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Preparation, Relative Toxicity and Therapeutic Efficacy in Mice and Rats of Liposomal HA-1-92, a New Oxohexaene Polyene Macrolide Antibiotic

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Abstract

HA-1-92, a new polyene oxohexaene macrolide antibiotic isolated from *Streptomyces* CDRIL-312, was incorporated into liposomes containing phosphotidyl choline and cholesterol.

The liposomal incorporated HA-1-92 considerably decreased toxicity when compared with free HA-1-92 in mice. Liposomal HA-1-92 showed improved pharmacokinetic profiles in rats. When administered to aspergillosis- and cryptococcosis-infected Balb/c mice, liposomal HA-1-92 showed increased antifungal activity, compared with free HA-1-92, with improved survival rate and decreased colony-forming units in lung, liver, spleen and kidney.

These results suggest that liposomal HA-1-92 is more effective than free HA-1-92 in controlling experimental aspergillosis and cryptococcosis in Balb/c mice.

During a soil-screening programme for antifungal antibiotics, *Streptomyces* CDRIL-312 was found to produce a polyene oxohexaene macrolide antibiotic, HA-1-92. HA-1-92 exhibited very good antifungal activity in-vitro but much less activity in-vivo (Harindran 1996). Most of the reported antifungal antibiotics, including HA-1-92, are toxic when administered by the parenteral route.

Increased toxicity and the lack of bioavailability of polyene antibiotics at the infected site have been the major problems in patients receiving antifungal agents, and these limiting factors often lead to suboptimal therapy (Allen 1997). For a long time liposomes (Bangham et al 1965) have attracted attention as potential drug delivery systems due to their ability to alter the pharmacokinetics of the associated drug (Hwang 1987; Allen et al 1995).

Previous reports on liposomal hamycin, a polyene antibiotic discovered at our research centre, and liposomal amphotericin B have shown improved absorption and reduced toxicity in animal experiments (Lopez Berestein et al 1984; Mehta et al 1991; Moonis et al 1993). Consequently, we have developed a liposomal preparation of HA -1-92 and undertaken a comparative study of its toxicity and therapeutic efficacy.

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Materials and Methods

Chemicals

HA-1-92 was prepared in our laboratories. Phosphotidyl choline and cholesterol were from Sigma Chemicals (St Louis, MO). All other reagents used were of analytical grade.

Preparation of liposomes

Liposomes were prepared with phosphotidyl choline/cholesterol as described by Surolia et al (1975). Briefly, the lipids (molar ratio 8 : 2) were dissolved in chloroform in a round-bottom flask, and a known concentration of a solution of HA-1-92 prepared in methanol was added. The lipid HA-1-92 mixture was reduced to dryness in a rotatory evaporator and kept in a dessicator overnight. The resulting thin dry film was resuspended in 0·15 M saline and sonicated for 30 min under nitrogen using a MSE Ultrasonicator. Non-associated HA-1-92 was removed by extensive dialysis against 0·15 M NaCl. The amount of HA-1-92 intercalated into liposomes was determined

spectrophotometrically at 404 nm. A sample of the liposomal preparation was dissolved in methanol (Lopez Berestein 1987) and injected onto an HPLC system using a C-18 reverse phase column (4 mm × 25 cm, Altech, USA). The solvent system was citrate buffer (pH 5·3)/acetonitrile (60 : 40), the flow rate was 1 mL min^{-1}, and a UV detector was set at 380 nm (Harindran 1996).

Preparation of free HA-1-92 suspension

HA-1-92 suspension was prepared by making a stock solution of hamycin (200 mg in methanol) mixed with 0·9% NaCl containing 1% Tween-80. The suspension was sonicated in a water bath sonicator for 30 min under nitrogen to completely disperse HA-1-92.

LD50 determination

Acute LD50 of free and liposomal HA-1-92 was determined in Balb/c mice by the intravenous route described by Ahmad et al (1989).

Serum concentration of free and liposomal HA-1-92

Wistar albino rats (150–180 g) of either sex were divided into two groups of 60 rats. One group received 0·5 mg kg^{-1} HA-1-92 (free drug) intravenously, and the other group 0·5 mg kg^{-1} liposomal incorporated HA-1-92 intravenously. To determine the serum concentration of HA-1-92, the animals were placed under light ether anaesthesia and blood samples drawn directly from the heart, at 0, 0·5, 1, 2, 3, 4, 8, 12, 16, 20 and 24 h. For each interval five rats were sampled.

The blood samples were kept at room temperature for 30 min. Samples were then centrifuged to obtain clear serum. Butanol was added to the serum in a 1 : 2 volume and then shaken vigorously for 30 min and centrifuged. The clear upper layer of butanol was aspirated and read at 404 nm on a spectrophotometer to determine the HA-1-92 serum concentration.

To serve as a control, a known concentration of drug was added to the pooled serum of untreated rats and the extraction efficiency calculated. Accordingly an appropriate correction factor was added to the test samples. The mean serum concentration from five rats (at each interval) was plotted against time. The half-lives of free and liposomal HA-1-92 were also determined.

Mice model of cryptococcosis

Sixty male Balb/c mice (20–25 g) (obtained from the National Institute of Virololgy, Pune, India) were infected with 0·25 mL *Cryptococcus neoformans* cell suspension (7 × 10^6 cells mL^{-1}) in normal saline via the caudal vein. The infected mice were divided into three groups. Group one received free HA-1-92 (0·5 mg kg^{-1}, i.v.), group two received liposomal HA-1-92 (0·5 mg kg^{-1}, i.v.), and group three received physiological saline. Free and liposomal HA-1-92 were administered on alternate days, starting from the third day of infection for 13 days. The progress of the infection and death of any animals were monitored for 13–16 days. Survival rate was recorded until the last dose. The mice were killed two days after the last dose and the fungal load determined, in terms of colony-forming units (CFU), in lung, liver, kidney and spleen.

Mice model of aspergillosis

The animal model for aspergilosis was established as described by Ahmad et al (1989). Forty five male Balb/c mice (20–25 g) were infected with 0·25 mL *Aspergillus fumigatus* spore suspension (2 × 10^7 cells mL^{-1}) in physiological saline via the caudal vein. After 2 h, mice were divided into three groups of 15. The first group of mice received liposomal HA-1-92 (0·5 mg kg^{-1}, i.v.) and the second group received free HA-1-92 (0·5 mg kg^{-1}, i.v.). The third group (control) received physiological saline. Survival of mice for seven days after therapy was recorded. The CFU in lung, liver, kidney, spleen and brain was determined by killing the mice on the 7th day after therapy.

CFU determination

The in-vivo antifungal activity was evaluated on the basis of survival rate of animals and the number of CFUs in homogenates of lungs, liver, kidney, spleen and brain. The organs were excised aseptically, washed with physiological saline, and then homogenized in saline. A 25-fold serial dilution was placed in Sabourauds dextrose agar. The colonies were counted after 48-h incubation at 37°C.

Statistical analysis

The CFU data were statistically evaluated by analysis of variance of one-way classification with unequal frequencies (Snedecor & Cochran 1968). The heterogeneity of means for the various organs was tested by the F ratio of treatment variance to the experimental error variance. The survival data were analysed using Chi-squared with Yates correction and by Fisher's exact test. LD50 was cal-

culated by the method of Litchfield & Wilcoxon (1949).

Results

The amount of HA-1-92 encapsulated into liposomes was determined spectrophotometrically. It was found in initial experiments that encapsulation efficacy of HA-1-92 decreased when the HA-1-92 to lipid ratio was greater than 1 : 10. Therefore, total lipid (phosphatidyl choline + cholesterol) to HA-1-92 ratio was fixed at 1 : 10 (8 + 2 : 10) which resulted in maximum encapsulation efficiency (89·7 ± 3·0%) of HA-1-92 (Table 1). Considering the encapsulation efficiency, appropriate corrections were made in HA-1-92 liposomal dosage preparations for the animal studies.

Table 1. Entrapment of HA-1-92 into liposomes.

Drug (mg)	Lipid (mg)	Ratio of phosphotidyl choline : cholesterol	% entrapment
1	30	8 : 2	65·45 ± 8·6
1	30	7 : 3	64·50 ± 2·8
1	45	8 : 2	89·74 ± 2·9
1	45	7 : 3	84·89 ± 5·6
1	45	10 : 1	54·2 ± 6·8

Entrapment values are mean ± s.d. and denote average of six readings.

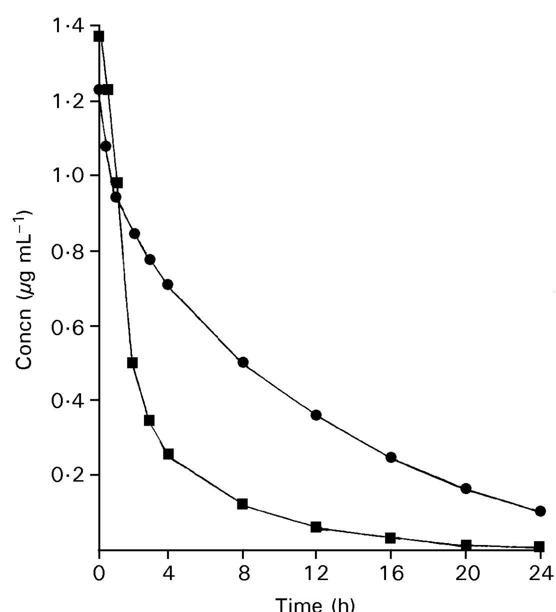

Figure 1. Blood concentration–time profile of HA-1-92 following administration of liposomal HA-1-92 (●) and free HA-1-92 (■) in rats.

Figure 1 shows the time course of the serum concentration of both free and liposomal HA-1-92. Rats treated with liposomal HA-1-92 (0·5 mg kg^{-1}) showed significantly higher serum concentration compared with animals treated with free HA-1-92 (0·5 mg kg^{-1}). The calculated half-lives of liposomal HA-1-92 and free HA-1-92 were 445 and 181 min, respectively.

The LD50 values of liposomal and free HA-1-92 are shown in Table 2. The animals treated with liposomal HA-1-92 showed mild toxic symptoms compared with the animals treated with free HA-1-92 (Table 2).

C. neoformans-infected mice treated with liposomal HA-1-92 shown significant reduction in CFU values in lung, kidney and spleen compared with control animals (Table 3). Treatment with free HA-1-92 showed significant reduction in CFU values in lung only compared with control mice (Table 3). It was observed that control animals infected with *Cryptococcosis neoformans* showed more than 50 and 90% mortality after 9 and 15 days, respectively. Treatment with free or liposomal HA-1-92 increased the survival by 20 and 30%, respectively. Aspergillosis mice administered free HA-1-92 had significantly decreased CFU in brain and spleen compared with control mice (Table 4). Infected animals receiving liposomal HA-1-92 showed significant reduction in CFU in lung and liver, and no CFU were observed in brain, spleen or kidney (Table 4). All the control mice infected with *A. fumigatus* died within five to seven days. Infected mice treated with free or liposomal HA-1-92 showed 10 and 40% survival, respectively.

Discussion

Liposomes have a number of properties which make them versatile drug carriers for either water-soluble or lipid-soluble drugs (Bangham et al 1965; Ozato et al 1978; Haran et al 1993; Allen 1997). Liposomes are composed of substances with low intrinsic toxicity and can be formulated in a large range of sizes and chemical composition. Liposomal formulation of amphotericin B has been shown to reduce the severe nephrotoxicity of amphotericin B in patients with systemic fungal infection (Tollemar et al 1995) and also in animal models (Ahmad et al 1989). The development of liposomal formulations has been instrumental in eradicating infections from patients unable to tolerate the dose intensity of the free drug necessary to control their infection (Graybill 1991; Heinemann et al 1994; Tollemar et al 1995; Russo et al 1996). When

Table 2. Acute toxicity studies of liposomal HA-1-92 and free HA-1-92 in mice.

	LD50 ($mg\,kg^{-1}$) in rats	19/20 Confidence limits in mice	Behavioural observation
Free HA-1-92 intravenously	12·5 ± 0·3	7·25 ± 0·2	Respiratory distress, loss of muscle tone. Mortality due to respiratory distress.
Liposomal HA-1-92 intravenously	41·5 ± 0·3 $P < 0{\cdot}001$	29·5 ± 0·25 $P < 0{\cdot}001$	Liposomal HA-1-92-treated mice showed mild to moderate toxic effects observed with free HA-1-92

Table 3. Colony-forming units (CFU) of *Cryptococcus neoformans* in different organs and percent survival of infected mice and effect of chronic treatment with free and liposomal HA-1-92.

Treatment	Tissue/organ	Log CFU $(g\,tissue)^{-1}$ (n = 3)	Percentage of survival 15 days after the therapy (n = 15)
Control without drug administration	Lung	3·919 ± 0·4	None survived
	Liver	3·301 ± 0·3	
	Kidney	3·919 ± 1·2	
	Spleen	3·863 ± 1·4	
	Brain	4·033 ± 1·5	
Free HA-1-92 (0·5 $mg\,kg^{-1}$, i.v.)	Lung	3·029 ± 0·02*	20 ± 0
	Liver	3·322 ± 0·02	
	Kidney	3·681 ± 0·18	
	Spleen	3·477 ± 0·2	
	Brain	3·792 ± 0·4	
Lipsomal HA-1-92 (0·5 $mg\,kg^{-1}$, i.v.)	Lung	2·602 ± 0·4*	40 ± 5·0
	Liver	3·278 ± 0·1	
	Kidney	2·875 ± 1·0*	
	Spleen	2·845 ± 4·8*	
	Brain	3·477 ± 1·1	

The values are expressed as mean ± s.e. from two separate experiments. Analysis of variance of one-way classification between the treatment means was heterogeneous and the *t* values (two-tailed) were significant at $*P < 0{\cdot}05$.

Table 4. Colony-forming units (CFU) of *Aspergillus fumigatus* in different organs and percent survival of infected mice and effect of chronic treatment with free and liposomal HA-1-92.

Treatment	Tissue/organ	Log CFU $(g\,tissue)^{-1}$ (n 3)	Percentage of survival 7 days after the therapy (n = 20)
Control without drug administration	Lung	2·317 ± 0·7	None survived
	Liver	2·618 ± 0·5	
	Kidney	2·705 ± 1·4	
	Spleen	3·161 ± 1·7	
	Brain	2·415 ± 1·8	
Free HA-1-92 (0·5 $mg\,kg^{-1}$, i.v.)	Lung	2·105 ± 8·2	10 ± 5·0
	Liver	2·477 ± 12·8	
	Kidney	2·795 ± 21·3	
	Spleen	2·985 ± 27·4*	
	Brain	2·098 ± 9·3*	
Lipsomal HA-1-92 (0·5 $mg\,kg^{-1}$, i.v.)	Lung	1·750 ± 3·6†	40 ± 5·0
	Liver	1·018 ± 1·8†	
	Kidney	Nil†	
	Spleen	Nil†	
	Brain	Nil†	

The values are expressed as mean ± s.e. Analysis of variance of one-way classification between treatment means was heterogenous and *t*-test values (two-tailed) were significant, $*P < 0{\cdot}05$ and $\dagger P < 0{\cdot}01$.

administered parenterally in the form of liposomes, hamycin has been shown also to be less toxic and to be effective in eradicating systemic fungal infections such as aspergillosis and cryptococcosis in animals (Mehta et al 1991; Moonis et al 1993).

In this study we have shown that HA-1-92, a new oxohexaene polyene macrolide antibiotic (Harindran 1996), when incorporated into liposomes showed low toxicity (as revealed by LD50 determination), improved pharmacokinetic profile (half-life) and therapeutic efficacy in systemic experimental fungal infections, as compared with free HA-1-92 in animal experiments. These effects could be due to intrinsic characteristics of the cell membrane, which ultimately facilitate the specific transporter of the drug. Our results are in agreement with other reports (Ozato et al 1978; Pagano & Weistein 1978; Fry et al 1979; Fry & Goldman 1982; Mazerski et al 1986). Several investigators are of the opinion that macrophages serve as secondary drug carriers for liposomal drugs (Margan et al 1985), and simultaneously decreasing the free drug concentration in other sensitive vital host tissues reduces the associated toxic effects. Our observations lead us to suggest that liposomal incorporated HA-1-92 acts as a depot from which the free HA-1-92 is released to the site of infection, largely as a result of enhanced capillary permeability in the region of the infection. Inevitably this particular event facilitates the achievement of significant improvement in the therapeutic index of liposomal HA-1-92.

The possible mechanisms by which liposomal HA-1-92 offers protection against *A. fumigatus* and *C. neoformans* infections may include its selective uptake by macrophages, and since it is an oxohexaene polyene macrolide antibiotic, known to act at membrane level, it might initiate macrophage activation.

The results demonstrate the potential usefulness of liposomes in improving the therapeutic index of HA-1-92 in the treatment of experimental fungal infection by reducing its toxicity and also by improving its pharmacokinetic profiles. Liposomal HA-1-92 may be effective in different systemic fungal infections, such as candidiasis, aspergillosis, systemic mycoses in bone marrow transplant recipients and cryptococcal meningitis in patients with AIDS after its pre-clinical toxicology evaluation.

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