Brazilian Journal of Medical and Biological Research (2000) 33: 841-846
ISSN 0100-879X Short Communication

Novel methods for the encapsulation of meglumine antimoniate into liposomes

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Abstract

The antimonial drug, meglumine antimoniate, was successfully encapsulated in dehydration-rehydration vesicles and in freeze-dried empty liposomes (FDELs). High encapsulation efficiencies (from 28 to 58%) and low weight ratios of lipids to encapsulated antimony (from 1:0.15 to 1:0.3) were achieved. These formulations, contrary to those obtained by conventional methods, can be stored as intermediate lyophilized forms and reconstituted just before use. The efficacy of FDEL-encapsulated meglumine antimoniate was evaluated in hamsters experimentally infected with *Leishmania chagasi*. A significant reduction of liver parasite burdens was observed in animals treated with this preparation, when compared to control animals treated with empty liposomes. In contrast, free meglumine antimoniate was found to be inefficient when administered at a comparable dose of antimony. This novel liposome-based meglumine antimoniate formulation appears to be promising as a pharmaceutical product for the treatment of visceral leishmaniasis.

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Research supported by CNPq (No. 521010/97), FAPEMIG (Nos. CEX1079/95, CBS1721/95, and CBS2418/96) and PRONEX (No. 3075).

Received November 25, 1999
Accepted April 10, 2000

Key words
- Liposomes
- Glucantime
- Meglumine antimoniate
- Leishmaniasis
- Encapsulation

In the seventies, a major advance occurred when it was found that liposome-encapsulated antimonial drugs were hundreds of times more effective than unencapsulated ones for the treatment of experimental visceral leishmaniasis in mice, hamsters and dogs (1,2). Similar results were obtained with other antileishmanial agents (3,4) and other vesicular systems made from nonionic surfactants, instead of phospholipids (5). This spectacular effect of liposome encapsulation was attributed to the sustained release properties of liposomes and to their natural tendency to be cleared from the circulation by the fixed macrophages of the liver, spleen and bone marrow, which are the major sites of parasite infection. It was therefore expected that liposome formulations would improve the use of antimonials, enabling a reduction in drug dose and therapy duration. However, much effort still has to be devoted to turn the experimental liposome preparations into pharmaceutical products (6).

Two different methods have been proposed so far for the encapsulation of antimonial drugs in liposomes. One method consists of the hydration of a thin film of lipids with a solution of the drug (7). The other method, known as reverse-phase evaporation procedure, involves the formation of a

water-in-oil emulsion using the drug solution as aqueous phase followed by evaporation of the organic solvent, which results in a phase change and the formation of a vesicle suspension (8). The main advantage of the latter method, compared to the former, is that it yields higher efficiencies of drug encapsulation and higher ratios of encapsulated drug to lipid. These characteristics are important since the efficacy of liposome-encapsulated antimonials was previously shown to depend on the quantity of drug entrapped (9). These characteristics also mean that a lower quantity of lipid has to be injected in order to introduce the same quantity of antimonial, which makes the treatment safer and more economical. Nevertheless, liposomes prepared by the reverse-phase evaporation procedure may be toxic at high doses due to unavoidable residual traces of organic solvent in the final liposome formulation. Furthermore, the resulting liposome preparations can be stored only as aqueous suspensions. In this condition, however, a significant leakage of the drug occurred with time from the encapsulated aqueous phase into the continuous aqueous phase. For instance, a typical liposomal formulation prepared by the reverse-phase evaporation procedure released more than 26-48% of the originally encapsulated drug when stored for 7 weeks at 25°C (8). Such instability is actually unacceptable from a pharmaceutical point of view (6). Attention should also be paid to the chemical stability of the antimonial compound in such conditions. Indeed, in the specific case of meglumine antimoniate, a recent study (10) has suggested that this drug is a complex mixture of various antimony complexes, which change as a function of time and dilution.

In the present study, we have investigated some novel methods for the encapsulation of meglumine antimoniate into liposomes. In addition to the conventional thin film hydration method (7), two alternative methods were evaluated: i) a method that produces dehydration-rehydration vesicles (DRVs) (11), which involves mixing the drug solution with a suspension of pre-formed liposomes in water, freeze-drying the resulting mixture and rehydrating it in a controlled manner, and ii) a method that produces freeze-dried empty liposomes (FDELs) (12), which involves a controlled hydration, with the drug solution, of a lyophilized powder of empty liposomes. We report that these alternative methods display several advantages over conventional ones. They allow for the encapsulation of meglumine antimoniate with high encapsulation efficiency and they avoid stability problems during storage. The most promising formulation has been tested and found to be highly effective against experimental visceral leishmaniasis in hamsters.

Meglumine antimoniate (Glucantime) was obtained from Rhodia Brasil Ltda. (São Paulo, SP, Brasil), as an aqueous solution sealed in an ampoule. The antimony concentration was assessed at 75 ± 7 g/l by atomic absorption spectroscopy using a Hitachi Z8200 spectrophotometer.

The encapsulation of meglumine antimoniate into multilamellar vesicles (MLVs) was performed according to the thin film hydration procedure, as previously described (7), with the following modifications. A chloroform solution of a mixture of 72 mg L-α-distearoylphosphatidylcholine (DSPC; Sigma Chemical Co., St. Louis, MO, USA), 28 mg cholesterol (CHOL, Sigma) and 10 mg dicetylphosphate (DCP, Sigma) was added to a round bottomed flask in the presence of 3-mm glass beads. A DSPC/CHOL/DCP molar ratio of 5:4:1 was therefore chosen. Using a rotary evaporator, the organic solvent was removed at 50°C for 1 h. MLVs were produced by hydration at 55°C of the thin lipid film with the solution of meglumine antimoniate (0.8 ml) and rotation of the flask for 15 min. The resulting suspension was diluted with 2.5 ml phosphate-buffered saline (PBS; 0.15 M NaCl and 10 mM phosphate, pH 7.2) and then centrifuged (10,000

g, 30 min, 4°C) to separate unencapsulated drug from liposomes. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

The encapsulation of meglumine antimoniate into DRVs was performed as previously reported (11). Briefly, MLVs were prepared from DSPC/CHOL/DCP (molar ratio of 5:4:1), as described above, using distilled water instead of meglumine antimoniate as the hydration solution, at a final lipid concentration of 55 g/l. The suspension of MLVs was subsequently submitted to ultrasound at 55°C using a 3-mm probe-sonicator (ultrasonic liquid processor, Misonix Inc., Farmingdale, NY, USA). Remaining MLVs and contaminating metal from the probe were eliminated by centrifugation at 10,000 *g* for 10 min. The resulting suspension of small unilamellar vesicles (SUVs) was then mixed with the solution of meglumine antimoniate at the final lipid to Sb weight ratio of 1:0.58. The mixture was immediately frozen and then dried overnight. Rehydration of the dried powder was performed at 55°C as follows: 20% of the original SUV volume of distilled water was added, and the mixture was vortexed and incubated for 30 min at 55°C; 20% of the volume of PBS was similarly added, and the mixture was vortexed prior to the addition of 160% of the original SUV volume of PBS and incubated for 30 min at 55°C. Drug-containing DRVs were separated from the free drug by centrifugation at 10,000 *g* for 30 min at 4°C. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

The encapsulation of meglumine antimoniate into FDELs was performed as previously described (12) with the following modifications. SUVs were prepared from DSPC/CHOL/DCP (molar ratio of 5:4:1) as described above at a final lipid concentration of 55 g/l. This suspension was frozen and subsequently dried overnight. Rehydration of the dried powder was performed with meglumine antimoniate solution as follows: 40% of the original SUV volume of meglumine antimoniate solution was added (corresponding to a lipid/Sb weight ratio of 1:0.58), and the mixture was vortexed and incubated for 30 min at 55°C; 40% of the PBS volume was similarly added, and the mixture was vortexed prior to the addition of 120% of the original SUV volume of PBS and incubated for 30 min at 55°C. Drug-containing FDELs were separated from the free drug by centrifugation at 10,000 *g* for 30 min at 4°C. The liposome pellet was then washed twice with 8 ml PBS and finally resuspended in 2 ml PBS.

To determine the amount of antimonial drug encapsulated in liposomes, antimony was evaluated in the liposome pellets and in the supernatant of the first centrifugation. These samples were placed in a solution of nitric acid and heated to dryness. This step was then repeated until the complete digestion of organic material. The final dry product was re-dissolved in a 1:1 mixture of concentrated hydrogen chloride and water, and boiled for 1 h. Finally, antimony content was determined by atomic absorption spectroscopy. The encapsulation efficiency (%E) of meglumine antimoniate was calculated as follows: %E = 100 x quantity of encapsulated antimony/(quantity of encapsulated antimony + quantity of unencapsulated antimony).

Young 60-80 g Golden Syrian hamsters (*Mesocricetus auratus*) were used to maintain the parasite and were used throughout the experiments. The *Leishmania chagasi* strain MHU/BR/70/BH46 was used in this study. Parasites were obtained by homogenizing fragments of liver and spleen from a freshly killed hamster which had been infected for approximately 90 days.

In order to evaluate the antileishmanial activity of the liposomal preparation, 4 groups of 10 hamsters were infected intraperitoneally with 0.1 ml of the suspension of *L. chagasi* amastigotes (approximately 10^8 para-

sites). Seventeen days after infection, animals were treated intraperitoneally with i) meglumine antimoniate entrapped in DSPC/CHOL/DCP FDELs (Lglu) at 60 mg Sb/kg, ii) free meglumine antimoniate (Glu) at 100 mg Sb/kg, iii) empty liposomes (Lemp), and iv) PBS (untreated). After 55 days, animals were sacrificed, and impression smears of liver and spleen were taken. Smears were fixed with methanol, stained with 10% Giemsa (Gibco) and the number of amastigotes per 1000 host cell nuclei was counted. For each animal, about 500 host cell nuclei in the liver as well as in the spleen were evaluated for the presence of amastigotes.

Entrapment data (Table 1) indicated that the 'DRV method' is the most efficient method for the encapsulation of meglumine antimoniate into liposomes. The 'FDEL method' gave a lower encapsulation efficiency than the DRV one, but a higher level (2.5-fold) than the conventional thin film hydration method. These results were obtained using a lipid quantity of 14 mg and an initial lipid to Sb weight ratio of 1:0.58. When the lipid quantity was increased in the FDEL preparation while maintaining constant the drug to lipid ratio, an increase in encapsulation efficiency was observed. For instance, when 140 mg of lipid was used, the encapsulation efficiency of meglumine antimoniate into FDELs reached 50 ± 10%.

Table 1 - Glucantime encapsulation into multilamellar vesicles (MLVs), dehydration-rehydration vesicles (DRVs) and freeze-dried empty liposomes (FDELs).

[a]N = 3. [b]14 mg of lipid used.

Liposome preparation	Percentage of entrapped Glucantime (mean ± SD)[a]	Weight ratio of lipid to encapsulated Sb
MLVs	12.5 ± 1	1:0.074
DRVs[b]	42 ± 2	1:0.25
FDELs[b]	31 ± 3	1:0.18

Table 2 shows that treatment of *Leishmania chagasi*-infected hamsters with Lglu reduced parasite burdens significantly in the liver, when compared to treatment with Lemp. On the other hand, no significant difference in parasite burden was observed between the groups treated with either empty liposomes or free meglumine antimoniate (100 mg Sb/kg) and the control group. More strikingly, all animals treated with Lglu showed less than 70 amastigotes per 1000 host cell nuclei either in the liver or in the spleen, and some animals (4 out of 9) seemed to be free of parasites (Table 2). This observation is in contrast with the results obtained with the other groups in which all animals showed parasites, and at a much higher level.

The aim of the present study was to look

Table 2 - The effect of liposome-encapsulated meglumine antimoniate (Lglu, 60 mg Sb/kg), free meglumine antimoniate (Glu, 100 mg Sb/kg) and empty liposomes (Lemp) on *L. chagasi* parasite burdens of liver and spleen in hamsters.

*P<0.05 compared to the Lemp group (one-way ANOVA and Tukey's test).

Treatment	Mean number of amatigotes/1000 host cell nuclei ± SD		Number of animals with amatigotes/1000 host cell nuclei <70 (total number)		Number of animals with no parasite detected (total number)
	Liver	Spleen	Liver	Spleen	
Lglu	9 ± 15*	13 ± 25	10 (10)	9 (9)	4 (9)
Glu	1800 ± 2000	3200 ± 4800	1 (11)	0 (10)	0 (11)
Lemp	2500 ± 2600	6700 ± 9700	0 (8)	0 (7)	0 (8)
None	1600 ± 1900	7600 ± 8100	1 (8)	0 (7)	0 (8)

for alternative methods for the encapsulation of meglumine antimoniate into liposomes, that do not suffer from the limitations of conventional ones. From the point of view of encapsulation efficiency, the methods that produce DRVs and FDELs appeared to be as efficient as the reverse-phase evaporation method that was claimed to produce encapsulation efficiency in the range of 38 to 57% (8). Moreover, comparable ratios of lipid to Sb were achieved.

In order to avoid stability problems during storage, such as those encountered with aqueous liposome suspensions, the DRV and FDEL preparations may be stored as intermediate lyophilized products: DRVs as a liposome-drug mixture and FDELs as preformed empty liposomes. The final rehydration step may be performed just before administration, using i) water and saline in the case of DRVs and ii) meglumine antimoniate solution in the case of FDELs. A significant advantage of the 'FDEL method' is that it does not expose the drug to lyophilization, thereby reducing the risk of chemical alteration of the drug. Moreover, the rehydration step was found to be much easier in the case of FDELs (data not shown). Finally, the FDEL preparation did show the expected high antileishmanial activity in hamsters infected with *Leishmania chagasi*.

Conventional therapy with meglumine antimoniate involves a multiple dosing regimen of a minimum of 28 daily intramuscular or intravenous injections of 20 mg Sb/kg (13). According to pre-clinical studies in experimental animals, liposome-encapsulated meglumine antimoniate is at least 200-fold as active as the free drug (1). These data suggest that a treatment consisting of four doses of 2 mg Sb kg^{-1} $week^{-1}$ with meglumine antimoniate encapsulated into liposomes might be satisfactory, since each dose is 70-fold lower than the cumulative dose of antimony administered in one week by conventional treatment. It is noteworthy that if the preparation is used without eliminating the 50% fraction of unencapsulated drug, then the quantity of unencapsulated drug administered would represent 2 mg Sb kg^{-1} $week^{-1}$, which is insignificant when compared to the dose administered in the conventional treatment. This observation means that the process of preparation of DRVs and FDELs might be simplified by the omission of the last centrifugation/separation step.

Another important point that needs to be addressed is the cost of treatment with the liposomal drug when compared to conventional therapy. In a treatment using the FDEL preparation, much lower doses of antimony would be used and the cost would be determined mainly by lipids. Assuming that the cost of lipid is US$50 per g and that of meglumine antimoniate is US$2 per 5 ml ampoule, a four-week treatment with 2 mg Sb kg^{-1} $week^{-1}$ of liposomal meglumine antimoniate would cost US$120, whereas conventional treatment (20 mg Sb kg^{-1} day^{-1}) costs about US$200.

In conclusion, we observed that FDEL-entrapped meglumine antimoniate is a suitable formulation for the treatment of visceral leishmaniasis: the preparation exhibited high antileishmanial activity, did not suffer from stability problems and was cost-effective. It is expected that treatment with this preparation would allow for a lower number of injections and a lower dose of antimony when compared to conventional treatment. Therefore, the treatment, in addition to its higher efficiency, should be safer and more comfortable.

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