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Antimicrobial activity of cephaloridine in the presence of crude and purified preparations of bacterial β-lactamases before and after liposome encapsulation

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Summary

The possible beneficial effect of liposome entrapment of sensitive antibiotics against enzymatic degradation by Gram-negative and Gram-positive bacterial β-lactamases was examined. Cephaloridine was entrapped in unilamellar liposomes composed of egg lecithin, dicetyl phosphate and cholesterol (molar ratio 7:2:1), prepared by the ether injection method. Cephaloridine-loaded liposomes were incubated for different time intervals in pH 7.4 phosphate-buffered saline at 37°C. The rate of loss of entrapped material due to the effects of leakage and degradation was found to be minimal for up to 6 h incubation. Drug-entrapped liposome samples were exposed to various purified and crude bacterial β-lactamase preparations with different substrate profiles and enzyme activities. Substantial protection from β-lactamases was achieved in every case when the rate of loss of entrapped cephaloridine was compared with a solution of cephaloridine containing empty (buffer-containing) liposomes. These findings were postulated to be due to drug adherence onto the outside of liposome membrane and an enzyme concentration dependent disorder of the lipid bilayer packing. The integrity of the unilamellar liposome membrane was investigated by monitoring the leakage of liposome-entrapped amaranth incubated in the presence of purified and crude β-lactamase preparations. The latter was adversely affected in the presence of the bacterial culture filtrate containing induced β-lactamase.

Introduction

Entrapment in liposomes has been proposed as a means of protecting chemically and metabolically labile drugs from degradation. Liposome encapsulation of certain orally inactive macromolecules such as insulin (Patel and Ryman, 1981) and blood coagulation factor VIII (Hemker et al., 1980) have resulted in modest improvements in oral activity. The protection of insulin from the attack of digestive enzymes in the gastrointestinal tract, as opposed to the transmucosal passage of the intact liposomally entrapped form, was suggested as a mechanism by which the improved absorption of insulin in liposomes was obtained (Weingarten et al., 1981; Patel et al., 1985; Kimura, 1988).

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The in vitro protection of insulin from degradative action of three digestive enzymes (pepsin, α-chymotrypsin, and trypsin) was demonstrated for insulin associated with or entrapped in positively charged liposomes (Weingarten et al., 1985).

Several studies have reported on the enhanced antimicrobial activity of liposomally entrapped antibiotics in bacterial cultures (Hodges et al., 1981; Stevenson et al., 1981; Nacucchio et al., 1985). Chowdhury and co-workers (1981) reported the growth inhibitory effect of liposome entrapped penicillins against some penicillin-resistant bacteria in vitro. A possible beneficial effect of liposomal entrapment is protection of sensitive antibiotics against enzymatic degradation by bacterial β-lactamases. This may prolong the period in which effective drug levels are maintained in the body fluids before reaching the target cells.

The present work was undertaken to examine the extent of liposomal protection of drugs using antibiotics and β-lactamases as a model system. Cephaloridine-loaded unilamellar liposomes were selected for study due to their high captured volume per mole of lipid and the low rates of diffusion of cephaloridine across liposome bilayers and susceptibility to a wide range of β-lactamases (Kimura et al., 1980; Barbas et al., 1982).

Material and Methods

Purified egg lecithin grade I was obtained from Lipid Products (South Nutfield, Surrey, U.K.), dicetyl phosphate, cholesterol, and amaranth were purchased from Sigma London Chemical Co. (Dorset, U.K.). Sephadex G-50 and blue dextran 2000 were obtained from Pharmacia (Uppsala, Sweden). *Bacillus cereus* β-lactamases were purchased from Genzyme Biochemicals (Maidstone, Kent; approx. 300 mU/ml (1 unit of β-lactamase is that amount of enzyme which will catalyze the hydrolysis of 1 μM of substrate per min at 30°C, pH 7.0; β-lactamase I measured using benzylpenicillin and β-lactamase II using cephalosporin C in the presence of Zn^{2+}) and 30 mU/ml of β-lactamases I and II, respectively). Cephaloridine sterile powder, methicillin, benzylpenicillin, and partially purified β-lactamase preparations: *Escherichia coli* TEM (*E. coli* TEM: 420 μM cephaloridine catalyzed/ml per h at 37°C), *Enterobacter cloacae* P99 (*Ent. cloacae* P-99: 470 μM cephaloridine catalyzed/ml per h at 37°C), and *Staphylococcus aureus* PC-1 (*S. aureus* PC-1: 650 μM ampicillin catalyzed/ml per h at 37°C) were gifts from Glaxo Laboratories (Greenford, Middlesex). All other chemicals, reagents or HPLC grade were obtained from BDH Chemicals (Poole, Dorset).

Encapsulation of cephaloridine in unilamellar vesicles

The ether injection method for the preparation of unilamellar liposomes was modified from that of Deamer and Bangham (1978). 2.0 ml of a chloroform solution containing 2.77 μmol/ml of egg lecithin, 0.82 μmol/ml of dicetyl phosphate (DCP) and 0.41 μmol/ml of cholesterol (molar ratio 7:2:1) were dried under nitrogen to a thin film in a round bottom flask. The lipid film was dissolved in 2.5 ml ether and 1 ml of this solution was injected at a rate of 0.02 ml/min into a solution of 26 mg cephaloridine in 4 ml of pH 7.4 phosphate-buffered saline (PBS) at 60°C.

A 30 cm $\times$ 1 cm gel chromatographic column (Amicon, Wright Scientific, Glocs., U.K.) packed with Sephadex G-50 (100–300 μm) was washed with PBS plus additional salt (0.94 g/l) prior to the addition of the 4 ml of liposome suspension. The same solution was used to elute the sample. The liposome fraction was separated from the external cephaloridine by monitoring the absorbance of the column effluent at 253 nm, using an 80 μl flow-through cell and an SP6-500 spectrophotometer (Pye Unicam). This procedure was repeated twice.

The concentration of entrapped cephaloridine was determined by HPLC using a Spectra Physics SP 8100 liquid chromatograph, SP 8440 detector set at 255 nm, and an SP 4100 computing integrator (St. Albans, Herts., U.K.). The stationary phase was Hypersil ODS (5 μm) prepacked in a 25 cm $\times$ 4.9 mm (i.d) column (Hichrom House, Reading, Berks.). The mobile phase consisted of

70% (v/v) 0.1 M acetate buffer pH 4.5 and 30% (v/v) acetonitrile. A 1 ml aliquot of the liposome fraction was mixed with an equal volume of 50% (v/v) propanol to rupture the liposomes and 10 μl of the mixture was injected into the pre-heated column at 45°C, at a flow rate of 2 ml/min. Cephaloridine concentration was determined from the standard curve prepared from known drug concentrations ($0.4–0.6 \times 10^{-3}$% w/v) in PBS in the presence of 0.5 mg/ml egg lecithin and propanol (25%, v/v). The calibration curve constructed showed linear responses ($r > 0.999$). Preliminary experiments from our laboratory showed that the stability and the extent of chemical degradation of cephaloridine in PBS and in the presence of buffer containing liposomes (as determined by the HPLC method) closely agreed with the extent of its antimicrobial activity in similar mediums as determined by a conventional microbiological assay method.

The formation of vesicles was confirmed by optical microscopy (Laborlux 12, Leitz, Germany) with a particle diameter of 1.5 μm corresponding to 50% of the cumulative volume frequency distribution determined by a Coulter counter TAII particle sizer (Coulter Electronics Ltd, Luton, Beds.).

Drug release study

The rate of loss of liposomally entrapped cephaloridine due to leakage and degradation of entrapped material was determined in order to establish the incubation period during which these effects were insignificant. Liposome suspensions were incubated at 37°C, and 1 ml samples were withdrawn immediately prior to incubation (i.e. at time zero) and at different time intervals. After passage through the gel column, the eluted liposome fractions were freeze-dried (Edwards), reconstituted with 0.5 ml distilled water and 0.5 ml propanol (50%, v/v), and the residual cephaloridine was determined using the HPLC method described previously. This experiment was repeated three times.

Experiments with partially purified β-lactamases

Purified *B. cereus* broad spectrum β-lactamase mixture and partially purified *E. coli* TEM, *Ent. cloacae* P-99, and *S. aureus* PC-1 enzymes were used. From two separate batches, 0.5-ml aliquots of the suspension of cephaloridine-containing liposomes were incubated at 37°C with 0.25 ml of the bacterial β-lactamases (suitably diluted with PBS) for different time intervals, up to a maximum of 6 h. At the end of each incubation period, 0.75 ml of acetonitrile was added to the reaction mixture to inhibit the β-lactamase and the concentration of unhydrolysed cephaloridine was determined by HPLC. Acetonitrile did not adversely affect the stability of cephaloridine (data not shown).

A control experiment was performed in which buffer-containing liposomes were suspended in a solution of cephaloridine at the same concentration as that found in the test suspension at time zero and were similarly exposed to the β-lactamases and analyzed.

Experiments with the crude β-lactamase preparation

Pure bacterial cultures of *S. aureus* NCTC 8511 in nutrient broth (Oxoid) were induced to produce β-lactamase by the addition of several antibiotic solutions. β-lactamase production was checked by the addition of 1 ml of nitrocefin solution in phosphate-buffered saline (2.4×10^{-2}% w/v) to an equal volume of the bacterial culture. Nitrocefin is a chromogenic cephalosporin which is yellow and changes to red upon cleavage of its β-lactam ring. The culture flask with added methicillin at 1 mg/ml level showed the most activity. This was centrifuged at 38000 $\times g$ for 30 min and the supernatant was filtered through a 0.45 μm pore size disposable filter holder (Schleicher and Schuell, East Molesey, Surrey).

From two separate batches, 0.5-ml aliquots of the liposome-entrapped cephaloridine suspensions were incubated with an equal volume of the bacterial culture filtrate for different time intervals. At the end of each incubation period, samples were treated with acetonitrile to stop the enzymatic reaction and to rupture the liposomes. The residual cephaloridine was determined by the HPLC method. Standard solutions of cephaloridine in the presence of the same final

concentration of culture filtrate were also prepared. A control experiment using buffer-containing liposomes suspended in a solution of cephaloridine at the same final concentration as that found entrapped at time zero was simultaneously carried out and was similarly analyzed.

Evaluation of the integrity of unilamellar liposome membrane on exposure to purified β-lactamase and bacterial culture filtrate

Amaranth, a red dye, is well retained by liposomes (unpublished report) and is not subject to hydrolysis as a result of the action of β-lactamases. Its leakage from the liposomes caused by instability or breakdown of the vesicle membrane was monitored spectrophotometrically. 1-ml aliquots from two separate batches of amaranth-containing liposome suspensions were separately incubated for 6 h at 37°C with 0.5 ml of diluted *B. cereus* and crude β-lactamase preparations. Control samples of the liposome suspension were simultaneously incubated with 0.5 ml of PBS. At the end of the incubation period, the external amaranth was separated from that remaining within the liposomes by sephadex gel chromatography. The eluted liposome fractions were made up to 5 ml with PBS and the absorbance readings were taken at 513 nm by first rupturing the liposomes with propanol.

Results and Discussion

Drug release study

Fig. 1 shows that for up to 6 h incubation at 37°C, the extent of drug loss due to the effects of leakage and degradation of liposomally entrapped cephaloridine was minimal (i.e. 5% loss of initial drug concentration). The liposome suspension showed reduced optical turbidity over the 24 h period, indicating breakdown of the vesicles themselves. Kimura and co-workers (1980) studied the permeability of the liposomal membrane to eight β-lactam antibiotics: ampicillin, amoxicillin, cephalothin, cephaloridine, cepharadine, cephalexin, ceftezol, and cefazolin. Release rate constants of these antibiotics from sonicated egg lecithin liposomes were determined

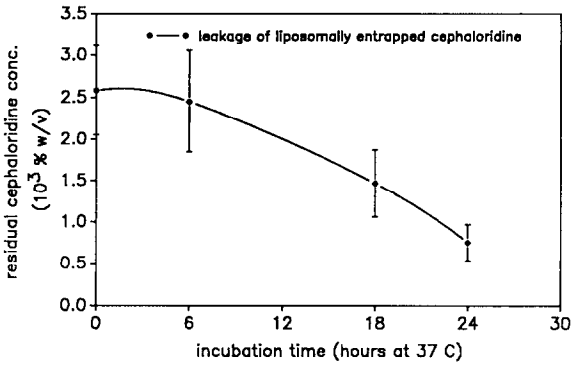

Fig. 1. Rate of loss of liposome-entrapped cephaloridine due to leakage and degradation of entrapped drug. Each value is the mean of three separate experiments.

by a dynamic dialysis method at 37°C. Cephaloridine showed the lowest release rate constant (2.2×10^{-4} min^{-1}), indicating that it was well retained by egg lecithin liposomes. In this study, a semilogarithmic plot of the residual cephaloridine concentration against time (data not shown) gave a similarly low release rate constant of 1.56×10^{-4} min^{-1}.

Experiments with partially purified β-lactamases

Each enzyme preparation was suitably diluted with PBS so that a reaction time of not greater than 6 h was obtained for the complete breakdown of a 2.5×10^{-3}% w/v solution of cephaloridine in buffer. These same enzyme dilution factors were used in experiments with the liposomally entrapped cephaloridine.

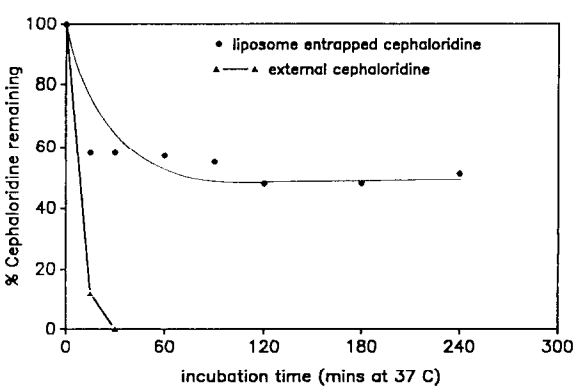

Fig. 2. Effect of *B. cereus* β-lactamase (dilution×500) on liposome-entrapped and free cephaloridine.

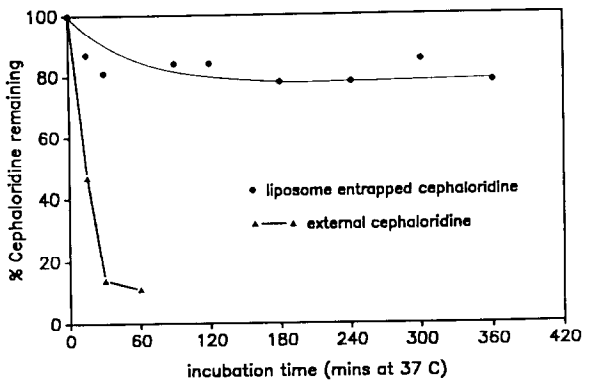

Fig. 3. Effect of *E. coli* TEM β-lactamase (dilution × 100) on liposome-entrapped and free cephaloridine.

Figs 2–4 illustrate the relative rates of hydrolysis of cephaloridine inside and outside unilamellar liposomes on exposure to the purified Gram-negative and Gram-positive bacterial β-lactamases (curves were obtained by second-order polynomial regression). The P-99 enzyme (*Ent. cloacae*) is a cephalosporinase whereas the TEM (*E. coli*) and the *B. cereus* enzymes are broad spectrum β-lactamases, exhibiting approximately equal penicillinase and cephalosporinase activities. In every case, the degradation kinetics appeared to be biphasic in that an initial rapid fall in the concentration of entrapped antibiotic occurred in the first 15 or 30 min of the incubation period, and was followed by a protracted period in which the concentration showed little further change. Unentrapped cephaloridine in the control experiments lost over 90% of its initial activity over the same time period. This indicated that irrespective of the type of enzyme used, liposome entrapment resulted in a substantial degree of protection of cephaloridine from enzymatic breakdown. The early fall in concentration of cephaloridine may be due to the hydrolysis of adsorbed drug on the exterior surface of the liposomes, which would contribute to the total amount of drug associated or entrapped by the vesicles. Di Giulio and co-workers (1991) reported that the release kinetics of ampicillin from liposomes of various lipid composition at neutral pH exhibited a biphasic feature, where the first rapid loss of ampicillin was attributed to the desorption of the drug from the outer shell of the liposomes. In the present study, the heterogeneous size distribution of the liposome suspension also may have contributed to the biphasic degradation kinetics, whereby a certain proportion of smaller vesicles released entrapped cephaloridine rapidly as compared with the larger ones.

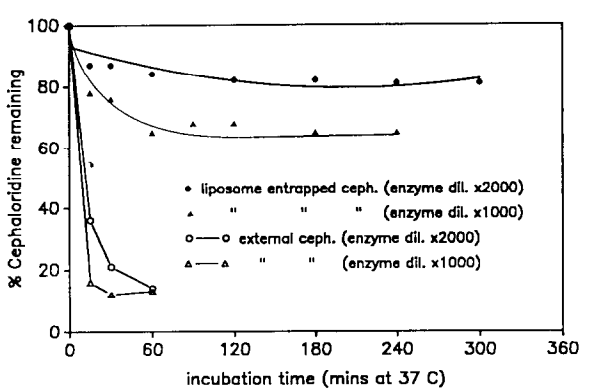

Fig. 4. Effect of *Ent. cloacae* P-99 β-lactamase (dilution × 1000 and × 2000) on liposome-entrapped and free cephaloridine.

When the same batch of liposome suspension was exposed to the P-99 β-lactamase at twice the concentration of that for the experiment in Fig. 4, the pattern of activity loss was similar, but the levelling out occurred at approx. 65% of the original concentration as compared with 80%. This indicated that the rate and the extent of the initial fall in concentration of liposome entrapped cephaloridine were dependent on the concentration of external enzyme at the two levels tested (i.e. dilution × 1000 and × 2000). One explanation for this effect is that when the concentration of the exogenous β-lactamase was doubled, the rate of enzymic degradation of external cephaloridine adsorbed to the lipid surface was increased with little or no change in the rate for liposome-entrapped material. The effect of different concentrations of the *B. cereus* and the TEM enzymes on entrapped cephaloridine was not examined.

It was previously shown that in the absence of the enzyme neither leakage of cephaloridine nor its degradation was a significant factor for the duration of the incubation period used in these

experiments. Although there is no direct evidence of the mechanism of degradation kinetics involved, the above findings may be due to a combination of factors such as drug adherence onto the outside of liposome membrane and leakage of antibiotic induced by association of enzyme with the bilayer membrane. The latter, however, is unlikely because enzyme-induced liposome membrane instability might be expected to result in continuous leakage of drug from within, rather than the abrupt change in the rate of drug loss which is particularly evident in Figs 2–4. The possibility exists that the enzyme only causes temporary disorder of the bilayer packing and membrane integrity is spontaneously restored during continued incubation. Hunt (1981), in a study of the kinetics of ^{14}C sucrose release from multilamellar liposomes incubated in human plasma, reported that plasma caused a rapid rise in liposome membrane permeability which then declined non-linearly. This was presumed to be because of a rearrangement of membrane lipids and adsorbed proteins to form their most stable configuration.

The PC-1 enzyme (*S. aureus*), a penicillinase, did not readily hydrolyse cephaloridine. At the highly concentrated enzyme level (dilution factor $\times 50$), a sustained rather than an abrupt decrease in the concentration of entrapped cephaloridine was obtained throughout the 6 h incubation period (see Fig. 5). This was interpreted to be due to a continued diffusion of cephaloridine across

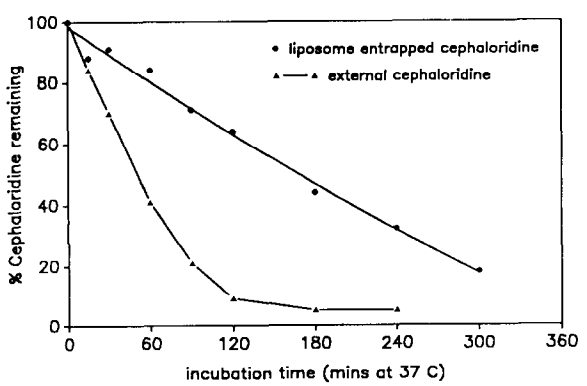

Fig. 5. Effect of *S. aureus* PC-1 β-lactamase (dilution ×50) on liposome-entrapped and free cephaloridine.

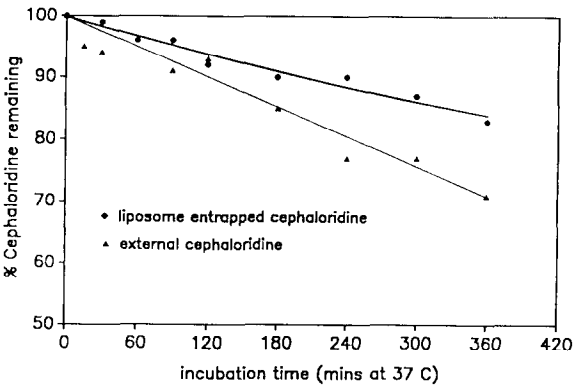

Fig. 6. Effect of *S. aureus* culture filtrate containing induced β-lactamase on liposome-entrapped and free cephaloridine.

the liposome membrane, presumably caused by protein-induced instability or breakdown of the liposome membrane, due to exposure to high concentrations of the enzyme.

Experiments with the crude β-lactamase preparation

The level of induced β-lactamase (penicillinase) activity in the *S. aureus* culture filtrate was measured visually by the change of colour of nitrocefin solution from yellow to red and was determined to be much lower than that of the purified *S. aureus* PC-1 enzyme preparation. Fig. 6 indicates that low levels of sustained hydrolysis of cephaloridine were determined for both the entrapped and control experiments. This corresponded to 20 and 30% loss of initial drug activity, respectively. The culture filtrate contained many dissolved solutes and leakage of entrapped cephaloridine was suspected to occur as a result of osmotic fragility and instability or breakdown of the liposome bilayer structure. The amaranth leakage studies described below also examined the effect of incubation with bacterial culture filtrate on the liposome membrane permeability.

Integrity of unilamellar liposome membrane on exposure to purified β-lactamase and bacterial culture filtrate

Leakage of liposome entrapped amaranth, a marker substance, was determined after 6 h incubation with the crude *S. aureus* and purified *B.*

cereus β-lactamases. The control experiment consisted of incubation with phosphate-buffered saline. In the experiment with the purified enzyme, only 13% of the original amount of entrapped amaranth diffused across the liposome membrane, whereas the comparable figure for the *S. aureus* culture filtrate was 66%. No leakage of amaranth was observed in the control experiment. These findings indicated that no significant breach of the integrity of liposome membrane occurred as a result of exposure to *B. cereus* β-lactamase, whereas the massive diffusion of amaranth from liposomes in the presence of the bacterial culture filtrate was attributed to an increase in permeability or disruptive interactions of the latter with membrane components. This was consistent with the results previously obtained for the cephaloridine studies.

Entrapment of β-lactamase in lipid vesicles has previously been described by Barbas and coworkers (1982) who conducted experiments to determine the permeation rate of nitrocefin and cephamandole across the lipid membrane as a means of assessing β-lactam penetration into cells of *Pseudomonas aeruginosa*. They observed negligible hydrolysis of the antibiotics when the vesicles were prepared from lipids and lipopolysaccharides rather than reconstituted bacterial membranes containing porins. The authors concluded that the permeation of the β-lactams involved movement through hydrophilic channels of the porins. In a similar study by Kobayashi (1982), purified cephalosporinase from *Citrobacter freundii* was entrapped in liposomes prepared from lipids and protein complexes (porins) isolated from the outer membrane of *E. coli*. Diffusion of several β-lactam antibiotics was determined by measuring the rate of substrate hydrolysis. Negligible enzymatic activity was detected with control liposomes lacking porins. These findings indicated that the entrapped β-lactamase did not structurally interfere with the lipid membrane bilayer, and are in agreement with the data reported in this study that neither the purified β-lactamases nor the cephaloridine readily diffused through the liposome membrane. In the present system, it was postulated that the addition of the bacterial β-lactamases created perturbations or instabilities in the liposome bilayer packing, enhancing some leakage of the entrapped cephaloridine. In addition, enzymic degradation of membrane-associated cephaloridine may have contributed to the rapid phase of drug loss.

There are several reports in the literature of liposomes in antimicrobial therapy, both in vivo and in vitro. Beneficial effects in terms of enhanced antimicrobial activity or elevated blood levels have been reported (Gregoriadis, 1973; Bonventre and Gregoriadis, 1978; Bakker-Woudenberg et al., 1988; Reed, 1988; Mehta, 1989). Although the results of this study have no bearing upon the intrinsic interaction between the antibiotic-containing liposome and the bacterial cell, they do indicate the possibility that liposome entrapment may prolong the period in which the effective levels are maintained in the body fluids in the case of drugs susceptible to extracellular degradation before reaching the target cells.

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