μ g tobramycin or 300 μ M BiEDT in free or liposomal formula was significantly lower ($P < 0.001$) than the control (Fig. 7). The effect of the liposomal formulation in lowering bacterial load was significantly higher than that of the free formulation ($3.06 \pm 0.13 \log_{10}$ CFU/lung versus $4.67 \pm 0.33 \log_{10}$ CFU/lung; $P < 0.001$), as shown in Fig. 7.

Levels of active antibiotic in the lungs, kidneys, and sera of treated rats. The tobramycin concentration was $25.1 \pm 1.48 \mu$ g/mg of lung at 24 h after administration of the last doses of LipoBiEDT-TOB. We did not detect tobramycin in the kidneys or sera of the rats treated with the liposomal formulation. In addition, we did not detect any active tobramycin in the lungs or sera, but we found $6.5 \pm 5.3 \mu$ g/mg tobramycin in the kidneys of the rats treated with free BiEDT-TOB (Fig. 8).

Effect of LipoBiEDT-TOB on IL-8 production. We investigated whether LipoBiEDT-TOB could reduce the level of IL-8. The level of IL-8 was reduced from 72.93 ± 28.81 pg/ml in lungs in

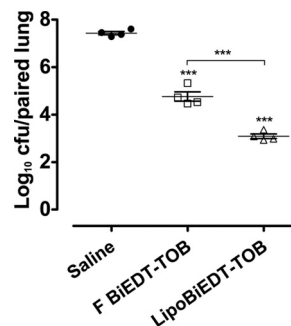


FIG 7 Effects of free (F) BiEDT-TOB or LipoBiEDT in the chronic lung infection model. Rats were inoculated with agar beads containing 10^6 CFU of *Pseudomonas aeruginosa*. After the bacteria were grown for 4 days, saline (filled circles), free BiEDT-TOB (open squares), or LipoBiEDT-TOB (filled triangles) was intratracheally administered at 300 μ g/ml/kg for 3 days. Lungs were then harvested and homogenized for analysis. Each column represents the mean $\pm$ SEM of four animals. ***, $P < 0.001$ for comparisons with the control as well as between groups.

the saline-treated group to 9.50 ± 1.31 and to 6.92 ± 2.13 pg/ml in the LipoBiEDT-TOB-treated and free BiEDT-TOB-treated groups, respectively (Fig. 9a). Free BiEDT-TOB slightly reduced IL-8 release into serum, to 34.29 ± 14.80 pg/ml, compared to 58.75 ± 9.86 pg/ml sera from the saline-treated group, whereas only 0.44 ± 0.29 pg/ml of IL-8 was detected in sera of LipoBiEDT-TOB-treated animals (Fig. 9b).

DISCUSSION

Many studies have described the efficacy of inhaled tobramycin on lowering *P. aeruginosa* pulmonary infection in CF patients (59). The high dose required and the prolonged use of tobramycin has raised investigators' concerns about its toxicity. Encapsulation of antimicrobial agents in liposomes has proven to increase their efficacy (60, 61). Bismuth has emerged as a therapeutic agent against gastrointestinal infection caused by *H. pylori* (62). Introducing BiEDT at a subinhibitory concentration resulted in reduced alginate and lipopolysaccharide production, as well as inhibition of adherence of *P. aeruginosa* to epithelial cells and secretion of virulence factors (47). Furthermore, previous results from our laboratory indicated that coencapsulation of BiEDT into liposomal-loaded tobramycin increased the killing effect on *P. aeruginosa* as well as diminishing AHL production and bacterial

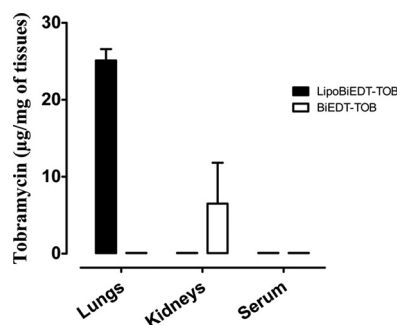


FIG 8 Measurements of active tobramycin levels. Tobramycin concentrations in the lung and the kidney homogenates of rats chronically infected with *P. aeruginosa* were evaluated by microbiological assay. Tissues were removed at 24 h after the last treatment with LipoBiEDT-TOB or free BiEDT-TOB. Bars represent means $\pm$ SEM of four animals.

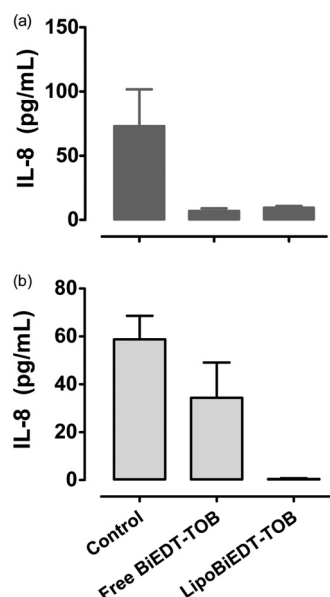


FIG 9 Concentrations of IL-8 in lungs (a) and in sera (b) of rats infected with *P. aeruginosa* and treated with the control, free BiEDT-TOB, or LipoBiEDT-TOB, as determined with an enzyme-linked immunosorbent assay. Each column represents the mean $\pm$ SEM for four animals.

adherence to human lung epithelial cells (52, 53). Herein, we have demonstrated that LipoBiEDT-TOB at a subinhibitory concentration is able to debilitate QS signaling molecule production and secretion of virulence factors, including protease, chitinase, and lipase *in vitro*. In addition, we examined the *in vivo* bactericidal efficacy and the anti-inflammatory property of LipoBiEDT-TOB in a rat model of pulmonary infection.

The MIC results reported here indicate significant differences between free and liposomal BiEDT-TOB. The MIC of LipoBiEDT-TOB was 16-fold lower than the MIC of tobramycin alone and 4-fold lower than the MIC of free BiEDT-TOB. These values are in agreement with previous observations on improved susceptibility of resistance Gram-negative strains to liposomal polymyxin B (60). Since exposing bacteria to the subinhibitory concentration of free or LipoBiEDT-TOB did not prevent *P. aeruginosa* from growing (Fig. 1), we investigated their potential effects on inhibition of clinical isolate *P. aeruginosa* communication and virulence factor production. The secretion of AHL molecules, which play an important role in regulating the production of several virulence factors, was reduced compared to the control with either free or LipoBiEDT-TOB up to 1/16 the MICs (Fig. 3). LipoBiEDT-TOB was able to reduce AHL production 29% at 1/8 the MIC, whereas production was reduced 19% by free BiEDT-TOB at 1/8 the MIC compared to control. Exposing *P. aeruginosa* to free BiEDT-TOB at 1/4 the MIC resulted in a 50% reduction in AHL, whereas LipoBiEDT-TOB at 1/4 the MIC led to an approximate 71% reduction compared to the control. However, comparing free and liposomal formulations, LipoBiEDT-TOB was found to be more effective at concentrations four times lower than free BiEDT-TOB, based on qualitative (Fig. 2) and quantitative (Fig. 3) measurements. Studies have reported that tobramycin at subinhibitory concentrations is able to decrease *N*-3-oxo-dodecanoyl-homoserine lactone and *N*-butanoylhomoserine lactone once tobramycin gains access to interact with bacterial ribosomes (36,

37). Another study reported improved efficacy of tobramycin with BiEDT in liposomes (53); thus, LipoBiEDT-TOB provides a greater advantage in reducing 3-oxo-dodecanoylhomoserine lactone and *N*-butanoylhomoserine lactone production levels by enhancing tobramycin penetration into the cell to interact with ribosomes. This interaction might result in downregulation of QS genes (36).

LipoBiEDT-TOB also reduced the level of virulence factors, including lipase (Fig. 4), chitinase (Fig. 5), and protease (Fig. 6), at a concentration four to eight times lower than free BiEDT-TOB with respect to the corresponding untreated control levels. It is not yet clear, however, how LipoBiEDT-TOB exerts its effect to reduce virulence factors. Tobramycin inhibits protein synthesis in *P. aeruginosa* (36). BiEDT is known to inhibit alginate and lipopolysaccharides, as well as to cause blebbing of the *P. aeruginosa* cell wall (47). Furthermore, transmission electron microscopy has provided evidence of the fusion of LipoBiEDT-TOB and the penetration of tobramycin into the cell wall of *P. aeruginosa* (63). Collectively, BiEDT in liposome form facilitates the uptake of loaded antibiotic, and it might thereby promote downregulation of QS and virulence factor gene expression or reduce their post-transcription synthesis (63).

Many investigators have reported studies employing intratracheal administration of liposome-loaded drugs, such as deguelin (64), insulin (65), tobramycin (66), small interfering RNA, antisense oligonucleotides, and anticancer drugs (67), into the lungs of rodents. The liposomal delivery system and intratracheal route satisfy three therapeutically preferred goals in pulmonary infection: (i) sustained release of an antibiotic from liposomes, which increases the residence time of the drug; (ii) reduction of antibiotic toxicity; (iii) direct targeting of the drug to the site of infection. The data reported here demonstrate that chronic respiratory infection caused by *P. aeruginosa* can be decreased by *in situ* administration of liposome-coencapsulated BiEDT and tobramycin. Three treatments with LipoBiEDT-TOB (300 mg/liter/kg for tobramycin and 300 μ M/kg for BiEDT) reduced *P. aeruginosa* in the lungs. We used a clinical isolate strain embedded on agar beads to initiate a chronic lung infection. Such retention apparently prevents physical elimination of the bacteria and ensures stimulation of host defenses typical of CF. Bacterial counts in the lungs showed a 2.7-log reduction in CFU in the free BiEDT-TOB-treated group compared to control, whereas LipoBiEDT-TOB reduced the bacterial counts by approximately 4.3 logs compared to the control. The increased efficacy of LipoBiEDT-TOB can be explained by its enhanced penetration of the encapsulated formula through the bacterial outer membrane, likely through a mechanism of fusion (60). Previous works by others have shown improved bactericidal activities of liposome-encapsulated antibiotics specific to *P. aeruginosa* (68, 69).

The microbiological analysis of the liposomal antibiotic in the lungs indicated the presence of 25 $\times$ the MIC for active tobramycin after 24 h of antibiotic therapy. However, no active tobramycin was detected at 24 h when the animals were treated with the free drug. Despite the fact that 25 $\times$ the MIC was detected in the lungs, the animals' lungs treated with LipoBiEDT-TOB remained infected. A previous study speculated that the persistent infection with liposomal antibiotic treatment might be due to the high stability of liposome lipid compositions, the protection of bacteria by agar beads, or a portion of the agar beads injected being preserved in the bronchial tree

(58). Since our formula consists of DSPC and cholesterol with a phase transition temperature of 55°C (70), the high stability of the vesicle might not allow the release of tobramycin at a sufficient concentration to ensure complete eradication. Also, use of agar beads to induce chronic infection might contribute to the presence of infection. The microbiological assay also showed no active tobramycin in the kidneys of the LipoBiEDT-TOB-treated group, but we found tobramycin accumulation in the kidneys of the free BiEDT-TOB-treated group. There was no active tobramycin detected in plasma when the antibiotic was administered in liposomes. Our findings agree with those of previously published reports (58, 71) and with the notion that the half-life of tobramycin in sera of humans and rodents is around 2 h after intravenous or intratracheal administration (58, 72). Likewise, our liposomal formulation results are in agreement with data reported by other researchers who investigated the efficacy of liposomal antibiotics against *P. aeruginosa* respiratory infection (73, 74), and this could suggest an advantage in reducing the nephrotoxicity associated with tobramycin treatment (73).

Tobramycin is known to have both antibacterial and anti-inflammatory activities (75, 76). Our results indicated the benefits of administration of LipoBiEDT-TOB intratracheally on *P. aeruginosa* infection and showed lowered inflammation by reduced IL-8 levels in lungs and sera. Although the exact mechanism of tobramycin as an anti-inflammatory drug is not well known, tobramycin has been shown to protect epithelial lung cells against myeloperoxidase by binding to anionic cell surfaces and neutralizing hypochlorous acid, which participates in tissue damage (77, 78). However, since the local inflammatory response is in agreement with pulmonary infection (79), the significant decrease in *P. aeruginosa* counts in lungs may be explained by the beneficial aspects of LipoBiEDT-TOB.

In conclusion, LipoBiEDT-TOB modulated the production of QS, virulence factors, and IL-8 and could highly enhance the treatment of chronic pulmonary infection in CF patients.

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We have no conflicts of interest to declare.

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