UNIVERSIDADE FEDERAL DO RIO GRANDE DO NORTE

CENTRO DE CIÊNCIAS DA SAÚDE

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE

ANÁLISE *IN VITRO* DA ATIVIDADE ANTIFÚNGICA E DE TOXICIDADE DA ANFOTERICINA B PRÉ-AQUECIDA

MIGUEL ADELINO DA SILVA FILHO

Natal/RN

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DISSERTAÇÃO APRESENTADA AO PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE, COMO PARTE O REQUISITO PARA OBTENÇÃO DO TÍTULO DE MESTRE.

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Natal

2011

DEDICATÓRIA

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RESUMO

A anfotericina B micelar (AmB) é um importante agente antimicrobiano utilizado contra infecções fúngicas sistêmicas. No entanto, seu uso é limitado devido a sua alta toxicidade. Formulações de anfotericina B em estruturas lipídicas são menos tóxicas, porém apresentam elevado custo. A AmB em solução aquosa possui formas monoméricas e agregadas, estes últimos são os responsáveis pelas reações adversas. Este trabalho avaliou a AmB desoxicolato de sódio (AmB-DOC) e AmB aquecida (AmB-DOC-H) em relação a sua toxicidade e atividade, bem como perfil de seu espectro em quatro concentrações diferentes (50mg.L^{-1}, 5mg.L^{-1}, 0,5mg.L^{-1} e 0.05mg.L^{-1}). O aquecimento controlado das soluções AmB levou a alterações no espectro de 327nm AmB (agregados) para 323nm (superagregados). Além disso, quando as amostras foram submetidas ao processo de diluição, apresentam o mesmo comportamento espectrofotométrico: ambas as amostras diminuem o pico das formas auto-associadas até apresentarem apenas monômeros com pico em 409nm, o que sugere um processo de liberação de monômeros por concentração-dependente. No que diz respeito à toxicidade, AmB-DOC e AmB-DOC-H apresentaram comportamentos distintos: a taxa de liberação de hemoglobina de hemácias para AmB-DOC, em altas concentrações, foi muito superior a AmB-DOC-H, cujos valores tendem a zero ($p < 0,05$). Já em relação à liberação de K^+ ambas as amostras apresentaram perfil semelhante. Sobre a atividade, AmB-H mantém o mesmo perfil da AmB-DOC. Em suma, a AmB-DOC-H é muito menos tóxica do que AmB-DOC e mantendo a atividade. Sendo este procedimento uma alternativa simples, de baixo custo e segura para, em um o futuro próximo, ser utilizada no tratamento de infecções fúngicas sistêmicas.

Palavras Chaves: Anfotericina B, Farmacotoxicidade, *in vitro*, micelas, Superagregados, Tecnologia Farmacêutica.

1 – INTRODUÇÃO

As infecções sistêmicas graves, principalmente as causadas por microorganismos oportunistas, constituem a maior complicação em pacientes neutropênicos, portadores de doenças neoplásicas, e de outras imunodeficiências (1-3). A disponibilidade aliado ao uso indiscriminado cada vez mais crescente de antimicrobianos de amplo espectro tem aumentado consideravelmente a incidência de infecções fúngicas sistêmicas graves (3).

A anfotericina B (AmB) é um fármaco utilizado no tratamento da maioria das infecções fúngicas sistêmicas, principalmente em pacientes imunocomprometidos (3-11). A AmB, na sua forma comercial tradicional (Fungizon®) e seus similares, consiste de uma mistura desta molécula com um sal biliar, o desoxicolato de sódio, formando uma solução micelar. Ela também é freqüentemente utilizada como terapia empírica em pacientes com granulocitopenia, que apresentam febre de origem desconhecida (3, 4, 12, 13).

Sabe-se que a alteração da permeabilidade em membranas está relacionada com a capacidade da AmB em formar canais transmembrana, enquanto o efeito lítico está relacionado com a ação peroxidativa em nível de membrana (14). Desta forma, foi realizado estudos comparativos entre as formas monoméricas, agregadas e uma nova, “superagregadas”, para uma investigação das vantagens destas últimas formas, uma vez que elas são mais estáveis por sofrerem um índice consideravelmente menor de auto-oxidação (8), além do que os agregados solúveis e a forma monomérica são mais eficientes na indução da permeabilidade de potássio em membranas que contêm colesterol, como as células humanas, do que os superagregados (15, 16).

Em virtude da elevada incidência de reações adversas, principalmente, febre, calafrios e nefrotoxicidade, o uso clínico da AmB é frequentemente limitado (3, 12, 17, 18). Entretanto, tal limitação está suscitando a realização de vários estudos, visando ao desenvolvimento de novas formulações de **AmB**, dentre as quais pode-se destacar a sua incorporação a estruturas lipídicas, tais como: lipossomas (2, 4, 19-22), complexação com sulfato de colesterol, microemulsões (7, 23), entre outros.

Apesar dos benefícios trazidos por estas novas formulações, estudos demonstram que a maior desvantagem desses sistemas refere-se aos custos, tendo em vista que podem ser onerosos quando comparados àqueles de preparação convencional (3, 11, 24, 25).

Desenvolver novas formulações eficazes de AmB, com menor toxicidade e de baixo custo, continua a ser um grande desafio. Recentemente (8), descobriram que o aquecimento moderado do Fungizon®, Anfotericina B pré-aquecida (AmB-DOC-H), permite um rearranjo molecular de maior tamanho, formadas pela condensação das formas monoméricas e agregadas resultando em “superagregados”, que têm demonstrado maior estabilidade química, mantendo sua eficácia nas células fúngicas e menor toxicidade em células de mamíferos (8). Assim, é preciso dar sequência ao estudo, uma vez que permitirá um conhecimento mais amplo sobre esse novo sistema para consolidá-lo como uma nova estratégia terapêutica.

2 – REVISÃO DA LITERATURA

2.1 Características da célula fúngica

Os fungos possuem estruturas típicas de células eucarióticas. A célula possui um complexo citosol que contém microvesículas, microtúbulos, ribossomos, mitocôndrias, aparelho de *Golgi*, núcleo, um retículo endoplasmático com dupla membrana e outras estruturas. O núcleo dos fungos, envolvido por uma membrana, contém praticamente todo o DNA celular, além de possuir um verdadeiro nucléolo rico em RNA (26, 27).

Há, envolvendo o citosol, uma membrana denominada plasmalema, composta de glicoproteínas, fosfolipídios e ergosterol. O fato de os fungos possuírem ergosterol é importante, pois a maioria das estratégias antifúngicas baseia-se na presença de ergosterol nas suas membranas (27)

Os fungos possuem uma parede celular rígida, de múltiplas camadas, imediatamente externa à plasmalema. A parede celular, complexa sob o ponto de vista estrutural e bioquímico, contém quitina, um homopolímero da N-acetilglicosamina, como sua base estrutural. Sobre a quitina há camadas de glucanos, manoproteínas e outros polissacarídeos complexos associados a polipeptídeos (26, 27)

Esses microorganismos apresentam tamanhos variados; o gênero *Candida*, cepa utilizada nos experimentos, quando em forma de levedura, apresenta um diâmetro de 4μm (27).

Tomando como base experiências clínicas, esses microorganismos podem provocar problemas de Saúde Pública extremamente sérios, dentre eles a *aspergilose*, uma infecção que causa a morte de aproximadamente 70% dos

pacientes submetidos a transplantes de órgãos ou de medula, contaminados com filamentos de *Aspergillus fumigatus*. Para tratamento da *aspergilose*, existem somente dois medicamentos disponíveis, comercialmente: o Fungizon® e o Itraconazol®. Devido à ocorrência frequente da toxicidade e das cepas resistentes, registra-se uma busca incessante pela síntese e estudo das propriedades dos novos glicopeptídios ativos (28).

Convém ressaltar que a identificação de *Candida tropicalis* ocorre em menor frequência que a *Candida albicans*, contudo, em certos hospitais, a *Candida tropicalis* pode ser a espécie de *Candida* isolada com maior predominância nos espécimes clínicos (29).

2.2 Características da Hemácia

As hemácias são discos bicôncavos com diâmetro entre 7 e 8 μm, com um volume médio de 90fL ($1fL=10^{-15}L$) e uma superfície média de 160μm (30). A estrutura da membrana plasmática é composta de aproximadamente 52% de proteínas, 40% de lipídios e 8% de carboidratos. As maiores quantidades de componentes lipídicos são: colesterol não-esterificado e fosfolipídios, presentes em quantidades equimoleculares (31-33). Os fosfolipídios da membrana possuem a seguinte composição: fosfatidilcolina, esfingomielina, fosfatidiletanolamina, fosfatidilserina, ácido fosfatídico e fosfatidilinositol (31, 32).

A membrana plasmática da hemácia apresenta maior quantidade de colesterol que a das demais células. Comparando o percentual de colesterol que integra a composição lipídica das diferentes membranas celulares pode-se destacar: a hemácia – 23%, mielina – 22%, hepatócito – 17%, retículo endoplasmático – 6% e

mitocôndria – 3% (34). Essa característica faz da hemácia um ótimo modelo para avaliar a toxicidade da AmB.

A hemácia possui a hemoglobina, uma importante proteína intracelular (33). A hemoglobina possui três funções essenciais; transporte de oxigênio dos pulmões para os tecidos, transporte de CO_2 dos tecidos para os pulmões e o tamponamento do sangue (35).

A principal função das hemácias consiste em transportar a hemoglobina (32, 36). Além disso, por conter grande quantidade de anidrase carbônica, catalisa a reação entre o dióxido de carbono e a água aumentando, assim, a velocidade desta em milhares de vezes. Como a hemoglobina nas células também é um excelente tampão ácido – base, as hemácias são responsáveis pelo tamponamento do sangue total (36).

2.3 Anfotericina B

A anfotericina B (AmB) é um fármaco pertencente ao grupo dos antibióticos poliênicos de ação antifúngica obtida inicialmente a partir de culturas de *Streptomyces nodosus* isoladas de amostras vegetais em decomposição no solo à margem do rio Orinoco na Venezuela em 1955 (37, 38). A molécula foi descoberta, em 1955, por Gold e colaboradores dos laboratórios Squibb, Estados Unidos (37). A AmB apresenta uma cadeia complexa, com uma massa molar igual a 924,1 daltons (39). Sua fórmula molecular é $C_{47}H_{73}NO_{17}$ e a estrutural está apresentada na Figura 1.

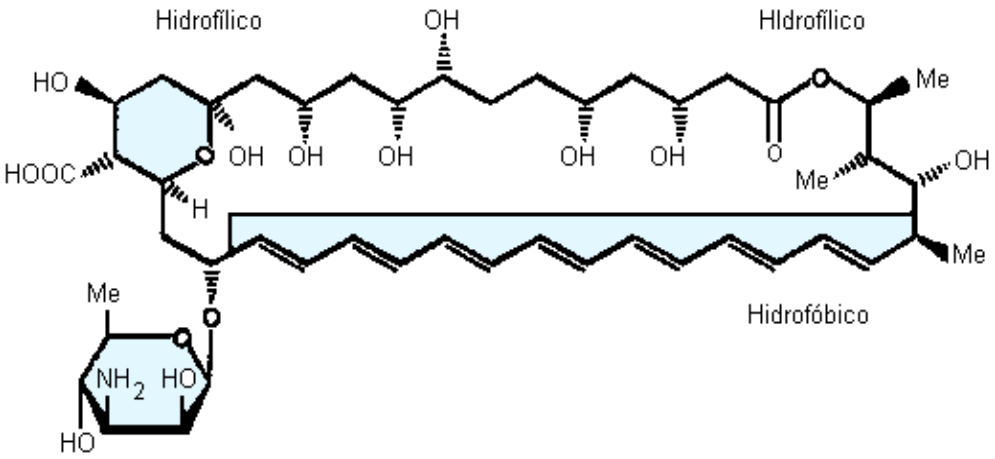

Figura 1: Fórmula estrutural da Anfotericina B (AmB)

A AmB é um pó amarelo alaranjado, sensível a luz e ao calor, inativa em baixos valores de pH, praticamente insolúvel em água, solúvel em dimetilsufóxido (DMSO), dimetilformamida e em propilenoglicol, muito levemente solúvel em metanol e praticamente solúvel em etanol (37). Além disso, a sua solubilidade é dependente do pH e devido a complexidade da molécula ela apresenta uma variação interna de pKa (5,7 para –COOH e 10 para NH_2) (40, 41).

A molécula da AmB consiste de um grande anel macrolídeo fechado por lactonização, formado por uma rígida cadeia lipofílica contendo sete duplas ligações (heptaeno) e, no lado oposto, por uma cadeia polihidroxilada com sete grupos hidroxilas livres o que lhe confere uma característica anfifílica. Em uma das extremidades da molécula, encontra-se um resíduo micosamina com um grupo amino livre formado por uma ligação α-glicosídica. A AmB tem aproximadamento 24Å de comprimento, o que corresponde a meia camada de fosfolípideo (42).

A AmB age se ligando aos esteróis da membrana plasmática [ergosterol (células fúngicas) e colesterol (células de mamíferos)] levando a formação de poros através das membranas lipídicas (43-45). A AmB e o esterol forma um complexo (AmB-esterol) perpendicular ao plano da bicamada da membrana, com o ácido

carboxílico formando ligações de hidrogênio com a porção hidrofílica do esterol da membrana com consequente formação de um poro iônico com as hidroxilas direcionadas para o centro do poro e, consequentemente, com a porção heptaeno voltada para a ligação com os esteróis (43). Sabe-se que o poro formado na verdade é um “meio poro”, pois se forma um poro na camada superior e outro na inferior da membrana e que eles são perfeitamente móveis dentro de cada metade da bicamada. Para formar um poro transmembranar os dois “meio poro” precisam se alinhar (46). A formação destes poros iônicos causam uma despolarização e aumento da permeabilidade da membrana levando a uma liberação de cátions monovalentes como o potássio (K^+) e pequenas moléculas inorgânicas do citoplasma, o que é responsável direta ou indiretamente pelo efeito letal à célula pela AmB (43, 44). Outro mecanismo de ação extremamente importante na ação lítica da AmB é o dano oxidativo produzido quando ela se liga aos esteróis da membrana. Provavelmente, a AmB provoca uma cadeia de reações oxidativas e estes metabólitos oxidados podem atuar como fatores de estimulação dos macrófagos, contribuindo para a ação antifúngica (43). Como os mecanismos de ação da AmB não é específico, apesar de ser mais seletivo para as células fúngicas, este fármaco induz muitos efeitos tóxicos (43, 44).

A AmB apresenta um amplo espectro de ação agindo tanto em fungos leveduriformes como *Candida, Malassezia, Saccharomyces* e *Trichosporon*, como em filamentosos como *Histoplasma, Coccidioides, Blastomyces, Paracoccidioides, Aspergillus, Scedosporium, Sporothrix, Paecilomyces, Penicillium, Fusarium, Bipolaris, Exophiala, Cladophialophora, Absidia, Apophysomyces, Cunninghamella, Mucor, Rhizomucor, Rhizopus* e *Saksenaea*. Apesar de algumas cepas de *Candida albicans, C. tropicalis, C. parapsilosis, C. lusitaniae, T. beigelli, Malassezia furfur,*

Scedosporium apiospermum e *S. prolificans*, *Fusarium* spp. e *Sporothrix schenckii* apresentarem resistência à AmB, muitas delas são raramente vista clinicamente e, sobre tudo, o desenvolvimento de resistência à AmB não tem sido um fator significante no tratamento de pacientes (47). A resistência à AmB é causada por um decréscimo na quantidade de ergosterol do fungo ou por uma mudança no alvo lipídico gerando uma redução na ligação da AmB ao esterol da membrana fúngica (47).

Esta complexa molécula apresenta dois tipos de toxicidade: (1) aguda (relacionada à infusão do fármaco) e (2) crônica (relacionada a doses repetidas). A toxicidade relacionada à infusão é caracterizada por febre, arrepios, calafrios, mal-estar, dor generalizada, vômito, náusea, dor de cabeça, hipóxia, hipotensão, hipertensão, hipotermia, arritmias cardíaca e infiltrado pulmonar. Estas reações são provavelmente associadas com a indução das citocinas pró-inflamatórias pela AmB e pela liberação de TNF-α dos macrófagos (48). A toxicidade crônica é responsável pelo aparecimento de hipocalemia, hipernatremia, diurese aumentada, hipomagnesemia, efeitos tóxicos sobre a medula óssea (anemia, leucopenia e trombocitopenia) e disfunção renal (nefrotoxicidade). A principal reação adversa provocada pela AmB é a nefrotoxicidade (a taxa de incidência de nefrotoxicidade provocada pela AmB está entre 49-65%) e essa reação é devido a um componente funcional relacionado à vasoconstrição renal, reduzindo o fluxo sanguíneo normal e a taxa de filtração glomerular, e um resultado da interação direta entre a molécula e as células tubulares (48, 49).

A grande quantidade e incidência de reações adversas da AmB despertou vários estudos com o intuito de reduzi-las. Uma maneira eficaz na redução destas reações adversas é a sua incorporação em estruturas lipídicas, como lipossomas

(AmBisome®), complexos lipídicos [ABLC (Abelcet®)], dispersões coloidais [ABCD (Amphotec® e Amphocil®)] e microemulsões (AmBME) (9, 48, 50).

Apesar das inúmeras formulações já desenvolvidas, ainda não existe uma alternativa ideal, economicamente viável para um sistema assistencial de saúde pública. O estudo dos superagregados de AmB-DOC-H é oportuno uma vez que tornará disponível mais dados científicos a respeito de sua viabilidade para produção de uma nova forma farmacêutica, já que até agora existem dados promissores quanto a sua maior atividade e menor toxicidade.

3 – ANEXAÇÃO DO ARTIGO

Título do artigo: How do micelles can be rebuilt by heating process?

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ORIGINAL RESEARCH

Evaluation of superaggregate micelle state of amphotericin B

Silva-Filho et al

How do micelle systems can be rebuilt by heating process?

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Abstract: The aim of this work was to evaluate how an aqueous micellar system containing Amphotericin B (AmB) and sodium deoxycholate (DOC) can be rebuilt

after heating treatment. Also a review of the literature about the new physicochemical and biological properties of this new system was carried out. Afterwards, heated (AmB-DOC-H) and unheated (AmB-DOC) micelles were subsequently diluted at four different concentrations (50, 5, 0.5 and 0.05mg.L^{-1}) to perform the physicochemical study and, then, the pharmacotoxicity assay, in which two cell models were used for the *in vitro* experiments, Red Blood Cells (RBC) from human donors and *Candida parapisilosis* (*Cp*). While potassium (K^+) and hemoglobin leakage from RBC were the used parameters to evaluate the acute and chronic toxicity, respectively, the efficacy of AmB-DOC and AmB-DOC-H were assessed by K^+ leakage and cell survival rate from *Cp*. The spectral study revealed a slight change on the aggregate peak from 327nm to 323nm for AmB-DOC-H compared to AmB-DOC. Concerning the toxicity, although AmB-DOC and AmB-DOC-H presented different behavior for hemoglobin leakage, AmB-DOC produced higher leakage than AmB-DOC-H at high concentrations (from 5mg.L^{-1}) with values tending to zero. However, concerning K^+ leakage, both AmB-DOC and AmB-DOC-H, showed similar profile for both cell models, RBC and *Cp* ($p<0,05$). AmB-DOC-H and AmB-DOC also revealed similar profile of activity against *Cp* with equivalent survival rate. In short, the AmB-DOC-H showed much less toxicity than AmB-DOC, but remained as active as the late one against fungal cell. Therefore, the results highlight the importance of this new procedure as simple, inexpensive and safe alternative to produce a new micelle system for treatment of systemic fungal infections.

Keywords: micelles; nanotechnology; pre-heated amphotericin B; superaggregates

Introduction

Amphotericin B (AmB) is a water insoluble compound widely used to effectively treat systemic fungal infections.[1] However, its utilization as antifungal agent has been limited by high acute and chronic toxicity, characterized by chills, fever, nausea, vomiting, and mainly nephrotoxicity.[2-4]

Due to amphiphilic nature of the molecule and its poor solubility in water, AmB is able to self-associate in water media and form supramolecular aggregates. However, in aqueous media monomers and soluble and insoluble aggregates of AmB coexist, the latter one being responsible for the toxic effects.[5-8]

Moreover, when AmB in solution is below than a minimal concentration of self-association the toxicity against fungi is still observed, but the human toxic effect decreases.[2,3]

In the current model of AmB selective toxicity, soluble monomeric AmB is able to form ion channels in ergosterol-rich fungal membranes, but a soluble self-associated oligomer damages sterol-free and cholesterol-containing membranes.[9,10] Also, the oligomers seem to be susceptible to oxidation, which may enhance their toxicity.[11] Therefore, the way in which AmB is cleared from the body play an important role on its toxicity and activity. In fact, the widely accepted mechanism of action of AmB described above is true for micelle systems, in which AmB molecules bind to a surfactant presents a high degree of freedom and can easily leave the surfactant molecule and self-associate.

However, the micellar deoxycholate system of AmB (AmB-DOC), a well known commercial antifungal product (Fungizon®), has been used in clinical practice for over 45 years for the treatment of several systemic fungal infections.[2,12] Unfortunately, AmB-DOC shows a concentration dependent profile of toxicity and its

selectivity depends on its aggregation state. Clinically, nefrotoxicity is frequently observed.[13]

Aiming to reduce the AmB-DOC toxicity, some lipid formulations such as liposomes and other AmB lipid-associated forms were developed.[14-18] These systems are able to immobilize the AmB monomers preventing self aggregation and slowly release monomers to the media, and, therefore, are quite active only against fungal cells. In spite of these formulations present a far superior therapeutic index compared to AmB-DOC, they present a high cost and their spread clinical use is inaccessible for many countries, where fungal-related diseases are rampant. In fact, only AmB-DOC is widely used in some developing countries, and after the ninety's, when Fungizon® was no more patent protected, this product started to be manufactured for several brands as a generic product.

It is known in the literature that the AmB-DOC, forms a new type of aggregates when subjected to controlled heat, which are less susceptible to oxidative degradation and less selective for the cholesterol in mammalian cell membrane, thus making it less toxic.[19-25] In fact, the heating of AmB-DOC solutions generates "superaggregates" in which the toxicity is significantly reduced while keeping their antifungal activity. However, a recent work conduced in our research group revealed that AmB micellar systems made by different brands present different profiles of activity and toxicity, probably due to the variation in the formation of the structure drug-surfactant micelle system due to changes on the manufacturing process or because of the quality of the used haw material (our unpublished results).

The aim of this work was to evaluate the relationship between the controlled heat treatment, the absorption spectra and the pharmacotoxicity of a Brazilian brand

AmB-DOC in aqueous micellar solutions before and after heat treatment. In this way, a new process to rebuilt pre-formed micelle system can be achieved.

Material and methods

Preparation of the AmB-DOC samples

The stock solution of AmB-DOC, purchased from Cristália - Brazil, at $5x10^{-3}$M (5,000mg.L^{-1}) was prepared adding 10mL of water for injection into the vial. Each vial of AmB-DOC contained 50mg of AmB, approximately 41mg of sodium deoxycholate, and phosphate buffer, pH 7.4.

Samples of AmB-DOC were prepared with different concentrations by successive dilutions 1:10 of AmB-DOC stock solution in order to obtain the concentrations of 5 x 10^{-5}M (50mg.L^{-1}), 5 x 10^{-6} M (5mg.L^{-1}), 5 x 10^{-7}M (0.5mg.L^{-1}) and 5 x 10^{-8}M (0.05mg.L^{-1}). The heated AmB (AmB-DOC-H) was prepared by treatment with controlled heat of AmB-DOC stock solution at 70℃ for 20 minutes in a thermostatic bath. Then, dilutions as for AmB-DOC were prepared to obtain the similar study concentration samples.

Spectral study

Scanning spectra of both AmB-DOC and AmB-DOC-H at the four before mentioned concentrations were taken by using a UV-VIS Spectrophotometer (Biochrom Libra S32, Cambridge, United Kingdom). The optical path of the quartz cuvettes used was 0.1, 1, and 10cm for the concentrations of 5 x 10^{-5}M (50mg.L^{-1}); 5 x 10^{-6}M (5mg.L^{-1}), and 5 x 10^{-7}M (0.5mg.L^{-1}) and 5 x 10^{-8}M (0.05mg.L^{-1}), respectively. These paths were chosen to obtain spectra with absorbance values less than 0.8. Their molar extinction coefficients (ε) were calculated using the Beer-

Lambert equation. All spectra were recorded at 25 ± 0.1ºC with a 300- 450nm range.[6]

Preparation of Red Blood Cells (RBC) suspension

This study was previously approved by the Ethical Research Committee of the Federal University of Rio Grande do Norte, protocol number 002/2009. In order to minimize sources of variability, one healthy adult female donor, who gave her written informed consent before participating in the study, provided all normal human RBCs for the *in vitro* experiments. Five mL of venous blood was collected in sterile EDTA [1mg/mL, ethylene-diamine-tetraacetate at 10%(w/v), Labtest, Lagoa Santa, Brazil] syringes and promptly centrifuged (Refrigerated centrifuge, ALC, Model PK121R, Milan, Italy) in tubes at 1,100g for 10 min at 4ºC. Plasma was carefully aspirated and the exposed buffy coat was removed and discarded. The RBCs were washed three times by centrifugation (1,100g for 5 min at 4ºC) and suspended in 5 volumes of normal saline [NaCl at 0.9% (w/v), B. Braun, São Paulo, Brazil]. They were then resuspended in 4mL of saline, counted in a Neubauer™ chamber, and resuspended again until desired concentration ($5x10^7$cells.mL^{-1}) was achieved. The cells were used in the same day of collection.[17]

Preparation of Candida parapsilosis suspension

A strain of *Candida parapsilosis* (*Cp*) ATCC (22019) was maintained on Sabouraud- Dextrose-Chloramphenicol agar (SDC, MicroMed, São Paulo, Brazil) at room temperature and passaged monthly. Before experiments, an inoculum from the culture was transferred to a SDC agar scope, and incubated at 37ºC for 16-18h. The fungal cells were then washed three times with normal saline, resuspended, counted

in the central reticule of a Neubauer™ chamber, and resuspended again to obtain the desired cell concentration ($5x10^7$cfu.mL^{-1}).[17]

Evaluation of the AmB-DOC and AmB-DOC-H toxicity

Four mL of RBCs ($5x10^7$cells.mL^{-1}) were incubated for 1 h at 37ºC with the vehicle control or with different concentrations (50, 5, 0.5 and 0.05mg.L^{-1}) of both AmB-DOC and AmB-DOC-H. The RBCs were then centrifuged for 5 min at 1,100*g* and washed three times with normal saline. The pellet of RBC was lysed by adding 4mL of distilled water and stirred and centrifuged (1,100*g* for 10 min) in order to remove membranes. Potassium ion (K^+) content of the supernatant was determined using a Flame Photometer 7000 (910M Analyser, São Paulo, Brazil) calibrated with K^+ reference at 5mEq.L^{-1}. Hemoglobin was estimated from its absorption at 540nm recorded on a UV-VIS Spectrophotometer (Biochrom Libra S32, Cambridge, United Kingdom). The total K^+ and hemoglobin content was measured for the control RBCs tubes. Release was calculated as the difference between control and treated cells and expressed as a percentage of the total hemoglobin or K^+ content. At least three different experiments were performed with each formulation and each experimental point was performed in triplicate.[26]

Evaluation of the AmB-DOC and AmB-DOC-H efficacy

Two mL samples of fungal suspension containing $5x10^7$cfu.mL^{-1} were incubated for 1 h at 37ºC with both AmB-DOC and AmB-DOC-H at the concentrations of 50, 5, 0.5 and 0.05mg.L^{-1}. Cells were centrifuged for 10 min at 2,200g, washed three times in normal saline, and 2mL of purified water were added to the pellet of fungal cells. An aliquot of this pellet was lysate by heating for 5 min at 100ºC, centrifuged to

remove membranes and free K^+ was measured. The K^+ leakage was calculated similarly to the RBCs. For the cell viability evaluation, 100µL aliquots of appropriate dilution of the fungal pellet were seeded, in duplicate, onto agar plates and incubated at 37ºC. The number of colony-forming units (CFU) was counted at 24 and 48 h and expressed as a percentage of those obtained from a control inoculum incubated without AmB-DOC or AmB-DOC-H. Three different experiments were performed with each formulation and each experimental point was performed in duplicate.[26]

Statistical analyses

The statistical results were performed with ANOVA and T test to analyze the variation response in the same group and in different groups, respectively using the Prim 4 for Windows 4.02 (GraphPad Software, USA).

Results

Aggregation state behavior of AmB-DOC and AmB-DOC-H

The AmB-DOC-H showed spectra similar to AmB-DOC which were concentration dependent (Figure 1 and Figure 2).

At low concentration, such as 5.10^{-8}M, the AmB presents a spectrum with three well defined absorption bands, with λ_{max} at 363, 385 and 408nm, and a shoulder around 347nm, which is similar to that obtained with organic solvents like methanol, for the monomeric AmB. However, by increasing the concentration of AmB a new band appeared at λ_{max} 327nm at the expense of the bands recorded in the longer wavelengths (363, 385 and 408nm), especially the one characteristic of the monomeric AmB specie at 408nm, which is responsible by the biological activity against fungal cells [10]. The band at 327nm was reported in the literature as indicative

of the presence of self-associated species of AmB. This band was also sensitive to AmB concentration being very well defined at high concentrations as in the range of 10 x 10^{-7} to 10^{-5}M.[10]

Concerning the AmB-DOC-H spectra, the band at 327nm was slightly blue shifted to 323nm after heating. Several authors stated that this band is characteristic of the formation of AmB superaggregates.[20] It is important to note that the variation in the molar extinction coefficient of AmB at λ_{max} 323nm and the peaks at 363, 385 and 408nm are related to variation on the equilibrium between the monomeric and aggregated species of AmB, which displays an isosbestic point at 347nm. See scheme.

$$AmB_m \underset{}{\overset{K}{\rightleftharpoons}} AmB_{sa} \qquad \text{(scheme 1)}$$

Where the underline letters m and sa refer to the monomeric and self-associated AmB, respectively, while K is the equilibrium constant between the AmB species.

This equilibrium shows that at low concentrations prevails the monomeric species of AmB_m, but when the concentration is increased the equilibrium is shifted favoring the predominance of the self-aggregated AmB_{sa}.

The most important difference among the AmB-DOC and AmB-DOC-H spectra occurred at the concentration of 5 x 10^{-7}M, in which the band at 323nm presented a high molar extinction coefficient ($\varepsilon = 71{,}00\tilde{0}$) compared to the one assigned for AmB-DOC ($\varepsilon = 48{,}666$). It should be emphasize that the spectra at 5 x 10^{-8}M were given to illustrate the tendency of concentration dependence. In fact, they should not be considered on a quantitative basis because of the weakness of

the signals at low wavelength region (maximum absorbance at 408nm was 0.071 for AmB-DOC).

The data of the Figures 1 and 2 shows that the recorded spectral differences can be attributed to different interaction of AmB with the surfactant aggregates provoked by controlled heating, and such changes remained over the whole range of concentrations.

In vitro toxicity assay for AmB-DOC and AmB-DOC-H

The toxicity of both AmB-DOC and AmB-DOC-H against RBCs were quite interesting. Concerning K^+ leakage both profiles were quite identical (Figure 3). In fact, no significant K^+ leakage was observed at 0.05mg.L^{-1} (5 x 10^{-8}M). At higher concentrations such phenomenon completely changes. At 0.5mg.L^{-1} (5 x 10^{-7}M) both products induced strong permeability of the cell membrane as reflected by a K^+ release of around 50% (59.59 ± 0.41% and 50.12 ± 0.9 % for AmB-DOC and AmB-DOC-H, respectively). At the highest concentrations of 5mg.L^{-1} (5 x 10^{-6}M) and 50mg.L^{-1} (5 x 10^{-5}M) were the aggregate specie of AmB predominates both systems presented a total release of K^+.

Both AmB-DOC and AmB-DOC-H presented no significant hemoglobin leakage below 0.5mg.L^{-1} (Figure 4). While the AmB-DOC-H showed no toxic effect on the whole range of the tested concentrations (0.05 to 50mg.L^{-1}, $p<0.001$), the AmB-DOC, revealed a sharp increase in the toxicity from 0.5mg/L^{-1} reaching to full cell lysed from 5mg.L^{-1} with total hemoglobin leakage to external media.

In vitro activity assay for AmB-DOC and AmB-DOC-H

The *in vitro* activity against *Cp showed that* both AmB-DOC and AmB-DOC-H were very effective (Figure 5) and that the profile of K^+ release was quite similar (Figure 3). In fact, a significant K^+ leakage was found at the lowest concentration ($0.05mg.L^{-1}$) reaching around 85% at the highest concentration. In this assay AmB-DOC-H was slightly more active than AmB-DOC over the whole range of concentration. For example, at $5mg.L^{-1}$ AmB-DOC-H presented a K+ release of 85.92 ± 0.85% while AmB-DOC showed a release of 79.75 ±1.96%. At $50mg.L^{-1}$ these values were 88.38 ±0.70% and 83.98 ± 3.86%, respectively. However, such values were not statistically significant.

These data are in agreement with the results of cell survival rate, which indicated for both AmB_DOC and AmB-DOC-H a significant decrease in the rate of survival cell at concentrations up to $0.5mg.L^{-1}$. At this concentration range the AmB-DOC-H showed higher antifungal activity than the unheated AmB. For example, at the concentration of $0.05mg.L^{-1}$ AmB-DOC-H was able to kill 53.72 ± 5.17% whereas AmB-DOC killed only 32.31 ± 1.40%. Considering the standard deviation, in the range of concentrations from $0.5mg.L^{-1}$ to $50mg.L^{-1}$ the CFU survival rate profile were similar for both preparations. Finally, at $50mg.L^{-1}$ both products were able to kill the totality of the fungal cells.

Discussion

Micelle systems have been used as nanotechnologic carriers for more than 60 years. Particularly for AmB, this system is used since 1951 when Bartner et al. studied the incorporation of AmB molecule into a micellar sodium deoxycholate (DOC), in a molar ratio of approximately 1:2 AmB:DOC, respectively.[27]

The idea that a micellar complex could change its physicochemical property by heating was completely undiscovered since the first works of Ernst et al [28]. Almost twenty years after Gaboriau et al[20] produced a deep study about this phenomena by discovering the super-aggregated form of AmB. This new AmB specie not only present an important change on the absorption and circular dichroism spectra of AmB, which is related with its physicochemical state, but also reveal a biological difference when compared to the unheated micelle. Therefore, it can be estimated that the industrial production of a micellar system could be very influenced by the manufacturing process, which can modify the surfactant-molecule binding and generates different physicochemical forms.

The results of this study show that differently from the unheated form, the Brazilian micelle system present a quite similar physicochemical profile to the European one when heated at 70ºC for 20 minute, generating a superaggregated spectroscopic specie, which was characterized by a band at 323nm on its spectra. Because this band appeared at the expenses of the ones at 327 and 408, the presence of the superaggregated form can be suggested as a result from the condensation of the aggregated and monomers species of the AmB. This explanation is in complete agreement with the study of Gaboriau et al using an European AmB-DOC (Fungizon®).[20] Different from the AmB aggregate specie, the dissociation of this super-aggregated form into monomers occurs at lower AmB concentrations.[29] Therefore, it can be inferred that the AmB super-aggregated specie may be considered as a reservoir of monomeric AmB specie that releases only a limited amounts of monomeric AmB in the aqueous media. As a consequence, the concentration of monomeric AmB might be below its critical aggregation concentration, which is around 10^{-6}M, and thus, the drug can remain in its

monomeric form. This organization would be able to bind to ergosterol of fungal cells, but inactive to bind cholesterol of the RBCs.[10]

In fact, the variation of patterns of activity against ergosterol-containing fungal cells regarding the toxicity against cholesterol-containing mammalian cells and its dependence on the AmB molecular presentation has been reported by several research groups.[1,6,30,31] It was well established that the monomeric specie of AmB is less effective than aggregated form to induce the permeability of membrane containing cholesterol to potassium leak.[32] The AmB aggregated form was shown to trigger permeability changes in RBC membranes and to induce cytotoxic events.[10] Such correlation was also maintained in several *in vivo* studies.[33-35]

The present study reveals that the equilibrium between the different species of AmB in aqueous media may change and, therefore, its overall activity or toxicity could be changed. The spectral evaluation of both AmB-DOC and AmB-DOC-H clearly demonstrates that the behavior of AmB molecules was modified. For example, the concentration dependence, characteristic of AmB-DOC, disappeared at the concentration of 5×10^{-7}M. In fact, at this concentration the AmB super-aggregated band, located at 323nm, was maintained, whereas the proportion of the monomeric form remained low (Figure 2). This suggests that the AmB molecules were strongly bound to the surfactant molecule and the micelle system has a greater strength than the unheated ones. Only at the lowest concentration, 5×10^{-8}M, both products present a similar spectrum in which the monomeric band has the same shape and similar intensity, reflected by almost the same value of ε.

The reservoir model hypothesis suggested for the super-aggregated form of AmB was demonstrated by the *in vitro* studies. When the target cells were human RBCs, AmB-DOC-H was unable to hemoglobin leakage over the whole range of

doses tested (Figure 4). This demonstrated that the existing aggregated formed at 5 x 10^{-6}M and 5 x 10^{-5}M was completely different from the AmB-DOC-H product. On the other hand, the activity of both products was quite similar when the cell model was a membrane containing ergosterol like in the *Candida parapisilosis* .

The statistical comparison among the different concentrations of AmB for both, AmB-DOC and AmB-DOC-H products, by the ANOVA test was significant ($p < 0.001$), which confirms that the dilution process induced a decreased on the pattern of activity for all samples. Additionally, the t student test showed that the toxicity assay against RBCs, represented by the release of K^+ and hemoglobin, was significantly different from AmB-DOC-H, which was much smaller than AmB-DOC ($p < 0.05$).

The probably mechanism behind this pattern of activity could be due to the high chemical stability of the AmB super-aggregates, which are less susceptible to the peroxidative process than the aggregated form and, therefore, present less affinity to membranes containing cholesterol than the unheated preparation.[8,22] Also,[8,12] this protection against the peroxidation process allow the AmB super-aggregated form act as a controlled release of the monomeric AmB specie , which is able to maintain its antifungal activity.[10] Therefore, as suggested in the literature, a complete resculpting of AmB aggregates probably occurs with the heat-inducing process, and this phenomenon induce changes in the AmB distribution and interaction with various serum fractions.[29]

A rationale mechanistic for the similar efficacy and lower toxicity of AmB-DOC-H than AmB-DOC previously proposed reports that AmB-DOC can be rapidly converted from its aggregated to form a protein-bound monomer in the presence of human serum albumin, whereas AmB-DOC-H demonstrates greater stability by

persisting as a stable inactive aggregate.[12] Moreover, the increased therapeutic index of the AmB-DOC-H also may be result from greater phagocytosis of the superagregates, owing to their larger size (600nm), which allows them to be efficiently engolfed by the macrophages and to be transferred to the site of infection in the case of internal cell infections like leishmaniosis.[19] On the other hand, it was also shown that the citotoxicity may be decreased due to a reduction the interaction of AmB-DOC-H with the kidney cell membranes.[36]

The benefits of the pre-heating process of AmB-DOC (Fungizon®) was also studied in persistently leukopenic mice with severe invasive candidiasis, concluding that higher dosages of AmB-DOC-H, 3 versus 0.8mg.L^{-1} of body weight, were tolerated than of AmB-DOC, resulting in significantly improved therapeutic efficacy.[37] In contrast, the cytotoxicity of AmB-DOC-H against pig kidney cells was evaluated by Bartlett et al who discovery a decrease on the AmB renal cytotoxicity without modifying its antifungal activity.[25] In the same way, Bau et al[38] showed the benefits of the patent-free heated AmB product to be used by public-health authorities or a reactive non-governmental organization for treatment of leishmaniasis[23] and other neglected diseases.

Other important studies were carried out with the AmB-DOC (Fungizon®) heated form. For example, a recent work evaluated the fluorescence of AmB aggregates for the heated and unheated form. The authors concluded that not only the monomer and dimmer AmB states, but also the aggregates and super-aggregates forms present different spectra.[39] Therefore, the fluorescence technique could also be used to characterize the AmB different state forms. On the other hand, Rogers et al studied the cytokine and chemokine response elicited by AmB-DOC-H in comparison to AmB-DOC in the human monocyte cell line THP-1.[24] They

concluded that AmB-DOC produced dose-dependent increases in interleukin (IL)-1β, IL-1α, tumor necrosis factor-α, macrophage inflammatory protein (MIP)-1α and MIP-1β and that AmB-DOC-H induced cytokine and chemokine production at a lower level than those observed with the corresponding concentrations of AmB-DOC.[24]

Conclusion

Heat treatment of Fungizon® was shown to modify the aggregation state of the AmB desoxicholate micellar system. The main physicochemical change on this system concerns the generation of an AmB super-aggregate specie in which its appearance is concomitant with the disappearance of both normal aggregates and monomeric AmB species.

Three decades ago Ernst et al[28] have shown the thermally induced increase in aggregate size of Fungizon® aqueous solution at 10^{-5}M. This phenomenon was more evidenced from 60^{o}C and increased rapidly at higher temperatures. In aqueous solution at 70^{o}C the apparent mass of the aggregates was 500-fold larger than the one at 20^{o}C.[20] Therefore, the term super-aggregate used for this new species of AmB is completely justified.[20]

All the results together suggest that the AmB-DOC-H, from a Brazilian industrial company, was able to reduce the toxicity of its unheated form (AmB-DOC) probably by changing the AmB aggregation state, similarly to the micelle systems produced abroad. Also, it demonstrates that a simple way to rebuilt micelles systems and generates new entities at a nanoscale domain is possible by simple heating. Therefore, we can speculate that this strategy open a way to create nanocarriers by changing the manufacturing parameters and process of micelles production.

In short, the heat treatment of Fungizon® solutions showed to be a simple and inexpensive alternative to treat patients with systemic fungal infections since AmB-DOC-H have demonstrate to be less toxic to mammalian cells while keeping its activity against fungal cells and protozoa microorganisms. Such statement is completely supported not only by the results here presented, but also by the set of results found on the literature, which was extensively discussed here.

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Disclosures

The author reports no conflicts of interest in this work.

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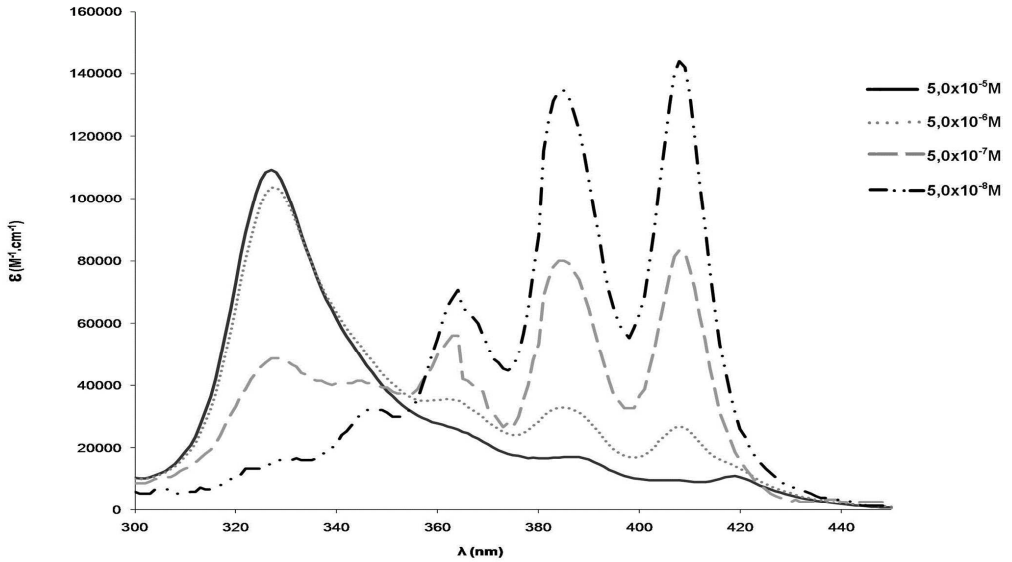

Figure 1 Concentration-induced changes in the AmB-DOC spectra at 25ºC at $5x10^{-5}$M.

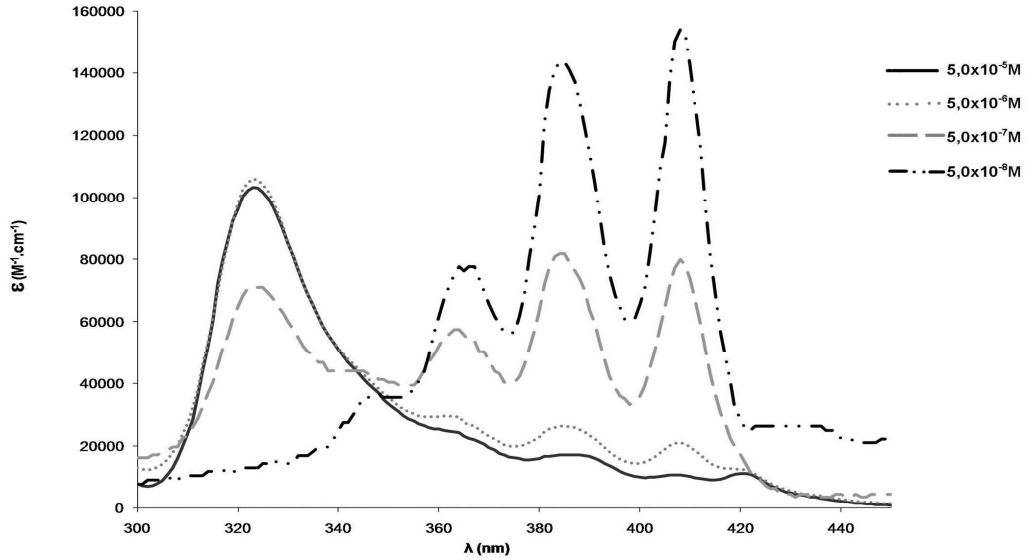

Figure 2 Concentration-induced changes in the AmB-DOC-H spectra at 25ºC at $5x10^{-5}$M.

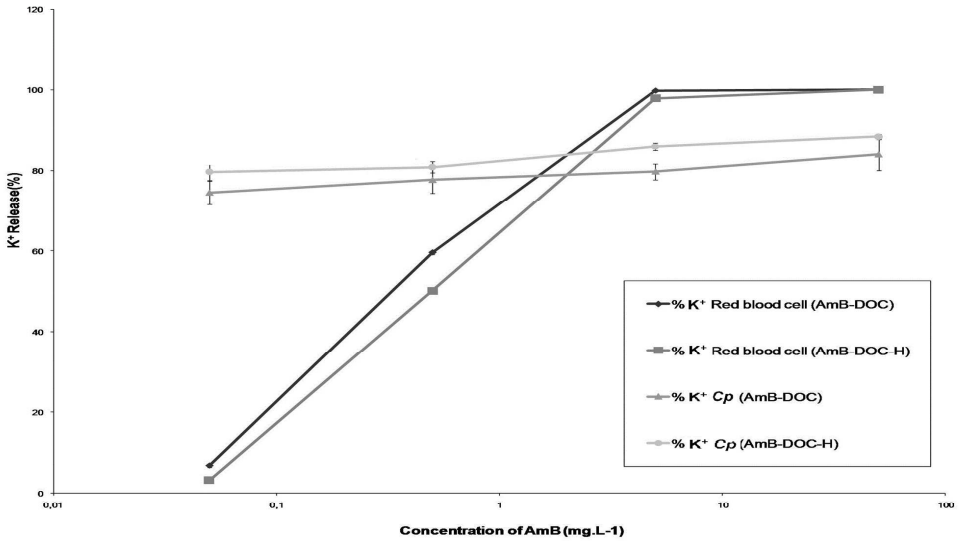

Figure 3 *In vitro* release of potassium from human RBCs and *C. parapsilosis* induced by AmB-DOC and AmB-DOC-H.

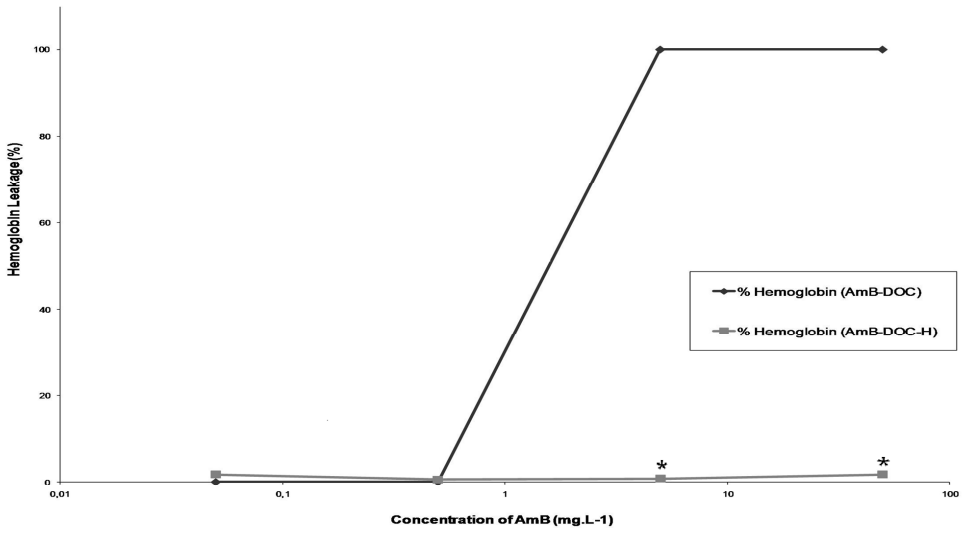

Figure 4 *In vitro* release of hemoglobin from human RBCs induced by AmB-DOC and AmB-DOC-H.

Notes: Each point on the figure is the mean (±SD) of three determinations. *Significant difference between both products ($p<0.001$).

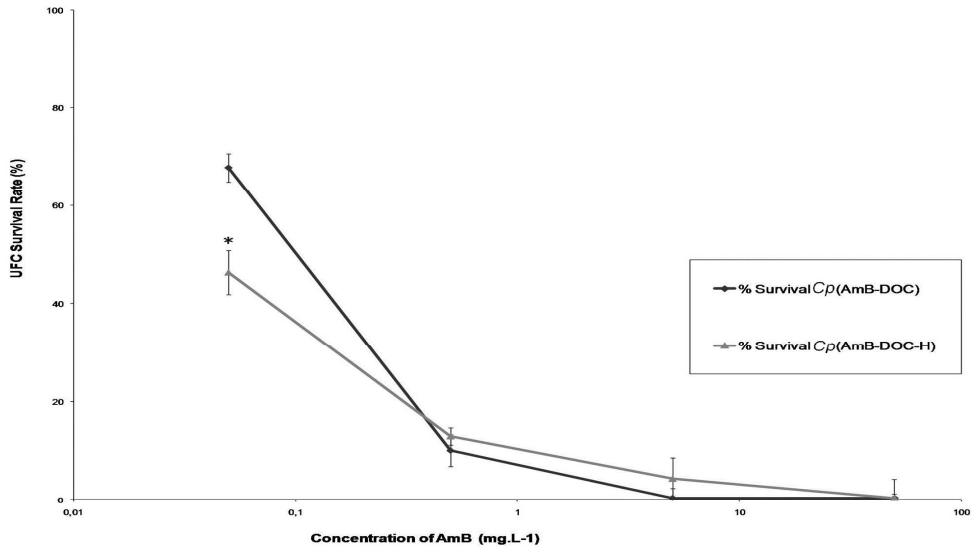

Figure 5 *In vitro* antifungal activity of AmB-DOC and AmB-DOC-H on *C. parapsilosis*.

Notes: Each point on the figure is the mean (±SD) of three determinations. *Significant difference between both products ($p<0.001$).

4 – COMENTÁRIOS, CRÍTICAS E SUGESTÕES

O desenvolvimento de novos sistemas de fármacos tem aumentado nos últimos anos. Inúmeros produtos têm sido desenvolvidos com a finalidade de melhorar o tratamento de doenças, principalmente no que diz respeito ao aumento da eficácia e redução da toxicidade.

O estudo destes produtos envolve uma grande quantidade de profissionais de áreas de atuação diferentes. Dentre eles podemos citar o farmacêutico, o médico, o químico, o biofísico, o biólogo e os engenheiros comprovando a multidisciplinaridade desse processo.

A anfotericina B é um fármaco que se enquadra nas necessidades expostas além de possuir propriedades físico-químicas particulares evidenciando a necessidade de desenvolvimento tecnológico de novas formulações, uma vez que este fármaco ainda apresenta eficientes resultados terapêutico sendo o maior obstáculo para o largo emprego clínico a sua toxicidade.

Este trabalho teve como objetivo a produção de superagregados de anfotericina B assim como a sua avaliação farmacotoxicológica *in vitro* por metodologia dominada pela nossa base de pesquisa e apresentada no artigo. Combinando isto com uma extensa revisão bibliográfica, nossa equipe, aprimorou o pensamento científico alinhado com a tecnologia de ponta apresentada por outros grandes pesquisadores nos permitindo uma melhor explicação sobre efeitos de atividade e toxicidade das diferentes formas de agregação da molécula assim como a sua relação com o desoxicolato de sódio ressaltando a diminuição dos parâmetros de toxicidade pelo tratamento de aquecimento controlado.

O desenvolvimento do mestrado nos permitiu obter novas idéias como o desenvolvimento do processo de produção de superagregados na apresentação de pó para re-solubilização, utilizando a tecnologia de liofilização, assim como estudos *in vivo* de fases I, II e III para efetiva utilização clínica desse novo sistema tanto para o tratamento de infecções fúngicas sistêmicas como para a leishmaniose. Sendo assim os próximos temas a serem abordados por nossa equipe de pesquisa que agora já possuímos conhecimento técnico-ciêntífico suficiente para o desenvolvimento destes novos projetos.

Particularmente, durante todo o meu mestrado obtive um amadurecimento científico que sou muito grato aos meus orientadores e que pretendo seguir crescendo e aprendendo a fazer pesquisa científica de ponta para melhor contribuir com o crescimento técnico-científico do nosso país. Também sou grato ao convívio que experimentei durante toda a minha formação acadêmica e durante o mestrado com todos do laboratório, evidenciado o espírito de companheirismo e familiar do nosso laboratório.

Pretendo ingressar no doutorado e continuar desenvolvendo as próximas fases deste projeto assim como possíveis novas oportunidades que o tema venha me propiciar para meu completo desenvolvimento científico e, em um futuro próximo, tornar-me um professor pesquisador para oferecer as mesmas oportunidades que me foram dadas.

5 – APENDICE

Produção bibliográfica

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7 – ABSTRACT

The aim of this work was to evaluate how an aqueous micellar system containing Amphotericin B (AmB) and sodium deoxycholate (DOC) can be rebuilt after heating treatment. Also a review of the literature about the new physicochemical and biological properties of this new system was carried out. Afterwards, heated (AmB-DOC-H) and unheated (AmB-DOC) micelles were subsequently diluted at four different concentrations (50mg.L^{-1}, 5mg.L^{-1}, 0.5mg.L^{-1} and 0.05mg.L^{-1}) to perform the physicochemical study and, then, the pharmacotoxicity assay, in which two cell models were used for the *in vitro* experiments, Red Blood Cells (RBC) from human donors and *Candida parapisilosis* (*Cp*). While potassium (K^+) and hemoglobin leakage from RBC were the used parameters to evaluate the acute and chronic toxicity, respectively, the efficacy of AmB-DOC and AmB-DOC-H were assessed by K^+ leakage and cell survival rate from *Cp*. The spectral study revealed a slight change on the aggregate peak from 327nm to 323nm for AmB-DOC-H compared to AmB-DOC. Concerning the toxicity, although AmB-DOC and AmB-DOC-H presented different behavior for hemoglobin leakage, AmB-DOC produced higher leakage than AmB-DOC-H at high concentrations (from 5mg.L^{-1}) with values tending to zero. However, concerning K^+ leakage, both AmB-DOC and AmB-DOC-H, showed similar profile for both cell models, RBC and *Cp* ($p<0,05$). AmB-DOC-H and AmB-DOC also revealed similar profile of activity against *Cp* with equivalent survival rate. In short, the AmB-DOC-H showed much less toxicity than AmB-DOC, but remained as active as the late one against fungal cell. Therefore, the results highlight the importance of this new procedure as a simple, inexpensive and safe alternative to produce a new kind of micelle system for treatment of systemic fungal infections.

Keywords: micelles; nanotechnology; pre-heated amphotericin B; superaggregates.