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Liposomal formulation of netilmicin

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Abstract

Netilmicin, a semisynthetic aminoglycoside (Wright, J.J., J. Chem. Soc. Chem. Commun., 6 (1976) 206–208), was encapsulated in two different kinds of liposomes: simplified dehydration-rehydration vesicles and extruded vesicles. These formulations were characterized and their behaviour was evaluated in one animal model.

Liposomal netilmicin formulations, prepared with the lipid composition phosphatidylcholine:cholesterol:phosphatidylinositol in a molar ratio of 5:1:4, exhibit high encapsulation efficiencies (> 65%) and high drug/lipid ratios (> 32 μg/μmol), without alteration of in vitro biological activity. A substantial reduction of in vivo acute toxicity was found for liposomal netilmicin (LD_{50} > 50 mg/kg) compared with the free form (LD_{50} = 20–28 mg/kg). The half-life circulation time of liposomal netilmicin formulations was greatly prolonged (12–18-fold) compared to the free form. The increase of liposomal netilmicin pharmacological activity was evaluated by the increase of survival in an in vivo model of peritonitis infection (from 33% to 83% prophylactically and from 17% to 50% therapeutically). The increase on the therapeutic index of liposomal netilmicin, suggests the possibility of reduction the frequency of administration and monitorization of netilmicin as well as safety during prolonged treatments. © 1997 Elsevier Science B.V.

Keywords: Netilmicin; Phosphatidylinositol; Liposomal netilmicin formulations; Pharmacokinetics; Acute toxicity; Pharmacological activity; Peritonitis model

1. Introduction

The aminoglycosides are a family of bactericidal polycation antibiotics which contain amino sugars in glycoside linkages (Sande and Mandell, 1983; Soares, 1985). Netilmicin sulphate (NET) (Sch 20569) is a semisynthetic aminoglycoside synthesized from sisomicin, differing in structure by an ethyl group at the 1-amino position of the deoxystreptamine ring (Miller et al., 1976; Wright, 1976; Guay, 1983). The aminoglycosides inhibit protein synthesis in a variety of microorganisms

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by binding bacterial ribosomes (Craig et al., 1983; Pancoast, 1988; Prista and Sousa, 1990).

Aminoglycoside antibiotics are essential and widely used, therapeutically and prophylactically, in the treatment of serious infections caused by Gram-negative microorganisms. They are particularly useful in the treatment of infections due to organisms which are resistant to many of the currently available antibiotics (e.g. tuberculosis, septicaemia, peritonitis, pneumonia, urinary tract infections) (Luft, 1978; Guay, 1983; Sande and Mandell, 1983; Soares, 1985).

The use of NET, as other aminoglycosides, causes severe side-effects such as oto- and nephrotoxicity which are major drawbacks to their utilization in critically-ill patients. The ototoxicity effects can involve both cochlear and vestibular toxicity. The nephrotoxicity effects include abnormalities of tubular resorption and renal morphology. The mechanism underlying the toxicities of the aminoglycosides may be associated with the ability of the aminoglycosides to bind to polyphosphoinositides found in inner ear and kidney tissues (Marche et al., 1987; Walker and Duggin, 1988; Mattie et al., 1989; Van Bambeke et al., 1993). It has been postulated that phosphatidylinositol diphosphate serves as a receptor for the drugs and renders these tissues more sensitive than others to this family of drugs (Lodhi et al., 1980; Feldman et al., 1982; Sastrasinh et al., 1982; Knauss et al., 1983). To reduce the associated toxicities, some authors have been concentrated on these supposed aminoglycoside receptors (Lipsky and Lietman, 1982; Janoff et al., 1990).

Also, free aminoglycosides are rapidly eliminated from the body by glomerular filtration (in an active and unchanged form), exposing renal tubules to potentially toxic concentrations (Craig et al., 1983; Pancoast, 1988).

Liposomal formulations of aminoglycosides have been suggested as a way to achieve higher potency pharmaceutical preparations with reduction of the dose and thus causing less tissue injury upon administration. Liposomes have shown evidence of protection against the toxicity effects (Bally et al., 1988; Swenson et al., 1990). When these antibiotics are encapsulated in liposomes, they are taken up primarily by the liver and spleen, and only a small fraction of the drug is excreted acutely by urine (Morgan and Williams, 1980). Therefore, the risk of renal tubular damage should be reduced substantially.

The encapsulation of gentamicin (Nacucchio et al., 1988; Fierer et al., 1990; Nightingale et al., 1993), streptomycin (Stevenson et al., 1983; Fountain et al., 1985), amikacin (Düzgünes et al., 1988; Cynamon et al., 1989), has been described in the past as having superior therapeutic efficiency when compared to the free forms of the antibiotic. However, these liposome-aminoglycoside formulations are obtained with high initial drug/lipid ratios. No references were found of NET encapsulation in liposomes.

Our purpose is to prepare liposomal formulations highly loaded with NET, with high encapsulation efficiency, high final drug/lipid ratio, low toxicity and high pharmacological activity.

2. Materials and methods

2.1. Reagents

L-α-Phosphatidylcholine (PC) (from egg yolk, 99%), L-α-phosphatidylinositol (PI) (from bovine liver, 98%), stearylamine (SA) (90%), cholesterol (Chol) (99%) and cyclophosphamide (ISOPAC) were purchased from Sigma Chemical (München, Germany). Netilmicin sulphate (NET) in a lyophilized pure form was generously provided by Schering-Plough Farma Lda. (Portugal). Mucin bacteriological (from hog gastric mucosa), Bacto nutrient broth, Bacto nutrient agar, tryptic soy broth, tryptic soy agar and Mueller-Hinton broth were obtained from Difco Laboratories (Detroit, USA). All other reagents were of analytical grade.

2.2. Bacteria

Bacillus subtilis (stock strain 6633) and *Escherichia coli* (stock strain 25922) were obtained from the ATCC (Rockville, Maryland, USA). *B. subtilis* was grown in Bacto nutrient broth and on Bacto nutrient agar at 37°C. *E. coli* was grown in tryptic soy broth and on tryptic soy agar at 37°C.

2.3. Animals

Adult males CD-1 Swiss mice (weighing 20–25 g) were obtained from Gulbenkian Institute of Sciences, Oeiras, Portugal. They were housed 8–10 per cage and allowed free access to food and water.

2.4. Liposome preparation

Simplified dehydration-rehydration vesicles (sDRV) were prepared according to Cruz et al. (1989). In brief, a thin film of lipid mixtures was hydrated with an aqueous NET solution, forming multilamellar vesicles. The liposome suspension was lyophilized on a freeze dryer at 25 mTorr overnight. The resulting powder was rehydrated, to a final osmolarity of 300 mOsm, in two successive steps: first with addition of 5% dextrose in one-tenth of the volume of NET solution used, followed by vigorous vortexing and a 30-min stabilizing rest period at room temperature; secondly, the volume was brought up to the initial volume with 0.154 M NaCl, followed by a 10 min stabilizing period at room temperature. Liposomes were then diluted 10-fold with 0.154 M NaCl and separated from unencapsulated NET by ultracentrifugation (washed three times at $118\,000 \times g$ for 10 min). The final sDRV pellet was resuspended in the initial volume of drug solution with 0.154 M NaCl.

The extruded vesicles (VET) were made from the sDRV suspension obtained after the rehydration. After a 10-fold dilution with 0.154 M NaCl, the resulting suspension was extruded under pressure through 400 and 200 nm polycarbonate membranes (Nuclepore), using an Extruder device from Lipex Biomembranes (Vancouver, BC, Canada). The final extruded solution was ultracentrifuged, at $177\,000 \times g$ for 30 min. The final VET_{200} pellet was resuspended in the initial volume of drug solution with 0.154 M NaCl.

The mean diameter of liposomes was determined by a quasi-elastic laser light scattering in a Malvern Zeta Sizer 3 (UK).

The liposome encapsulation parameters used were: NET to lipid ratio (N/L), NET recovery (percentage of the ratio between final liposome associated drug to total initial drug) and encapsulation efficiency (EE) (percentage of the ratio between final to initial N/L ratio) (Cruz et al., 1989, 1993).

2.5. Phospholipid analysis

Phospholipid concentration was determined by the phosphate assay of Fiske and Subbarow (1925), modified by King (1932).

2.6. Netilmicin analysis

The concentration of NET in the initial and final liposome suspension was determined by the spectrophotometric assay of Satake et al. (1960), after the disruption of the lipid membranes with 20% Triton X-100.

2.7. MBC test

The minimum bactericidal concentration test was based on the common broth (Mueller-Hinton broth) microdilution method of susceptibility testing (Schoenknecht et al., 1985). The MBC is considered the lowest concentration of antibiotic which kills at least 99.9% of the original inoculum (2.5×10^4 bacteria/ml) after 24 h of incubation at 37°C.

2.8. Granulocytopenic mice

Granulocytopenia was produced in mice, by two intraperitoneal injections of cyclophosphamide, 150 and 100 mg/kg at 4 and 1 day before infection, respectively (Gerber et al., 1986). We checked for granulocytopenia in blood smears of 5–8 mice chosen randomly. No more than three granulocytes per smear were found.

2.9. Antibacterial activity

The antibacterial activity in blood (collected from mice), of free and liposomal NET, was determined (after disruption of lipid membranes with 20% Triton X-100) by using an agar disk diffusion assay with *B. subtilis* as the indicator organism (Anhalt, 1985; Lorian, 1986). A control

Table 1
Effect of liposomal charge on encapsulation efficiency (sDRV)

Lipid composition (molar ratio)	$(N/L)_{initial}$ (μg/ml)	$(N/L)_{final}$ (μg/μmol)	EE (%)	Recovery (%)
PC/Chol/SA (7:2:1)	5.2 ± 0.1	2.6 ± 0.1	50 ± 2	38 ± 1
PC/Chol/PI (7:2:1)	6.1 ± 0.2	4.0 ± 0.1	68 ± 3	50 ± 3

Initial lipid concentration: 15–16 μmol/ml.
Initial drug concentration: 78–95 μg/ml.
Values shown are the means from four independent experiments ± S.D. Each experiment was run at least in triplicate.

with 20% Triton X-100 alone was used to determine its effect on bacterial growth.

2.10. Pharmacokinetic studies

Pharmacokinetic studies were performed on normal and granulocytopenic mice. The animals were injected intravenously via the lateral tail vein with bolus doses of free and liposomal NET (4 mg/kg). Blood was collected at fixed times, post-dose, from the retroorbital sinus of mice, in tubes with EDTA as anticoagulating agent, for later bioassay. The pharmacokinetic parameters were determined by an automatic stripping procedure (PKCALC, B.B.N., Software Products written by R.C. Shumaker, 1987).

2.11. In vivo acute toxicity

Groups of ten mice were injected intravenously with free and liposomal NET in a range of doses from 10 to 50 mg/kg. The number of surviving animals per dose was determined and the dose which killed 50% of the animals (LD_{50}) was performed according to the Reed and Muench method (Cleeland and Grunberg, 1986).

2.12. Pharmacological activity

To evaluate the therapeutic efficiency of liposomal NET formulations, an animal model of peritonitis was set up. The infection was established by inoculating, intraperitoneally, with a rapidly lethal dose (death in 24–48 h) of *E. coli* (1×10^8 bacteria/ml) suspended in 5% hog mucin. Groups of six mice were used. Two types of treatment with free and liposomal NET were assayed: prophylactically (given intravenously by bolus injection, 24 h before infection) or therapeutically (given intravenously by bolus injection, immediately post-infection, 0 h). The therapeutic dose was 10-fold lower than the dose used prophylactically (2 mg/kg). The control group was injected with 0.154 M NaCl. The survival was calculated by the ratio between the number of surviving animals to the total number of animals treated, after 24 days.

3. Results

3.1. Effect of liposomal charge on netilmicin encapsulation

To study the effect of liposomal charge on NET encapsulation parameters, negatively and positively charged liposomes were prepared: PC/Chol/PI and PC/Chol/SA, respectively, in 7:2:1 molar proportions. The type of liposomes used were sDRV. The results are shown in Table 1. With the addition of the negatively charged lipid, PI, to PC/Chol mixture, the entrapment efficiencies are higher than 65% and recoveries are of the order of 50%, while much smaller values of those parameters were found for PC/Chol/SA. So, in the aim of this work the lipid composition PC/Chol/PI was selected to further experiments.

3.2. Effect of initial NET/lipid ratio on encapsulation efficiency

The above selected lipid composition (PC/Chol/PI) in the same molar proportion (7:2:1), was used to study the behaviour of NET encapsulation, in

Table 2
Effect of initial NET/lipid ratio on encapsulation efficiency (sDRV)

Lipid composition (molar ratio)	$[NET]_{initial}$ (μg/ml)	$(N/L)_{initial}$ (μg/μmol)	$(N/L)_{final}$ (μg/μmol)	EE (%)	Recovery (%)
PC/Chol/PI (7:2:1)	100 ± 3	6.7 ± 0.4	4.3 ± 0.4	64 ± 6	46 ± 5
	200 ± 18	14.9 ± 2.3	9.9 ± 0.4	69 ± 12	63 ± 8
	400 ± 2	25.6 ± 2.5	17.9 ± 2.0	70 ± 7	49 ± 3
	600 ± 27	35.4 ± 1.5	25.5 ± 0.9	72 ± 5	46 ± 3
	650 ± 18	41.2 ± 3.1	17.7 ± 1.8	44 ± 7	35 ± 3
	800 ± 45	52.6 ± 5.2	9.7 ± 0.4	19 ± 2	13 ± 1

Initial lipid concentration: 14–17 μmol/ml.
Values shown are the means from three independent experiments ± S.D. Each experiment was run at least in triplicate.

sDRV, as a function of initial drug concentration (Table 2). By increasing the initial NET concentration, for the same initial lipid concentration, we observed that both encapsulation efficiency and final NET/lipid ratio have increased to the initial NET concentration of 600 μg/ml. They are directly proportional to initial NET/lipid ratio. For values of initial NET/lipid ratio higher than 35 μg/μmol a substantially reduction of both parameters was observed, which means that a vesicle saturation with drug was achieved. The selected initial concentration was 600 μg/ml of NET and will be used for further studies.

3.3. Effect of phosphatidylinositol on encapsulation parameters

In view of obtaining liposomes highly loaded with NET, liposomes type VET_{200} were made with lipid and NET concentrations 4-fold greater than the ones used in the first studies. The lipid mixture PC/Chol/PI was prepared in several molar proportions, increasing the molar ratio of PI from 10 to 40% of the total lipid. The results (Fig. 1) show that parameters of encapsulation are increased when PI molar ratio is raised. With the formulation PC/Chol/PI in molar proportions 5:1:4, we entrapped larger amounts of drug (1618 ± 16 μg/ml) with higher recoveries (63 ± 2%). These results allow to perform in vivo studies with liposomal formulations, as they require higher doses of entrapped NET.

Liposomes type sDRV with the above described initial experimental conditions were compared with VET_{200}. The encapsulation parameters that characterized the two types of vesicles selected to the in vivo tests are described in Table 3.

3.4. Effect of liposomal encapsulation on the biological activity of netilmicin

The MBC of free and liposomal NET for the strain of *E. coli* were both 4 μg/ml. No changes in biological activity of NET were detected after encapsulation.

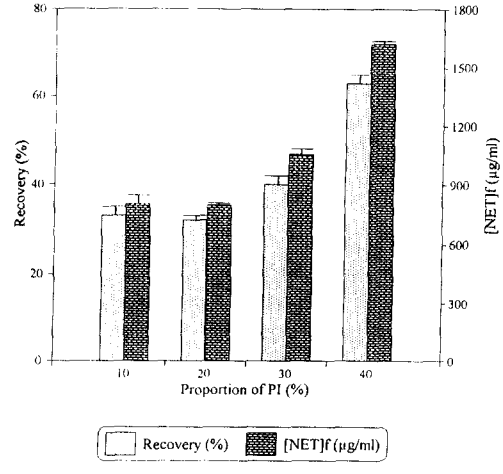

Fig. 1. Effect of phosphatidylinositol (PI) on encapsulation parameters (VET_{200}). The effect of PI was assessed for liposomes composed of PC/Chol/PI with different molar proportions of PI: 7:2:1 (240 ± 30 nm), 7:1:2 (216 ± 22 nm), 6:1:3 (197 ± 13 nm), 5:1:4 (200 ± 10 nm). Initial lipid concentration: 62–66 μmol/ml. Initial drug concentration: 2.4–2.6 mg/ml. Values shown are the means from six independent experiments ± S.D. Each experiment was run at least in triplicate.

Table 3
The selected formulations for in vivo studies

Formulation	Diameter (nm)	$(N/L)_{initial}$ ($\mu g/\mu mol$)	$(N/L)_{final}$ ($\mu g/\mu mol$)	EE (%)	Recovery (%)
sDRV	900 ± 95	69 ± 13	49 ± 11	66 ± 1	56 ± 4
VET_{200}	200 ± 10	40 ± 2	34 ± 2	85 ± 3	63 ± 2

Lipid composition: PC/Chol/PI (5:1:4).
Initial lipid concentration: 49–64 μmol/ml.
Initial drug concentration: 2.3–3.1 mg/ml.
Values shown are the means from three independent experiments $\pm$ S.D. Each experiment was run at least in triplicate.

3.5. Pharmacokinetic studies

The pharmacokinetic parameters are shown in Table 4. For a dose of 4 mg/kg, the half-life circulation time was greatly prolonged by VET_{200} formulations (137.6 min) when compared to the free drug (12.1 min). The larger increase observed for granulocytopenic animals (219.0 min) was probably attributable to their low phagocyte capacity. For sDRV formulations, in normal mice, the half-life time was much lesser (30.5 min) than for VET_{200}, as expected for larger vesicles which are rapidly captured by the mononuclear phagocytic system (MPS). The circulation time of NET has increased after encapsulated in liposomes, which is particularly important for treatments with high doses.

Table 4
Pharmacokinetic parameters

Animals (mice)	Formulation	$t_{1/2}$ (min)	V_{SS} (ml)
Normal	Free NET	12.1	15.7
	sDRV NET	31.1	0.2
	VET_{200} NET	137.6	2.9
Granulocytopenic	Free NET	11.1	15.9
	VET_{200} NET	219.0	2.9

$t_{1/2}$, half-life time.
V_{SS}, volume of distribution at the steady state.
Dose: 4 mg/kg
Initial lipid concentration: 62–66 μmol/ml.
Initial drug concentration: 2.4–2.6 mg/ml.
Values shown are the means from six independent experiments. Each experiment was run at least in triplicate.

3.6. In vivo acute toxicity

The acute toxicity evaluation was made with both free and liposomal NET (VET_{200}). For free NET, the LD_{50} was 24 ± 4 mg/kg. In contrast, for liposomal NET, LD_{50} is greater than 50 mg/kg, since for this dose 100% of the animals survived without any sign of anaphylactic shock. A higher dose was not possible to administer due to technical difficulties to inject highly concentrated liposomes or bigger volumes.

3.7. Pharmacological activity/peritonitis model

The infected control mice began to die 12 h after inoculation and all were dead within 48 h. The survival rate is shown in Fig. 2. Animals treated with VET_{200} formulations as prophylactic

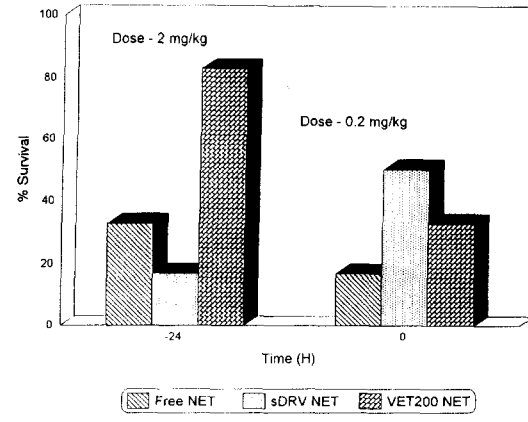

Fig. 2. Pharmacological activity of NET formulations (peritonitis model). Free or liposomal NET formulations (sDRV and VET_{200}) were administrated intravenously with a single dose of 2 mg/kg (-24 h) or 0.2 mg/kg (0 h).

show survivals of 83%, while those treated with sDRV show even smaller survivals (17%) than those treated with free NET (33%). This might be due to smaller residence time of sDRV resulting in negligible concentrations when infection was induced. By contrast when animals are treated at the same time as infection induction, sDRV allow higher survivals (50%) than VET_{200} (33%), as these vesicles are fastly taken by infected cells. For free NET survival rate was only 17%.

4. Discussion

The aim of this work was to prepare liposomal NET formulations with high incorporation efficiency, low toxicity and high pharmacological activity. For that purpose we encapsulated NET in two different kinds of liposomes: one with larger diameter (sDRV), theoretically with higher capture by the reticulum endothelial system (RES), and another one with smaller diameter (VET), theoretically with longer circulation time (Allen and Everest, 1983).

The high entrapment and the intraliposomal concentrations of drug appropriate for in vivo studies were achieved by increasing the PI proportion up to 4 in 10 total lipid. The presence of this specific phospholipid (a polianionic molecule) that interacts electrostatically with NET (policationic molecule) is connected with the higher values obtained for the encapsulation parameters (Table 3). This affinity resembles the specific interactions observed at renal membranes (proximal tubules brush border membranes) and which has been associated with the nephrotoxicity of aminoglycosides (Sastrasinh et al., 1982; Walker and Duggin, 1988; Mattie et al., 1989). PI residues are known as specific sites of toxicity (Lodhi et al., 1980; Knauss et al., 1983). So, the immobilization of NET in such vesicles will allow the direct blockage of the membrane binding sites. The liposomal-PI will compete with renal-PI to the drug (Alving, 1988). Under these conditions, a reduction of toxicity and enhanced therapeutic index of this form of NET is expected.

The NET formulations described herein are a significant improvement over earlier protocols (Bally et al., 1988; Janoff et al., 1990; Karlowsky and Zhanel, 1992). These formulations are less concentrated than those related in Bally et al. (1988) PCT Patent. While these authors achieved recoveries of only 40% for high drug/lipid ratios (6.2–12.4 mg/100 mg lipid), our recovery is higher than 60% starting from very low drug/lipid ratios (0.47–5.6 mg/100 mg lipid). Further more, these authors use at least 50 mg/ml of lipid concentration with an optimal at 100–400 mg/ml. Our range goes from 10–100 mg/ml with an optimal at 40 mg/ml. We do not need so much lipid to have good encapsulation, as it is specific. Janoff et al. (1990), in a US Patent, proposed that aminoglycosides could be complexed with phosphatidylinositolphosphates prior to administration to reduce interactions between the drug and the endogenous toxicity receptor. He also discusses the possibility of administering these drug-lipid complexes as liposomes. The difficulty remains in formulating stable liposomes with charged lipids such as phosphatidylinositol in the presence of water, as would be necessary for most aminoglycosides. This aim was achieved in the present work. Further more, the drug portion of the drug-lipid conjugate is exposed to the patient and may become separated from the conjugate in the patient.

In the review article by Karlowsky and Zhanel (1992), different types of liposomal aminoglycosides (gentamicin, amikacin and tobramycin) were compared. In spite of several lipid composition use in the literature, none uses our lipid composition with the same molar proportions, and particularly with so high PI proportion. Also NET was not so far immobilized in liposomes.

The high encapsulation efficiencies achieved with low initial drug/lipid ratios indicate that the liposomal NET formulations prepared are also highly effective as therapeutic agents. The results of pharmacological activity obtained, demonstrate a high efficiency, better than the other published liposomal aminoglycoside formulations, tested in several animal models (Karlowsky and Zhanel, 1992), although it is difficult to extrapolate the activity of different formulations tested in different animal models. Our formulation seems to have higher therapeutic activity as, with very low

dose (2 mg/kg in prophylactic and 0.2 mg/kg in treatment) survivals of 83% and 50% were obtained respectively (Fig. 2).

Evidence for different mechanism of action of the liposomal formulations used are showed by our results (Fig. 2). The larger size liposomes, sDRV, were the most effective formulation when used therapeutically, probably because as they are avidly taken by the RES organs, they can be internalized by infected cells of these organs. The intraphagocytic killing of bacteria is increased by liposomally delivered aminoglycosides. This might occur by direct delivery of the encapsulated drug into the phagocyte cells by liposomes (Dees and Schultz, 1990; Bakker-Woudenberg et al., 1993). By contrast, as sDRV have smaller residence time, they are not good as prophylactics, because they can not stay in the organisms enough time for that. The mechanism of VET_{200} (smaller size liposomes) action seems to be the opposite: as they have longer residence times in circulation they showed substantially greater efficiency when used prophylactically.

The therapeutic dose for treatment in clinical is 4 mg/kg in free form. The tests we have done were performed with 20-fold less therapeutic dose (0.2 mg/kg). So, our dose is minimal, which represents a high advantage, in reducing drug toxicity. It is possible that this therapeutic activity could be enhanced by increasing the dose.

The results here described indicate that liposomal NET formulations are effective agents in the prophylactic or therapeutic treatment of serious infections caused by Gram-negative bacteria.

The liposomal NET formulations produced may be administered at higher concentrations, and as a result, efficiency is enhanced, without fear of increased toxicity. The prolonged action of liposomal NET may also allow a reduction in frequency of drug administration and monitoring of drug concentration as well as associated medical care. Tests to check the nephrotoxicity of free and liposomal NET are in progress.

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