Iranian Journal of Pathology (2009)4 (4), 151 -156

Original Article

In Vitro Bactericidal Activity of Encapsulated Amikacin in Liposome

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ABSTRACT

Background and Objectives: **The most common problems limiting the medical use of aminoglycosides have been the nephro- and oto-toxicities as well as the increasing bacterial resistance. Encapsulation of drugs into liposomes enhances their efficacy while reducing their toxicities. The aim of this study was to evaluate the antimicrobial activity of free and liposomal amikacin.**

Material and Methods: **Encapsulated amikacin into liposome was prepared by sonication. The drug contained in the liposome was measured by HPLC after lysis of vesicles by 0.2% Triton X-100. The amikacin kinetic released from liposomes in the presence of normal human pooled plasma was also evaluated. The MICs of this drug for *Pseudomonas. aeruginosa* (ATCC 27853), *Escherichia. coli* (ATCC 25922), *Streptococcus. faecalis* (ATCC 29212) and *Staphylococcus. aureus* (ATCC 29213) were determined and compared to those of the respective free drug using a broth dilution method.**

Results: **In the presence of plasma, liposomal retention of amikacin was 80.25 ± 0.55% ($P \leq 0.05$) after 1 h of incubation and then remained nearly constant over a 24 h period of the study. The encapsulation efficiency of liposomal preparation was 24.36% ± 0.14 ($P \leq 0.05$) of the initial amount of the drug in solution. The MICs of liposomal amikacin against all bacterial strains tested were lower than MICs of free amikacin.**

Conclusion: **The amikacin appears a promising approach in the management of bacterial infections and should be further evaluated *in vivo* experiments.**

Key words: Amikacin, *E coli*, Liposome, *Pseudomonas aeruginosa*, *Staphylococcus aureus*

Introduction

Aminoglycoside antibiotics are used alone or in combination with penicillin or cephalosporin to treat various serious infections caused by aerobic Gram-negative bacteria or aerobic Gram-positive cocci (1). However, their nephrotoxicity, ototoxicity and neuromuscular paralysis limit their clinical application (2-5). In addition, bacterial resistance to aminoglycosides is increasing, in particular resistance that results from plasmid-mediated elaboration of aminoglycoside- degrading enzymes (6, 7). A delivery system that reduces the drugs toxicity while increasing their therapeutic index is a great interest

Received: 17 December 2008
Accepted: 1 May 2009
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and liposomes can provide the benefits (8). Liposomes are colloidal vesicles ranging from a few nanometers to several micrometers in diameter (9).

The main reasons for their use include sustained drug release, reduced dose requirement and/or possibility of administering a larger dose with reduced toxicity and increased sensitivity of bacteria to the liposome-encapsulated drug by preventing enzymes, immunological and chemical inactivation (10).

Hydrophilic drugs such as aminoglycosides can be entrapped in aqueous compartments of liposomes, whereas hydrophobic drugs are incorporated in their lipid bilayer (11). The encapsulation of aminoglycosides into liposomes markedly alters their pharmacokinetics, increases half-lives and area under the curves and causes a shift in drug accumulation from the kidney to other organs, thus potentially reducing nephrotoxicity (12). We have then incorporated amikacin in liposome, composed 1, 2- dimyristoyl-*sn*-glycero- 3-phosphocholine (DPPC) and cholesterol, and assessed this activity against selective Gram-positive and Gram-negative bacteria. The liposomal encapsulation efficiency and kinetic release of this drug was also evaluated.

Material and Methods

Antimicrobial agent, lipids, and reagents

Amikacin sulphate, o-phthaldialdehyde, Triton X-100 and 2-mercaptoethanol were obtained from Sigma-Aldrich Chemie, Steinhein, Germany. DPPC and cholesterol were from Lipoid Co, Germany. Sodium carbonate, potassium di-hydrogen phosphate, sodium hydroxide, 2-propanol and boric acid were purchased from Panreac, Barcelona. Methanol was obtained from Merck Darmstad, Germany. Amikacin solution was freshly prepared on the day of use with appropriate allowance for drug potency.

Bacterial strains

Bacterial strains of *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), *Streptococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 29213) were used as microorganisms. For experimentation, these organisms were inoculated on to blood agar plates and incubated for 20 h at 37 ºC.

Preparation of liposomes

Multilamellar liposomes (MLV) containing amikacin were prepared from a lipid mixture of DPPC and cholesterol in molar ratio of 2:1 by the method as previously reported (13). In brief, after evaporation of the chloroform with a rotary evaporator (Heidolph Rotavapor, laborata 4000 efficient, Germany) under vacuum at 30 ºC, the thin lipid film formed on the vessel wall was then dispersed by agitation in 6 ml of an aqueous solution of amikacin at a concentration of 10 mg/ml. The lipid suspensions, submerged in ice-bath, were sonicated for 40 s in an ultrasonic bath (Bandelin Sonorex, RKH10H, Germany). Unencapsulated antibiotic was removed by centrifugation (60,000 g for 45 min at 4 ºC). The supernatant was carefully collected using a Pasteur pipette. The pelleted vesicle was resuspended in saline and washing was repeated twice. The final pellet was resuspended in 6 ml of distilled water and assay for drug activity and content. For this experiment, the size and homogeneity of liposomal suspensions were determined by negative-stain electron microscopy as previously reported (14), and were found to be uniform (50 to 100 nm) and reproducible for this preparation tested. Amikacin in liposomes was measured by the HPLC assay after disrupting the lipid membranes with 0.2% (v/v) of Triton X-100. Empty liposomes were prepared in the same manner but without amikacin.

High Performance Liquid Chromatography (HPLC) analysis

The chromatographic equipment consisted of a Shimadzu pump (mod. LC-10AD), a Shimadzu system controller (mod. SCL-10A), and a Shimadzu auto injector (mod. SIL-10AXL) coupled to a Shimadzu fluorescence detector (mod SDV 30 plus). Data collection was accomplished with a Shimadzu chromatography data system Class VP version 6.0. Reverse phase Kromasil 100 C-18 columns (15 x 0.46 cm) (Tecnokroma. Barcelona, Spain) of 5 mm particle size were used. The mobile phase was composed of a mixture (69:31 v/v) of methanol, water, and 2.2 g of EDTA tripotassium salt (Sigma, Steinhein, Germany) (15). This mobile phase was prepared daily, filtered in a Supelco vacuum system (mod. 7-8094) with a 0.45 mm nylon filter (Whatman. Malstone, U.K.),

and degassed in a Shimadzu ultrasound bath (mod. RKH10H). Flow rate during the assays was 1.5ml/min and λ exc was 360 nm and λ emi was 435 nm. The process was carried out in a temperature-controlled bath at 37ºC (16).

Encapsulation efficiency

Encapsulation efficiency was calculated as the percentage of antibiotic incorporated in liposomes relative to initial total amount of antibiotic in solution.

Encapsulation drug release kinetics of liposomes

Normal human pooled plasma (preheated at 37 ºC) were supplemented with either amikacin in liposomal form at concentration 500µg/ml an incubated at 37 ºC under 5% CO2 (to maintain the plasma pH at close to physiological levels 7.4 with mild constant agitation) (17). At 0 (after incubation of liposomes in pooled plasma), 1, 2, 3, 4, 5, 6, 7, 8 and 24 hours after the addition liposomal antibiotics, the sera samples were removed (by centrifugation at 60,000 g for 45 min) and antibiotic concentrations in supernatants were determined by HPLC assay. Released of antibiotic was expressed as a percentage of liposomal retention of the initially encapsulated antibiotic added at 0 h.

Minimal inhibitory concentrations determination

The Minimal inhibitory concentrations *(*MICs) of free and liposomal antibiotics for all strains were determined by standard macro broth dilution technique as recommended by CLSI (formerly NCCLS) (18). Briefly, serial dilutions of free or liposome encapsulated antibiotics (0.5, 1, 2, 4, 8, 16, 32, 64, 128, 256, 512, and 1,024 µg/ml) in Mueller-Hinton broth were prepared. Bacterial suspensions were then added to each tube to achieve final inoculums of 1×10^5cfu/ml. The lowest concentrations of antibiotic formulations that inhibited the visible bacterial growth after 24 h at 37 ºC were defined as Minimal inhibitory concentrations.

Data analysis

The results are expressed as means ± SEM (Standard Errors of the Means) obtained from three independent experiments. Comparisons were made by paired student's *t*-test, and $P \leq 0.05$ value was considered significant.

Results

Entrapment efficacy

Entrapment efficacy as a percentage of the initial amount of the drug added to phospholipids. Encapsulation rate of amikacin was 24.36% ± 1.14 of the initial amount of drug in solution.

Antibiotic release kinetics

Fig. 1 present in vitro release kinetic data on amikacin from liposomes in presence of normal human pooled plasma incubated at 37 ºC with mild agitation. One hour after the liposomes were added to plasma, approximately 80% to 90% of amikacin was retained (80.25 ± 0.55%). Once drug release occurred, the antibiotic in plasma was maintained at a plateau, which was stable over a time span of 24 h for this antibiotic (Fig. 1).

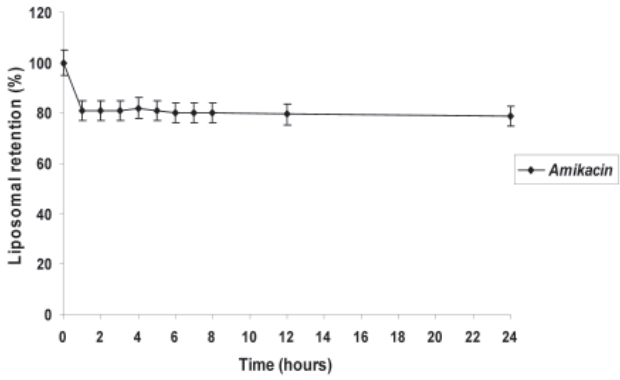

Fig.1: Liposomal amikacin retention of liposome. Results are given as means ± SEM of three separate experiments

Antimicrobial activity of free and liposomal amikacin

The MICs values of amikacin in both free and liposomal forms for *E. coli, P. aeruginosa, S. faecalis and S. aureus* are shown in Table 1. The MICs of liposomal amikacin were lower than those free amikacin against *E. coli, S. faecalis, S.aureus* and *P. aeruginosa* decreased 3-, 3-, 2- and 2-folds, respectively. Empty liposomes have no effect on bacterial growth. The combination of empty liposomes and free amikacin had an antimicrobial activity similar to that of respective free amikacin.

Table 1: In vitro antimicrobial activity of free and liposomal amikacin against on selective laboratory strains of bacteria

Drug / Bacterial strains	Minimum inhibitory concentration (µg/ml)		
	F-AMK	L-AMK	F-AMK+ E- LIPO
E. coli (ATCC 25922)	4	0.5	4
P. aeruginosa (ATCC 27853)	2	0.5	2
S. faecalis (ATCC 29212)	256	32	256
S. aureus (ATCC 29213)	4	1	4

Minimal inhibitory concentrations (MICs) were determined by standard broth dilution technique. The MIC was recorded to be the lowest concentration of the drug that prevented visible growth and expressed in µg/ml. F-AMK: free amikacin; L-AMK: liposomal amikacin; E-LIPO: empty Liposome.

Discussion

The use of liposomes as a carrier for delivering antibiotics and other materials has been widely studied (14). However, the main problem associated with their use is either their high persistent material retention or their premature drug release before they attain, in sufficient quantities, the target site. In addition, when encapsulation efficiency is low, high liposomal volumes are required to achieve clinical dosages.

In this report, we evaluated the potential of incorporation of amikacin into liposomes formulated from DPPC and cholesterol. The results presented demonstrate that this antibiotic can be encapsulated into these liposomes. Incubation of the encapsulated antibiotic with pooled plasma maintained at 37 ºC fewer than 5% CO2, to maintain the pH at 7.4 to approximate the physiological conditions making this system an appropriate in vitro model for predicating circulating liposome leakage *in vivo* (19), showed that approximately 20% of the encapsulated amikacin was released from these liposomes. Spontaneous release of this antibiotic was negligible after 1 h and 2.5% after 24 h, as indicated by the control incubation in plasma-free medium. The liposomal permeability can be minimized by the inclusion cholesterol into liposomal structure (20) as it has been done in our liposomal preparation by adding approximately 16% cholesterol. In the present investigation, liposome encapsulation enhanced the antibacterial activity of amikacin against *E. coli, S. faecalis, S. aureus* and by 3-, 3-, and 2-fold, respectively, whereas it reduced the antibacterial activity against *P. aeruginosa* by 2-fold.

Other studies have shown improved efficacy of liposomal antibiotics of different formulations (21-23). For instance, tobramycin encapsulated into negatively charged fluid liposome displayed stronger bactericidal activity than the free drug (23). Nacucchio *et al.* demonstrated that encapsulation of piperacillin in liposomes prepared with phosphatidylcholine and cholesterol (molar ratio, 1:1) protected the drug from hydrolysis by staphylococcal β-lactamase as well. Several hypotheses including reduced electrostatic repulsion of liposomal antibiotics or protection of the drugs from bacterial enzymes may explain the mechanism of enhanced antimicrobial activities of liposomal formulations (24, 25). The major mechanisms conferring antibiotic resistance is preventing the drug from entering the cell, rapid extrusion of the drug, and enzymatic inactivation of the drug or alteration of its molecular target (26). Later studies suggest that mechanisms of resistance work synergistically with decreased permeability and/or the presence of membrane-associated energy driven efflux systems playing an important role in the phenomenon. For example, it is well known that in Gram-negative bacteria, both enzymatic modifications of the drug and impermeability of the outer membrane contribute to resistance (27). These variable effects of amikacin encapsulated on Gram-negative and Gram-positive bacteria (Table 1) are probably due to their fusional interaction leading to variable changes in properties of liposomal and bacterial membrane permeability due to phospholipids transfer from liposome to bacterial cells (28). Additionally, the molecular configuration of antibiotics within liposomes could have played a

role in this interaction and rate of release (28).

In our study the combination of free antibiotic and empty liposomes had an antibacterial activity similar to that if free antibiotics alone. This result suggests that no interaction had occurred between amikacin and phospholipids used.

Conclusion

The encapsulation rate of this drug appear to be interesting and over 75% of liposomal amikacin remain encapsulated over the time-period studied. The encapsulation of amikacin into this liposomal formulation increases its antibacterial activity against all organisms studied, appear to be a promising approach in the management of Gram-negative and Gram-positive bacterial infections, and should be developed for further evaluation *in vivo* experimental studies.

Acknowledgements

This work was supported by a grant from Islamic Azad University, branch of Boroujerd, Iran. The authors declare that they have no Conflict of Interests.

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