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Biological characterization of L-asparaginase liposomal formulations

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Abstract The biological properties of preselected liposomal formulations of L-asparaginase (L-ASNase) were studied. Pharmacokinetics studies showed that encapsulation in large liposomes (sDRV; median diameter 1,249 nm) decreased the circulation time of the enzyme, whereas encapsulation in small liposomes (VET: median diameter 158–180 nm) prolonged it by a factor of up to 10. Liposome encapsulation in either VET or sDRV prevents the induction of anti-asparaginase antibodies and mitigates the anaphylatic reaction, as no death occurred in animals presensitized and challenged with liposomal formulation, in contrast to animals treated with the free enzyme. The antitumor activity was also enhanced by liposome encapsulation. The survival of animals bearing P1534 tumors was prolonged by a factor of 2 after treatment with selected liposomal formulations as compared with free enzyme.

Key words Liposomal L-ASNase · In vivo tests · L-ASNase · Liposomes · Leukemia · Therapeutic system

Introduction

L-Asparaginase (L-ASNase, L-asparagine amidohydrolase EC 3.5.1.1.) is an anticancer agent whose activity was demonstrated in 1961 by Broome [4]. It exerts its mechanism of action by depletion of the L-asparagine circulating in the blood, an amino acid that is necessary for malignant cells to proliferate. L-ASNase is currently a standard agent for the treatment of children with acute lymphoblastic leukemia. Its use, however, is limited by severe allergic reactions, namely, fever, skin rashes, chills, and anaphylaxis [12, 17]. To overcome these limitations, several approaches have been tried, ranging from encapsulation of the enzyme in liposomes [5, 16], chemical modification combined with encapsulation in liposomes [10], and covalent linkage to polyethylene glycol (PEG) [8, 14].

We have recently developed liposomal formulations of L-ASNase with high entrapment efficiency and stability in biological fluids. Lipidic compositions consisting of mixtures of phosphatidylcholine, cholesterol, and either stearylamine, phosphatidylinositol, or monosialoganglioside resulted in a high encapsulation efficiency (40% and 98% in VET and sDRV, respectively), high stability in saline and human serum (65–90% after 48 h), and considerable preservation of enzymatic activity (74–98%). The liposomal formulations were found to be less toxic than the free enzyme against Chinese hamster ovarium (CHO) cells in vitro [5]. Because L-ASNase exerts its effect in the circulation, liposomes with prolonged circulation half-lives and decreased uptake by the MPS (mononuclear phagocytic system) should be a rational choice. In this paper we present the pharmacokinetic parameters, acute toxicity, and antitumor activity of free L-ASNase and L-ASNase encapsulated in different types of liposomes.

Materials and methods

Egg phosphatidylcholine (PC), bovine liver phosphatidylinositol (PI), bovine brain monosialoganglioside (GM1), cholesterol (Chol), and stearylamine (SA) were obtained from Sigma Chemical CO. (München, Germany). L-ASNase (Elspar) was purchased from Merck Sharp & Dohme. All other reagents were of analytical grade. P1534 lymphoma cells were supplied by the National Cancer Institute-Frederick Cancer Research Facility DCT Tumor Repository. Adult male CD-1 Swiss mice and male BDF1 mice (weight

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20–25 g) were obtained from the animal house of the Gulbenkian Institute of Sciences (Oeiras, Portugal).

Liposome preparation

sDRV liposomes: simplified dehydration-rehydration vesicles

The method has been described elsewhere [5]. Briefly, the lipid mixture was dried under a nitrogen stream. The lipidic film was hydrated with an aqueous solution of L-ASNase. The multilamellar liposomes formed were lyophilized overnight and rehydrated with a solution of 0.3 *M* mannitol in a volume of one-tenth of the final volume. The rehydration was completed with a solution of 0.154 *M* NaCl. The nonencapsulated L-ASNase was removed by three cycles of 30 fold dilution with 0.154 *M* NaCl and centrifugation at 38,000 g for 30 min. The final liposome pellet was resuspended in a solution of 0.154 *M* NaCl.

VET liposomes: extruded vesicles

VET liposomes were prepared, as like the sDRV liposomes, by drying the lipidic mixture under a nitrogen stream. The procedure was identical to that described for sDRV liposomes until the rehydration step. The liposomal preparation was then diluted 10 times with saline and the liposomes were subsequently filtered using an Extruder device (Lipex Biomembranes Inc., Vancouver, Canada). Liposomes were extruded sequentially through polycarbonate membranes (Nucleopore Corporation) of 0.8, 0.6, 0.4, 0.2, and 0.1 μm pore size one time each under a nitrogen pressure of 100–500 psi. The liposomes collected after extrusion with the last membrane (0.1 μm) were concentrated by ultracentrifugation at 250,000 *g* for 90 min.

Liposome characterization

The encapsulation efficiency (EE) was defined as the percentage of the ratio between the final protein-to-lipid (P/L) ratio and the initial (P/L) ratio. The protein was determined using the method described by Lowry et al. [13] after disruption of the liposomes with Triton X-100 and sodium dodecyl sulfate (SDS) [20]. Lipid determinations were performed using the method of Fiske and Subbarow [6] as modified by King [11]. The activity of L-ASNase in the free and liposomal forms was determined by the method described by Jayaram et al. [9] after disruption of the liposomes with Triton X-100.

The stability of liposome-encapsulated L-ASNase was assessed by measuring the enzymatic activity [9] occurring in the pelleted vesicles (184,000 $g \times 90$ min centrifugation) at different times after incubation in 0.154 *M* NaCl diluted 1: 2 (v/v) in human serum at 37°C in relation to the enzymatic activity recorded before incubation. The size distribution of the vesicles was determined by dynamic laser light scattering (ZetaSizer 3, Malvern, UK).

Biological activity of liposome-encapsulated L-ASNase

Pharmacokinetics of liposomal L-ASNase

Pharmacokinetics studies were performed in male CD-1 Swiss mice weighing 20–25 *g*. Animals were injected intravenously in the tail vein with a 400 U/kg dose of free or liposomal L-ASNase. Circulating blood levels of enzyme activity were determined in blood samples collected at selected times from the retroorbital vein in tubes containing ethylene diaminetetraacetic acid (EDTA). For sDRV liposomes, blood samples were drawn at 5, 7, 15, 20, and 30 min. For free L-ASNase, blood was drawn at 15 and 30 min and at 1, 2, 4, 6, and 8 h, and for VET liposomes an additional sample was drawn at 12 h after liposome administration.

L-ASNase activity was measured in whole-blood samples previously incubated in 1% Triton X-100 at 37°C for 30 min. Four animals were used for each time point. The mean values obtained for each time point were used to calculate the pharmacokinetic parameters by an automatic stripping procedure (PKCALC, B.B.N., software products written by R.C. Shumaker, 1987). L-ASNase activity was determined by the method described by Jayaram et al. [9].

In vivo toxicity

For toxicity studies, CD-1 Swiss mice were sensitized with three subcutaneous injections of 2,000 or 4,000 U/kg each of free or liposomal enzyme on days 0, 10, and 20. On day 30, animals were challenged with the same dose of free or liposomal L-ASNase given intravenously (in the tail vein). The liposome composition was PC: Chol: SA at a molar ratio of 7: 2: 0.25. Mice were observed for 3 days and deaths were recorded.

In vivo antitumor activity

For in vivo antitumor activity studies, BDF1 mice were inoculated subcutaneously with 5×10^6 P1534 lymphoma cells, whose growth is inhibited by L-ASNase [15]. Treatment started on day 10 after tumor inoculation by intravenous administration. Treatment consisted of five 800-U/kg doses given every 2 days of free L-ASNase or liposomal L-ASNase of different lipid composition, namely, PC: Chol: Sa (VET and sDRV), PC: Chol: Pl, and PC: Chol: GM1. Control animals were treated with saline using the same schedule. Results were expressed as %T/C (ratio between the median survival of treated animals and the median survival of control animals expressed in percent). Animals alive at 4 months were recorded as being cured.

Results

Characterization of liposomal L-ASNase

Table 1 shows the size, encapsulation efficiency, (P/L) protein-to-lipid ratio, retention of enzymatic activity, and human serum stability recorded for different liposomal formulations of L-ASNase. sDRV liposomes (1,249 nm in mean diameter) showed an almost 100% retention of enzymatic activity and a mean encapsulation efficiency of 72%, whereas extruded vesicles (VET; mean diameter ranging from 158 to 180 nm) showed encapsulation efficiencies ranging from 34% to 36% and enzymatic activity exceeding 93%. Extruded vesicles showed a 78–90% retention of activity after 48 h of incubation in human serum versus 65% for sDRV.

Pharmacokinetics studies

The blood clearance and pharmacokinetic parameters of free and liposomal L-ASNase are shown in Table 2.

Table 1 Characteristics of different liposomal L-ASNase formulations[a]

Lipid composition (molar ratio)	Liposome size diameter (nm)	Encapsulation efficiency (%)	Activity retention (%)	Enzimatic activity in human serum at 48 h (%)
PC:Chol:SA (7:2:0.25) sDRV	1,249 ± 249	72.5 ± 7.2	99.4 ± 0.9	65 ± 1.5
PC:Chol:SA (7:2:0.25) VET100	158 ± 11	36.7 ± 0.5	99.3 ± 0.9	90 ± 1.8
PC:Chol:Pl (10:5:1) VET100	180 ± 19	36.7 ± 4.0	99.3 ± 0.9	78 ± 2.4
PC:Chol:GM1 (10:5:1) VET100	170 ± 12	34.7 ± 3.4	93.7 ± 3.3	–

[a] Recorded data are the mean values of at least 3 experiments

Table 2 Pharmacokinetic parameters[a] (*MRT* Mean residence time, AUC_{total} area under the plasma concentration-time curve, Clp plasma clearance, Vd_{ss} volume of distribution at steady state, $t_{1/2}$ half-life)

Formulation	MRT (h)	AUC_{total} (U h m^{-1})	Clp (ml/h)	Vd_{ss} (ml)	$t_{1/2}$ (h)
L-ASNase	2.9	23.1	0.434	1.266	1.96
PC:Chol:SA sDRV (7:2:0.25)	0.3	0.7	14.445	3.466	0.11
PC:Chol:SA VET100 (7:2:0.25)	11.1	58.5	0.171	1.904	9.35
PC:Chol:PI VET100 (10:5:1)	15.4	92.0	0.109	1.678	11.04
PC:Chol:GM1 VET100 (10:5:1)	34.4	108.1	0.120	4.140	28.75

[a] The pharmacokinetic parameters were obtained by nonlinear regression analysis: dose 400 U/kg, number of mice for each point 4, drugs L-ASNase and L-ASNase incorporated in liposome types sDRV and VET, inoculation intravenous

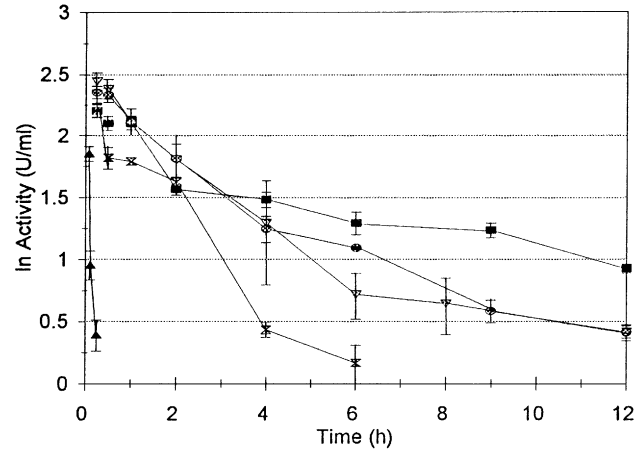

Fig. 1 Pharmacokinetics of different L-ASNase formulations: VET 100 PC:Chol:PI (*black squares*), VET 100 PC:Chol:GM1 (*shaded circles*), VET 100 PC:Chol:SA (*shaded triangles*), SDRV PC:Chol:SA (*black triangles*), and free L-ASNase (*shaded hourglasses*)

Curves generated for all formulations are shown in Fig. 1. The incorporation of L-ASNase in liposomes type VET increased the mean residence time (MRT) of the free enzyme by a factor ranging from 4 to 11, depending on the lipid composition. However, the MRT of sDRV liposomes was lower than that of free L-ASNase. The area under the plasma concentration-time curve (AUC_{total}) increased by a factor of 2–5 for VET liposomes and decreased for sDRV liposomes. The plasma clearance showed a 4 fold reduction for VET liposomes and a 30 fold increase for sDRV liposomes.

Toxicity studies

Free L-ASNase was toxic, with 17% and 83% of presensitized animals dying as a result of the i.v. administration of 2,000 and 4,000 U/kg, respectively. After a second challenging dose, 80% of the animals that had remained alive after the first challenge dose died (Table 3). The encapsulation of L-ASNase in liposomes reduced the acute toxicity. No death was observed when the sensitizing agent was the enzyme in free form and the challenging agent was the liposomal form involving either VET or sDRV of lipidic composition PC: Chol: SA. When liposomal L-ASNase was used as the sensitizing and challenging agent the survival was 100% for both kinds of liposomes used, for both doses, and for first- and second-challenge administration. In contrast,

Table 3 Effect of liposome encapsulation on acute toxicity[a] (*i.m.* Intramuscular, *i.v.* intravenous)

Experiment	Animals *n*	Dead *n* (%)
Free L-ASNase		
Dose 2,000 U/kg		
i.m. Free L-ASNase		
1st i.v. Free L-ASNase	6	1 (17)
2nd i.v. Free L-ASNase	5	4 (80)
Dose 4,000 U/kg		
i.m. Free L-ASNase		
1st i.v. Free L-ASNase	6	5 (83)
Liposomal L-ASNase (VET100)		
Dose 2,000 U/kg		
i.m. Free L-ASNase		
1st i.v. Liposomal L-ASNase	6	0 (0)
2nd i.v. Liposomal L-ASNase	6	0 (0)
i.m. Liposomal L-ASNase		
1st i.v. Free L-ASNase	6	1 (17)
2nd i.v. Free L-ASNase	5	1 (20)
i.m. Liposomal L-ASNase		
1st i.v. Liposomal L-ASNase	6	0 (0)
2nd i.v. Liposomal L-ASNase	6	0 (0)
Liposomal L-ASNase (sDRV)		
Dose 4,000 U/kg		
i.m. Free L-ASNase		
1st i.v. Liposomal L-ASNase	6	0 (0)
i.m. Liposomal L-ASNase		
1st i.v. Free L-ASNase	6	4 (67)
i.m. Liposomal L-ASNase		
1st i.v. Liposomal L-ASNase	6	0 (0)

[a] Inoculation was done subcutaneously for sensitizing and intravenously for challenging; doses 2,000 and 4,000 U/kg, drugs L-ASNase and liposome types VET- and sDRV-entrapped L-ASNase, lipid composition PC: Chol: SA (7 : 2 : 0.25)

the free enzyme given intravenously to animals sensitized with the liposomal enzyme was as toxic as it was to animals sensitized with free L-ASNase (17–67% mortality). However, increased survival (80%) was observed after a second challenge dose of free L-ASNase in animals sensitized with VET L-ASNase, whereas only 20% of the animals survived when free L-ASNase was the sensitizing agent. These results indicate that the liposomes are more effective in preventing the allergic reaction in a sensitized animal than in preventing the sensitization.

Antitumor activity studies

Table 4 shows the antitumor activity of free and liposomal formulations of L-ASNase. At intravenous doses of 400 and 800 U/kg the antitumor activity of the enzyme encapsulated in sDRV liposomes was lower than that of the free enzyme. In contrast, treatment with the enzyme encapsulated in VET liposomes resulted in a 2-fold enhancement of the % TC and in a marked increase in the number of cured animals as compared with injection of the free enzyme.

Table 4 Antitumor activity[a]

Formulation	Dose (U/kg)	Cured animals	%T/C[b]
Free L-ASNase	400	2 of 6	382
	800	2 of 10	392
sDRV	400	1 of 6	131
PC: Chol: SA	800	0 of 10	135
VET100	400	2 of 6	244
PC : Chol : SA	800	5 of 9	> 700
VET100	400	2 of 9	200
PC : Chol : PI	800	7 of 10	> 700
VET100	400	1 of 7	163
PC : Chol : GM1	800	9 of 10	> 700

[a] Tumor P1534, inoculation subcutaneous, treatment every 2 days 5 injections i.v. starting on day 10, doses 400 and 800 U/kg, drugs L-ASNase and liposome type VET-entrapped L-ASNase, control group median survival 19 days

[b] %T/C = (median survival of treated animals/median survival of control animals) × 100

Discussion

In previous work [5], different liposomal formulations of L-ASNase were prepared. Some of the formulations displaying high encapsulation efficiency, high stability in saline and human serum, high preservation of enzymatic activity, and reduced in vitro toxicity were selected for biological evaluation.

The increase in circulating half-life ($t_{1/2}$) observed for small liposomes (VET) as compared with large liposomes (sDRV) is in accordance with early reports in the literature for other systems [1, 2, 19]. That the $t_{1/2}$ of VET is higher than that of the free enzyme is important since the target of the enzyme is the circulating subtract, L-asparagine. The $t_{1/2}$ was dependent on the lipid composition of liposomes increasing when SA combined with PC and Chol was replaced by PI or GM1. These findings are in agreement with previous observations [18] and result from shielding of the negative charge by some hydroxyl groups surrounded by bulky neutral, hydrophilic groups, yielding a decrease in the uptake of liposomes by the MPS [1–3, 7, 18]. In addition, in the case of GM1, the lipid results in liposomes of high rigidity that are not easily destroyed in the circulation [1]. The $t_{1/2}$, however, is shorter when the native enzyme is incorporated in liposomes (present work) than when a derivatized form (palmitoyl-L-ASNase) is used with the same type of liposomes [10].

Accordingly, the MRT and AUC are lower for the liposomal native enzyme than for the derivatized enzyme. This might be due to stabilization of the liposomal membrane by the derivatized enzyme, which, because of its hydrophobic nature, is located in the lipid matrix of liposomes and, therefore, is partially exposed to the external milieu [10].

The toxicity studies presented in this paper indicate that the encapsulation of native L-ASNase in liposomes (types VET and sDRV) prevents the immune response. This effect has been described elsewhere [16]. The free enzyme, due to its hydrophilic nature, is encapsulated in the internal aqueous space of the liposomes. The mitigation of the allergic reaction by the liposomal native enzyme is different from that by the liposomal hydrophobic, derivatized enzyme, with which some immunogenic reactions were observed. In the case of the derivatized enzyme the lipid composition of liposomes is important since it modulates the accommodation of the enzyme in the lipid matrix and its presentation to the immune system. Studies with PEG-L-ASNase showed a lack of significant toxicity for this form of the enzyme in dogs [14] and humans [8], although a few patients developed anaphylaxis and bronchospasm [8]. Our results cannot easily be compared due to differences in the animals used and to our use of pre-sensitized animals and our measurement of acute toxicity, but we can conclude that our formulations are immunogenically safe.

The increased antitumor activity obtained for liposomal L-ASNase is closely related to the pharmacokinetic parameters. Treatment with sDRV liposomes, which are quickly taken up by the MPS, resulted in a shorter period of survival as compared with the free enzyme. The survival increased by a factor of 2 when long-circulating liposomes were used. The number of cured animals also increased when VET liposomes were used at a dose of 800 U/kg. At lower doses (400 U/kg), liposomal L-ASNase was not effective. In contrast, the acylated form of L-ASNase (palmitoyl-L-ASNase) entrapped in some liposomal formulations is more effective than the free enzyme (L-ASNase) [10]. This is probably due to the possibility that this enzyme acts both when bound to the liposomes and after their disruption [10]. The antitumor activity of PEG-L-ASNase has been studied in dogs and is not higher than that of the free enzyme [14]. Our VET L-ASNase formulations seem superior since they result in an increase in the % T/C.

The results of this study show that as compared with previous formulations described in the literature, our selected VET formulations containing L-ASNase are immunogenically safe and more effective than the free enzyme currently used in the clinic. These formulations are candidates for further development.

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References

1. Allen TM (1989) Stealth liposomes: avoiding reticuloendothelial uptake. In: Lopez-Berestein G, Fidler I (eds) Liposomes in the therapy of infectious diseases and cancer. (New series, vol 89) Alan R. Liss, New York, pp 405–415
2. Allen TM (1994) The use of glycolipids and hydrophilic polymers in avoiding rapid uptake of liposomes by the mononuclear phagocyte system. Adv Drug Deliv Rev 13 : 285–309
3. Allen TM, Hansen C, Rutledge J (1989) Liposomes with prolonged circulation times: factors affecting uptake by reticuloendothelial and other tissues. Biochim Biophys Acta 981: 27–35
4. Broome JD (1961) Evidence that L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. Nature 191 : 1114–1115
5. Cruz MEM, Gaspar MM, Lopes F, Jorge JS, Perez-Soler R (1993) Liposomal L-asparaginase: in vitro evaluation. Int J Pharm 96 : 67–77
6. Fiske CH, Subbarow Y (1925) The colorimetric determination of phosphorus. J Biol Chem 66 : 375–400
7. Gabizon A, Papahadjopoulos D (1988) Liposome formulations with prolonged circulation time in blood and enhanced uptake by tumors. Proc Natl Acad Sci USA 85 : 6949–6953
8. Ho DH, Brown NS, Yen A, Holmes R, Keating M, Abuchowski A, Newman RA, Krakoff IH (1986) Clinical pharmacology of polyethylene glycol—L-asparaginase. Drug Metab Dispos 14 : 349–352
9. Jayaram HN, Cooney DA, Jayaram S (1974) A simple and rapid method for the estimation of L-asparaginase in chromatographic and electrophoretic effluents: comparison with other methods. Anal Biochem 59 : 327–346
10. Jorge JCS, Perez-Soler R, Morais JG, Cruz MEM (1994) Liposomal palmitoyl-L-asparaginase: characterization and biological activity. Cancer Chemother Pharmacol 34 : 230–234
11. King EJ (1932) The colorimetric determination of phosphorus. Biochem J 26 : 292–297
12. Land VJ, Sutow WW, Fernbach DJ, Lane DM, Williams TE (1972) Toxicity of L-asparaginase in children with advanced leukemia. Cancer 30 : 339–347
13. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193 : 265–275
14. MacEwen GE, Rosenthal RC, Fox LE, Loar AS, Kurzman ID (1992) Evaluation of L-asparaginase: polyethylene glycol conjugate versus native L-asparaginase combined with chemotherapy. J Vet Intern Med 6 : 230–234
15. Mashburn LT, Wriston JC Jr (1964) Tumor inhibitory effect of L-asparaginase from *Escherichia coli*. Arch Biochem Biophys 105 : 450–452
16. Neerunjun D, Gregoriadis G (1976) Tumor regression with liposome-entrapped asparaginase: some immunological advantages. Bichem Soc Trans 133–134
17. Oettgen HF, Phyllis AS, Morton KS, Leeper RD, Tallal L, Charlotte CT, Clarkson BD, Golbey RD, Krakoff IH, Karnopsky DA, Murphy M, Burchenal JH (1970) Toxicity of *E. coli* L-asparaginase in man. Cancer 25 : 253–278
18. Park YS, Maruyama K, Huang L (1992) Some negatively charged phospholipid derivatives prolong the liposome circulation in vivo. Biochim Biophys Acta 1108 : 257–260
19. Senior JH (1987) Fate and behavior of liposomes in vivo: a review of controlling factors. CRC Crit Rev Ther Drug Carrier Syst 3 : 123–193
20. Wang CH, Smith RL (1975) Lowry determination of the protein in the presence of Triton X-100. Anal Biochem 63 : 414–417