Similarity between the *in vitro* activity and toxicity of two different fungizone™ / lipofundin™ admixtures

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ABSTRACT - Purpose: Amphotericin B (AmB), an antifungal agent that presents a broad spectrum of activity, remains the gold standard in the antifungal therapy. However, sometimes the high level of toxicity forbids its clinical use. The aim of this work was to evaluate and compare the efficacy and toxicity *in vitro* of Fungizon™ (AmB-D) and two new different AmB formulations. **Methods**: three products were studied: Fungizon™, and two Fungizon™ /Lipofundin™ admixtures, which were diluted through two methods: in the first one, Fungizon™ was previously diluted with water for injection and then, in Lipofundin™ (AmB-DAL); the second method consisted of a primary dilution of AmB-D as a powder in the referred emulsion (AmB-DL). For the in *vitro assay*, two cell models were used: Red Blood Cells (RBC) from human donors and *Candida tropicallis* (*Ct*). The in vitro evaluation (K^+ leakage, hemoglobin leakage and cell survival rate-CSR) was performed at four AmB concentrations (from 50 to 0.05mg.L^{-1}). **Results**: The results showed that the action of AmB was not only concentration dependent, but also cellular type and vehicle kind dependent. At AmB concentrations of 50 mg.L^{-1}, although the hemoglobin leakage for AmB-D was almost complete (99.51), for AmB-DAL and AmB-DL this value tended to zero. The p = 0.000 showed that AmB-D was significantly more hemolytic. **Conclusion**: The Fungizon™-Lipofundin™ admixtures seem to be the more valuable AmB carrier systems due to their best therapeutic index presented.

KEYWORDS: Fungizon™. Lipofundin™. amphotericin B. Fungal infection. Drug delivery. Red Blood Cells

Introduction

The effectiveness of amphotericin B (**AmB)** in the treatment of systemic fungal infections, whose incidence has been considerably increasing in patients with immunodeficiency, has been attracting several researchers' interest in the world. Attempts have focused on finding a formulation as effective as FungizonÔ, but inducing a low toxicity level and presenting a smaller cost than others commercially available formulations. An alternative could be a new delivery system based on the lipid emulsions used for parenteral nutrition in clinical practice (Intralipid™ or Lipofundin™). This system has been showing to reduce the **AmB** toxicity with little damage in its efficacy[1]. In fact, the reduction of the toxicity of FungizonÔ - IntralipidÔ admixtures was observed in several clinical trials[1,6]. In 2002, an *in vitro* evaluation of FungizonÔ and FungizonÔ/ Lipofundinä admixture revealed that the latter one was less toxic against red blood cells, presenting no hemolytic activity[7].

Recently, our group has shown that the way in which FungizonÔ is incorporated into the emulsion (Intralipid™ or Lipofundin™) did not change the profile of activity or toxicity found for the **AmB** admixtures[7,8]. Therefore, the response against mammalian or fungal cells was remained.

The aim of this work was to correlate the efficacy and toxicity of FungizonÔ (**AmB-D**) and two AmB emulsion admixtures against two cell models, a cholesterol containing membrane and an ergosterol one.

Methods

In this research work, two cell models were used for the *in vitro* assay: Red Blood Cells (**RBC**), from human healthy donor, and *Candida tropicalis* (***Ct***). Representing respectively a mamalian (cholesterol containing) and a fungal (ergosterol containing) model. Potassium ($\mathbf{K^+}$) and hemoglobin leakage from **RBC** were monitored, respectively, as a measure of acute and chronic toxicity. K^+ leakage or cell survival rate (**CSR**) from ***Ct***, was used to evaluate the pharmacological activity of the products. Three pharmaceutical products were evaluated: **AmB-D**, a micellar solution of **AmB** containing sodium deoxycholate and marketed as Fungizonä (Bristol - Myers Squibb, São Paulo/SP - Brazil), and two preparations obtained through the admixture of Fungizonä and a parenteral emulsion (Lipofundinä LCT/MCT - 20%, B.BRAUN - Brazil). Based on the literature, such admixtures were prepared by two methods:

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in the first one, **AmB-D** was previously reconstituted with distilled water for injection and then, added to the parenteral emulsion (**AmB-DAL**) (9-11). The second method consisted of a primary dilution of AmB-D as a powder in the referred emulsion (**AmB-DL**) (12). For each preparation, the concentrations of 50, 5, 0.5 and 0.05 mg.L^{-1} of **AmB** (5.10^{-5}, 5.10^{-6}, 5.10^{-7}, 5.10^{-8}M, respectively) were used for the in vitro profile of activity or toxicity. To evaluate the effectiveness and toxicity of AmB, 4mL of RBC (5×10^{7} cell/ml) or 2mL of ***Ct*** (5×10^{7} cfu/ml) suspension were incubated by one hour at 37ºC with **AmB-D**, **AmB-DL** or **AmB-DAL**, respectively. Each experiment was accomplished in triplicate and repeated three times (7, 13). The results were expressed in percentage of hemoglobin and K^{+} leakage by the **RBC** and the percentage of K^{+} release and **CSR** for ***Ct***.

Statistical analysis

All potassium, hemoglobin release and CFU viability data were expressed as the mean ± SD. Statistical analysis was performed using ANOVA test and the significance was defined as $P<0.05$.

Ethics

This study was performed by written informed consent from the female healthy donor.

Results

The action of **AmB** was not only concentration dependent (Figures 1 to 3), but also cellular type (Figure 2 and 3) and vehicle kind dependent (Figures 1 to 3). **AmB** at the concentrations of 50 mg.L^{-1} induced an almost complete (99.51) hemoglobin leakage for **AmB-D.** However, for **AmB-DAL** and **AmB-DL** this value tended to zero (Figure 1). In fact, the p = 0.000 showed that **AmB-D** was significantly more hemolytic.

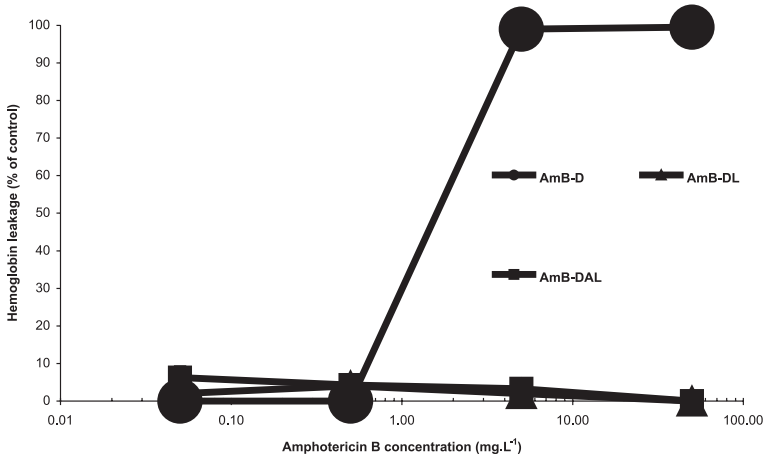

FIGURE 1 – Average of percentage and standard desviation of hemoglobin leakage from **RCB** under action of **AmB-D**, **AmB-DL** or **AmB-DAL,** respectively

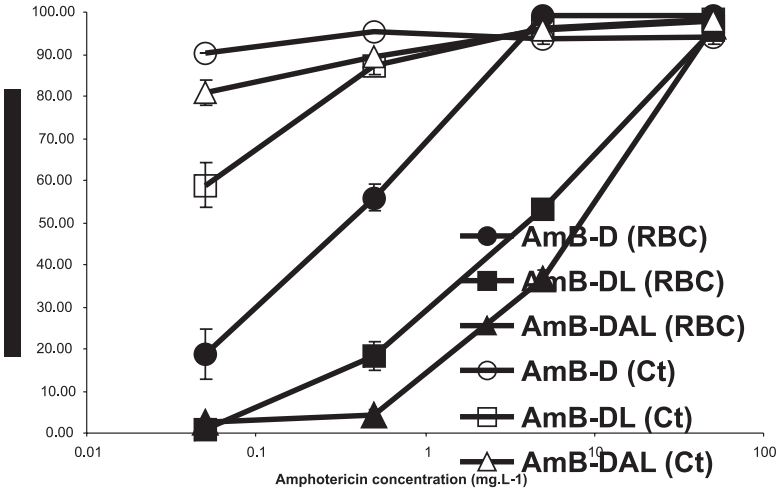

FIGURE 2 – Average of percentage and standard desviation of $\mathbf{K^{+}}$ leakage from **RCB** and ***Ct*** under action of **AmB-D**, **AmB-DL** or **AmB-DAL,** respectively.

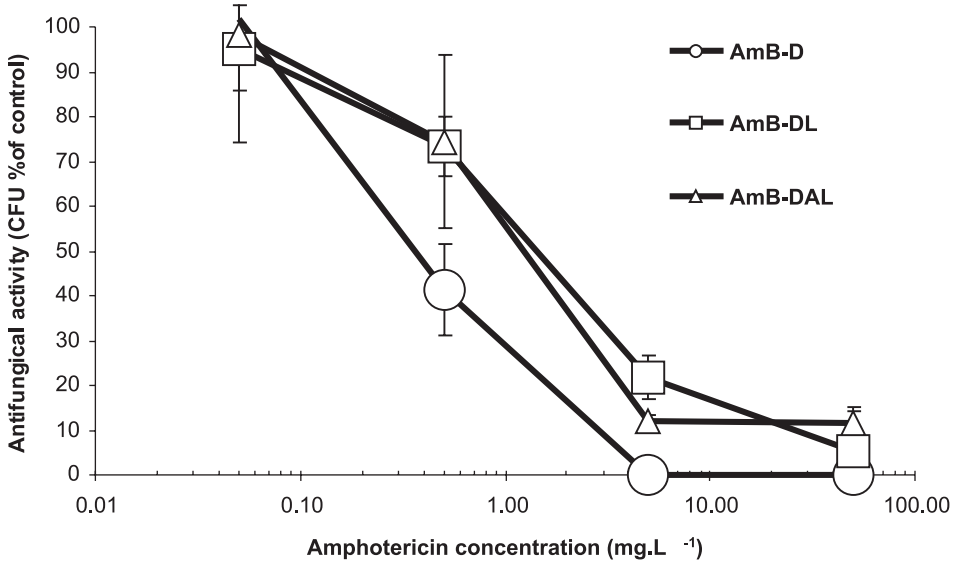

FIGURE 3 – Average of percentage and standard desviation of **CSR** from ***Ct*** under action of **AmB-D**, **AmB-DL** or **AmB-DAL,** respectively

Concerning K+ leakage, **AmB-D** was also more toxicic against **RBC** than **AmB-DAL** and **AmB-DL** (Figure 2)**.** Although at AmB concentration of 5 mg.L^{-1} 100% of leakage was observed for **AmB-D**, for **AmB-DAL** and **AmB-DL** products it tended to 50%. In fact, the K^+ release induced by **AmB-D** was significantly different from **AmB-DAL** and **AmB-DL** ($p<0.0001$).

When the cell model was the fungal one, a similar profile of activity was detected for all tested products, but after incubation with **AmB-D** at 0.05 mg.L^{-1} a larger K^+ release (Figure 2) was found compared to the **AmB-DAL** and **AmB-DL** ($p<0.0001$).

Such activity profile was also confirmed by the **CSR** data. **AmB-D**, **AmB-DAL** and **AmB-DL** showed a high effectiveness from 0.5 mg.L^{-1} that tended to 0% as soon as a ten fold concentration was achieved (Figure 3). Additionally, the **CSR** showed that whereas **AmB-D** was able to kill all seeded fungal cells from a concentration of 5 mg.L^{-1}, **AmB-DAL** and **AmB-DL** were discreetly less effective showing a small number of CFU (Figure 3) not statistically significant, at 50 mg.L^{-1}.

Discussion

The development of new drug delivery systems for **AmB** remains a great challenger for several pharmaceutical research groups. The aim is to develop less toxic formulations by changing the **AmB** physico-chemical behavior, and consequently, its biological properties *in vitro* and *in vivo*. In fact, the main mechanism of action (and toxicity) of this molecule consists on the formation of ion chanels by its interaction with the membrane sterols[14]. Depending on the size of the ion chanels formed, they allow the leakage of the internal constituents like potassium and hemoglobin on the mamallian cells. This is followed by a disturbance in the enzyme activity of the cells inducing their death.

Lipidic systems like liposomes or emulsions are able to strongly interact with the **AmB** molecule and change its physico-chemical properties. This is mainly due for the formation of reservoir systems that release monomeric species of **AmB**, which are less toxic to mammalian cells, but high actif against fungal cells[15]. Therefore, the use of an *in vitro* methodology that contemplates both cell models seems to be a valuable tool for the evaluation of new **AmB** delivery carriers.

On the other hand, the mixture of parenteral emulsion with Fungizon™ has been largely accepted as a way to reduce the AmB toxicity in the clinical therapy. In fact, several clinical trials reveal the efficacy of such strategy[2,4,12]. However, additional studies should be addressed to this "new pharmaceutical entity" to clearly understand its mechanism of action. Recentrly we have developed some physico-chemical studies on this AmB-lipid admixture system and showed, by spectrophotometric studies, that the presence of oligomeric **AmB** species is reduced in such preparation[7].

This work permitted us to evaluate the real correlation between two different **AmB**-Lipofundin™ admixtures, and by consequence, to determine if the way how the mixture should be carried out interferes in their final *in vitro* behavior of **AmB.** The data presented above (Figures 1 to 3) confirm the results that were published previously by our group[7,8]. They relate the influence of Fungizone™ / Lipofundin™ admixtures in decreasing **RCB** hemolysis. Besides, they indicate that although these admixtures reduced the permeability (K+ leakage) on **RBC** membranes, they increase such permeability against ergosterol containing membranes, and also promoting a slight decrease in their percentage of **CSR**.

All the results together suggest that parenteral emulsions can be able to reduce the Fungizon™ toxicity probably by changing the behavior of the **AmB** molecule into the dispersed emulsion system. The profile of activity, which was similar to

other lipid-based forms of **AmB**[1], indicates that AmB-emulsion admixtures could be an eligible carrier for such molecule. These systems may have some practical advantages. To name a few, they do not include expensive semi-synthetic lipids and are currently in use on clinical trials.

Conclusion

This paper showed that the methodology adopted to evaluate the pharmacological action and toxicity of different **AmB** carrier systems was fully satisfactory. The results showed that the action of **AmB** was not only concentration dependent, but also cellular type and vehicle kind dependent. The Fungizon™-Lipofundin™ admixtures seem to be a good system for future use due to their best therapeutic index presented. However, it is important to stand out that the **AmB** admixtures should be considered as a new pharmaceutical dosage form; therefore, their use should be made with caution and after additional pre-clinical and clinical studies to validate them.

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RESUMO – Objetivo: A anfotericina B é um agente antifúngico de largo espectro bastante empregado na terapia antifúngica. Entretanto, esta molécula apresenta um alto nível de toxicidade que, na maioria das vezes, impede o seu uso contínuo na terapêutica médica. O objetivo deste artigo foi comparar a eficácia e a toxicidade *in vitro* do Fungizon™ (AmB-D) e de dois sistemas carreadores de AmB. **Métodos**: Três produtos foram avaliados: o Fungizon™ , e dois sistemas oriundos da mistura entre o Fungizon™ e o Lipofundin™ , uma emulsão de uso parenteral. Tais sistemas foram obtidos por duas técnicas: Na primeira diluiu-se previamanete o Fungizon™ com água para injetáveis e em seguida inseriu-se o Lipofundin™ (AmB-DAL); o segundo método consistiu na diluição extemporânea do Fungizon™ com a referida emulsão (AmB-DL). Dois modelos celulares foram empregados no estudo: os eritrócitos (RBC) oriundos de doadores humanos e a *Candida tropicalis* (*Ct*). A avaliação in vitro (liberação de K^+ e hemoglobina, e o índice de sobrevivência celular-CSR) foi realizado com quatro concentrações de AmB (entre 50 e 0.05mg.L^{-1}). **Resultados:** Os resultados demonstram que a ação da AmB não só foi dependente da concentração como também variou de acordo com o modelo celular e o veículo que diluiu o Fungizon™ . Nas concentrações de 50 mg.L^{-1}, apesar da liberação de hemoglobina ser quase que total para AmB-D (99.51), para a AmB-DAL e AmB-DL este valor tendeu a zero. Um p = 0.000 demonstrou que AmB-D foi significativamente mais hemolítico. **Conclusão**: A mistura Fungizon™ -Lipofundin™ aparenta ser um bom sistema para carrear a AmB tendo em vista seu elevado índice terapêutico demonstrado.

DESCRITORES: Fungizon™ . Lipofundin™. Anfotericina B. Infecção fúngica. Sistemas de liberação de fármacos. Eritrócitos

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