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In Vivo Synergistic Interaction of Liposome-Coencapsulated Gentamicin and Ceftazidime

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ABSTRACT

Antimicrobial agents may interact synergistically. But to ensure synergy in vivo, the drugs should both be present at the site of infection at sufficiently high concentrations for an adequate period of time. Coencapsulation of the drugs in a drug carrier may ensure parallel tissue distributions. Since liposomes localize preferentially at sites of infection, this mode of drug delivery could, in addition, increase drug concentrations at the focus of infection. The therapeutic efficacy of gentamicin and ceftazidime coencapsulated into liposomes was examined by monitoring survival in a rat model of an acute unilateral pneumonia caused by antibiotic-susceptible and antibiotic-resistant *Klebsiella pneumoniae* strains. It is shown that administration of gentamicin in combination with ceftazidime in the free form either as single dose or as 5-day treatment resulted in an additive effect on rat survival in both models. In contrast, targeted delivery of liposome-coencapsulated gentamicin and ceftazidime resulted in a synergistic interaction of the antibiotics in both models. Consequently, liposome coencapsulation of gentamicin and ceftazidime allowed both a shorter course of treatment at lower cumulative doses compared with administration of the antibiotics in the free form to obtain complete survival of rats. Liposomal coencapsulation of synergistic antibiotics may open new perspectives in the treatment of severe infections.

Administration of combinations of antimicrobial agents is frequently used in clinical practice to increase therapeutic efficacy. Efficacy may be increased by broadening the antimicrobial spectrum of the treatment, preventing the emergence of resistant strains, reducing toxicity, eliminating multiresistant microorganisms, and/or enhancing bacterial killing by exploiting the synergistic interaction of a specific drug combination (Barriere, 1992; Schimpff, 1993; Shlaes et al., 1993). To ensure a synergistic drug interaction in vivo, the drugs should both be present at the site of infection at sufficiently high concentrations for an adequate period of time (Den Hollander et al., 1998; Join-Lambert et al., 1998; Strenkoski-Nix et al., 1998; Mouton et al., 1999). Due to the differences in physicochemical properties between the various antimicrobial agents, the pharmacokinetics and tissue distributions of these agents vary substantially. A significant interaction of antibiotics at the infectious focus resulting in a synergistic activity is therefore not guaranteed. The use of a drug carrier containing both antibiotics could enforce a parallel tissue distribution of both of the encapsulated agents. In addition, the use of a targeted drug carrier (including liposomes) may increase the concentrations of the drugs at the site of infection, which would further strengthen the synergistic drug interaction. In this respect, coencapsulation of antibiotics in liposomes may open new perspectives.

Liposomes have been widely investigated as targeted drug carriers in infectious diseases. Liposomes have been shown to localize selectively at the infected target site in a variety of experimental models of infection (Oyen et al., 1996; Awasthi et al., 1998a,b; Dams et al., 1999a,b; Schiffelers et al., 1999). The selective localization appears to be the result of the locally increased capillary permeability allowing local liposome extravasation (Allen, 1997; Boerman et al., 1998). Up to now, only liposomes containing a single antimicrobial agent have been investigated. The aim of the present study was to coencapsulate two antibiotics, gentamicin and ceftazidime, that have documented synergy in vitro (Giamarellou et al., 1984) into liposomes and examine their therapeutic efficacy

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ABBREVIATIONS: CZ, ceftazidime; GN, gentamicin; MIC, minimal inhibitory concentration; cfu, colony-forming unit; LE, liposome-encapsulated; D_a D_b, dose of agent A alone or agent B alone, respectively, needed to produce a desired effect; d_a d_b, doses in a combination of agent A and agent B, respectively, that produce the same effect; AUKC, area under killing curve; ANOVA, analysis of variance.

in vivo by monitoring survival in a rat model of an acute unilateral *Klebsiella pneumoniae* pneumonia. Both an antibiotic-susceptible and an antibiotic-resistant *K. pneumoniae* strain were studied.

Materials and Methods

Liposome Preparation. Polyethylene glycol-coated long-circulating liposomes were used, as previous studies have demonstrated that this liposome type shows substantial localization at the site of infection in the investigated model (Schiffelers et al., 1999). Liposomes were prepared as described previously (Schiffelers et al., 1999). Appropriate amounts of the indicated lipids partially hydrogenated egg phosphatidylcholine (Asahi Chemical Industry Co. Ltd., Ibarakiken, Japan), cholesterol (Sigma Chemical Co., St. Louis, MO), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[polyethylene glycol-2000] (Avanti Polar Lipids, Alabaster, AL) in a molar ratio of 1.85:1.00:0.15, respectively, were dissolved in a mixture of chloroform and methanol. After evaporation of the solvent under constant rotation and reduced pressure, the lipid mixture was dried under nitrogen, dissolved in 2-methyl-2-propanol (Sigma Chemical Co.) frozen by immersing in ethanol (−40°C), and freeze-dried overnight. The resulting lipid film was hydrated for 2 h in aqueous solutions of appropriate concentrations of ceftazidime (CZ) (Glaxo-Wellcome, Zeist, The Netherlands) or gentamicin (GN) (Duchefa Biochemie, Haarlem, The Netherlands). For coencapsulation of the drugs in liposomes, the CZ solution was added first, followed by the GN solution. Lipid concentration was diluted to a final concentration of 100 μmol of total lipid per milliliter using Hepes/NaCl buffer pH 7.4 (10 mM Hepes) (Sigma Chemical Co.) and 135 mM NaCl (Merck, Darmstadt, Germany). The lipids were sonicated for 8 min with an amplitude of 8 μm using a 9.5-mm probe in an MSE Soniprep 150 (Sanyo Gallenkamp PLC, Leicester, UK) to obtain long-circulating liposomes with a mean particle size of 100 nm. Particle size distribution was measured using dynamic light scattering, detected at an angle of 90° to the laser beam on a Malvern 4700 System (Malvern Instruments Ltd., Malvern, UK). In addition to the mean particle size, the system reports a polydispersity index (a value between 0 and 1). A polydispersity index of 1 indicates large variations in particle size, a reported value of 0 means that size variation is apparently absent. All liposome preparations used had a polydispersity index below 0.3. Unencapsulated GN and/or CZ was removed by ultracentrifugation of the liposomes in two changes of Hepes/NaCl buffer at 265,000*g* for 2 h at 4°C. Phosphate concentration was determined spectrophotometrically according to Bartlett (Bartlett, 1959). Total (liposome-encapsulated and free) and free (unencapsulated) GN and/or CZ was measured using a diagnostic sensitivity test agar (Oxoid, Basingstoke, UK) diffusion test with *Staphylococcus aureus* Oxford strain (ATCC 9144) (CZ-resistant) and an *Escherichia coli* strain (clinical isolate, GN-resistant) as the indicator organism for GN and CZ, respectively, as described previously (Bakker-Woudenberg et al., 1995). For total (unencapsulated and encapsulated) drug measurements, liposomes were disintegrated by 0.1% v/v (final concentration) Triton X-100 (Janssen Chimica, Geel, Belgium). Less than 10% of the GN and/or CZ was shown to be unencapsulated after ultracentrifugation. The validity of the agar diffusion test for the determination GN and CZ concentrations in the combination was ascertained in a separate experiment. Enzymatic inactivation of GN using aminoglycoside-acetylating enzyme (Den Hollander et al., 1996) or of CZ using β-lactamase (Koch-Light Ltd., Haverhill, UK) yielded similar inhibitory zones as without deactivation of either one of the antibiotics, thus showing the possibility to measure one drug at a time in the combination.

Bacterial Strains. The susceptible *K. pneumoniae* (ATCC 43816, capsular serotype 2, MIC = 0.5 μg/ml for both GN and CZ) was used. The MIC was determined by plating an inoculum of 10^4 cfu/spot on Mueller-Hinton agar (Difco, Detroit, MI) plates containing 2-fold dilutions of GN or CZ, according to Woods and Washington (1995). The resistant *K. pneumoniae* (MIC = 32 μg/ml for GN and 16 μg/ml for CZ) was obtained by culturing the susceptible strain in Mueller-Hinton broth (Difco) containing increasing concentrations of CZ. The MIC was determined according to the method described above. The resulting CZ-resistant strain was conjugated with an *E. coli* R176 strain (clinical isolate) that produced a plasmid encoding for an aminoglycoside-acetylating enzyme. In this way, a strain resistant to both GN and CZ was obtained. The stability of the GN/CZ-resistant phenotype in vitro was checked by culturing the bacteria five times in succession in antibiotic-free medium, followed by determination of the MIC. All of 100 tested bacterial colonies remained resistant to both antibiotics.

Checkerboard Titrations. Checkerboard titrations were performed with GN and/or CZ at the indicated concentrations in Mueller-Hinton broth of 37°C in a total volume of 3 ml. An inoculum of 5×10^5 susceptible or resistant *K. pneumoniae* cfu/ml in the logarithmic phase of growth was used. Tubes were incubated for 24 h at 37°C, and (the absence of) microbial growth was determined macroscopically. Each titration was performed in triplicate.

Time-Kill Curves. Time-kill curves were performed with GN and/or CZ at the indicated concentrations in Mueller-Hinton broth of 37°C in a total volume of 3 ml. An inoculum of 5×10^5 susceptible or resistant *K. pneumoniae* cfu/ml in the logarithmic phase of growth was used. Samples were taken at 0, 1, 2, 4, 6, and 24 h after addition of the inoculum. Number of bacteria in the samples was determined by making serial dilutions in phosphate-buffered saline 4°C. Two hundred microliters of each dilution was plated on tryptone soy agar plates and incubated overnight at 37°C. Colonies were counted. Each curve was determined in triplicate.

Unilateral Pneumonia. The animal experiments ethical committee of the Erasmus University Medical Center Rotterdam approved the experiments described in this study. Female albino RP/AEur/RijHsd strain albino rats, 18 to 25 weeks of age, body weight 185 to 225 g (Harlan, Horst, The Netherlands) with a specified pathogen-free status were used. A left-sided unilateral pneumonia was induced as described previously (Bakker-Woudenberg et al., 1982). In brief, rats were anesthetized and the left primary bronchus was intubated. Through the tube, 0.02 ml of a saline suspension containing 10^6 susceptible *K. pneumoniae* was inoculated. Inoculated bacteria were in the logarithmic phase of growth. For the resistant *K. pneumoniae* strain the inoculum was adjusted to 2×10^8 to establish a median survival of untreated controls that was comparable between both models. Rats were housed individually. In vivo stability of the phenotype of the *K. pneumoniae* was checked by culturing dilutions of homogenized left lung tissue obtained at 24 h after bacterial inoculation (the starting point of therapy) on Mueller-Hinton plates. Colonies were isolated and MIC was determined as described above on Mueller-Hinton plates. All of 100 tested colonies of both the susceptible and resistant strain had a stable phenotype, regarding GN and CZ-susceptibility, after inoculation in vivo.

Treatment was started at 24 h after bacterial inoculation. Controls were left untreated. GN and/or CZ was administered either as a single dose or as multiple doses every 12 h. In case of combination of GN and CZ, the drugs were injected with an interval of 5 min. Liposome-encapsulated gentamicin or ceftazidime (LE-GN or LE-CZ, respectively) or liposome-coencapsulated gentamicin and ceftazidime (LE-GN-CZ) was administered either as a single dose or as multiple doses every 24 h. The formulations were injected intravenously into the tail vein. Survival of rats was examined every day until 14 days after bacterial inoculation. The MIC of the *K. pneumonia* bacteria recovered from deceased rats was determined as described above and similar to that of the inoculated bacteria.

Statistical Analysis. To identify synergy, in vitro and in vivo, the effect of a drug combination was compared with the expected effect for each of the drugs alone. This method to identify synergy, also known as the isobole or iso-effect curve-method, has been validated by Berenbaum (1989). The method is based on the equation $d_a/D_a +$

$d_b/D_b = I$, where D_a and D_b are the doses of agent A alone and agent B alone, respectively, needed to produce a desired effect. The terms d_a and d_b are the doses in a combination of agent A and agent B, respectively, that produce the same effect (iso-effect). If no interaction between agent A and agent B is present, or in other words the effects of agent A and agent B are additive, the interaction index (I) = 1. Deviations indicate synergy ($I < 1$) or antagonism ($I > 1$). Curves, resulting from the checkerboard titrations were compared with lines describing absence of drug interaction using the *F* test. Survival between experimental groups was compared by the log-rank test. Area under the time-kill curve (AUKC) was calculated using the trapezoid rule. Analyses were performed using GraphPad Prism 3.00 software (GraphPad Software Inc., San Diego, CA). AUKCs were compared by one-way analysis of variance (ANOVA) corrected for multiple comparisons using the Bonferroni method.

Results

Checkerboard Titrations. The results of the checkerboard titrations with the susceptible strain and resistant strain are shown in Fig. 1, A and B, respectively. The shape of the best-fitted curve in Fig. 1, A and B, is concave up and describes the relationship significantly better than the line that would represent the relationship in absence of drug interactions (*F* test, $p < 0.0001$ for both curves). Thus, CZ and GN act synergistically against both *K. pneumoniae* strains.

Time-Kill Curves. The time-kill curves of the susceptible strain and the resistant strain are shown in Fig. 2, A and B, respectively. Bacterial density for both strains rapidly increased to a plateau of 10^9 bacteria/ml in absence of antibiotics. For the susceptible strain, GN alone, at a concentration of 0.3 μg/ml, initially reduced bacterial numbers. After 4 h of incubation >99% of bacteria were killed. However, between 6 and 24 h of incubation, bacterial outgrowth was observed to 10^7 bacteria/ml. Similar results were obtained with CZ alone at a concentration of 0.3 μg/ml. Incubation of 0.15 μg/ml GN and 0.15 μg/ml CZ in combination reduced bacterial numbers more efficiently. After 4 h of incubation >99.99% of bacteria were killed, whereas after 24 h of incubation the bacterial density was 10^5-fold lower compared with the single agent incubations. The AUKC values of the time-kill curves are shown in Table 1. As the AUKC value of the combination is significantly lower than the AUKCs of the single agent incubations (ANOVA, $p < 0.05$ for both GN and CZ), and thus $d_{GN}/D_{GN} + d_{CZ}/D_{CZ} < 1$, it can be concluded that GN and CZ display a synergistic interaction against the susceptible *K. pneumoniae* (Berenbaum, 1989).

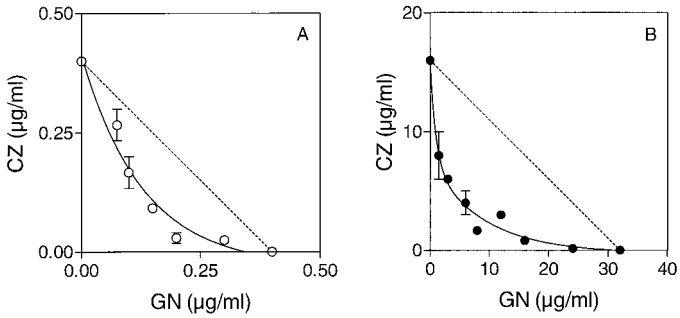

Fig. 1. Checkerboard titrations of GN and CZ against the susceptible *K. pneumoniae* (A) and the resistant *K. pneumoniae* (B). The symbols represent the lowest concentrations of GN and CZ that resulted in absence of bacterial growth. The curve shows the best fit through the symbols, whereas the dotted line represents the relationship that would be obtained in absence of drug interactions. Both experiments were performed in triplicate.

For the resistant strain, GN alone, at a concentration of 16 μg/ml, initially stabilized bacterial numbers, but at 24 h after incubation bacterial density had increased to the control level. CZ alone, 8 μg/ml, also stabilized bacterial counts initially, but eventually bacterial outgrowth was observed to 10^7 bacteria/ml after 24 h of incubation. The combination of GN and CZ at concentrations of 8 and 4 μg/ml, respectively, initially killed and then stabilized bacterial counts throughout the study period of 24 h. As the AUKC value of the combination was significantly lower compared with those of the single agent incubations (ANOVA, $p < 0.001$), and thus $d_{GN}/D_{GN} + d_{CZ}/D_{CZ} < 1$, GN and CZ showed a synergistic interaction against the resistant *K. pneumoniae* (Table 1).

Rat Survival in Susceptible *K. pneumoniae* Pneumonia Model. The results of the in vivo survival experiments with rats infected with the susceptible *K. pneumoniae* are shown in Fig. 3. Maximum survival after a single dose of free GN alone or free CZ alone was 50% (Fig. 3A). The maximum dose for free GN alone was 20 mg/kg and for free CZ alone 200 mg/kg, because 2-fold higher doses caused acute toxicity (local irritation at the site of injection for CZ and convulsions for GN). As the combination of free GN and free CZ did not increase survival compared with an equivalent dose of free GN alone or free CZ alone, $d_{GN}/D_{GN} + d_{CZ}/D_{CZ} > 1$, which would suggest antagonism. However, as the single dose treatment never resulted in survival >50%, these data seem more indicative for the conclusion that treatment is too short to have sufficiently prolonged concentrations at the site of infection for the drugs to exert their maximum effect on rat survival.

By prolonging treatment to 5 days and administering the antibiotics every 12 h, survival is increased (Fig. 3B). Free GN alone showed a steep dose-response relation between 0.63 mg/kg/day (0% survival) and 1.25 mg/kg/day (100% survival), explaining why intermediate drug doses were also studied. Free CZ alone showed a response of 0% survival up to 100% at doses ranging from 12.5 to 100 mg/kg/day. Looking at iso-effective doses of free GN alone and free CZ alone (e.g., 1.05 mg GN/kg/day and 50 mg CZ/kg/day), each resulting in 60 to 70% survival, shows that combination of half of these iso-effective doses for free GN and free CZ (i.e., 0.53 mg GN/kg/day combined with 25 mg CZ/kg/day) results in a similar survival percentage (60%). As a result, $d_{GN}/D_{GN} + d_{CZ}/D_{CZ} \approx 1$, indicating that there is no interaction between free GN and free CZ in vivo at the 5-day treatment schedule.

Using the liposome-encapsulated antibiotics, the survival data obtained with single doses of either LE-GN alone, LE-CZ alone, or coencapsulated LE-GN-CZ were completely different (Fig. 3C). A single dose of LE-GN alone produced a dose response with 0% survival at a dose of 1.25 mg/kg, increasing to complete survival for the 20-mg/kg dose. Whereas with LE-CZ, 0% survival was obtained after administration of 0.38 mg/kg. Survival increased gradually to 100% for 12 mg/kg. Looking at iso-effective doses for LE-GN and LE-CZ alone (e.g., 5 mg of LE-GN/kg or 3 mg LE-CZ/kg) each resulting in 60 to 67% survival, shows that coencapsulation of half of these iso-effective doses (i.e., LE-GN-CZ 2.5/1.5 mg/kg, respectively) showed a significantly better survival (100%) (Log-rank test, $p < 0.05$). Consequently, $d_{LE\text{-}GN}/$

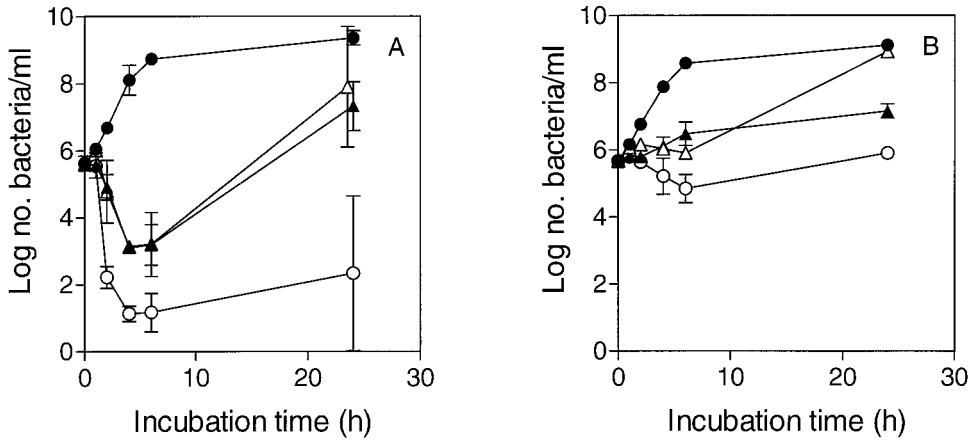

Fig. 2. Time-kill curves of the susceptible *K. pneumoniae* (A) and the resistant *K. pneumoniae* (B). A, susceptible bacteria were incubated without antibiotics (●), 0.3 μg/ml GN (△), 0.3 μg/ml CZ (▲), or 0.15 μg/ml GN in combination with 0.15 μg/ml CZ (○). B, resistant bacteria were incubated without antibiotics (●), 16 μg/ml GN (△), 8 μg/ml CZ (▲), or 8 μg/ml GN in combination with 4 μg/ml CZ (○). Both experiments were performed in triplicate.

TABLE 1
Log AUKC values for the susceptible and the resistant *K. pneumoniae* strain
Bacteria were incubated with indicated concentrations of GN, CZ, or GN in combination with CZ.

Susceptible		Resistant	
Drug (μg/ml)	AUKC[a]	Drug (μg/ml)	AUKC[a]
	log no. bacteria × h/ml		*log no. bacteria × h/ml*
Control	10.4 ± 0.1***	Control	10.2 ± 0.1***
GN (0.3)	8.9 ± 1.7*	GN (16)	9.9 ± 0.1***
CZ (0.3)	8.2 ± 0.6*	CZ (8)	8.3 ± 0.1***
GN/CZ (0.15/0.15)	5.9 ± 0.2	GN/CZ (8/4)	7.0 ± 0.2

[a] Experiment was performed in triplicate. Results are expressed as mean ± S.D. ANOVA corrected for multiple comparisons using the Bonferroni method, significant differences compared with the combination regimen are noted as *** $p < 0.001$, * $p < 0.05$.

$D_{LE\text{-}GN} + d_{LE\text{-}CZ}/D_{LE\text{-}CZ} < 1$, revealing a synergistic interaction of liposome-coencapsulated GN and CZ.

Rat Survival in Resistant *K. pneumoniae* Pneumonia Model. The results of the in vivo survival experiments with rats infected with the resistant *K. pneumoniae* are shown in Fig. 4. Administration of single doses of the free drugs, alone or in combination, at the maximum tolerated dose did not yield survival (data not shown). Prolongation of treatment to 5 days with the free drugs administered every 12 h increased survival. Yet, free GN alone at the maximum tolerated dose of 40 mg/kg/day showed only 40% survival. With free CZ alone a nearly complete dose-response relation could be obtained at doses ranging from 50 (0% survival) to 400 mg/kg/day (90% survival). Looking at iso-effective doses of free GN alone and free CZ alone (e.g., 40 mg GN/kg/day or 100–200 mg CZ/kg/day) each resulting in 30 to 50% survival, shows that combination of half of these iso-effective doses of GN and CZ (i.e., 20 mg GN/kg/day combined with 50 or 100 mg CZ/kg/day) did not increase the survival percentage significantly (20–70%) (Fig. 4A). Consequently, $d_{GN}/D_{GN} + d_{CZ}/D_{CZ} \approx 1$, indicating that there is no interaction between free GN and free CZ.

In contrast, treatment for only 2 days with LE-CZ alone showed 0% survival at a dose of 3 mg/kg/day, and complete survival was already obtained at a dose of 24 mg/kg/day. LE-GN alone at the maximum administered dose of 40 mg/kg/day did not produce survival. However, at this dose of 40 mg/kg LE-GN survival of rats was significantly prolonged compared with the controls ($p < 0.01$). Liposomal coencapsulation of GN and CZ improved survival compared with LE-GN alone or LE-CZ alone. LE-GN-CZ, at a dose of 10 and 12 mg/kg/day, respectively, already produced complete survival, which was obtained for LE-CZ alone at 24 mg/kg/day and for LE-GN alone at a dose that exceeded 40 mg/kg/day (probably by far) (Fig. 4B). Consequently, $d_{LE\text{-}GN}/D_{LE\text{-}GN} + d_{LE\text{-}CZ}/D_{LE\text{-}CZ} < 1$, thus showing a synergistic interaction of liposome-coencapsulated GN and CZ. Similar reasoning shows a synergistic interaction for LE-GN-CZ at doses of 10 combined with 6 mg/kg/day as well as 10 combined with 3 mg/kg/day CZ, respectively.

Discussion

Treatment with a combination of antimicrobial agents may improve therapeutic efficacy over single agent treatment as a result of synergistic drug interactions. Synergistic drug interaction in vitro has been clearly shown for various drug combinations. For a synergistic drug interaction to occur in vivo, the drugs in the combination should be present at the site of infection at sufficiently high concentrations for an adequate period of time. Theoretically, simultaneous drug delivery to the target site could strengthen synergistic interactions. Interestingly in this respect, targeted liposomal delivery of single antimicrobial agents has demonstrated superior therapeutic efficacy over conventional antimicrobial treatment in a number of experimental infection models (Wasan and Lopez-Berestein, 1995; Bergers et al., 1995; Fielding et al., 1998). The superior efficacy is attributable to the increased concentration of the drug at the site of infection as a result of the targeted drug delivery. Up to now, only

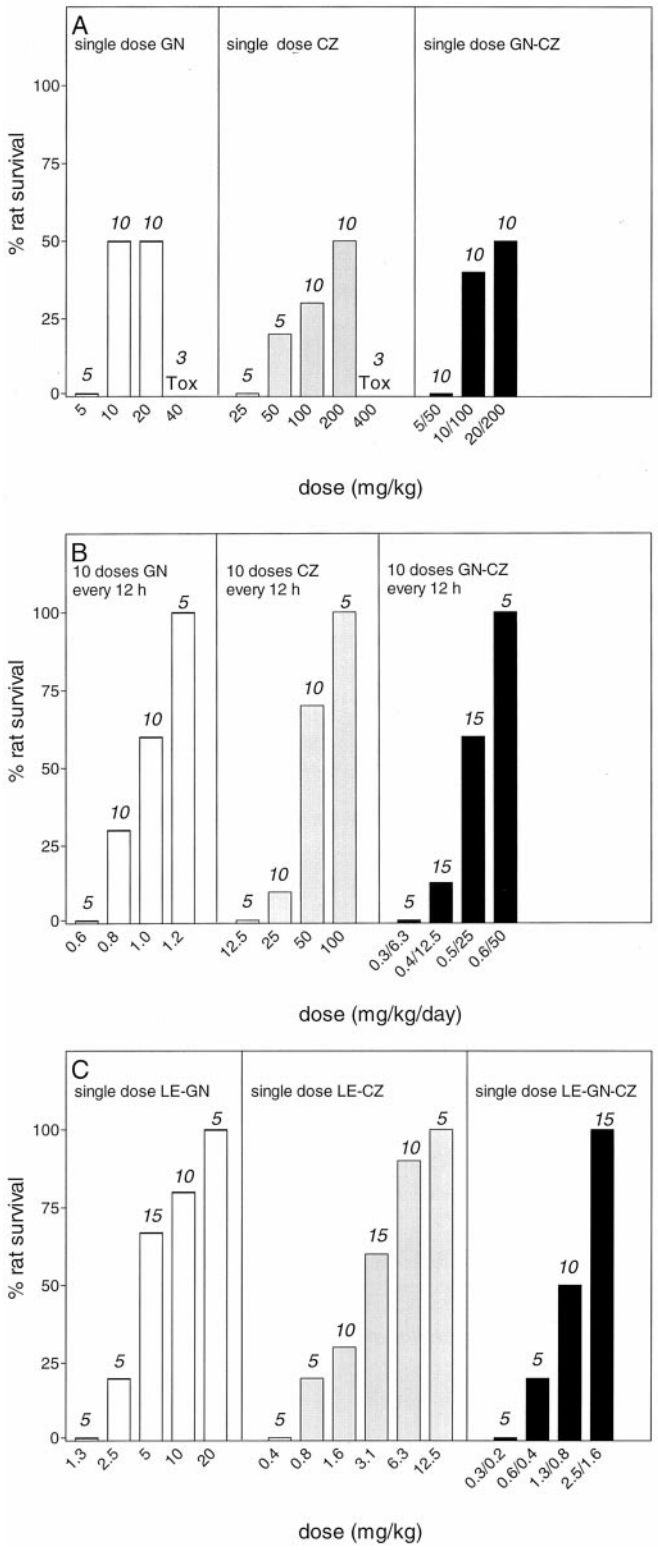

Fig. 3. Percentage of rat survival at 14 days after inoculation of the susceptible *K. pneumoniae* in the left lung. Rats were treated at 24 h after bacterial inoculation with a single dose of free GN (□), free CZ (▨), or GN and CZ (■) (A); 10 doses every 12 h of GN (□), CZ (gray bars), or GN and CZ (■) (B); single dose of LE-GN (□), LE-CZ (▨), or LE-GN-CZ (■) (C). Number of animals per experimental group in italics.

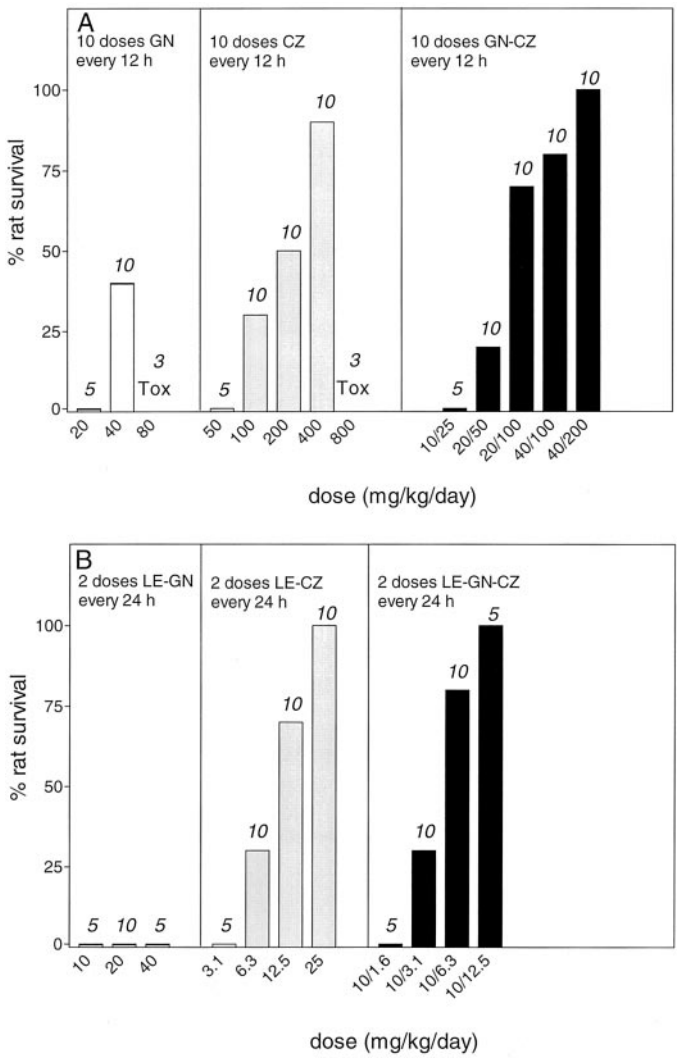

Fig. 4. Percentage of rat survival at 14 days after inoculation of the resistant *K. pneumoniae* in the left lung. Rats were treated at 24 h after bacterial inoculation with 10 doses every 12 h of free GN (□), CZ (▨), or GN and CZ (■) (A); two doses every 24 h of LE-GN (□), LE-CZ (▨), or LE-GN-CZ (■) (B). Number of animals per experimental group in italics.

single agent liposome preparations have been investigated. The present study aimed to investigate the therapeutic efficacy of liposome-coencapsulated antimicrobial agents in vivo in a rat model of pneumonia caused by an antibiotic-susceptible strain or antibiotic-resistant strain of *K. pneumoniae*. The results of the present study show that targeted liposomal delivery of GN and CZ results in a synergistic interaction of these antibiotics in vivo. Importantly, the synergistic interaction was present in the animals infected with the susceptible strain as well as the animals infected with the resistant strain. In contrast, administration of the combination of the antibiotics in the free form, although showing synergy in vitro, displayed only an additive effect in both in vivo models. Synergy in vivo was not observed. As a result, by use of liposome-coencapsulated GN and CZ, 100% survival can be obtained using a shorter treatment schedule and lower total drug exposure compared with treatment with the free drugs.

The interaction between GN and CZ against both the susceptible and resistant *K. pneumoniae* was first examined in vitro by performing checkerboard-titrations and time-kill experiments. Both in vitro assays show that GN and CZ acted synergistically against both the susceptible strain and the resistant *K. pneumoniae* strain. The in vitro synergistic in-

teraction of GN and CZ, or in general aminoglycosides and β-lactam antibiotics, has been reported earlier. The interaction is suggested to be due to the limited penetration of aminoglycosides into bacteria to effect bacterial killing and the ability of β-lactams to increase that penetration (Davis, 1982).

To investigate whether GN and CZ can act synergistically in vivo, rats were infected with either the susceptible or the resistant *K. pneumoniae* strain, and survival was monitored for 14 days. At single doses of either free GN alone or free CZ alone a maximum survival of 50% could be obtained in rats infected with the susceptible strain. Combination of single doses of free GN and free CZ did not improve survival compared with an equivalent single dose of either free GN alone or free CZ alone. Likely, treatment at a single dose of GN and CZ is too short and thus adequate concentrations at the site of infection are too transient for synergistic interactions to have an effect on survival.

To increase therapeutic efficacy, treatment with the free drugs was prolonged to 5 days and both antibiotics were administered every 12 h. Using this dosing schedule, complete survival could be obtained with either free GN alone or free CZ alone against the susceptible *K. pneumoniae* infection. The effects of free GN combined with free CZ on rat survival in this 5-day treatment schedule, however, are merely additive. Synergism was not detected. This result was unexpected as, in vitro, GN and CZ acted synergistically against both *K. pneumoniae* strains and synergism between aminoglycosides and β-lactams in vivo has been reported (Pefanis et al., 1993; Mimoz et al., 1998). The discrepancy between in vitro and in vivo data is possibly the result of the rapidly changing concentrations of the antibiotics at the site of infection in the rats compared with the constant drug concentrations in the in vitro incubations (Den Hollander et al., 1998; Join-Lambert et al., 1998). Seemingly, the pharmacokinetics and tissue distributions of free GN and free CZ in rats (Acred, 1983; Nassberger and De Pierre, 1986; Swenson et al., 1990; Granero et al., 1998) do not provide adequate drug concentrations at the site of the *K. pneumoniae* infection in a timely manner for synergistic drug interactions to occur. Consequently, the assessment of in vitro synergistic interactions does not guarantee in vivo synergy to occur predictably.

The results obtained with the liposome-encapsulated antibiotics contrast favorably with the results obtained with the free antimicrobial agents. Single doses of LE-GN alone or LE-CZ alone were shown to be highly effective, as complete survival could be obtained in the susceptible *K. pneumoniae* infection. Apparently, the simultaneous targeted delivery of LE-GN-CZ results in higher GN and CZ concentrations at the target site for prolonged periods of time, enabling synergistic drug interactions to occur. A single dose of liposomal coencapsulated agents produced complete survival at a comparable GN-exposure and a 170-fold reduced CZ body exposure, compared with 10 injections of the free drug combination.

To investigate the strength of the synergistic drug interaction after administration in the coencapsulated form, comparative studies were also performed in rats infected with the resistant *K. pneumoniae* strain. In this model, survival in a 5-day treatment schedule could only be obtained with doses of free GN alone or free CZ alone that were well over the clinically recommended doses. Combinations of free GN with free CZ were again just additive. In contrast, administration of two doses of LE-CZ alone of 24 mg/kg/day already resulted in complete survival. LE-GN alone was less effective as two doses of 40 mg/kg/day failed to increase survival. Yet, liposome-coencapsulation of GN and CZ resulted in significantly improved survival compared with the expected efficacy based on the dose-response relations of LE-GN alone and LE-CZ alone, demonstrating that the synergistic interaction was strong enough to overcome infection with a resistant *K. pneumoniae* infection. Two doses of liposome-coencapsulated GN and CZ produced complete survival at a 10-fold lower GN exposure and 40-fold lower CZ exposure compared with 10 injections of the free GN-CZ combination.

In conclusion, the present study demonstrates that targeted delivery of GN and CZ by liposome-coencapsulation results in synergistic drug interactions in a susceptible as well as resistant *K. pneumoniae* pneumonia model. In these models, synergistic interaction of a combination of free GN and free CZ could not be demonstrated. The application of multiple antimicrobial agents coencapsulated into liposomes could be a valuable contribution to the treatment of severe bacterial infections.

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