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International Journal of Pharmaceutics 315 (2006) 59–66
INTERNATIONAL JOURNAL OF PHARMACEUTICS
www.elsevier.com/locate/ijpharm

In vitro antimicrobial activity of liposomal meropenem against *Pseudomonas aeruginosa* strains

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Received 7 November 2005; received in revised form 2 February 2006; accepted 10 February 2006
Available online 6 March 2006

Abstract

Twelve lipid formulations of liposomal meropenem were tested on six drug-susceptible and two drug-resistant *Pseudomonas aeruginosa* strains to determine their antibacterial activity. Cationic liposomes, especially PC/DOPE/SA 4:4:2 and PC/DOTAP/Chol 5:2:3, were more effective than anionic ones against sensitive isolates as the MICs of those formulations were two to four times lower than those of the free drug. None of the studied liposomal forms of meropenem exhibited bactericidal activity against isolates, which are drug-resistant due to low permeability. Even Fluidosomes® (liposomes made of DPPC/DMPG 18:1), which demonstrated fusion with *P. aeruginosa* membranes, showed 4–16 times higher MICs for sensitive and resistant strains than did the free meropenem.

Keywords: *Pseudomonas aeruginosa*; Liposomes; Meropenem

1. Introduction

Pseudomonas aeruginosa, a Gram-negative opportunistic pathogen, can cause serious nosocomial infections, especially wound infections and respiratory diseases, including cystic fibrosis (Bodey et al., 1983; Revathi et al., 1998). These rod-like bacteria are responsible for hospital-acquired infections and are most often multidrug resistant (Emori and Gaynes, 1993; Mayon-White et al., 1988). They are naturally resistant to many antibiotics and chemotherapeutics due to the low permeability of their outer membrane (Nikaido, 1989; Putman et al., 2000). They are present in nosocomial isolates shown to be unsusceptible to almost all antibiotics (Carmeli et al., 2001; Livermore, 2001; Siami et al., 2001). Only a few antimicrobial agents (e.g. carbapenems) show antibacterial activity against this species. The mechanisms of *P. aeruginosa* resistance to carbapenems are well known (Livermore, 2001; Livermore and Woodford, 2000; Quinn et al., 1986; Senda et al., 1996) and involve: (i) drug inactivation by carbapenemases activity (Livermore, 2001; Livermore and Woodford, 2000; Senda et al., 1996), (ii) prevention of drug influx related to the loss of membrane OprD porin (Fung-Tomc et al., 1995; Livermore, 2001), and (iii) active extrusion of drug from the cell by an efflux pump system containing the proteins OprM and OprN (Livermore, 2001; Masuda et al., 1995). In the case of strains resistant due to low permeability, liposomal drug formulations were developed to increase the bactericidal efficacy of antibiotics (Beaulac et al., 1998; Omri and Ravaoarinoro, 1996; Omri et al., 2002; Puglisi et al., 1995; Sachetelli et al., 2000). The liposomal forms promoted effective interaction between bacteria and drug, increased the lifetime of the entrapped antibiotic, and reduced systemic drug absorption (Beaulac et al., 1998). Various liposomal forms of fluroquinolones, aminoglycosides, and polymyxin B demonstrated reductions in MIC compared with the free drug against Gram-positive and Gram-negative bacteria (Beaulac et al., 1998; Omri and Ravaoarinoro, 1996; Omri et al., 2002; Puglisi et al., 1995). Tobramycin encapsulated in Fluidosomes® showed efficient in vitro antimicrobial activity against *P. aeruginosa*, *Burkholderia*

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doi:10.1016/j.ijpharm.2006.02.017

cepacia, *Escherichia coli*, and *Stenotrophomonas maltophilia* strains (Beaulac et al., 1998). Sachetelli proved that antibiotics in Fluidosomes® were able to overcome bacterial resistance related to the permeability barrier and enzymatic hydrolysis by a fusion process between liposomes and bacterial membranes (Sachetelli et al., 2000).

The present study was designed to evaluate: (i) the entrapment efficiency of various fluid and rigid liposome formulations, (ii) the in vitro activity of meropenem encapsulated in liposomes against susceptible *P. aeruginosa* strains, (iii) the in vitro activity of meropenem encapsulated in liposomes against *P. aeruginosa* strains which are meropenem resistant due to low permeability or efflux, and (iv) the in vitro activity of meropenem encapsulated in liposomes against *P. aeruginosa* strains resistant to meropenem due to the production of carbapenemases.

2. Materials and methods

2.1. Chemicals

Meropenem (MEM) was obtained from Astra Zeneca UK Ltd. (Macclesfield, UK).

1,2-Dioleoyloxy-3-trimethylammonium-propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), phosphatidylcholine (PC), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (DMPG), and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) were purchased from Northern Lipids Inc. (Vancouver, BC, Canada). Octadecylamine (SA) was obtained from Sigma–Aldrich Chemie GmbH (Steinheim, Germany) and cholesterol (Chol) from E. Merck (Darmstadt, Germany). HPLC solvents were supplied by J.T. Baker (Deventer, Holland).

2.2. Encapsulation of meropenem in liposomes

The lipid compositions of the various liposomes used in the experiments are provided in Table 1. Appropriate amounts of lipids dissolved in chloroform (10 mg/ml) were mixed in a 100 ml round-bottom flask. By evaporating the organic solvent at 40 °C, a thin film of dry lipid was deposited on the inner wall of the flask. Residual solvent was removed under high vacuum applied for at least 1 h. The dry lipid films (with 30 mg of total lipid) were hydrated by adding 1 ml of meropenem solution (35 mg in 150 mM NaCl). Hydration was performed at a temperature maintained above the phase transition temperature of the main liposome lipid (20 °C for PC, 50 °C for DPPC, and 66 °C for DSPC) and was facilitated by adding two 5 mm glass beads and vortexing the liposomal suspension. To enhance drug encapsulation efficiency, the FAT procedure was performed by freezing the MLVs in liquid nitrogen and thawing the samples in a water bath (six to seven cycles) at the same temperature that was employed in the formation of the MLVs. Unilamellar liposomes were prepared by extrusion through two stacked polycarbonate filters of 100 nm pore size (Nucleopore, Whatman) at 20, 50, or 66 °C on a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, BC, Canada). Non-encapsulated drug was removed from the liposomes on a Sephadex® G-50 fine (1 cm × 20 cm) column equilibrated with 150 mM NaCl solution. Opalescent liposome fractions were collected and the drug and lipid concentrations were then determined.

The quantification of meropenem in liposomes was performed by the HPLC method.

Meropenem-encapsulated liposomes (50 μl) were dissolved in 950 μl of methanol. The chromatographic system consisted of a Waters 600 Controller, 717plus Autosampler, 996 Photodiode Array Detector, and a Knauer Lichrosorb-100, RP-18.5 μm, 4.6 mm/25 mm column. The data were collected and processed by a Millennium 32 Chromatography Manager. The chromatographic mobile phase consisted of 12% (v/v) acetonitrile and aqueous phosphate buffer (10 mM, pH 6.65). Twenty-μl samples were injected onto the HPLC column and run with a flow rate of 1.5 ml/min. The 210–350 nm spectral range was collected for drug identification and a wavelength of 300 nm was chosen for its quantification. The results were calculated based on a standard curve prepared for the meropenem amount range of 0.2–1.0 μg.

Liposome size (multimodal analysis, volume weighted) and zeta potential were routinely determined on a Zetasizer 5000 (Malvern Instruments Ltd., Malvern, UK). Lipid concentration was determined colorimetrically with ammonium ferrothiocyanate (Stewart, 1959).

Table 1
Properties of liposomes containing meropenem

Liposome composition	Size analysis (nm)	Zeta potential analysis in PBS (mV)	MEM encapsulation efficiency (%)	Drug:lipid ratio (w:w)
PC/DOTAP 9:1 (m/m)	142 ± 12	+8.3 ± 1.0	5.6 ± 0.8	1:10.71
PC/DOTAP 8:2 (m/m)	129 ± 6.2	+18.8 ± 1.8	7.2 ± 1.1	1:8.33
PC/Chol/SA 5:3:2 (m/m)	107 ± 3.3	+24.2 ± 1.2	4.9 ± 0.7	1:12.25
DPPC/Chol/SA 5:3:2 (m/m)	119 ± 5.2	+22.5 ± 0.6	4.3 ± 1.1	1:13.95
DSPC/Chol/SA 5:3:2 (m/m)	152 ± 11	+22 ± 4	4.55 ± 0.8	1:13.5
PC/DOPE/SA 4:4:2 (m/m)	110 ± 2.2	+18.5 ± 0.3	4.5 ± 0.4	1:13.3
PC/DOTAP/Chol 5:2:3 (m/m)	121 ± 3.8	+20.7 ± 2.2	5.7 ± 0.5	1:10.5
DPPC/DMPG 18:1 (w/w)	120.7 ± 2.8	−8.5 ± 1	4.9 ± 0.7	1:12.25
PC/DMPG 8:2 (m/m)	122 ± 4.4	−24.5 ± 1.2	3.7 ± 0.4	1:16.2
DMPC/Chol/PS 4:3:4 (m/m)	115 ± 4	−31.5 ± 1.6	7.2 ± 1.3	1:8.7
PC/PS 8:2 (m/m)	126 ± 5.7	−12.7 ± 1.5	6.1 ± 0.9	1:9.8
PC/DMPG/DOPE 6:2:2 (m/m)	121 ± 3.3	−22.2 ± 1.2	5.9 ± 0.7	1:10.17

2.3. Encapsulation of carboxyfluoroscein in liposomes and liposome permeability tests

A water-soluble fluorescent marker, 5,6-carboxyfluoroscein, was purified by the method of Ralston et al. (1981). For bilayer permeability tests, the liposomes PC/DOPE/SA 4:4:2 m/m and PC/DOTAP/Chol 5:2:3 m/m were prepared as follows: 5 mg of total lipids in 500 μl of chloroform were placed in 50 ml round-bottom flask and then a thin lipid film was prepared by evaporating the organic solvent under vacuum. The lipid film was then dried for 1 h under high vacuum. The dry lipid films were hydrated by adding 1 ml of a 100 mM 5,6-carboxyfluoroscein (CF) solution in a phosphate buffer solution (PBS), pH 7.2–7.4, and shaken with a vortex mixer. After formation of MLV liposomes, a FAT procedure (six cycles) was performed to equilibrate the CF concentration inside and outside the liposomes as in the case of liposomes carrying meropenem. The resulting FAT-MLV liposomes were then extruded 12 times through two stacked polycarbonate filters of 100 nm pore size (Nucleopore) at 20 °C on a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, BC, Canada). CF not entrapped was removed from the liposomes on a Sephadex® G-50 fine (1.2 cm × 20 cm) column equilibrated and eluted with PBS. Finally, for the bilayer permeability tests the liposomes were diluted 50-fold in PBS and incubated in the dark at 37 °C for 120 h. The percentage of CF released was determined fluorometrically (excitation 490 nm, emission 520 nm) on a Kontron SMF 25 fluorophotometer (Kontron Instruments, Italy) after 1, 3, 12, 24, 48, 96, and 120 h. CF release was calculated according to the equation

$$\%\ \text{of CF release} = 100 \times (F_t - F_0)/(F_\infty - F_0)$$

where F_0 is the initial fluorescence of the liposomal suspension (no CF released), F_∞ the fluorescent intensity of CF when the liposomes entrapping CF were completely disrupted by Triton X-100, and F_t the fluorescent intensity of CF liposomes at time t.

2.4. Drug leakage in the culture medium

To determine drug leakage into the culture medium, PC/DOTAP/Chol 5:2:3 and PC/DOPE/SA 4:4:2, the best liposomal compositions of meropenem-bearing liposomes, were prepared as described above from 20 mg of total lipids. After liposome extrusion and free drug removal, liposomes at a concentration of 10 mg of total lipid/ml were gently mixed with broth medium in a volume ratio of 1:10 and incubated at 37 °C for 24 h. For a determination of drug leakage, 200 μl samples were taken at times 0, 1, 3, and 24 h of incubation. Then free drug was separated from the liposomes using micro sephadex G-50 fine columns. The quantification of meropenem in the liposomes was performed by the HPLC method and the amount of drug was compared with the initial value in time 0. Liposomal fractions (100 μl) with encapsulated meropenem were dissolved in 900 μl of methanol. The chromatographic system comprised a Waters 600 Controller, 717plus Autosampler, 996 Photodiode Array Detector and Knauer Lichrosorb-100, RP-18.5 μm, 4.6 mm/25 mm column. The data were collected and processed by Millennium 32 Chromatography Manager. The chromatographic mobile phase consisted of 12% (v/v) acetonitrile and aqueous phosphate buffer (10 mM, pH 6.65). Twenty-μl samples were injected on HPLC column and run with the flow rate of 1.5 ml/min. The 210–350 nm spectrum range was collected for drug identification and the 300 nm wavelength was chosen for its quantification. The results were calculated based on a standard curve prepared for the meropenem amount range of 0.2–1.0 μg.

2.5. Bacterial strains

Seven clinical mucoid strains of *P. aeruginosa* (12/3, 82/3, 15/3, 9/5, 49/3, 14/3, and 133) were isolated from tracheal infections of patients hospitalised in the ICU ward of the Lower Silesian Centre of Paediatrics in Wroclaw, Poland. The microbiological isolation from the clinical samples was performed at the bacteriology department of the same hospital. As a reference, the *P. aeruginosa* ATCC 27853 strain from the American Type Culture Collection was used. Bacteria were stored at −70 °C in Trypticase Soy Broth (Becton Dickinson and Company, Cockeysville, MD, USA) supplemented with 20% glycerol.

2.6. Zeta potentials measurements of P. aeruginosa strains

Bacterial cells were washed twice in PBS by centrifugation and then diluted in PBS to attain a medium count rate as measured by the correlator of the Zetasizer 5000. The bacterial suspension was introduced into the zeta potential measuring cell and the program macro FFR (fast field reversal) was run to measure the zeta potential of the living bacterial cells. The measurements were performed at 25 °C.

2.7. Determination of imipenem susceptibility and carbapenamase production

Etest MBL strips consisting of imipenem or imipenem + EDTA (AB BIODISK, Solna, Sweden) for the in vitro detection of metallo beta-lactamases (MBL) were used. According to the manufacturer's recommendation, the presence of MBL was reflected by a reduction in imipenem (IPM) MIC of ≥ 3 $\log_2$ dilutions in the presence of EDTA or the appearance of a phantom zone or deformation of the IPM ellipse. A positive MBL test indicated the presence of beta-lactamases that hydrolyse carbapenems (Livermore, 2001; Livermore and Woodford, 2000; Senda et al., 1996).

2.8. Determination of bacterial susceptibility

The antimicrobial activity of liposome-encapsulated meropenem was determined in comparison with the free drug by determining the MICs of the *P. aeruginosa* strains. The MIC tests were performed by a broth microdilution method according to the NCCLS standards (NCCLS, 2000). Serial two-fold dilutions from 0.06 to 64 μg/ml of the free and the liposomal drug in PBS, pH 7.4, were prepared. Immediately after preparation, 100 μl of each of the 11 MEM concentrations were placed into microtitre wells. For the experiments, the

bacterial strains were inoculated onto blood agar plates, incubated for 18 h at 35 °C, and then diluted in PBS to the optical density equal to the McFarland No. 0.5. Ten microlitres of bacterial culture was diluted 1:10 (approximate concentration: 10^7 cells/ml) and added to the microtitre wells containing the drug solution and incubated at 35 °C. After 2 h, 100 μl of cation-adjusted Mueller Hinton II Broth (MHB) (Becton Dickinson and Company, Cockeysville, MD, USA) was added to each well. The final concentration of microorganisms was 5×10^5 cfu/ml. The plates were then incubated for 18 h at 35 °C. MIC was defined as the lowest concentration of meropenem at which no visible growth of bacteria was observed after 18 h.

Positive controls (growth) consisted of bacteria in broth and bacteria with empty liposome in broth. Negative controls (sterility) consisted of uninoculated broth and each of the drug/liposome dilutions in broth. Each assay was repeated three times and three additional times on a different day to ensure reproducibility of results.

In vitro killing curves were determined in the presence of 0.5 μg/ml (MIC) and 0.125 μg/ml (1/4 MIC) of free meropenem and meropenem encapsulated in PC/DOTAP/Chol 5:2:3 liposomes for the *P. aeruginosa* ATCC 27853 strain. The growth control consisted of bacteria in MHB alone. A control containing bacterial culture with empty liposomes and free drug was also carried out, but no differences from the growth with the free drug only were observed. Samples of 10 μl were collected after 0, 3, 6, and 16 h of incubation, serially diluted, and plated on Mueller Hinton II Agar (Biomerieux, France). The number of cfu/ml was determined after 18 h of incubation at 35 °C.

2.9. Statistical analysis

Statistical analysis was performed using the *t*-test for independent samples (Statistica 5.0 StatSoft, Poland).

3. Results

Seven cationic and five anionic liposome compositions were prepared (Table 1). The mean vesicle size was between 107 and 152 nm and the meropenem encapsulation efficiency was found to be in the range of 3.7–7.2%. For liposome preparation and drug encapsulation, the thin lipid film method was chosen as the routine method. Because of the characteristics of the meropenem molecule, no active drug-loading method could be applied. Other methods yielding high-efficiency encapsulation, such as the reverse-phase evaporation method, were not suitable because of the presence of residual toxic organic solvents in the liposome preparation. Another issue could be the use of the freeze-dried liposome rehydratation method, which often offers higher encapsulation efficiency than methods such as the thin lipid film method, but in the case of meropenem we obtained similar results when both methods were applied (data not shown). The encapsulation efficiency of the drug was thus relatively low, but similar results were obtained by other investigators (Lutwyche et al., 1998; Omri and Ravaoarinoro, 1996; Puglisi et al., 1995). The drug-to-lipids ratios were, however, high and varied from 1:9 to 1:18 (w/w), depending on the liposome formulation.

The antimicrobial sensitivity results for the tested strains are shown in Table 2. Three clinical strains (12/3, 82/3, 15/3) and *P. aeruginosa* ATCC 27853 showed susceptibility to meropenem and imipenem: their MICs were ≤4 μg/ml. Isolates 9/5 and 49/3 were resistant to both antibiotics (MICs ≥ 16 μg/ml), probably due to the low permeability of the bacterial cover (loss of membrane OprD porin) and the presence of the efflux pump system, because they did not produce carbapenemases. The 14/3 and 113 *P. aeruginosa* strains demonstrated susceptibility to meropenem, but resistance to imipenem. It was also shown that they produced carbapenemases. In the case of these two isolates it was difficult to establish if they exhibited a loss of membrane porin OprD at the same time (resistance to IPM and sensitivity to MEM) (Fung-Tomc et al., 1995; Livermore, 2001). Interesting results were obtained from the zeta potential measurements. Isolates susceptible to both carbapenems showed much lower potentials (≤−11.2 mV) than resistant ones, whose values were between −2.3 and −8.7 mV.

The bactericidal activity of cationic liposomes against the *P. aeruginosa* strains are presented in Table 3. The most efficient of the tested lipid formulations were fluid liposomes composed of PC/DOPE/SA4:4:2 and PC/DOTAP/Chol 5:2:3, as the MICs for the meropenem-sensitive strains were mostly two to four times lower than those of the free drug. The remaining cationic liposomes containing meropenem showed various antimicrobial activities on the tested *P. aeruginosa* isolates. Isolates 9/5, the IPM- and MEM-resistant 49/3, and the carbapenemase-producing 14/3 all exhibited much higher levels of resistance to all the cationic liposomal forms of meropenem than to the free drug. The MICs of the liposomes for these strains were

Table 2
Antibiotic susceptibility and zeta potential of *P. aeruginosa* strains

Bacterial strais	MIC of MEM (μg/ml)	MIC of IPM (μg/ml)	Carbapenemase production	Zeta potential (mV)
P. aeruginosa ATCC 27853	0.5^S	1^S	–	−13.0 ± 1.0
P. aeruginosa 12/3	1^S	1^S	–	−11.2 ± 0.9
P. aeruginosa 82/3	0.5^S	2^S	–	−13.3 ± 0.7
P. aeruginosa 15/3	0.5^S	1^S	–	−15.3 ± 0.9
P. aeruginosa 9/5	16^R	16^R	–	−4.9 ± 0.7
P. aeruginosa 49/3	16^R	32^R	–	−8.7 ± 0.5
P. aeruginosa 14/3	4^S	32^R	+	−2.3 ± 1.0
P. aeruginosa 113	2^S	16^R	+	−3.6 ± 1.1

MEM: meropenem; IPM: imipenem. According to NCCLS breakpoints—R: resistant; S: susceptible.

Table 3
MIC values of free MEM and cationic liposome-incorporated MEM against *P. aeruginosa* strains

Bacterial strains	Free MEM	PC/DOTAP 9:1	PC/DOTAP 8:2	PC/Chol/ SA 5:3:2	DSPC/Chol/ SA 5:3:2	DPPC/Chol/ SA 5:3:2	PC/DOPE/ SA 4:4:2	PC/DOTAP/ Chol 5:2:3
P. aeruginosa ATCC 27853	0.5	2	1	0.25	1	0.25	0.125	0.125
P. aeruginosa 12/3	1	32	32	4	0.5	2	1	0.25
P. aeruginosa 82/3	0.5	2	2	0.25	1	1	0.125	0.25
P. aeruginosa 15/3	0.5	4	2	0.5	1	1	2	0.25
P. aeruginosa 9/5	16	64	64	64	64	64	64	64
P. aeruginosa 49/3	16	64	64	64	64	64	64	64
P. aeruginosa 14/3	4	64	64	64	64	64	64	32
P. aeruginosa 113	2	8	8	16	2	8	1	2

≥4 times higher than for free meropenem. No significant differences were observed between the antimicrobial activity of the solid liposomes (DPPC/Chol/SA and DSPC/Chol/SA) and the fluid formulations. There was also no correlation between the zeta potentials of the cationic liposome formulations (Table 1) and the observed antimicrobial activities.

To verify that encapsulation of the meropenem is necessary to enhance the antimicrobial activity of this drug, killing curves were determined for the 1/4 MIC, MIC, and 4× MIC of the antibiotic. The growths of *P. aeruginosa* ATCC 27853 cultures exposed to free meropenem and encapsulated meropenem in one of the most efficient liposome formulations (PC/DOTAP/Chol 5:2:3) are presented in Figs. 1–3. Free meropenem in concentrations of 0.5 μg/ml (MIC) and 2 μg/ml (4× MIC) showed a significantly better bactericidal activity ($p < 0.05$) after 6 h of incubation (cfu/ml reduction by 10^3, both) than the liposomal form (cfu/ml reduction by a factor of 33 and 10^2, respectively). After 18 h of incubation, the decreases in the bacterial counts in MIC and 4× MIC concentrations of free and encapsulated meropenem were nearly the same (Figs. 2 and 3). In Fig. 1 are presented the killing curves of the *P. aeruginosa* ATCC 27853 strain exposed to free and encapsulated meropenem in a sub-MIC concentration (0.125 μg/ml). The free drug had statistically significantly ($p < 0.05$) greater bactericidal efficacy after 3 and 6 h of incubation, but after 18 h, bacterial growth reached 10^8 cfu/ml, similar to that of the control. We obtained significant

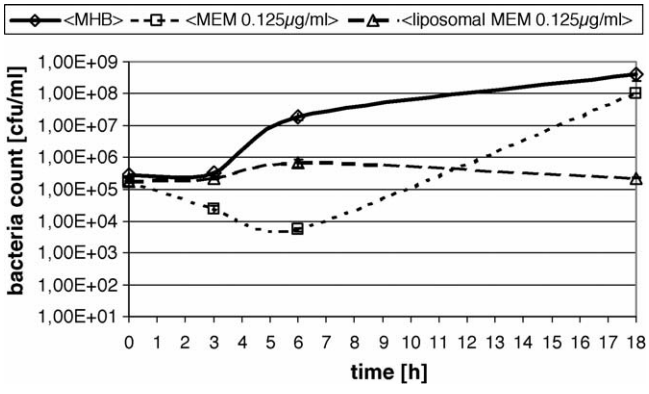

Fig. 1. Growth of bacteria exposed to 0.125 μg/ml of free meropenem (1/4 MIC) and meropenem encapsulated in PC/DOTAP/Chol 5:2:3 liposomes for *Pseudomonas aeruginosa* ATCC 27853 strain. Control was growth of bacteria in Mueller Hinton Broth (MHB). Data points represent means of the three samples ± S.D.

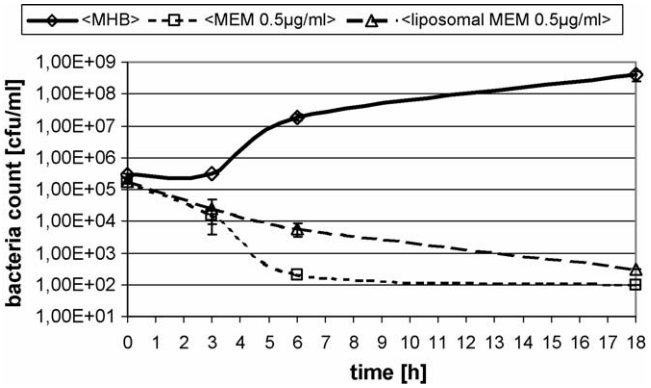

Fig. 2. Growth of bacteria exposed to 0.5 μg/ml of free meropenem (MIC) and meropenem encapsulated in PC/DOTAP/Chol 5:2:3 liposomes for *P. aeruginosa* ATCC 27853 strain. Control was growth of bacteria in Mueller Hinton Broth (MHB). Data points represent means of the three samples ± S.D.

inhibition of bacterial growth ($p < 0.05$) after 18 h of incubation using liposomal meropenem compared with the control growth and free meropenem. It has been proved that encapsulation of meropenem is necessary to enhance its antimicrobial activity. The sub-MIC concentration of antibiotic inhibited the growth of bacterial culture because the liposomes promoted effective interaction between bacteria and drug and increased the drug concentration close to the bacterial cells.

To determine the stability of the most efficient cationic liposome formulations (PC/DOPE/SA4:4:2 and PC/DOTAP/Chol

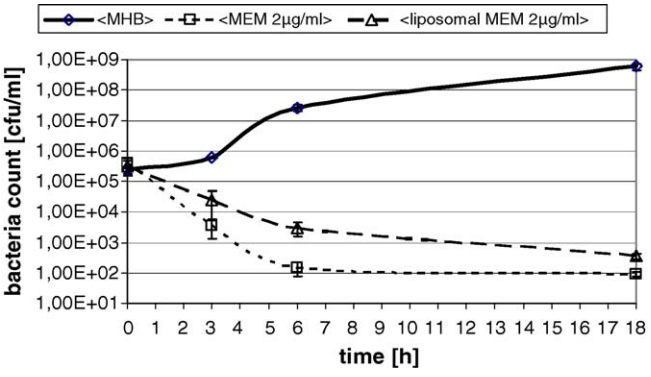

Fig. 3. Growth of bacteria exposed to 2 μg/ml of free meropenem (4× MIC) and meropenem encapsulated in PC/DOTAP/Chol 5:2:3 liposomes for *P. aeruginosa* ATCC 27853 strain. Control was growth of bacteria in Mueller Hinton Broth (MHB). Data points represent means of the three samples ± S.D.

Table 4
MIC values of free MEM and anionic liposome-incorporated MEM against *P. aeruginosa* strains

Bacterial strains	Free MEM	DPPC/DMPG 18:1	PC/DMPG 8:2	DMPC/Chol/PS 4:3:4	PC/PS 8:2	PC/DMPG/DOPE 6:2:2
P. aeruginosa ATCC 27853	0.5	4	0.5	2	1	0.5
P. aeruginosa 12/3	1	16	1	16	4	4
P. aeruginosa 82/3	0.5	4	0.5	2	2	1
P. aeruginosa 15/3	0.5	4	0.5	2	1	1
P. aeruginosa 9/5	16	64	64	64	64	64
P. aeruginosa 49/3	16	64	64	64	64	64
P. aeruginosa 14/3	4	64	32	16	8	8
P. aeruginosa 113	2	64	8	nd	nd	nd

nd: Not determined.

5:2:3), two release profiles were determined. As an indicator of liposome permeability, carboxyfluorescein release profiles were measured in PBS at 37 °C. The stability of both formulations in PBS (Fig. 4) was similar and liposome leakage reached approximately 6, 8, 14, and 24% after 1, 3, 12, and 24 h, respectively. The PC/DOTAP/Chol 5:2:3 formulation was more stabile after 48, 96, and 120 h of incubation (approximately 31, 55, and 67%, respectively) than the PC/DOPE/SA4:4:2 liposomes, where the release percentages reached 39, 71, and 81%, respectively. To check the drug leakage in the culture medium, the meropenem release profile from the liposome formulations was carried out in MHB medium at 37 °C (Fig. 5). Meropenem leakage from the PC/DOTAP/Chol 5:2:3 liposome compositions in the culture medium was similar to carboxyfluoroscein leakage in PBS and reached approximately 6, 13, and 23% after 1, 3, and 24 h, respectively. The PC/DOPE/SA4:4:2 formulation containing meropenem was less stabile in MHB and showed drug leakage in the range of 9, 18, and 33% after 1, 3, and 24 h of incubation time, respectively.

The MIC values of anionic liposome-incorporated MEM against *P. aeruginosa* strains are presented in Table 4. The lipid formulation DPPC/DMPG 18:1 (Fluidosomes®) had the least bactericidal efficacy against all strains. The MICs of liposomal meropenem were 4–16 times higher than those of the free drug. Similar MICs were obtained for other anionic liposomal forms compared with free antibiotic except for the resistant strains 9/5 and 49/3. Anionic formulations showed lower antibacterial activity than cationic ones, as the MICs for meropenem encapsulated in the anionic formulation were equal to or higher than the MICs of the free drug.

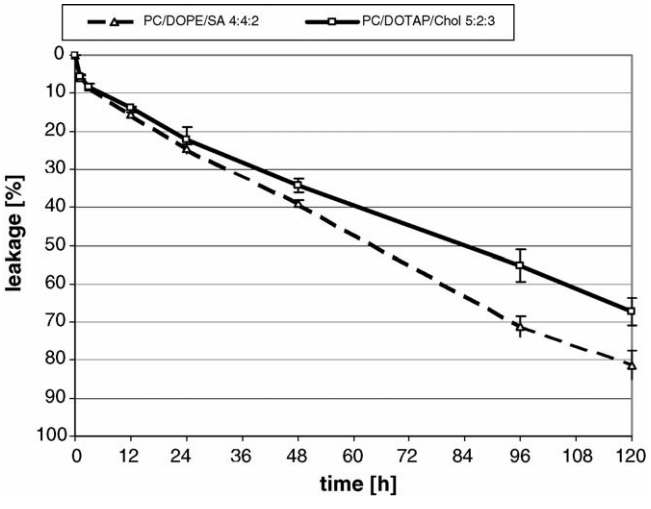

Fig. 4. Carboxyfluorescein release profiles from PC/DOTAP/Chol 5:2:3 and PC/DOPE/SA 4:4:2 liposome formulations in PBS at 37 °C.

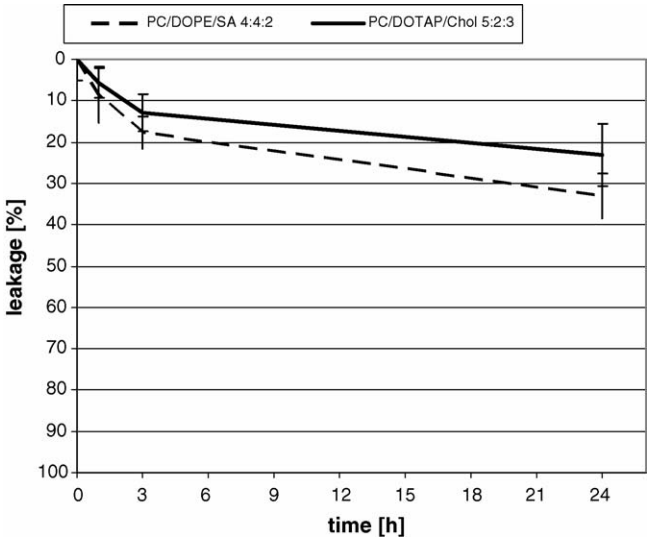

Fig. 5. Meropenem release profiles from PC/DOTAP/Chol 5:2:3 and PC/DOPE/SA 4:4:2 liposome formulations in MHB medium at 37 °C.

4. Discussion

P. aeruginosa is known as one of most drug-resistant bacterial pathogens. The resistance mechanism is largely based on the very low, non-specific permeability of its outer membrane or the presence of an efflux pump system removing drugs from the cell (Carmeli et al., 2001; Livermore, 2001; Nikaido, 1994; Siami et al., 2001). Fusional interactions between the bacterial membrane phospholipids and liposome-containing antibiotic could be promising in overcoming non-enzymatic drug resistance (Beaulac et al., 1998; Omri and Ravaoarinoro, 1996; Puglisi et al., 1995). Omri and Ravaoarinoro (1996) demonstrated an increase in bactericidal activity of netilmicin entrapped in liposomes (PC/Chol 7:1) against Gram-negative *E. coli*, *P. aeruginosa*, and *Stenotrophomonas* (*Xanthomonas*) *maltophilia*. The same authors (Omri et al., 2002) proved a higher antibacterial efficacy of the liposomal form of polymyxin B (DPPC/Chol 2:1) compared with the free drug against *P. aeruginosa* strains.

Beaulac and Sachetelli (Beaulac et al., 1998; Sachetelli et al., 2000) demonstrated fusion between Fluidosomes® (liposomes consisting of DPPC/DMPG 18:1) and *P. aeruginosa* cells. Tobramycin encapsulated in Fluidosomes® in a sub-MIC concentration showed decreases in the bacterial counts of *P. aeruginosa*, *E. coli*, *S. maltophilia*, and *B. cepacia.* In our study we tested the antibacterial activity of Fluidosomes® containing meropenem. This formulation exhibited the highest MICs for *P. aeruginosa* strains among all our tested liposomes (Table 4). The encapsulated meropenem probably interacts with lipids, changing the liposome properties, and inhibits the fusion process between liposome and bacteria.

Rigid liposome formulations have a good antibacterial activity both in vitro and in vivo against facultative and obligate intracellular pathogens. These liposomes are delivered to the infection site by the mononuclear phagocyte system (Deol and Khuller, 1997; Mehta et al., 1993; Salem and Duzgunes, 2003; Schiffelers et al., 2001; Wong et al., 2003). However, in the treatment of extracellular infection, eradication of *P. aeruginosa* was not observed when antibiotics in rigid liposome were used (Beaulac et al., 1996). In this study, the in vitro antibacterial activities of both rigid and fluid liposome were determined. Three formulations with increasing fluidity, i.e. DSPC/Chol/SA 5:3:2 < DPPC/Chol/SA 5:3:2 < PC/Chol/SA 5:3:2, were tested (Table 3). Rigid and fluid formulations showed various bactericidal effects acting in sub-MIC and MIC concentrations on sensitive *P. aeruginosa* strains.

We have determined the in vitro activity of meropenem encapsulated in liposomes against susceptible *P. aeruginosa* strains, against *P. aeruginosa* strains resistant due to low permeability or efflux, and against *P. aeruginosa* strains producing carbapenemases. The sensitive isolates showed low zeta potential values (Table 2) and the cationic liposome formulations were more effective than the anionic liposomes against these strains. The most bactericidal activity was shown by the fluid PC/DOPE/SA 4:4:2 and PC/DOTAP/Chol 5:2:3 formulations, as the MICs for the meropenem-sensitive strains were two to four times lower than those of the free drug (Table 3). We conclude from the CF and meropenem release profiles of these two liposome formulations (which reached 22–25% after 24 h of incubation at 37 °C) that sub-MIC antimicrobial efficacy is not correlated with rapid drug release from liposomal formulations, but rather from electrostatic interaction/fusion between the liposomes and the bacterial cell.

It is well known that cationic liposomes attach to the bacterial biofilm produced by staphylococci, *P. aeruginosa*, and oral bacteria (Sihorkar and Vyas, 2001). Liposomal vesicles are successfully used as the targeted carriers of antiseptic drugs in local applications. It seems very promising to apply fluid cationic formulations in the treatment of respiratory tract infections or to prevent bacterial biofilm formation when in vitro the most active PC/DOPE/SA 4:4:2 and PC/DOTAP/Chol 5:2:3 formulations exhibited bactericidal efficacy at concentrations two to four times lower than that of the free drug against *P. aeruginosa* strains.

Resistant isolates exhibited higher zeta potentials than did susceptible strains (Table 2), which was probably related to the differences in the composition of the outer membranes of their cells. Resistant *P. aeruginosa* strains showed increased unsusceptibility to all cationic and anionic liposomal formulations containing meropenem. Bacterial cells showing low permeability of the outer membrane probably have liposome binding properties different from those of meropenem-sensitive strains of *P. aeruginosa*.

The in vitro experiments were performed to determine the role of antibiotic carriers mainly in the targeting of bacterial cells. Liposomal formulations of antibiotics improve in vivo their pharmacokinetic properties and antimicrobial activity (Bakker-Woudenberg, 1995; Pinto-Alphandary et al., 2000). Prolonged plasma half-lives and enhanced retention at infected sites can help to eradicate drug-susceptible and -resistant strains using liposomal formulations of antibiotics. On account of these liposome properties, further investigation verifying the antimicrobial activity of meropenem liposomal formulations will be carried out in an animal model.

Acknowledgement

This study was supported by Polish Scientific Research Committee research grant No. 3P04B 01924.

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