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Liposomal palmitoyl-L-asparaginase: characterization and biological activity

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Abstract. A new derivative of L-asparaginase, palmitoyl-L-asparaginase (palmitoyl-L-ASNase), has been incorporated in liposomes. For this purpose we modified the dehydration-rehydration method and optimized the liposomal composition. The pharmacokinetics, toxicity, and in vivo antitumor activity against P1534 lymphoma of different liposomal palmitoyl-L-ASNase formulations were studied. Liposomal encapsulation of palmitoyl-L-ASNase as compared with free palmitoyl-L-ASNase resulted in a prolongation of the blood half-life (from 2.88 h to longer than 23.7 h), abrogation of acute toxicity, and preservation of in vivo antitumor activity.

Introduction

L-Asparaginase (L-ASNase; L-asparagine amido hydrolase, E.C.3.5.1.1.) is an antitumor agent active against human acute lymphoblastic leukemia [2, 4, 5, 25, 30]. However, its use is limited by severe side effects, mainly acute allergic reactions ranging from skin rashes to death secondary to anaphylactic shock [7, 8, 16, 22, 28, 31].

Liposomes have been used in the past as carriers of L-ASNase and showed to protect against acute allergic reactions [27], but the entrapment efficiency of the developed formulations has been low. We have developed a new derivative of L-ASNase, palmitoyl-L-ASNase [24], which can be easily incorporated in the lipidic compartment of liposomes. As such, this derivative is exposed partially to the external medium, giving rise to liposomes that evidence enzymatic activity in an intact form.

Palmitoyl-L-ASNase has never been entrapped in liposomes before. Due to its hydrophobic nature, we developed a method [9] involving the addition of lyophilized modified protein to preformed empty liposomes followed by a dehydration-rehydration procedure [21] and extrusion through polycarbonate filters. Studies aimed at optimizing the composition and size of the liposomes were performed. Physicochemical characterization and in vivo studies of the formulations were performed.

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Materials and methods

Materials. Egg phosphatidylcholine (PC), bovine liver phosphatidylinositol (PI), cholesterol (Chol), and stearylamine (SA) were obtained from Sigma, Spain. L-ASNase (Elspar) was obtained from M. S. D. Palmitoyl-L-ASNase was kindly prepared by Eng. Maria Bárbara Martins (INETI-DB, Bioquímica I, Queluz, Portugal). Mice were obtained from Gulbenkian Institute of Science, Oeiras, Portugal. P1534 lymphoma cells were kindly donated by Dr. Roman Perez Soler, M. D. Anderson Cancer Center (Houston, Texas, USA). All other chemicals were of reagent grade.

Preparation of palmitoyl-L-ASNase suspensions. To obtain a suitable formulation for the in vivo administration of free palmitoyl-L-ASNase, lyophilized modified enzyme was rehydrated in 0.5% Tween-80 and filtered through 0.2-μm polycarbonate filters.

Liposome preparation. Lipid was dried under N_2 stream. Water was added to form empty multilamellar vesicles, which were added to the lyophilized protein. Dehydration followed by rehydration in the presence of 0.3 *M* mannitol in a volume of one tenth of the final volume was subsequently performed. The liposomes were then resuspended in an excess volume of saline and extruded sequentially through polycarbonate filters ranging from 5.0 to 0.2 μm in pore size. Due to its hydrophobic nature, the nonincorporated protein precipitates at the outer surface of the liposomes. To remove the nonincorporated protein, the liposome suspension was ultracentrifuged for 90 min at 250,000 *g* (Beckman Ultracentrifuge L8 – 60M) in a saccharose gradient (from 0 to 40 mg/ml). The liposomes were collected at the middle of the gradient and the free protein at the bottom of the tube was neglected. To wash the saccharose out of the liposomes and to concentrate the liposomal preparation, a final ultracentrifugation was done at 250,000 *g* for 60 min.

Liposome characterization. Protein recovery (the ratio between the liposome-associated protein and the total amount of initial protein) was calculated according to the Lowry method [23] by measuring the amount of protein remaining after the disruption of liposomes with

Triton X-100 and sodium dodecyl sulfate (SDS) [32]. The specific activity of palmitoyl-L-ASNase was measured according to Jayaram et al. [18] in a Shimadzu UV3000 dual-wavelength spectrophotometer. Determination of the specific activity of palmitoyl-L-ASNase incorporated in liposomes was performed by the same method after the disruption of liposomes with Triton X-100. To determine the intact activity (AI) of these liposomes, no disruption was carried out, the enzymatic activity being evaluated in the presence of intact liposome suspension. Preliminary studies were performed to ensure that these vesicles were not permeable to asparagine (data not shown).

AI/AT (%) is the ratio between the specific activity of palmitoyl-L-ASNase incorporated in liposomes as measured without prior disruption of liposomes and the total specific activity of palmitoyl-L-ASNase as determined after disruption of the same liposome preparation with Tween-80. This ratio represents the specific activity of the palmitoyl-L-ASNase exposed to the external medium in relation to the total palmitoyl-L-ASNase incorporated in liposomes.

The phospholipid concentration was determined according to Fiske and Subbarow [13] as modified by King [20]. The encapsulation efficiency (EE) was calculated as the ratio between the final protein-to-lipid ratio (Pf/Lf) and the initial protein-to-lipid ratio (Pi/Li). Protein recovery represents the amount of liposome-associated protein in the final liposomal suspension in relation to the total amount of protein initially added. Liposome size was measured by quasi-elastic laser light scattering in a Malvern ZetaSizer 3 device.

Animal experiments. Pharmacokinetics studies were done in BDF_1 mice injected i.v. with 2,000 U/kg of the different formulations. Blood was collected from the orbital sinus at selected time points. Palmitoyl-L-ASNase activity was determined in total blood by the same method used in all activity studies.

Immunology studies were performed in CD_1 mice. Sensitization was achieved by s.c. administration of 2,000 U/kg liposomal palmitoyl-L-ASNase, palmitoyl-L-ASNase suspended in 0.5% Tween-80, and palmitoyl-L-ASNase suspended in 0.154 *M* NaCl on days 10, 20, and 30. At 10 days after the last injection, a challenge dose of 2,000 U/kg was given via the tail vein. Animals were observed for the development of either anaphylactic shock or death for 72 h.

For the antitumor activity studies, BDF_1 mice (groups of 8–10 animals) were inoculated s.c. with 5×10^6 P1534 lymphoma cells. Treatment was started on day 10 by the i.v. route. Animals received a total of five injections (given every other day) of palmitoyl-L-ASNase or liposome-incorporated palmitoyl-L-ASNase (400 and 2,000 U/kg). Control animals were treated with saline using the same schedule and route. Results were expressed as % T/C (the rate of survival of treated versus control animals × 100). Animals that were alive on day 96 were considered cured.

Results

Characterization of palmitoyl-L-ASNase formulations

On the basis of previous studies conducted by our group using native L-ASNase [10], we elected to work with the best lipidic compositions for the native enzyme, which are PC : Chol : PI in a molar ratio of 10 : 5 : 1 and PC : Chol : SA in a molar ratio of 7 : 2 : 0.25. Incorporation parameters were determined for these types of vesicles before (MLV) and after the extrusion procedure (VET_{200}). The results are shown in Table 1. The ratio of intact activity (AI) to total (destroyed) activity (AT) is the most important parameter since it reflects the activity of palmitoyl-L-ASNase exposed to the outer medium. It increased from about 30% for MLV to 51% and 74% in the two VET_{200} formulations. The particle sizes obtained were 422.7 ± 147.5 μm (MLV) and 208.6 ± 32.6 μm (VET_{200}) for PC : Chol : PI and 548.3 ± 417.9 μm (MLV) and 192.0 ± 69.3 μm (VET_{200}) for PC : Chol : SA.

Table 1. Incorporation parameters

	Recovery (%)	EE (%)	AI/AT (%)	Pf/Lf ($\times 10^3$)
PC : Chol : PI (MLV)	56 ± 9	80 ± 16	25 ± 4	18 ± 4
PC : Chol : PI (VET_{200})	36 ± 8	69 ± 19	51 ± 14	15 ± 5
PC : Chol : SA (MLV)	65 ± 7	86 ± 14	36 ± 4	19 ± 4
PC : Chol : SA (VET_{200})	43 ± 15	74 ± 12	74 ± 11	16 ± 6

The recovery, EE, final ratio between protein and lipids (Pf/Lf), and ratio of intact (AI) to total (destroyed) activity (AT) were determined for the two developed lipidic compositions before (MLV) and after the extrusion of liposomes (VET_{200}) as described in Materials and methods. All values are expressed in percent except Pf/Lf (w/w). Initial protein concentration, 0.250 mg/ml; initial lipid concentration, 16.5 μmol/ml

Table 2. Influence of the initial protein-to-lipid ratio on the incorporation parameters

	Recovery (%)	EE (%)	AI/AT (%)	Pf/Lf ($\times 10^3$)
PC : Chol : PI (VET_{200})	29 ± 7	46 ± 6	54 ± 14	29 ± 6
PC : Chol : SA (VET_{200})	44 ± 6	66 ± 10	46 ± 12	41 ± 10

The recovery, EE, Pf/Lf ratio, and AI/AT ratio were determined for the two developed lipidic compositions after the extrusion of liposomes (VET_{200}) as described in Materials and methods. All values are expressed in percent except Pf/Lf (w/w). Initial protein concentration, 2.87 mg/ml; initial lipid concentration, 66.0 μmol/ml

To obtain enough liposomal palmitoyl-L-ASNase for the animal studies it became necessary to increase the amounts of lipid and protein in the liposomal preparations. For that purpose, the initial ratio of 16.5 μmol/0.25 mg (lipid/protein) was modified to 66.0 μmol/2.87 mg. The incorporation parameters of the liposomal formulations made under these new conditions are presented in Table 2. For the VET_{200} liposomes containing PI, the incorporation parameters were: recovery, 29% ± 7%; EE, 46% ± 6%; and AI/AT, 54% ± 14%. For the VET_{200} liposomes containing SA, the incorporation parameters were: recovery, 44% ± 6%; EE, 66% ± 10%; and AI/AT, 46% ± 12%.

Pharmacokinetics

The blood clearance and pharmacokinetic parameters of liposome-entrapped (VET_{200}) and free palmitoyl-L-ASNase suspended in 0.5% Tween-80 are shown in Fig. 1 and Table 3. All pharmacokinetic parameters were obtained by an automatic stripping procedure (PKCALC; B. B. N. Software Products, written by R. C. Shumaker in 1987). The incorporation of palmitoyl-L-ASNase in liposomes increased the mean residence time (MRT) from 4.1 h to larger than 32 h. The area under the curve was also significantly increased from 77.4 U h ml^{-1} to higher than 650 U h ml^{-1}. The plasma clearance was reduced with liposome incorporation from 0.646 ml/h to values smaller than 0.076 ml/h. No change in the distribution volume (steady-state) was observed.

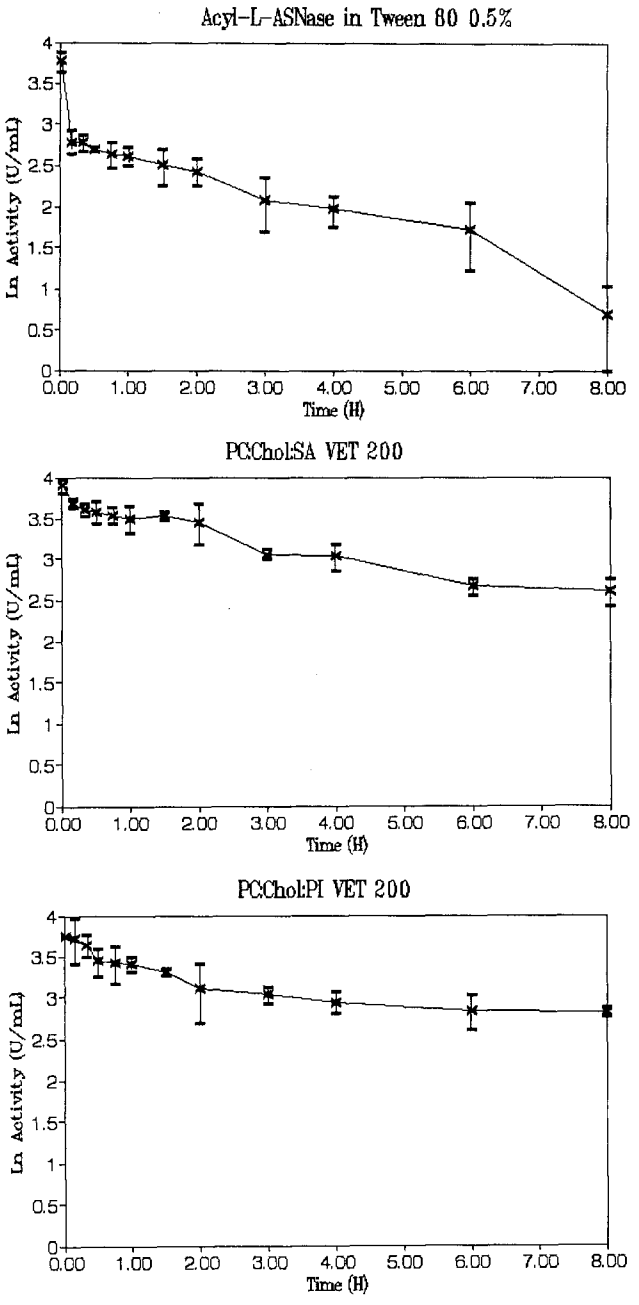

Fig. 1. Pharmacokinetics of palmitoyl-L-ASNase in 0.5% Tween-80 and of the liposomal formulations of palmitoyl-L-ASNase (VET_{200})

Table 3. Pharmacokinetic parameters

	MRT (h)	AUC_{total} (U h ml^{-1})	Clp (ml/h)	Vd_{ss} (ml)	$t_{1/2}$ (h)
Palmitoyl-L-ASNase in 0.5% Tween-80	4.1	77.4	0.646	2.64	2.88
PC:Chol:SA (VET_{200})	32.1	656.3	0.076	2.44	24.3
PC:Chol:PI (VET_{200})	33.1	740.9	0.067	2.24	23.7

The following pharmacokinetic parameters were obtained by nonlinear regression analysis: mean residence time (MRT), area under the curve (AUC_{total}), plasma clearance (Clp), volume of distribution at steady state (Vd_{ss}), and half-life ($t_{1/2}$). Delivered dose, 2000 U/kg

Table 4. Effect of liposome composition on acute toxicity

Experiment	Animals (*n*)	Shock (*n*)	Dead (*n*)
S: i.m., – Acyl-L-ASNase, 0.5% Tween-80 C: i.v., – Acyl-L-ASNase, 0.5% Tween-80	6	4 (66%)	1 (17%)
S: i.m., – Acyl-L-ASNase, 0.154 *M* NaCl C: i.v., – Acyl-L-ASNase, 0.154 *M* NaCl	6	4 (66%)	1 (17%)
S: i.m., – Acyl-L-ASNase, PC:Chol:PI (0.2 μm) C: i.v., – Acyl-L-ASNase, PC:Chol:PI (0.2 μm)	6	0 (0)	0 (0)
S: i.m., – Acyl-L-ASNase, PC:Chol:PI (0.2 μm) C: i.v., – Acyl-L-ASNase, 0.5% Tween-80	6	0 (0)	0 (0)
S: i.m., – Acyl-L-ASNase, 0.5% Tween-80 C: i.v., – Acyl-L-ASNase, PC:Chol:PI (0.2 μm)	6	0 (0)	0 (0)
S: i.m., – Acyl-L-ASNase, 0.5% Tween-80 C: i.v., – Acyl-L-ASNase, PC:Chol:SA (0.2 μm)	6	0 (0)	0 (0)
S: i.m., – Acyl-L-ASNase, PC:Chol:SA (0.2 μm) C: i.v., – Acyl-L-ASNase, PC:Chol:SA (0.2 μm)	6	6 (100%)	2 (33%)
S: i.m., – Acyl-L-ASNase, PC:Chol:SA (0.2 μm) C: i.v., – Acyl-L-ASNase, 0.5% Tween-80	6	6 (100%)	3 (50%)

S, Sensitizing agent; C, challenging agent

Toxicity studies

The toxicity studies (Table 4) showed that palmitoyl-L-ASNase either in 0.5% Tween-80 or in NaCl suspensions is as toxic as L-ASNase, with four of six animals developing anaphylactic shock and one animal dying as a result.

The liposomal formulation composed of PC:Chol:PI (VET_{200}) showed elimination of acute toxicity. When it was used as the sensitizing as well as challenging agent, no case of anaphylactic shock or death was observed in any of the six animals. When liposomal palmitoyl-L-ASNase was used only as the sensitizing or challenging agent, animals challenged or sensitized with palmitoyl-L-ASNase in 0.5% Tween-80 showed no anaphylactic shock or death.

By contrast, the liposomal formulation (VET_{200}) composed of PC:Chol:SA showed enhanced toxicity both when used as the sensitizing and the challenging agent (all six animals presenting anaphylactic shock, with two dying as a result) and when used as the sensitizing agent, with palmitoyl-L-ASNase in 0.5% Tween-80 serving as the challenging agent (all six animals presenting anaphylactic shock and three dying as a result).

Table 5. Antitumor activity of liposomal formulations incorporating palmitoyl-L-ASNase and of palmitoyl-L-ASNase solubilized in 0.5% Tween-80

Experiment	Animals cured	%T/C
Palmitoyl-L-ASNase:		
400 U/kg	5 of 10	>700
800 U/kg	5 of 7	>700
2000 U/kg	9 of 10	>700
4000 U/kg	5 of 8	>700
Liposomal palmitoyl-L-ASNase:		
PC:Chol:PI (0.8 μm):		
400 U/kg	4 of 8	>700
800 U/kg	6 of 10	>700
2000 U/kg	7 of 9	>700
4000 U/kg	6 of 9	>700
PC:Chol:PI (0.2 μm):		
400 U/kg	0 of 8	338
800 U/kg	2 of 8	273
2000 U/kg	7 of 9	>700
4000 U/kg	6 of 8	>700
PC:Chol:SA (0.2 μm)		
400 U/kg	4 of 8	>700
800 U/kg	5 of 9	>700
2000 U/kg	7 of 9	>700
4000 U/kg	5 of 8	>700

No anaphylactic shock or death was noted in animals sensitized with palmitoyl-L-ASNase in 0.5% Tween-80 that had received challenge injections of either of the two liposomal formulations.

In vivo antitumor activity studies of free and liposomal palmitoyl-L-ASNase

Table 5 shows the results of studies of the antitumor activity of palmitoyl-L-ASNase in 0.5% Tween-80 and of the two liposomal formulations against P1534 lymphoma. For palmitoyl-L-ASNase in 0.5% Tween-80, all doses yielded a good rate of relative survival (>700%, T/C). Similar results were obtained for the majority of the liposomal formulations, the exception being the formulation composed of PC:Chol:PI (VET_{200}), for which only doses exceeding 2,000 U/kg resulted in good survival.

Discussion

To minimize the immunogenic effects observed with the clinical use of a foreign protein such as L-ASNase, several approaches have been tried. The packing of L-ASNase in microcapsules has been described [6, 26], but this approach has serious limitations related to the accumulation of undegradable material in the body [15]. The use of liposomes has also been tried by several authors [12, 27, 29], but with limited success as judged from the galenic point of view or by biological behavior. In fact, the reported EE values have been low, ranging from 12% to 50% [12, 27, 29]. The use of large liposomes [19] results in short circulating times, and size reduction by sonication [11] is not recommended because it concomitantly reduces the enzyme activity and the EE. Other authors [17] have covalently linked the enzyme to polyethylene glycol (PEG), which results in an increased half-life but does not avoid hypersensitivity reactions.

The approach described in this paper offers several advantages over the above-mentioned approaches. The hydrophobic characteristics of palmitoyl-L-ASNase [24] have enhanced the possibility of incorporating this enzyme within the lipid bilayer of liposomes. The method described herein allows an efficient incorporation of the enzyme (Tables 1, 2), probably due to a better interaction between the enzyme (added in lyophilized form) and the lipid.

Evidence that palmitoyl-L-ASNase can be partially incorporated within the lipid matrix is provided by the enzymatic activity of intact liposomes. The activity of palmitoyl-L-ASNase exposed to the outer medium is quantified by the ratio of intact to total activity and is dependent on the type of vesicle used, being 30% for MLV and 51%–74% for the VET_{200} formulations. This increase is probably due to the increased outer surface area per milligram of lipid of smaller vesicles as compared with larger vesicles.

As the nonincorporated protein was previously removed by saccharose gradients, the external activity was due either to the partial exposure of the enzyme-active site to the external medium or to the adsorbed modified protein. This behavior is different from that of intact vesicles with water-soluble enzyme that do not display enzymatic activity (data not shown) under similar conditions.

Since it has been extensively reported that large multilamellar vesicles are rapidly cleared from the circulation and that L-ASNase exerts its biological activity by degrading a circulating substrate (L-asparagine), we chose small liposomes for therapeutic tests because of their longer circulating times [1, 14]. Due to technical difficulties and to the observed reduction in encapsulation with decreasing size, it was not possible to use liposomes smaller than 200 nm (Table 1), in part because of the hydrophobicity of the protein, which sticks to the extrusion filter (filtration of formulations through 100-nm-pore filters was very difficult, even using a 20-fold dilution).

The pharmacokinetics studies clearly indicate an increase in circulating time for VET preparations, independently of the lipidic composition used (MRT, 32 h), as compared with palmitoyl-L-ASNase (MRT, 4 h) and the nonmodified enzyme (MRT, 2.9 h; experimentally determined under the same conditions).

The toxicity studies suggest that the incorporation of palmitoyl-L-ASNase in liposomes alters the presentation of the enzyme to the immune system, depending on the lipid composition and surface charge. In fact, its incorporation in negatively charged vesicles such as PC:Chol:PI prevented the immune response to the modified enzyme; by contrast, the presentation of the enzyme in vesicles composed of PC:Chol:SA resulted in similar or increased immune reactions as compared with the free modified enzyme. It is also noteworthy that animals sensitized with palmitoyl-L-ASNase in 0.5% Tween-80 did not present anaphylactic shock or die when challenged with either of the enzymatic liposomal formulations. For incorporation of this hydrophobic enzyme, high amounts of lipids were needed (13 μmol/animal). Thus, the adjuvant effects of the lipids on

immunogenicity should be carefully considered in the future selection of lipid composition.

The antitumor studies showed that at doses equal to or higher than 2,000 U/kg, the liposomal formulations of palmitoyl-L-ASNase and the modified enzyme itself were equally active (%T/C, >700%; Table 5). Although smaller doses achieved some good survival rates, it can be seen from Table 5 that there were a significant number of dead animals.

All dead animals showed tumor invasion of the liver, pancreas, and spleen but no evidence of s.c. tumor. Histopathology studies of the liver, spleen, and pancreas of dead animals confirmed the massive invasion of these organs by tumor cells, with normal cells being almost totally absent in these organs. These findings indicate that even at low doses, all formulations were capable of suppressing the growth of the primary s.c. tumor. This treatment was not as successful against metastasis, probably due to insufficient amounts of the enzyme in the metastatized organs, in agreement with the increased circulating times of the studied formulations, and also because of the presence of metastatic disease in the animals at the time the treatment was initiated.

From all these observations, we can conclude that palmitoyl-L-ASNase entrapped in PC:Chol:PI liposomes may substitute for native enzyme in the treatment of acute lymphoblastic leukemia. By using liposomal formulations, an increase in the therapeutic index can be achieved; they decrease significantly the risk of anaphylactic shock and maintain the therapeutic activity as compared with the native enzyme.

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