International Journal of Pharmaceutics 358 (2008) 278–284

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Pharmaceutical Nanotechnology

Liposomal bismuth-ethanedithiol formulation enhances antimicrobial activity of tobramycin

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ARTICLE INFO

Article history:
Received 21 January 2008
Received in revised form 4 March 2008
Accepted 8 March 2008
Available online 15 March 2008

Keywords:
Liposomal-(bismuth ethanedithiol)
MICs
MBCs
Toxicity
Bacterial adhesion
Burkholderia cenocepacia
Pseudomonas aeruginosa
Staphylococcus aureus

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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doi:10.1016/j.ijpharm.2008.03.008

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).

Briefly, standard curves of diluted tobramycin as well as samples of free and permeabilized LipoBiEDT-TOB were prepared. Duplicate samples were transferred into the holes of the agar plates containing *S. aureus* ATCC 29213 as the recommended reference organism for antibiotic susceptibility analysis. As a control, the activity of LipoBiEDT alone (18.5 μM of bismuth) on the agar plate against the reference organism was also assessed (18.5 μM of bismuth was the maximum entrapping capacity of LipoBiEDT before adding the antibiotic to the formulation). Plates were incubated at 37 °C for 24 h and the inhibition zones were then measured. Encapsulation efficiency was calculated as follows:

$$\text{E.E.} = \left(\frac{\text{concentration of tobramycin}_{\text{Released}}}{\text{concentration of tobramycin}_{\text{Initial}}}\right) \times 100$$

2.7. Tobramycin stability within LipoBiEDT-TOB formulations

The stability of entrapped tobramycin in the LipoBiEDT-TOB formulation was determined after 0, 0.5, 1, 3, 6, 24, and 48 h incubation at 4 and 37 °C in PBS, as well as at 37 °C in pooled normal plasma. Samples at each time interval were harvested and centrifuged (100,000 × *g*, 20 min, 4 °C, Beckman L8-M Ultracentrifuge). Tobramycin concentrations in the supernatants were measured by the microbiological assay using the agar diffusion method (Halwani et al., 2007).

2.8. Determination of the minimum inhibitory concentrations (MICs) and the minimum bactericidal concentrations (MBCs)

The broth dilution method was used to determine the MICs and MBCs for tobramycin (Rukholm et al., 2006). Briefly, the reference strain *S. aureus* or clinical isolates of *P. aeruginosa* and *B. cenocepacia* were exposed to different dilutions of tobramycin (free or LipoBiEDT-TOB) and to a combination of tobramycin with BiEDT. The contribution of bismuth ethanedithiol to the MICs and MBCs was assessed by exposing the aforementioned bacterial strains to different concentrations of bismuth added in the form of BiEDT, LipoBiEDT, and LipoBiEDT-TOB with a starting concentration of 4096 μM, which is the minimum concentration used under our experimental conditions to observe death of resistant isolates. Drug-free bacterial cultures and broth medium alone were used as positive and negative controls, respectively.

2.9. Cell toxicity study

The human lung carcinoma epithelial cell line A549, was maintained in DMEM supplemented with 10% FBS without antibiotics. At sub-confluency, the cells were seeded into 96-well plates at a density of 5×10^3 cells/well for the MTT assay or into 24-well plates at a density of 2×10^4 cells/well for the LDH assay and were allowed to adhere overnight. The media was removed from the wells and replaced with 100 μL media containing BiEDT (10 or 20 μM) or LipoBiEDT-TOB equivalent. Concentrations of LipoBiEDT-TOB were determined based on BiEDT concentration. Controls included untreated cells containing media alone, cells treated with H_2O_2 (as a positive control), empty liposomes (to account for any effect of liposomes on cell viability (equal to 20 μM LipoBiEDT formula)), free tobramycin, as well as a combination of empty liposomes, BiEDT, and tobramycin. Plates were incubated for 24 h in 5% CO_2 at 37 °C.

The MTT assay is an effective colorimetric method for determining *in vitro* cytotoxicity and cellular viability (Berridge and Tan, 1993; Mosmann, 1983) by measuring mitochondrial activity in cells. After 24 h treatment, the wells were washed once with complete DMEM and once with DPBS, and replaced with fresh media. MTT dye (25 μL of 5 mg/mL of MTT dye in PBS) was added to each well and the plates were incubated in the dark for 4 h at 37 °C. MTT lysis buffer (a solution of 1:1, v/v dimethylformamide and water and 0.7 M sodium dodecyl sulphate) was then added to each well. The plates were further incubated in the dark overnight at 37 °C, prior to spectrophotometric measurement at 590 nm (reference at 625 nm). Blank absorbance values (wells containing media, MTT dye and lysis buffer) were subtracted from each value and cellular viability was expressed as a percentage.

The LDH level was also determined as a biomarker of cell membrane integrity. The cells were cultured and treated with equivalents of 20 μM of bismuth into BiEDT and LipoBiEDT-TOB; tobramycin alone was 0.2 mg/mL and controls as detailed above for 24 h. After the treatment, the plates were centrifuged (250 × *g* for 4 min, 25 °C), and the supernatant was transferred to a clean, 96-well plate. The LDH assay mixture was prepared according to the manufacturer's protocol (Sigma–Aldrich). The LDH solution was added at a volume equal to half of the total well volume of culture medium. The plates were kept in the dark at room temperature for 25 min. The absorbance was then read at 510 nm. Blank values (wells containing media and LDH solution) were subtracted from all readings. Cells treated with H_2O_2 were representative of 100% LDH released. All toxicity studies were representation of three separate experiments, performed in triplicate.

2.10. Effects of formulations on bacterial adhesion to human lung cells in culture

Adhesion of the mucoid strain of *B. cenocepacia* M13642R to A549 lung cells was studied by the method described elsewhere (Azghani et al., 2002). The monolayer of A549 cells were grown overnight in 35 mm culture dish, then washed twice with DPBS and incubated with 2 mL DMEM with no supplements prior to addition of bacteria. A fresh culture of *B. cenocepacia* was adjusted to 0.5 McFarland standard in DMEM (without supplements). Bacterial aliquots were incubated with BiEDT and LipoBiEDT in final concentrations of 2.5, 5, 10, and 20 μM of bismuth and LipoBiEDT-TOB (tobramycin concentrations were 49, 98, 196, and 392 mg/L), and empty liposomes (20.4 mg/ml of DSPC lipids). After an incubation period of 3 h at 37 °C, bacterial samples were added onto cell A549 monolayers as follows: cells were exposed to the control or treated bacteria for 3 h at 37 °C supplemented with 5% CO_2. The monolayers were then washed five times with DPBS to remove non-adhered bacteria. The cells were removed by a sterilized Cell Lifter (Costar, Fisher Scientific), resuspended in DPBS, and samples (100 μl) were plated on Mueller–Hinton agar plates. Bacterial colonies on triplicate agar plates were counted, after 24 h at 37 °C, to determine the number of adhered bacteria.

2.11. Data analysis

The data presented as mean ± S.E.M. of three independent experiments. Comparisons between individual groups were made by paired Student's *t*-test and $P \leq 0.05$ was considered significant. For multiple comparisons within and between groups, we used ANOVA with post-test Dunnett analysis. All analyses were perfomed using GraphPad Prism version 5.0.

3. Results

3.1. LipoBiEDT-TOB characterization

The average size of LipoBiEDT-TOB formulation was 908.0 ± 42.7 nm. The encapsulation of tobramycin in the LipoBiEDT formulation was measured to be 0.2 mg/mL. Atomic

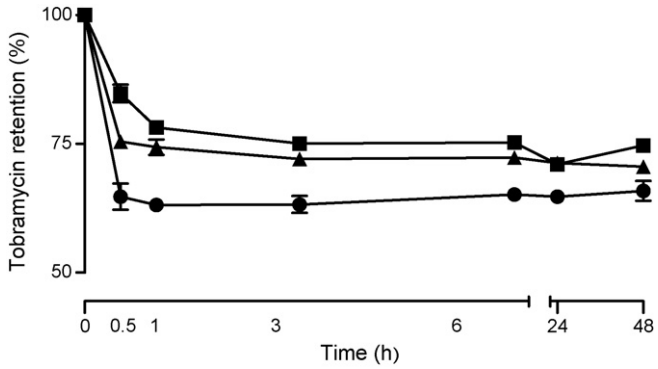

Fig. 1. Stability of LipoBiEDT-TOB formulation in PBS at 4 °C (square), 37 °C (triangle), and in plasma at 37 °C (circle). Data represent the mean ± S.E.M. of three different experiments. LipoBiEDT-TOB incubated in PBS at 4 °C retained significantly more antibiotics than those incubated in plasma at 37 °C ($P < 0.05$), and were similar ($P > 0.05$) with those incubated in PBS at 37 °C at the end of the study period.

absorption analysis showed that the concentration of bismuth incorporated into LipoBiEDT-TOB formulation was 10.6 ± 0.7 μM. The liposomal formulation retained high amounts of bismuth (>79.2%) in a 48 h period at 37 °C. Entrapped tobramycin in LipoBiEDT was significantly more stable in PBS at 4 °C than in PBS or plasma at 37 °C within the first 30 min of incubation (84.80, 75.5, and 64.75%, respectively, $P < 0.008$); thereafter, the levels of entrapped tobramycin were comparable among the different treatments (Fig. 1). Briefly, this treatment effect may be attributed to the release of tobramycin from the liposomes due to differences in temperature (4 °C vs. 37 °C) (Mugabe et al., 2006a) and/or plasma components causing an initial increase in liposome membrane permeability which then declines, presumably because of a rearrangement of membrane lipids and adsorbed proteins to form their most stable configuration (Hunt, 1982).

3.2. MICs and MBCs determination

The MIC and MBC values are presented in Tables 1 and 2. The MICs of LipoBiEDT-TOB formulation against *P. aeruginosa* and *B. cenocepacia* strains were significantly lower than that of the free tobramycin and combination of free tobramycin and BiEDT. For example, the MIC of free tobramycin for non-mucoid *B. cenocepacia* M13637 was 512 mg/L compared to 32 mg/L of the tobramycin-BiEDT and 0.25 mg/L of the tobramycin encapsulated in LipoBiEDT. The latter formulation was bactericidal at 8 mg/L. More impressive, was the decrease in the MICs of the LipoBiEDT-TOB formulation against the non-mucoid antibiotic resistant *P. aeruginosa* as well (0.25 mg/L vs. 1024 mg/L tobramycin) with a bactericidal concentration (MBCs) as low as 4 mg/L. Likewise, LipoBiEDT-TOB decreased the MIC of *B. cenocepacia* M13642R from 128 to 0.25 mg/L, and completely eradicated this strain at only 16 mg/L. These data indicated that the encapsulated tobramycin in LipoBiEDT formulation are significantly more effective against antibiotic resistant strains of both laboratory and clinical isolates of *P. aeruginosa* and *B. cenocepacia* than the free tobramycin.

S. aureus or clinical isolates of *P. aeruginosa* and *B. cenocepacia* strains were all highly resistant against BiEDT alone; however, encapsulated BiEDT in liposomal formulation significantly reduced the resistance of all strains but not to the same extent seen with the liposomal formulation containing tobramycin as well.

3.3. Toxicological evaluation of LipoBiEDT-TOB formulation

Recognizing the fact that bismuth is cytotoxic at concentrations of 10 μM or greater (Wu et al., 2002), the effects of BiEDT and

Table 1
Bactericidal activity of liposomal-bismuth formulation

Bacterial strains	Minimum inhibitory concentration (MIC)				
	Tobramycin (mg/L)	BiEDT (μM)	Tobramycin (mg/L) and BiEDT (4 μM)	LipoBiEDT formula (μM)	LipoBiEDT-TOB[a] (mg/L)
M13637	512	1024	32	32	0.25
M13642R	128	1024	64	64	0.25
PA-48913	1024	256	256	32	0.25
PA-48912-2	64	2048	8	8	0.25
PA-10145	1	2048	4	16	0.25
SA-29213[b]	2	256	1	32	0.25

MIC values for free tobramycin, BiEDT, a combination of BiEDT with free tobramycin, LipoBiEDT, and LipoBiEDT-TOB against resistant clinical isolates of *P. aeruginosa* and *B. cenocepacia*.

[a] Bismuth concentrations in LipoBiEDT-TOB formulations were 1 μM.

[b] *S. aureus*, ATCC 29213, was used to validate the method according to the CLSI standard.

Table 2
Bactericidal activity of liposomal-bismuth formulation

Bacterial Strains	Minimum bactericidal concentration (MBC)				
	Tobramycin (mg/L)	BiEDT (μM)	Tobramycin (mg/L) and BiEDT (4 μM)	LipoBiEDT formula (μM)	LipoBiEDT-TOB[a] (mg/L)
M-13637	1024	2048	64	64	8
M-13642R	256	2048	128	128	16
PA-48913	4096	512	512	64	4
PA-48912-2	128	4096	32	16	0.5
PA-10145	1	4096	8	32	4
SA-29213[b]	2	512	1	64	0.25

MBC values for free tobramycin, BiEDT, a combination of BiEDT with free tobramycin, LipoBiEDT, and LipoBiEDT-TOB against resistant clinical isolates of *P. aeruginosa* and *B. cenocepacia*.

[a] Bismuth concentration in LipoBiEDT-TOB formulations were 32, 64, 16, 2, 16, and 1 μM for M-13637, M-13642R, PA-48913, PA-48912-2, PA-10145 and SA-29213, respectively.

[b] *S. aureus*, ATCC 29213, was used to validate the method according to the CLSI standard.

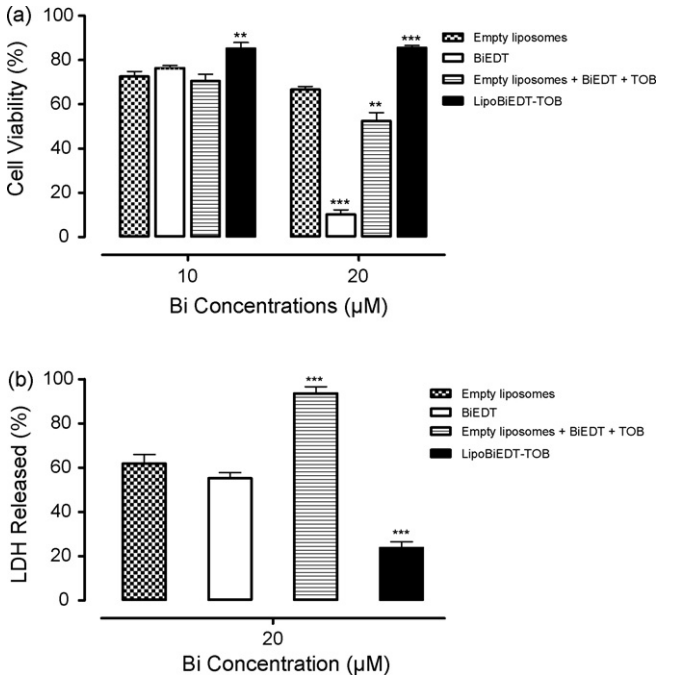

Fig. 2. Effect of LipoBiEDT-TOB, BiEDT, empty liposomes, and a combination of empty liposomes, BiEDT and TOB on (a) cell viability [MTT assay] and (b) LDH release in A549 lung epithelial cells. A549 lung epithelial cells were exposed to 10–20 μM of bismuth in BiEDT and LipoBiEDT-TOB, and empty liposome equivalents to LipoBiEDT-TOB. Data represent the mean ± S.E.M. of three independent experiments, performed in triplicate. (**) $P < 0.01$ was considered significant when comparing the mean value to the group treated with empty liposomes. (***) $P < 0.001$ was considered significant when comparing the mean value to the group treated with empty liposomes.

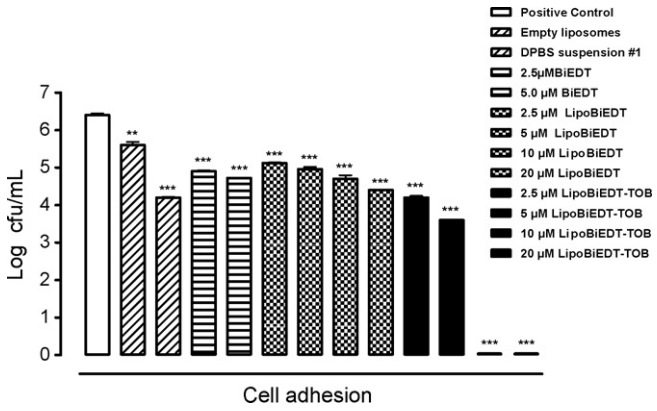

Fig. 3. Bacterial adhesion to lung cells: bacteria were pretreated with 2.5, 5, 10, and 20 μM of LipoBiEDT, 2.5, 5, 10, and 20 μM of LipoBiEDT-TOB, empty liposomes (equivalent to the amount used in the LipoBiEDT-TOB formulation) or the first post-treatment wash (DPBS suspension #1) of LipoBiEDT-TOB treatment and 2.5, and 5 μM of BiEDT. 3 h post-treatment, bacteria were added onto A549 lung cells and further incubated for 3 h. The monolayers were detached after five washes, suspended in DPBS, and plated on agar plates for CFU count. Data are the mean ± S.E.M. of three independent studies. (**) $P < 0.01$ was considered significant when compared with the mean value of the control group. (***) $P < 0.001$ was considered significant when compared with the mean value of the control group.

LipoBiEDT formulations on cell viability and cell membrane permeability were assessed in A549 cells by MTT (Fig. 2a) and LDH (Fig. 2b) assays.

Cells treated with 10 and 20 μM of free BiEDT reduced cell viability to 76.26 ± 1.23% and 10.14 ± 2.18%, whereas the LipoBiEDT-TOB preserved cell viability at 85.21 ± 2.67% and 85.63 ± 0.94%, respectively, as measured by the MTT assay. Cells treated with BiEDT resulted in significant increases in LDH release (55.20 ± 2.57%) while that seen following exposure to LipoBiEDT-TOB (23.71 ± 2.87% LDH) was comparable to that seen in untreated control cells (20.32 ± 1.43%). Cells treated with 0.2–0.4 mg/mL of free tobramycin displayed no differences compared to controls in MTT and LDH assays (data not shown).

3.4. Bacterial adhesion reduction

We examined the effect of LipoBiEDT-TOB formulation on adhesion of *B. cenocepacia* M13642R (mucoid) to A459 monolayers. As shown in Fig. 3, bacterial treatment with 20 μM LipoBiEDT reduced adhesion to lung epithelial cells by 2 logs. The adhesion was further reduced by the LipoBiEDT-TOB formulation containing only 5 μM of bismuth (Fig. 3). Interestingly, 10 μM LipoBiEDT-TOB formulation inhibited bacterial adhesion completely.

To rule out the possibility that the reduced adhesion of bacteria to A549 cells was not due to the presence of bacteria killed by the encapsulated tobramycin during the incubation period (6 h), we examined the bacterial viability in the DPBS suspension that was isolated following aspiration of non-adhered bacteria (DPBS suspension #1). As seen in Fig. 3, bacteria isolated in the aspirate of the first wash of the adhesion experiments demonstrated growth when plated in agar dish for 24 h at 37 °C suggesting that LipoBiEDT-TOB is effective in reducing bacterial adherence prior to any bactericidal effect.

Empty liposomes had a slight effect on bacterial adhesion compared to DPBS treated positive control, as seen in Fig. 3c. The BiEDT formula alone at concentrations higher than 5 μM caused monolayer detachment (data not shown). However, BiEDT at 2.5 and 5 μM caused considerable reduction in bacterial adherence to lung cells as seen in Fig. 3.

4. Discussion

Results from our previous studies demonstrate that encapsulation of tobramycin in liposomes is more effective than free tobramycin in eradicating Gram-negative bacteria as evidenced by the lower MICs (three to eightfold) (Halwani et al., 2007; Mugabe et al., 2006b). In this study, we provide evidence where the incorporation of bismuth-ethanedithiol into the liposomal tobramycin formulation further improved the antimicrobial capacity of the liposomal tobramycin against several non-resistant and resistant strains of Gram-negative bacteria (Tables 1 and 2). Bismuth is used as an over-the-counter antibacterial and antidiarrhetic drug as well as bactericidal agent in many *in vitro* studies (Domenico et al., 1997; Murata, 2006; Veloira et al., 2003). Also, it has been reported that bismuth-ethanedithiol (a) disturbs bacterial membrane integrity and prevents biofilm formation (Huang and Stewart, 1999; Wu et al., 2002) and (b) ethanedithiol lipophilicity enhances bismuth anchoring to liposomes, which could improve the delivery of bismuth inside bacterial cells and consequently enhance its bactericidal activity (Domenico et al., 2001; Veloira et al., 2003).

BiEDT was not effective in reducing MICs and MBCs while LipoBiEDT alone was effective against most Gram-negative bacteria but not to the same extent as that seen with LipoBiEDT-TOB. The inability of BiEDT to inhibit bacterial growth in our study is not surprising because the levels of bismuth used were at sub-inhibitory concentrations (1 μM) (Veloira et al., 2003). However, the effectiveness of sub-inhibitory concentrations of bismuth delivered as a liposomal formulation could be attributed to the inherent properties of liposomes to deliver bismuth inside the bacterial cells. Once inside the cell, bismuth acts as a metabolic poison, resulting in growth

inhibition and cell death (Wu et al., 2002; Dill and McGown, 1994). Recently, immunocytochemistry and electron transmission microscopic studies have shown that liposomes facilitate the penetration of antibiotics into resistant strains of *P. aeruginosa* (Alipour et al., 2008; Mugabe et al., 2006a). The limited antibacterial effectiveness seen following exposure of bacteria to a combination of free bismuth and tobramycin are also evidence to suggest that these agents can be delivered to the microorganisms more effectively when these are encapsulated in liposomes. Indeed, the LipoBiEDT-TOB was the most effective antibacterial formulation and it remains to be examined whether this treatment effect was attributed to the ability of bismuth to sensitize bacterial cells against tobramycin and/or the effect was attributed to the independent mechanism(s) of action of bismuth and tobramycin.

Free BiEDT at concentration of 10–20 μM rendered A549 human lung cancer cells nonviable as demonstrated by the results of this study and reported by others (Wu et al., 2002). On the other hand, exposure of A549 human lung cancer cells to the LipoBiEDT-TOB formulation reduced its toxicity, as determined by the MTT and LDH assays. In all cases, liposome encapsulation preserved cell viability in comparison to cells treated with free bismuth, tobramycin or empty liposomes. It has been shown that liposomes are used in delivering toxic drugs such as doxorubicin and paclitaxel in cancer chemotherapy (Angioli et al., 2007; Fetterly and Straubinger, 2003), thereby reducing cellular toxicity. Our data suggest that the entrapment of bismuth-ethanedithiol within the liposomal bilayers reduces the toxicity of BiEDT perhaps by reducing the availability of the agent within the cell membrane of cells (Desjardins et al., 2002).

P. aeruginosa or *B. cenocepacia* complex in CF lungs eventually form rough biofilm layers (Garcia-Medina et al., 2005; Saiman et al., 1990). The slime layers, limited oxygen, and lower bacterial metabolic activity interrupt antibiotic delivery and render the biofilm community resistant to chemotherapy (Walters et al., 2003). We examined the capability of LipoBiEDT-TOB formulation to reduce bacterial adherence to human lung cells in culture, while acknowledging the fact that this model does not represent the *in vivo* CF environment. Indeed, the combination of anti-adhesive properties of BiEDT and tobramycin and fusible properties of liposomes with bacterial cells prevented bacterial adhesion to A549 monolayers. Our immediate goal in this regard is to investigate the effectiveness of the formulation in reducing or preventing bacterial adhesion to lung cells in biofilm system and in chronic mice model of pulmonary infection.

In conclusion, LipoBiEDT-TOB is less toxic and its antimicrobial activity is superior to free tobramycin against *P aeruginosa* and *B. cenocepacia*. Also, this formulation prevents bacterial adhesion to human lung cells in culture and it has potential clinical applications in treating acute and chronic pulmonary infections in CF patients.

Acknowledgments

All clinical isolates were kindly provided by the Department of Microbiology, Memorial Hospital, Sudbury, Ontario, Canada. We thank Dr. Yuwei Chen, Paul Michael and Beverly Harper for their technical assistance. This work was partly supported by a research grant from the Ministry of Health of Saudi Arabia presented by Saudi Arabian Cultural Bureau in Ottawa for funding (M.H.) and LURF (Laurentian University Research Funds).

References

Alipour, M., et al., 2008. Antimicrobial effectiveness of liposomal polymyxin B against resistant Gram-negative bacterial strains. Int. J. Pharm. 355, 293–298.

Alkawash, M.A., et al., 2006. Alginate lyase enhances antibiotic killing of mucoid *Pseudomonas aeruginosa* in biofilms. Apmis 114, 131–138.

Angioli, R., et al., 2007. Liposome-encapsulated doxorubicin citrate in previously treated recurrent/metastatic gynecological malignancies. Int. J. Gynecol. Cancer 17, 88–93.

Azghani, A.O., et al., 2002. *Pseudomonas aeruginosa* outer membrane protein F is an adhesin in bacterial binding to lung epithelial cells in culture. Microb. Pathogen. 33, 109–114.

Bekersky, I., et al., 2000. Safety, toxicokinetics and tissue distribution of long-term intravenous liposomal amphotericin B (AmBisome): a 91-day study in rats. Pharm. Res. 17, 1494–1502.

Berridge, M.V., Tan, A.S., 1993. Characterization of the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT): subcellular localization, substrate dependence, and involvement of mitochondrial electron transport in MTT reduction. Arch. Biochem. Biophys. 303, 474–482.

Burkhardt, O., et al., 2006. Once-daily tobramycin in cystic fibrosis: better for clinical outcome than thrice-daily tobramycin but more resistance development? J. Antimicrob. Chemother. 58, 822–829.

Cordeiro, C., et al., 2000. Antibacterial efficacy of gentamicin encapsulated in pH-sensitive liposomes against an *in vivo Salmonella enterica* serovar typhimurium intracellular infection model. Antimicrob. Agents Chemother. 44, 533–539.

Cunha, M.V., et al., 2004. Studies on the involvement of the exopolysaccharide produced by cystic fibrosis-associated isolates of the *Burkholderia cepacia* complex in biofilm formation and in persistence of respiratory infections. J. Clin. Microbiol. 42, 3052–3058.

Desjardins, A., et al., 2002. Differential behaviour of fluid liposomes toward mammalian epithelial cells and bacteria: restriction of fusion to bacteria. J. Drug Target 10, 47–54.

Dill, K., McGown, E.L., 1994. The biochemistry of arsenic, bismuth and antimony. In: Patai, S. (Ed.), The Chemistry of Organic Arsenic, Antimony and Bismuth Compounds. John Wiley & Sons, New York, NY, pp. 695–713.

Domenico, P., et al., 1997. Enhancement of bismuth antibacterial activity with lipophilic thiol chelators. Antimicrob. Agents Chemother. 41, 1697–1703.

Domenico, P., et al., 2001. Activities of bismuth thiols against *staphylococci* and *staphylococcal* biofilms. Antimicrob. Agents Chemother. 45, 1417–1421.

Fetterly, G.J., Straubinger, R.M., 2003. Pharmacokinetics of paclitaxel-containing liposomes in rats. AAPS Pharm. Sci. 5, E32.

Garcia-Medina, R., et al., 2005. *Pseudomonas aeruginosa* acquires biofilm-like properties within airway epithelial cells. Infect. Immun. 73, 8298–8305.

Govan, J.R., Deretic, V., 1996. Microbial pathogenesis in cystic fibrosis: mucoid *Pseudomonas aeruginosa* and *Burkholderia cepacia*. Microbiol. Rev. 60, 539–574.

Halwani, M., et al., 2007. Bactericidal efficacy of liposomal aminoglycosides against *Burkholderia cenocepacia*. J. Antimicrob. Chemother. 60, 760–769.

Henry, D.A., et al., 2001. Phenotypic methods for determining genomovar status of the *Burkholderia cepacia* complex. J. Clin. Microbiol. 39, 1073–1078.

Huang, C.T., Stewart, P.S., 1999. Reduction of polysaccharide production in *Pseudomonas aeruginosa* biofilms by bismuth dimercaprol (BisBAL) treatment. J. Antimicrob. Chemother. 44, 601–605.

Hunt, C.A., 1982. Liposomes disposition *in vivo*. V. Liposome stability in plasma and implications for drug carrier function. Biochim. Biophys. Acta 719, 450–463.

Marier, J.F., et al., 2003. Liposomal tobramycin against pulmonary infections of *Pseudomonas aeruginosa*: a pharmacokinetic and efficacy study following single and multiple intratracheal administrations in rats. J. Antimicrob. Chemother. 52, 247–252.

Miller, M.B., Gilligan, P.H., 2003. Laboratory aspects of management of chronic pulmonary infections in patients with cystic fibrosis. J. Clin. Microbiol. 41, 4009–4015.

Moore, R.A., Hancock, R.E., 1986. Involvement of outer membrane of *Pseudomonas cepacia* in aminoglycoside and polymyxin resistance. Antimicrob. Agents Chemother. 30, 923–926.

Moskowitz, S.M., et al., 2005. Cystic fibrosis lung disease: genetic influences, microbial interactions, and radiological assessment. Pediatr. Radiol. 35, 739–757.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55–63.

Mugabe, C., et al., 2005. Liposome-mediated gentamicin delivery: development and activity against resistant strains of *Pseudomonas aeruginosa* isolated from cystic fibrosis patients. J. Antimicrob. Chemother. 55, 269–271.

Mugabe, C., et al., 2006a. Preparation and characterization of dehydration-rehydration vesicles loaded with aminoglycoside and macrolide antibiotics. Int. J. Pharm. 307, 244–250.

Mugabe, C., et al., 2006b. Mechanism of enhanced activity of liposome-entrapped aminoglycosides against resistant strains of *Pseudomonas aeruginosa*. Antimicrob. Agents Chemother. 50, 2016–2022.

Murata, T., 2006. Effects of bismuth contamination on the growth and activity of soil microorganisms using thiols as model compounds. J. Environ. Sci. Health A: Tox. Hazard. Subst. Environ. Eng. 41, 161–172.

Poole, K., 2002. Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. Curr. Pharm. Biotechnol. 3, 77–98.

Poole, K., 2004. Efflux-mediated multiresistance in Gram-negative bacteria. Clin. Microbiol. Infect. 10, 12–26.

Rukholm, G., et al., 2006. Antibacterial activity of liposomal gentamicin against *Pseudomonas aeruginosa*: a time-kill study. Int. J. Antimicrob. Agents 27, 247–252.

Saiman, L., et al., 1990. *Pseudomonas cepacia* adherence to respiratory epithelial cells is enhanced by *Pseudomonas aeruginosa*. Infect. Immun. 58, 2578–2584.

Schiffelers, R., et al., 2001. Liposome-encapsulated aminoglycosides in pre-clinical and clinical studies. J. Antimicrob. Chemother. 48, 333–344.
Senior, J., Gregoriadis, G., 1989. Dehydration-rehydration vesicle methodology facilitates a novel approach to antibody binding to liposomes. Biochim. Biophys. Acta 1003, 58–62.
Veloira, W.G., et al., 2003. *In vitro* activity and synergy of bismuth thiols and tobramycin against *Burkholderia cepacia* complex. J. Antimicrob. Chemother. 52, 915–919.
Vinion-Dubiel, A.D., Goldberg, J.B., 2003. Lipopolysaccharide of *Burkholderia cepacia* complex. J. Endotoxin Res. 9, 201–213.
Vyas, S.P., et al., 2005. Aerosolized liposome-based delivery of amphotericin B to alveolar macrophages. Int. J. Pharm. 296, 12–25.
Walters, M.C., et al., 2003. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. Antimicrob. Agents Chemother. 47, 317–323.
Wei, S., et al., 2006. Cystic fibrosis testing among Arab-Americans. Genet. Med. 8, 255–258.
Wu, C.L., et al., 2002. Subinhibitory bismuth-thiols reduce virulence of *Pseudomonas aeruginosa*. Am. J. Respir. Cell Mol. Biol. 26, 731–738.