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Current Drug Delivery, 2015, *12*, 000-000

Biochemical Characterization of GM1 Micelles-Amphotericin B Interaction

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Abstract: In this work a thorough characterization of the GM1 micelle-Amphotericin B (AmB) interaction was performed. The micelle formation as well as the drug loading occurs spontaneously, although influenced by the physicochemical conditions, pH and temperature. The chromatographic profile of GM1-AmB complexes at different molar ratios shows the existence of two populations. The differential absorbance of GM1, monomeric and aggregate AmB, allowed us to discriminate the presence of all of them in both fractions. Thus, we noted that at higher proportion of AmB in the complex, increases the larger population which is composed mainly of aggregated AmB. The physical behavior of these micelles shows that both GM1-AmB complexes were stable in solution for at least 30 days. However upon freeze-thawing or lyophilization-solubilization cycles, only the smallest population, enriched in monomeric AmB, showed a complete solubilization. *In vitro*, GM1-AmB micelles were significantly less toxic on cultured cells than other commercial micellar formulations as Fungizone, but had a similar behavior to liposomal formulations as Ambisome. Regarding the antifungal activity of the new formulation, it was very similar to that of other formulations. The characterization of these GM1-AmB complexes is discussed as a potential new formulation able to improve the antifungal therapeutic efficiency of AmB.

Keywords: Aggregation-states, amphotericin, drug-delivery, gangliosides, micelles, physicochemical characterization.

INTRODUCTION

Amphotericin B (Fig. **1a**) is the main antifungal agent used in the treatment of systemic fungal infections, despite having many problems with respect to toxicity and formulation. Furthermore, in recent years there has been a significant increase in fungal infections mainly related to the high frequency of organ transplantation, epidemics related to the acquired immunodeficiency syndrome (AIDS) and the high immunosuppression present in patients with cancer [1, 2].

Although experimental evidence is still lacking on how AmB exerts its action, the most widely accepted model involves the formation of AmB-sterol complexes in the cell membranes, which subsequently associate into a transmembrane barrel with a large -OH lined aqueous pore down the middle [3]. The complexes formed between AmB and ergosterol, the primary sterol of fungal cell membranes, are much stronger than those formed between the drug and cholesterol, principal sterol present in the cellular membranes of mammals, even though the structural differences between the two sterols are very subtle [4]. Recent reports show that the sterol side chain is essential for AmB selectivity toward ergosterol and for the activity of AmB-sterol ion channels. In addition, these authors emphasize the importance of the C7-double bond of the sterol B-ring for interaction with the drug [5, 6].

Some of the most important problems encountered in the clinical use of AmB are its serious side effects and the requirement of parenteral administration due to its poor absorption from the gastrointestinal tract. Besides, its structure comprising a hydrophobic heptane chain and a hydroxyl rich hydrophilic chain imparts an amphoteric nature to the molecule which results in a negligible solubility in aqueous and hydrophobic systems.

In recent years different formulations of amphotericin have been developed with the aim of reducing the complications associated with antifungal therapy, including renal toxicity and infusion-related adverse effects. The conventional formulation of amphotericin B is a freeze-dried powder, which on reconstitution yields a micellar solution of amphotericin B with the bile salt sodium deoxycholate (Fungizone Bristol Myers-Squibb, Princeton, NJ). However, the use of Fungizone is associated with side effects such as fever and chills, bile rigors, nausea, hematological intolerance and considerable nephrotoxicity [7]. The latter two drawbacks mentioned above have been overcome to a significant extent by lipid based formulations such as Ambisome (Liposomal Amphotericin B), Abelcet (dispersion of ribbon like amphotericin B - lipid complex) and Amphocil (colloidal dispersion of amphotericin B - cholesteryl sulphate complex) [8, 9]. The decrease in toxicity has been attributed to factors such as a

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1567-2018/15 $58.00+.00

different interaction with cell membranes, larger particle sizes, different pharmacokinetics, ormodified drug release. These formulations are based on expensive technologies such as the preparation of liposomes that increase significantly the cost of therapy [10]. Furthermore, it has been established in many cases that the reduction in AmB toxicity was accompanied by a considerable reduction in AmB activity [11, 12].

In recent years, researchers have been active in the development of new drug delivery systems to improve the properties of the therapeutic compounds, reduce undesirable side effects and increase efficacy. In this connection, micelles have attracted attention as potential drug carriers. Micelles are colloidal associations having regions with a strong anisotropy and a water soluble decreasing gradient that goes from the outside to the inside of them. These properties give micelles the capacity to solubilize a wide range of solutes. Another advantage is their size, large enough to avoid renal exclusion but small enough to evade capture by the reticuloendotelial system.

In previous studies, we demonstrated that lipid micelles of the monosialoglycosphinglolipid GM1 (Fig. **1b**) are able to spontaneously load high amounts of hydrophobic drugs as paclitaxel (Ptx) [13]. In this work, we characterized GM1-AmB interactions under different experimental conditions. We also evaluated the biological effect and *in vitro* toxicity of the drug in this micellar formulation compared to other available commercial formulations of AmB.

MATERIALS AND METHODS

Materials

Amphotericin B (AmB) with a purity of 95 % was a gift from NANOX S.A. Pig monosialoganglioside GM1 as a sodium salt with a purity of 94 % was a gift from TRB Pharma S.A. All other chemicals and solvents used were of analytical grade.

Methods

Preparation of GM1-AmB Micelles under Different Physicochemical Conditions

Stock solutions of GM1 with a concentration of 200 $mg.mL^{-1}$, were prepared in bi-distilled water as described in Leonhard *et al.* [13]. Stock solutions of AmB were prepared by dissolving the drug in dimethylsulfoxide (DMSO) at a final concentration of 30 $mg.mL^{-1}$. Stock solutions of AmB were prepared in darkness using nitrogen to protect it from oxidation.

Loading of AmB into GM1 micelles: The solution of AmB was slowly added to the solution of GM1 micellesin aqueous solution at pH 6 and 4 ºC in order to attain different GM1-AmB molar ratios, i.e.: 10/1; 5/1; 2.5/1; 1/1. After stirring at 600 rpm for 10 min, these mixtures were incubated at 4 ºC for 24 h in the dark. Unbound AmB was removed by centrifugation at 15,000 *x g* for 10 min and then samples were dialyzed for 24 h in the dark at 4 ºC using SpectraPor dialysis

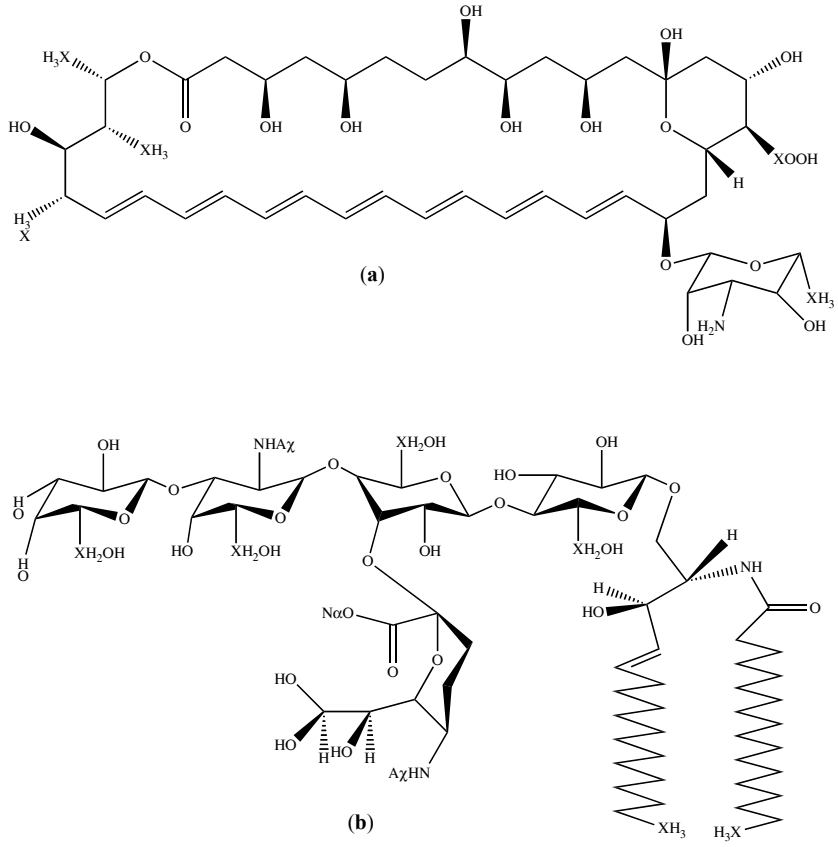

Fig. (1). Chemical structures of: a. AmB. b. GM1.

membranes with a MW cut-off of 10,000 Da to remove all DMSO.

Effect of temperature on the loading of AmB: The solutions of GM1 were incubated for 2 hours at 4, 25, 37 and 55 °C. Next, these solutions were mixed with AmB solution to reach GM1-AmB: 10/1; 5/1; 2.5/1; 1/1 molar ratios. Drug loading was performed at the same temperature for 2 hours before incubating the samples at 4 °C for 24 h. After incubation all samples were centrifuged at 15,000 *x g* for 10 min and dialyzed for 24 h at 4 °C to remove the DMSO.

Effect of pH on the loading of AmB: The solutions of GM1 were incubated for 1 h at pH 2, 5, 7 and 10, using 50 mM citrate-HCl and acetate buffers for pH 2 and 5, and phosphate and carbonate buffers for pH 7 and 10. Next, these solutions were mixed with AmB solution to reach GM1-AmB: 10/1; 5/1; 2.5/1; 1/1 molar ratios and incubated at 4 °C for 24 h. Finally, samples were centrifuged at 15,000 *x g* for 10 min and dialyzed against bi-distilled water for 24 h at 4 °C to remove buffers and DMSO.

Determination of GM1 Concentration

Ganglioside concentrations were measured by the modified colorimetric resorcinol assay described by Miettinen *et al.* [14].

Determination of AmB Concentration

AmB concentrations were controlled spectrophotometrically from its absorption at 405 nm after complete monomerization by dilution in methanol as described by Gaboriau *et al.* [15]. Spectroscopic determinations were obtained using a Perkin Elmer lambda 25 spectrophotometer.

The sample concentration was then obtained from a calibration curve from 1 to 10 $\mu g.mL^{-1}$ of AmB in 50 % v/v methanol.

Physicochemical Characterization of GM1-AmB Micelles

Chromatographic Analysis

Samples and controls were run on an Åkta Explorer 100 system (GE Healthcare) as described in Leonhard *et al.* [13]. An UV-detector at 227, 333 and 405 nm was used to follow the elution pattern of GM1 and AmB respectively. GM1 and AmB levels were quantified as described above.

Determination of Particle Size

Average particle size of the micelles was measured by dynamic light scattering (DLS), which was performed on a Delsa™ Nano Submicron Particle Size and Zeta Potential Particle Analyzer at a fixed scattering angle of 165º. Data were analyzed by DelsaNano Beckman Coulter software (version 2.2) with CONTIN analysis method.

Resistance to High Centrifugation Forces

GM1-AmB micelles were centrifuged at 100,000 *x g* for 1 h at 20 ºC in a XL-90 ultracentrifuge (Beckman Coulter Inc., USA). Immediately afterwards, the concentrations of soluble GM1 and AmB in the supernatants were determined as described above.

Stability in Solution

Solutions of GM1-AmB micelles were stored for 30 days at 4 ºC in the dark. Aliquots of samples were taken at various time periods and the concentration of soluble GM1 and AmB were determined as described above.

Effect of Freeze-thawing Cycles and Lyophilization

GM1-AmB micelles were frozen at -80 ºC and after 24 h samples were kept at room temperature for about 2 hours until complete thawing, centrifuged at 15,000 *x g* for 10 min and the concentration of soluble GM1 and AmB were determined as described above.

Moreover, lyophilized micelles were dissolved in their initial volume and filtered through a 0.22 µm pore and the concentration of soluble GM1 and AmB were determined as described above.

In vitro Studies

Hemolysis

Human whole blood from healthy volunteers was collected in tubes with EDTA and washed two times in 9 volumes of sterile 0.9 % NaCl saline solution. After each washing, cells were pelleted by centrifugation at 1,500 *x g* for 10 min and the supernatant was discarded. The final pellet was adjusted to 4 x 10^7 cells per ml. Hemolytic activity of AmB was studied by adding 1 vol of red blood cell suspension to 1 vol of GM1-AmB solution (with 10/1 to 1/1 molar ratios) containing AmB at 0.1 and 1.5 $mg.mL^{-1}$. Samples were incubated for 1 h at 37 ºC stirred at 500 rpm and then centrifuged at 1,500 *x g* during 10 min. The supernatants were collected for the analysis of the percentage of hemolysis by reading the absorption of free hemoglobin at 540 nm.

Cytotoxic Effect on Cell Cultures

Vero cells (African green monkey epithelial kidney) were maintained at 37°C with 5 % CO_2 in minimal essential medium (MEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS) (NATOCOR, Córdoba, Argentina), 2 mM L-glutamine and 10 $\mu g.mL^{-1}$ gentamicin (all culture reagents were from Invitrogen).

For the experiments, cells were seeded into wells of a 96-well plate and after 24 h of incubation 100 µL of serial dilutions from 0.5 to 200 $\mu g.mL^{-1}$ of GM1-AmB micelles (with 10/1 to 1/1 molar ratios), AmB in deoxycholate micelles (Fungizone) and liposomal-AmB formulation (AmBisome) were added to the wells and cultured for 24 h at 37 ºC. The cytotoxicity test was determined by MTT assay. In brief, 50 µL of MTT was added to the wells, the cells were cultured for additional 4 h at 37ºC. Then the medium was discarded and 100 µL dimethyl sulfoxide (DMSO) was gently added to the wells. The solubilized formazan was measured at 595 nm using microplate spectrophotometer (Anthos reader 2010). Each treatment was assayed in triplicate.

Effect of AmB on Candida Albicans

Minimum fungicidal concentrations (MFCs) of AmB were determined for *C. albicans* by the M27-A method [16].

Briefly, 100 µL of yeast inoculum at a concentration of 0.5 × 103 to 2.5 × 103 CFU.mL^{-1} were added to plates contained 100 µL of serial dilutions of the antifungal drug (as GM1-AmB, Fungizone and AmBisome formulations) per well and four drug-free medium wells for sterility and growth controls. After 24 h of incubation, the optical density of each microplate well was measured with a microplate spectrophotometer set at a wavelength of 620 nm (Anthos reader 2010). The MFC endpoint was defined as the lowest drug concentration resulting in a reduction of growth of 90% or more compared with growth of the control.

RESULTS

Solubilization of AmB in GM1 Micelles

We evaluated the ability of GM1 micelles to load AmB. For this purpose, solutions of GM1 from 8.4 to 84 mg.mL^{-1} were incubated with 5 mg.mL^{-1} of AmB to attain GM1-AmB molar ratios of 10/1; 5/1; 2.5/1 and 1/1.

As shown in (Fig. **2**), the maximum loading capability of GM1 micelles for AmB seems to reach a plateau at a molar ratio of about 2.5/1, in which it is possible to solubilize about 4 mg.mL^{-1} of the drug.

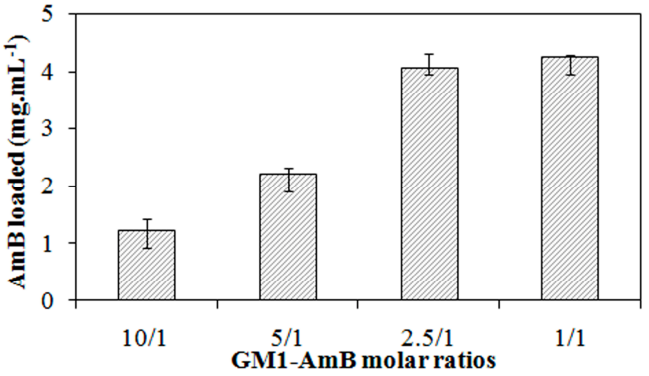

Fig. (2). Loading of AmB into GM1 micelles. The incorporation was done at 4 ºC for 24 h. Error bars indicate the SD of the mean (n = 3).

Effect of Temperature and pH on the Capacity of GM1 to Load AmB

Considering the singular conformational behavior of GM1 relative to temperature, having a bi-stable behavior between two states characterized by different aggregative properties and with a different degree of hydration [17-21], we evaluated the effect of temperature on the incorporation of AmB into GM1 micelles. A solution of GM1 was incubated for 2 hours at 4, 25, 37 and 55 °C before adding the AmB solution and then each tube was incubated for another 2 hours at the respective temperature.

As seen in (Fig. **3**), the optimum temperature for the incorporation of AmB was 4 °C and not 55 °C as might be expected to be due to the hydrophobic nature of AmB that could suffer more rejection in aqueous solutions at higher temperature.

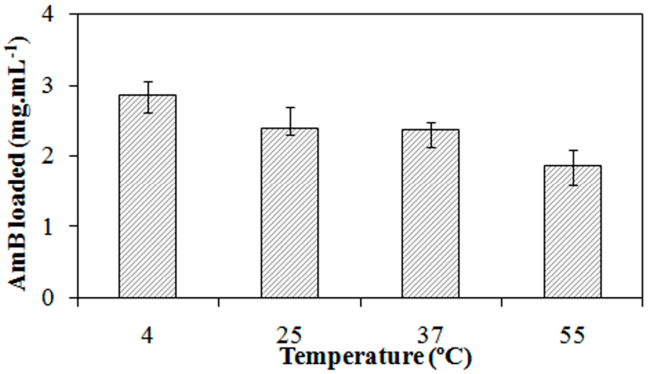

Fig. (3). Effect of temperature on loading of AmB into GM1 micelles. Error bars indicate the SD of the mean (n = 3).

Another factor that could modify the incorporation of AmB into GM1 micelles is the pH. Fig. (**4**) shows that the increase of the pH from pH 2 to pH 10, produces a sequential increase in the incorporation of AmB into GM1 micelles. This may be due to the generation of more soluble forms and with smaller degree of aggregation of the drug at alkaline pH, favoring their incorporation into the micelle.

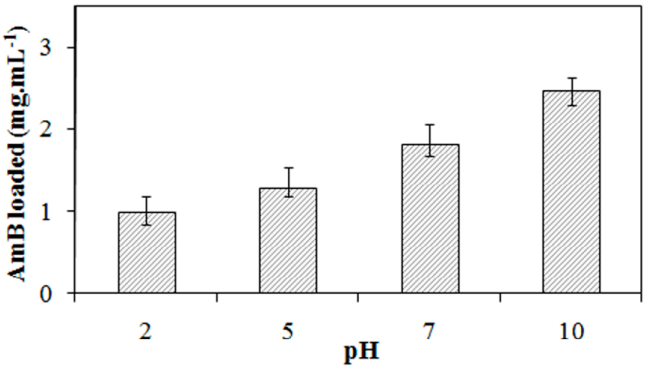

Fig. (4). Effect of pH on loading of AmB into GM1 micelles. Error bars indicate the SD of the mean (n = 3).

Spectroscopic Characterization of GM1-AmB Micelles

Various degrees of aggregation of AmB with a critical aggregation concentration of 1 µg.mL^{-1} have previously been described [22]. Since each form shows different absorption patterns when changing the polarity of the medium, spectrophotometric studies are a useful to determining the state of aggregation of the drug under different conditions. Thus, a UV spectrum of AmB in an organic solvent such as methanol, wherein the drug exists in monomeric form, shows the presence of four absorption bands with decreasing intensities at 405, 385, 365 and 344 nm (Fig. **5a**). As the water proportion increases, the self-associated structure is formed; the band at 344 nm undergoes a blue shift to about 333 nm and increases in intensity while the other three bands undergo red shifts to about 368, 390 and 420 nm respectively together with a reduction in intensity, indicating the presence of the self-aggregated form [23].

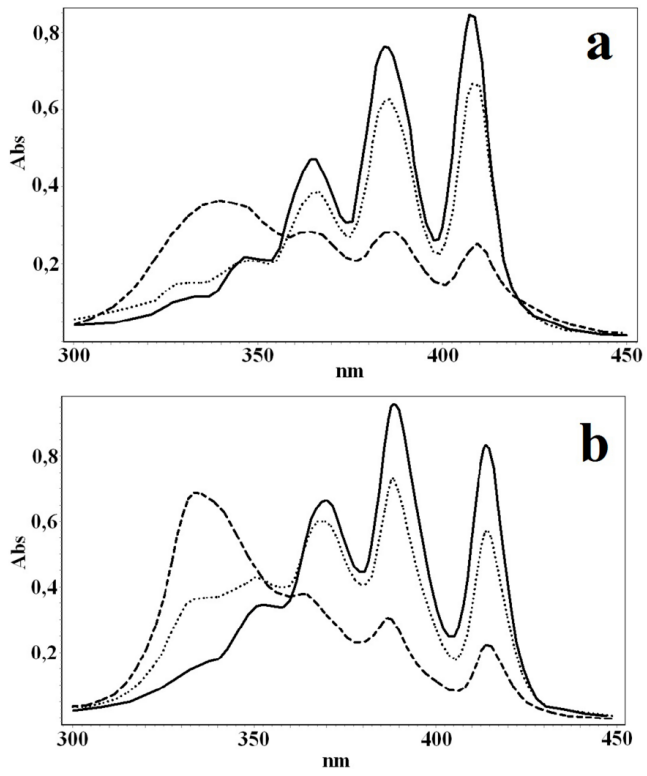

Fig. (5). Absorption spectrum of AmB in: a. Methanol/water mixtures with 4 % (▬ ▬ ▬), 40 % (••••••••••) and 100 % (▬▬▬▬) Methanol. b. GM1-AmB micelles of 10/1 (▬ ▪ ▬), 5/1 (▬▬▬▬), 2.5/1 (••••••••••) and 1/1 (▬ ▬ ▬) molar ratios.

Fig. (**5b**) shows the UV spectrum of GM1-AmB micelles at 1/1; 2.5/1; 5/1 and 10/1 molar ratios. It is observed that the absorption spectrum of AmB changes with the amount of AmB loaded into the micelle. It showed that from 10/1 to 5/1, AmB appeared in a monomeric state, while at2.5/1 the proportion of aggregated AmB increased which was even more accentuated at 1/1 molar ratio.

Structural Characterization of GM1-AmB Micelles

In order to gain further insight into the structure and thermodynamic equilibrium of the GM1-AmB nanostructures, we evaluated the elution profile of GM1-AmB complexes at different molar ratios using a size exclusion chromatography column and their average size by DLS.

The chromatographic patterns of GM1 at 227 nm show two elution peaks, the first one at about 11 mL, equivalent to a globular protein of 365 kDa, and the other at 22 mL corresponding to the monomers that are in equilibrium with the micelles (Fig. **6**).

For AmB detection, we chose 405 and 333 nm as representative wave-lengths of the monomeric and aggregate form of the drug respectively.

Fig. (**6**) shows the chromatographic profile of GM1-AmB at different molar ratios (a. 5/1; b. 2.5/1; c. 1/1). It is observed that GM1-AmB micelles eluted in two main positions, one with the V_0, which corresponds to structures having a molecular weight equal or higher than 600 kDa (9 mL), and the other at around 13 mL, which corresponds to the hydrodynamic radius of a globular protein of 180 kDa. However, the proportion of each population, as well as the components that make it up, changed substantially in each of the molar ratios studied. As the amount of AmB increases in the GM1-AmB complex, a displacement of the chromatographic elution profile to the population that elute with V_0 is observed, which is mainly composed of aggregates of AmB.

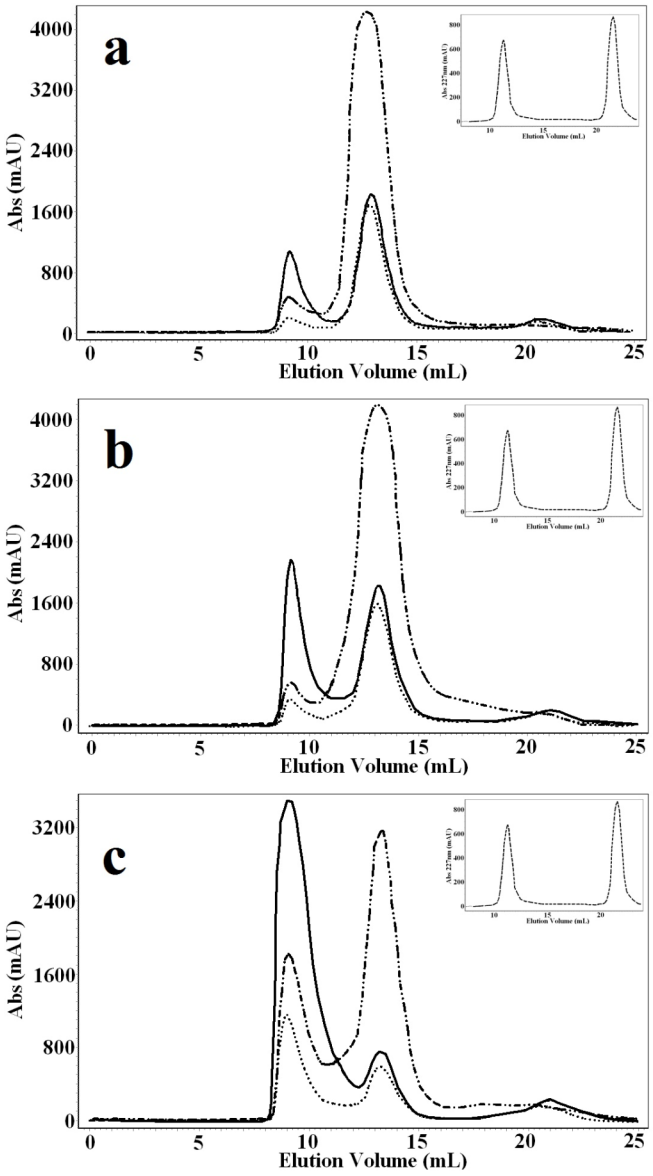

Fig. (6). Size-exclusion chromatographic patterns of GM1 micelles with different amounts of AmB: a. 5/1; b. 2.5/1 and c. 1/1. Control GM1 (▬ ▬ ▬) in the right corner of each chromatography was measured at 227 nm. GM1-AmB micelles were measured at 227 (••••••••••), 333 (▬▬▬▬) and 405 (▬ ▪ ▬) nm as representatives wave lengths of the GM1 component and the aggregate and monomeric form of the AmB, respectively.

Is worth mentioning that the peak corresponding to GM1 monomers practically disappear with the interaction with

AmB, suggesting a stabilization of the micellar state induced by the drug loaded. However, it can be observed a small amount of AmB aggregated eluting in this region, probably associated to GM1 monomers.

Moreover, the mean particle size, size distribution and polydispersity of GM1-AmB micellar complexes were measured by dynamic light scattering (DLS). Table **1** shows that the size of the complex differs according to the GM1-AmB molar ratio. At 10/1 and 5/1 molar ratios the average size of the micelles are 12.1 and 14.8 nm respectively, with a polydispersity index between 0.09 and 0.1. These results are not significantly different compared with that of the empty micelle of about 12.9 nm.

However, at 2.5/1 and 1/1, where the proportion of AmB in the complex is higher, a significant increase in the size of the structures is observed, reaching an average size of 132.9 and 165.5 nm respectively, with a polydispersity index that exceeds 0.3, indicating that there is great variability in size of the generated nanostructures. These results are in agreement with the chromatographic profiles described above and together, clearly defined that molar ratios below 2.5/1 are not considered viable due to the size of the aggregates as well as the great diversity that exist between them.

Table 1. Average size of micellar complexes by DLS.

GM1-AmB (molar ratio)	Mean Particle Size (nm)	Polydispersity index
10/1	12.1 ± 0.6	0.093
5/1	14.8 ± 0.5	0.100
2.5/1	132.9 ± 1.0	0.459
1/1	165.5 ± 0.9	0.311
1/0	12.9 ± 1.0	0.015

Physical Stability of GM1-AmB Micelles

We determined the stability of GM1-AmB complex in solution or after different treatment conditions like centrifugation, freeze-thawing, lyophilization and filtration.

All GM1-AmB formulations were stable for at least 1 month at 4 °C and more than 98 % of AmB remained in solution after centrifugation up to 100,000 *x g* for 1 hour.

On the other hand, at least 95 % of GM1-AmB complexes were re-solubilized after lyophilization except complexes formed with 1/1 molar ratio, where only 30 % could be reconstituted. In addition, after filtration through 0.22 µm, over 95 % of GM1-AmB complexes were recovered, except for the molar ratio 1/1 in which recovery did not exceed 89 %.

Instead, after freeze-thawing cycles, only complexes with a 5/1 molar ratio showed good resolubilization (> 95 %) while the rest of the complexes with different molar ratios showed resolubilization percentages between 82 and 88 % (data not shown).

Hemolytic Effect of GM1-AmB Micelles

The use of some AmB formulations (i.e. Fungizone®) is limited due to their hematological intolerance. For this reason, we evaluated the hemolytic activity of the GM1-AmB formulation and we observed no significant effect of the complex, with hemolysis lower than 2 % (data not shown).

Cytotoxicity and Biological Activity of GM1-AmB Micelles

We studied the in-vitro cytotoxicity of the GM1-AmB formulation compared with two commercially available formulations, AmB in deoxycholate micelles (Fungizone) and liposomal AmB (AmBisome). Fig. (**7**) shows that GM1-AmB micelle showed less toxicity than the AmB in deoxycholate and the liposomal formulation of AmB on Vero cells.

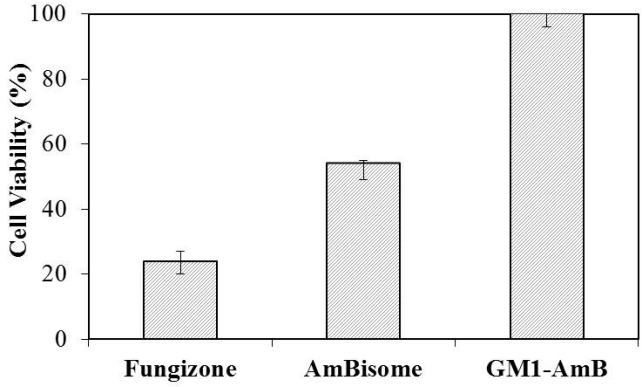

Fig. (7). *In-vitro* cytotoxic effects of GM1-AmB micelles (5/1 molar ratio), Fungizone and AmBisome on Vero cells at 37 °C and 24 h of incubation at AmB concentration of 200 µg.mL^{-1}. Error bars indicate the SD of the mean (n = 3).

Finally, we studied the biological activity of AmB in GM1 micelles against *Candida albicans* and we compared it with respect to the deoxycholate and liposomal formulations of the drug. As shown in (Fig. **8**), no significant differences in biological activity of the drug are noted in any of the formulations evaluated, with a MIC value of 0.5 µg.mL^{-1} in all cases.

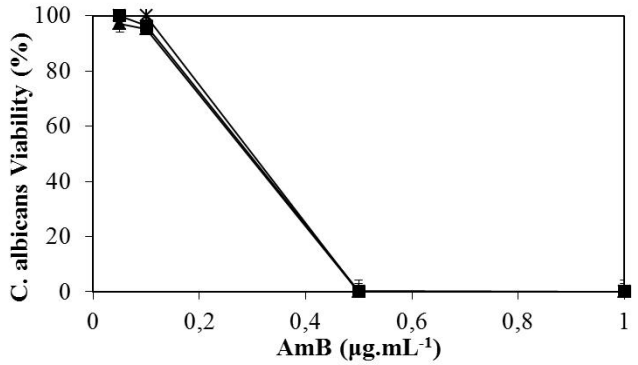

Fig. (8). *In-vitro* biological activity of AmB against *Candida albicans*. GM1-AmB: 5/1 molar ratio (—✳—), Fungizone (—■—) and AmBisome (—▲—). Error bars indicate the SD of the mean (n = 3).

DISCUSSION

The development of new and improved drug delivery systems for AmB remains a great challenge for several research groups. The aim is to develop less toxic formulations by changing AmB physicochemical behavior, and consequently, its biological properties *in vitro* and *in vivo*. One approach to decrease the toxicity of AmB is to develop new derivatives or formulations with lower aggregation.

In previous reports [13] we demonstrated that GM1 micelles are capable of spontaneously incorporate highly hydrophobic anticancer drugs like paclitaxel and docetaxel. The GM1-drug interaction was also widely characterized. Here, we characterized the interaction between GM1 micelles and AmB under different physicochemical conditions and the properties and behavior of these GM1-AmB micelles. Our results demonstrate that GM1-AmB complexes behavior is dependent on different factors, such as AmB concentration, GM1-AmB molar ratio, pH of medium and also the temperatures they have been exposed to.

A particular feature of this formulation is that the lipid-drug molar ratio plays a fundamental role in the final aggregation state of AmB. This effect was also reported in another lipidic formulations of AmB like liposomes closely related to the cholesterol content [24], and also in polymeric micelles dependent on the composition of the acyl derivative of (ethylene oxide)- block- poly (L-aspartate) micelles [25]. The self-aggregation capacity of AmB drastically affects its behavior. Moreover, it has been accepted that the aggregation state of AmB greatly affects its toxicity [26]. Therefore, it has been given great importance to the study of the effect produced by different self-aggregated AmB species as well as monomers against fungal and also mammalian cells [27].

In this sense, many of the results described in this paper appear to be closely linked to the different states of aggregation of the drug. Such is the case of the effect of temperature on the loading of AmB in micelles for example, where a favorable effect was observed at 4 °C and not at 55 °C as previously described with Ptx. This result could be explained by the fact that it has been observed that when the aqueous solution of AmB is heated, an increase in the aggregation state of AmB occurs (data not shown). Similar results were reported by Gaboriau *et al.* [15], who described super aggregate forms of AmB produced by high temperatures. These results suggest that under these aggregation conditions, the entry of AmB in the GM1 micelles is disfavored.

Also, a significant increase in the loading capacity of AmB is observed at pH 10, this effect does not respond to changes in the polar head of the gangliosides, which occurs near their pKa at pH between 2 and 5. Moreover, from standpoint of AmB, it might be expected that such mechanism be independent to the external pH being AmB a hydrophobic and non-ionic molecule. Again, in this case the result could be explained by changes in the aggregation state of AmB as a function of pH, since we have found that at more alkaline pH values, a shift occurs to the monomeric state which can facilitate AmB incorporation intoGM1 micelles (data not shown).

The characterization of the hydrodynamic radius and the size of GM1 micelles as a function of AmB incorporation showed drastic changes in the chromatographic pattern of the micelles. Initially, GM1 has two elution peaks, one with a hydrodynamic radius of 360 kDa, corresponding to the micellar aggregates, and one with a hydrodynamic radius of about 1.6 kDa, related to the elution of GM1 monomers in thermodynamic equilibrium with the micelles. After the incorporation of AmB, the 1.6 kDapeak disappears, demonstrating a shift of the monomer-micelle equilibrium to the aggregated form in presence of the drug. Moreover, there are two other peaks, one eluting with the V_0, with an apparent molecular weight greater than 600 kDa and a second peak of approximately 180 kDa (LMW) eluting at about 13 mL. In both peaks, three components can be observed: GM1 (227 nm), aggregated (333 nm) and monomeric AmB (405 nm). However, a gradual change in the relative amount of each component is observed as the proportion of AmB in the complex is increased. Up to molar ratios of 2.5/1, GM1 elutes mainly in the 180 kDa peak, together with a high proportion of monomeric AmB and also with a fraction of aggregated AmB. From this onward, a displacement of all components, and in particular of aggregated AmB to V_0 peak is observed, suggesting the formation of large aggregates of GM1, monomeric AmB and, mainly, aggregated AmB which is probably dragging the other two components.

It is worth mentioning the presence of a third small fraction peak of AmB aggregate around 1.5 kDa, very close to the monomers of GM1, that also grows with the proportional increase of AmB. This would reinforce the idea that the aggregated form of the drug might also be adsorbed to the few monomers of gangliosides that remined not micellar in each graphic.

These results are in agreement with those of DLS which showed that, at high molar ratios (10/1 and 5/1), the size is very similar to that of GM1 micelles alone. But, at molar ratio 2.5/1 and 1/1 the size of GM1-AmB micelles increase around 10 times. Moreover, other interesting fact in this relation is the polydispersity index of the different GM1-AmB micelles. Complexes with 10/1 and 5/1 molar ratio have a low polydispersity index that correlates with the fact that these structures have a predominant population with a molecular weight of about 180 kDa. In this fraction GM1 eluted mostly with monomeric AmB. In contrast, complexes with 2.5/1 and 1/1 molar ratios, eluting with V_0, show high polydispersity index which correlates with the presence of the large aggregates observed in the chromatographic elution profile.

The aggregation state of AmB also affects the stability of the micelle. While the stability over time and also after centrifugation at high speed shows no difference in the various molar ratios, the lyophilization does show marked differences. Lyophilized micelles of GM1-AmB to molar ratios from 10/1 to 2.5/1 were re-solubilized in aqueous solution up to 95 %, while those with a molar ratio of 1/1 were re-solubilized only up to 30 %.

Similar results were obtained for re-solubilization of GM1-AmB complex after freeze-thawing cycles. In this case, complexes with a molar ratio of 5/1 showed a high percentage of re-solubilization (> 95 %). But, at molar ratios of 2.5/1 and 1/1, the proportion of re-solubilization obtained was about 82 and 88 % respectively. These results suggest

that high molecular weight aggregates formed at higher proportions of AmB are unstable.

While it has long been described that the aggregation state of AmB is a critical factor associated with the biological activity and toxicity of the drug, there is still disagreement about which aggregation state is more toxic or has the more specific activity. Hamilton *et al.* [28] have a proposed that the toxicity of AmBis in part due to the existence of aggregates that formphase-separated domains in the lipid phase and the tendency for these aggregates to separate into a rigid phase within the cell membrane. Wójtowicz *et al.* [29] have also pointed out that those formulations used for pharmacological treatment that have the presence of aggregated AmB, usually correlated with drug-selective toxicity. Meanwhile, Espada *et al.* [30] concluded that the less toxic aggregation form of AmB was the poly-aggregated one. Hence, much effort to reduce the toxicity of AmB has focused on formulating the drug in association with a variety of amphipathic molecules [31, 32].

According to the results described in the present work, we cannot distinguish in the GM1-AmB formulation a differential effect of each of the aggregation states of the drug. While the amount of aggregate AmB differs in the different molar ratios of the complex, none of them had lytic effects on red blood cells. And, unlike the other two formulations tested, the AmB in micelles of GM1 at different molar ratios showed no toxicity on cells in culture.

Furthermore the evaluation of the biological activity of AmB against *Candida albicans* showed a very similar antifungal activity of GM1-AmB enriched in monomeric or aggregates, moreover we also found similar effect with other classical formulations tested, demonstrating that the decrease in the toxicity observed for the GM1-AmB formulation would not cause a concomitant loss of biological activity as occurs with other formulations with a diminished toxicity [12].

CONCLUSION

The results described in this work show that GM1 micelles appear to be able of incorporating AmB without any complex process, using completely aqueous processing conditions and achieving a high level of drug entrapment. On the other hand, these micelles with different aggregate forms of AmB show very low toxic effect on cells while retaining the same biological activity in-vitro as currently used commercial formulations. In this connection, it is interesting to remark the potential advantage of this formulation compared to the liposomal form, since previous reports have demonstrated that minor alterations of the process used for making the liposomes can also affect the product's toxicity [33, 34] which does not happen with the micelles.

However, further in-vivo experiments must be addressed to tackle the proposed AmB formulation presented here as an alternative to the currently used formulations.

CONFLICT OF INTEREST

The author(s) confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from CONICET to Drs. Beltramo and Bianco, PIP 11220100100502 and PIP 11220090100732 from CONICET. The authors declare no conflict of interest. RVA, IDB and DMB are research staff and VL is a fellow of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas).

PATIENT CONSENT

Declared none.

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Received: October 17, 2014 **Revised: January 06, 2015** **Accepted: March 07, 2015**