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Liposomal bismuth-ethanedithiol formulation enhances antimicrobial activity of tobramycin

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ABSTRACT

Pseudomonas aeruginosa and *Burkholderia cenocepacia* (formally, genomovar III genotype of *Burkholderia cepacia* complex) have emerged as serious opportunistic resistant pathogens in patients with cystic fibrosis (CF). We have developed a liposomal formulation containing bismuth-ethanedithiol (BiEDT) and tobramycin to overcome bacterial resistance. The stability of liposomal BiEDT-tobramycin (LipoBiEDT-TOB) was studied in phosphate buffered saline (PBS) and human pooled plasma at 4 and 37 °C. Minimal inhibitory concentrations (MICs) and minimal bactericidal concentrations (MBCs) for free tobramycin and LipoBiEDT-TOB against clinical isolates of *P. aeruginosa* and *B. cenocepacia* were determined by the broth dilution method. The toxicity profile and the influence on bacterial adhesion of LipoBiEDT-TOB formulation were determined using a human lung carcinoma cell line (A549). LipoBiEDT-TOB exhibited lower MICs than the conventional antibiotic (0.25 mg/L vs. 1024 mg/L) and eradicated this highly resistant bacterial strain of *P. aeruginosa* (PA-48913) at very low concentrations (4 mg/L vs. 4096 mg/L). LipoBiEDT-TOB was significantly less toxic when compared to the free BiEDT, as evaluated by the MTT and LDH assay. The LipoBiEDT-TOB formulation suppressed bacterial adhesion (*B. cenocepacia* M13642R) to A549 cells. These data suggest that the novel LipoBiEDT-TOB drug delivery system could be utilized as a new strategy to enhance the efficacy of existing antibiotics against resistant organisms that commonly affect individuals with chronic lung infections.

1. Introduction

Cystic fibrosis (CF) is an autosomal, recessive disease that affects primarily the Caucasian population, although other ethnic groups are also involved (Wei et al., 2006). CF is characterized by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene which results in the lack of functional chloride channels lining the epithelial surfaces of the lung (Moskowitz et al., 2005). It is the inability of CF patients' to effectively clear the viscous mucus from the pulmonary surface that provides an ideal environment for bacterial growth, including that of pathogenic Gram-negative *Pseudomonas aeruginosa* and *Burkholderia cepacia* complex. It is generally accepted that chronic bacterial infection and colonization are the primary stimuli for pulmonary inflammation and consequently compromised pulmonary function (Govan and Deretic, 1996). To date insufficient progress has been made toward gene therapy in hopes of altering the CFTR dysfunction (Moskowitz et al., 2005) and currently, aminoglycoside antibiotics such as tobramycin are used to treat the most resistant microorganisms threatening CF patients such as *P. aeruginosa* and *B. cepacia* (Burkhardt et al., 2006). However, this treatment strategy is limited due to changes in bacterial membrane permeability attributed to the alterations in exopolysaccharide (EPS) [an outer membrane component of *B. cepacia* and *P. aeruginosa*], known to play an important role in biofilm alginate production (Alkawash et al., 2006; Cunha et al., 2004) and reduction in cell surface anionic charges, rendering these organisms resistant to cationic antibiotics (Miller and Gilligan, 2003; Moore and Hancock, 1986; Poole, 2002, 2004; Vinion-Dubiel and Goldberg, 2003).

Optimizing existing therapies are among the primary strategies to overcome antibiotic resistance. Specific drug carriers, such as liposomes, may modify antibiotic release patterns at the site of infection and improve the overall drug uptake and residence time in the target organ (Alipour et al., 2008). Liposomal formulations improve drug efficacy and reduce drug-associated toxicity (Bekersky et al., 2000; Cordeiro et al., 2000; Mugabe et al., 2005,

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2006b; Vyas et al., 2005). In the case of CF, liposomal encapsulation of antibiotics allows higher drug concentrations to be specifically delivered to the lungs, thereby improving aminoglycoside action against these highly resistant pathogens. So far, it has been shown that liposomal tobramycin is effective in eradicating Gram-negative bacteria when compared to its free form (Marier et al., 2003). Data presented in our previous studies and those presented by other investigators indicate that liposomes increase intercellular drug concentrations through fusion with bacterial cell membrane (Alipour et al., 2008; Cordeiro et al., 2000; Mugabe et al., 2006b; Schiffelers et al., 2001). However, in order to optimize existing antibacterial treatments, co-administration of antibiotics with other antimicrobial agents might prove to be a more effective treatment. Recently, bismuth has emerged as a new bacterial sub-inhibitory agent (Veloira et al., 2003), and when combined with the solubilizer ethanedithiol, leads to decreases in antibiotic resistance through the inhibition of alginate production and bacterial respiratory enzymes, as well as through the suppression of biofilm formation (Huang and Stewart, 1999; Veloira et al., 2003).

In this study, the antibacterial efficacy of a new liposomal tobramycin formulation that is enriched with bismuth-ethanedithiol (BiEDT) was investigated. Data with regards to the stability, antibacterial activity, and toxicity of this novel formulation as well as its ability to modulate bacterial adhesion toward human lung cells are discussed.

2. Materials and methods

2.1. Chemicals and media

The liposomes were composed of 1,2-distearoyl-*sn*-glycero-3-phosphocholine (DSPC) (Northern Lipids, Vancouver, BC, Canada). Cholesterol, Triton X-100, 1,2-ethanedithiol $C_2H_6S_2$, propylene glycol $C_3H_8O_2$ (PG), bismuth nitrate [$Bi(NO_3)_3{\cdot}5H_2O$], MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide], and lactate dehydrogenase (LDH) assay kits were obtained from Sigma–Aldrich (Oakville, ON, Canada). Trypsin–EDTA, penicillin/streptomycin, tobramycin, Dulbecco's modified Eagle's medium-high glucose (DMEM) and Dulbecco's phosphate buffered saline (DPBS) were all obtained from Fisher Scientific (Ottawa, ON, Canada).

2.2. Cell culture

A549 human lung carcinoma epithelial cells were obtained from the American Type Culture collection (ATCC CCL-185, Manassas, USA) and cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum (FBS) without the addition of antibiotics for the toxicity study and with 1% penicillin/streptomycin for the adhesion study. The cells were grown to 85% confluence in 5% CO_2 at 37 °C and maintained using traditional cell culture techniques.

2.3. Organisms

Non-mucoid M13637 and mucoid M13642 strains of *Burkholderia cenocepacia* (genomovars III as indicated by the specific recA gene test (Henry et al., 2001)) and non-mucoid PA-48913 and mucoid PA-48912-2 strains of *P. aeruginosa* were isolated from sputum, saliva, and throat swab of CF patients at Sudbury Regional Hospital (Sudbury, Ontario, Canada). Laboratory strains of *P. aeruginosa* (ATCC 10145) were used to test tobramycin activity and *Staphylococcus aureus* (ATCC 29213) was used as quality control, as recommended by the Clinical and Laboratory Standards Institute (CLSI). All strains were stored in Mueller–Hinton broth at −80 °C (Becton Dickinson Microbiology Systems, Oakville, ON, Canada) supplemented with 10% glycerol. All strains were grown for 18 h in Mueller–Hinton broth prior to the experiments.

2.4. Liposomal BiEDT-tobramycin (LipoBiEDT-TOB) formulation preparation

The dehydration–rehydration technique was used to prepare multilamellar liposomal vesicles containing bismuth-ethanedithiol with entrapped tobramycin (Senior and Gregoriadis, 1989). For the preparation of the 1,2-ethanedithiol bismuth, bismuth nitrate was dissolved in methanol with the addition of 600 mM of sodium hydroxide (NaOH) (to maximize bismuth solubility); then, 1,2-ethanedithiol was added to the bismuth solution in 1:1 molar ratio [1,2-ethanedithiol was added to maintain the solubility of bismuth and facilitate the anchoring of bismuth within the liposomal membranes due to its lipophilicity] (Domenico et al., 1997). For the preparation of the liposomal vesicles, DSPC was dissolved with cholesterol (2:1 molar ratio) in chloroform with 40 μM bismuth-ethanedithiol being added to the dissolved lipids; this mixture was dehydrated by evaporation under controlled vacuum to form the lipid film (Buchi Rotavapor R 205, Buchi vacuum controller V-800, Brinkman, Toronto, Ont, Canada). Usually, this process completely removes the organic solvents (chloroform, methanol) used for the preparation of the formulations. The lipid film was then rehydrated with distilled water containing PG (1:1, w/v). The solution was sonicated for 5 min prior to the addition of tobramycin (8 mg/mL). The mixture was then sonicated for an additional 5 min. The LipoBiEDT formulation with encapsulated tobramycin (LipoBiEDT-TOB) was lyophilized as reported elsewhere (Mugabe et al., 2006b). To rehydrate, sterile water (10% of final volume before lyophilization) were added and the mixtures were incubated at 55 °C for 30 min. This step was repeated once more with PBS. Additional PBS were then added to form the original volume and incubated as above. The rehydrated LipoBiEDT-TOB formulations were centrifuged (100,000 × *g* for 20 min at 4 °C, Beckman L8-M Ultracentrifuge) and washed twice with PBS to remove the unencapsulated bismuth and tobramycin. The size of the homogenous LipoBiEDT-TOB formulation was determined with a Submicron Nicomp particle sizer (Model 270, Nicomp, Santa Barbara, CA, USA) as described elsewhere (Mugabe et al., 2006b).

2.5. Quantification of bismuth in liposomal formulations

The bismuth content of the LipoBiEDT-TOB formulation was measured by graphite furnace atomic absorption spectroscopy (GFAAS). Samples were lyophilized, weighed, and then transferred into Pyrex glass tubes. A total of 1 mL H_2O_2 (30%, w/w) and 4 mL HNO_3 (15N) was added and the samples were digested overnight at 25 °C. Samples were then subjected to hot plate digestion at 135–140 °C for 3 h and the volumes were adjusted to 25 mL with distilled water. Samples were then analyzed by GFAAS (PerkinElmer 5000).

To study the effect of tobramycin on encapsulation of bismuth inside LipoBiEDT-TOB formulation, three different samples of LipoBiEDT-TOB were incubated at 37 °C for 48 h with agitation at 250 rpm. Samples were centrifuged for 20 min (100,000 × *g*, 4 °C, Beckman L8-M Ultracentrifuge), the supernatants were discarded and the bismuth levels in the pellets were analyzed by GFAAS as previously described.

2.6. Tobramycin encapsulation efficiency (E.E.) within LipoBiEDT-TOB formulation

The agar diffusion test was used to determine the amount of encapsulated tobramycin in liposomes (Mugabe et al., 2006b).