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AVALIAÇÃO DA TOXICIDADE E EFICÁCIA DE NANOEMULSÕES CONTENDO ANFOTERICINA B PARA O TRATAMENTO DA LEISHMANIOSE VISCERAL

Belo Horizonte – MG

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Tese apresentada ao Programa de Pós-Graduação em Ciências Farmacêuticas da Faculdade de Farmácia da Universidade Federal de Minas Gerais, como requisito parcial à obtenção do título de Doutor em Ciências Farmacêuticas.

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Dedico esta tese à minha família: meus pais, irmãos e sobrinhas.

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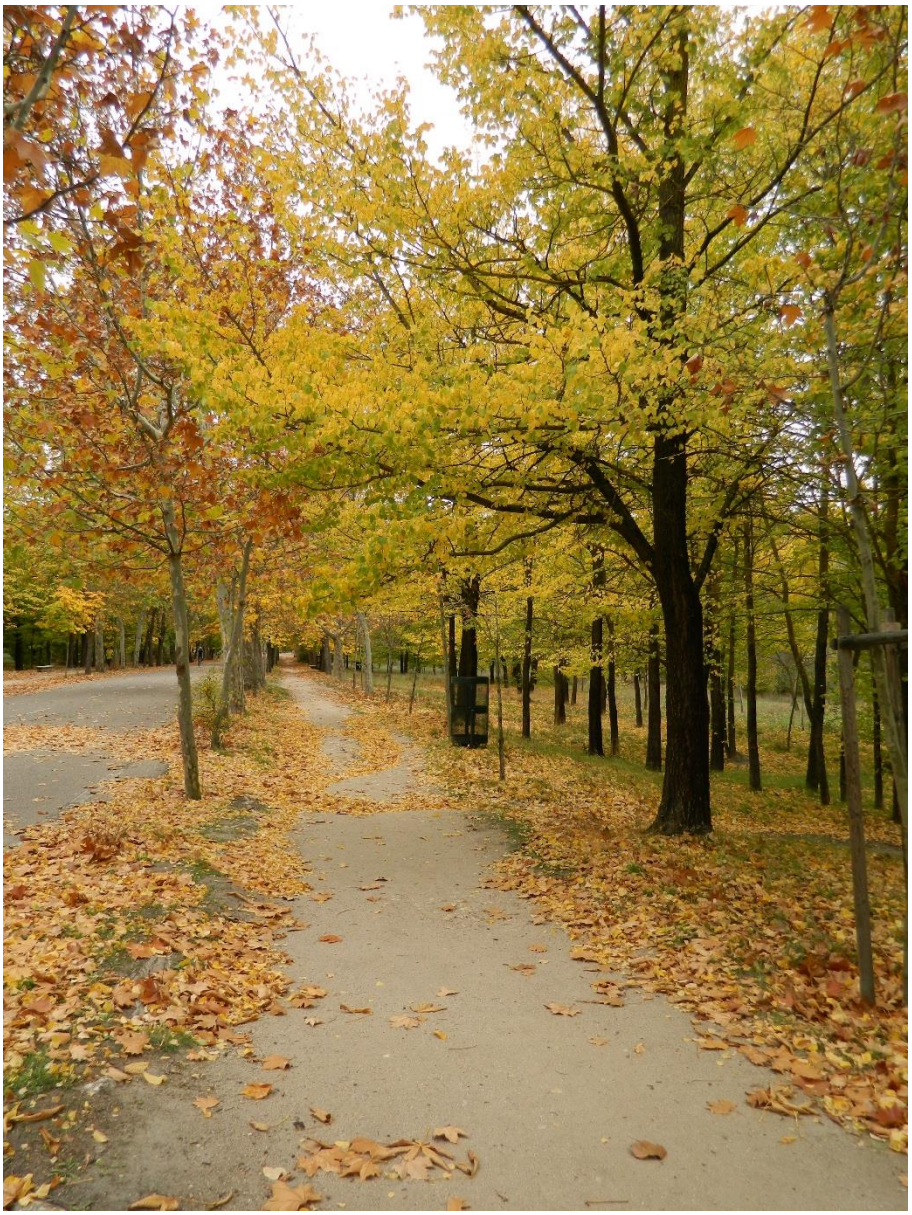
Fernando Teixeira de Andrade



RESUMO

A anfotericina B (AmB), fármaco de segunda escolha para o tratamento da leishmaniose visceral (LV), possui atividades antifúngica e antiparasitária de amplo espectro. No entanto, sua formulação convencional (C-AmB), a qual a AmB está associada ao desoxicolato de sódio, apresenta elevada toxicidade. Várias reações adversas estão associadas ao uso desta formulação, sendo a nefrotoxicidade e anemia as maiores limitações ao tratamento. Formulações lipídicas, como as nanoemulsões (NE), constituem uma alternativa interessante para contornar tais obstáculos. Assim sendo, este trabalho teve como objetivo otimizar, caracterizar, avaliar os efeitos tóxicos *in vitro* e *in vivo* e a atividade de NE contendo AmB (NE-AmB) em camundongos BALB/c experimentalmente infectados com *Leishmania (Leishmania) infantum chagasi.* A NE foi caracterizada pelo tamanho, índice de polidispersão (IP) e eficiência de encapsulação do fármaco (EE). A atividade hemolítica *in vitro* induzida pelas formulações de AmB foi avaliada pelo contato de células sanguíneas vermelhas. Os estudos de atividade em modelo murino de LV, a C-AmB e NE-AmB (1 ou 2 mg/kg/dia) foram administradas, por via intravenosa, em 5 dias alternados. Os estudos de toxicidade *in vivo* foram conduzidos em camundongos sadios, utilizando AmB (1 mg/kg) em 3 dias alternados. Análises bioquímicas (uréia nitrogenada (BUN) e creatinina) e histopatológicas foram realizadas, além das análises metabolômicas do plasma em 3 diferentes técnicas analíticas acopladas a espectrometria de massas (MS). Após aumentos gradativos na pressão no homogeneizador de alta pressão (HAP), a NE apresentou diâmetro dos glóbulos (~140 nm) e valores de IP < 0.2, inferiores aos obtidos anteriormente e EE próximo a 100%. A atividade hemolítica *in vitro* de C-AmB foi muito maior do que aquela observada para a NE-AmB. A NE-AmB e C-AMB, ambas a 1 mg/kg, reduziram significativamente a carga parasitária no fígado e baço em comparação com o controle, mas diferenças entre as duas formulações foram observadas somente quando a NE-AmB foi administrada a 2 mg/kg sem qualquer efeito agudo, ao contrário da C-AmB, a qual foi letal nessa dose. Os valores de BUN, creatinina e as análises histopatológicas não revelaram quaisquer alterações nas funções renais. Nas análises metabolômicas, observou-se que no grupo tratado com C-AmB, as vias metabólicas, como as

dos ácidos graxos poliinsaturados e do ácido araquidônico, foram as mais afetadas, sugerindo que a C-AmB pode estar associada a processos inflamatórios, stress oxidativo e isquemia renal. Estes dados demonstraram que a NE-AmB apresentou menor efeitos tóxicos *in vitro* e *in vivo* e apresentou eficácia *in vivo*, se constituindo, portanto, numa alternativa promissora para o tratamento da LV.

Palavras-chave: nanoemulsões, anfotericina B, leishmaniose visceral, metabolômica, efeitos tóxicos, atividade em modelo animal.

ABSTRACT

Amphotericin B (AmB), a second choice drug for the treatment of visceral leishmaniasis (VL), has a broad-spectrum of antifungal and antiparasitic activity. However, its conventional formulation (C-AmB), in which the AmB is associated to sodium deoxycholate, has high toxicity. Several adverse reactions have been linked to this formulation, like nephrotoxicity and anemia, and they are the major limitations for the treatment. Lipid formulations, such as nanoemulsions (NE), became an interesting alternative to overcome these drawbacks. Therefore, this study aimed to optimize, to characterize, to evaluate the *in vitro* toxic effects and to evaluate *in vivo* toxicity and activity of the AmB-loaded NE (NE-AmB) in BALB/c mice experimentally infected with *Leishmania (Leishmania) infantum chagasi*. The NE was characterized by the size, the polydispersity index (PI), and the drug encapsulation efficiency (EE). The hemolytic activity *in vitro* induced by AmB formulations was evaluated by their contact with red blood cells. The activity studies in a murine model of VL, C-AmB and NE-AmB (1 or 2 mg/kg/day) were administered intravenously on 5 alternate days. The *in vivo* toxicity studies were conducted in healthy mice, using AmB (1 mg/kg) on 3 alternate days. Biochemical analysis, blood urea nitrogen (BUN) and creatinine, and histopathology were carried out. Metabolomic analysis of the plasma on 3 different platforms coupled to mass spectrometry (MS) were also performed. After gradual increases in pressure in High Pressure Homogenizer (HAP), the NE had the droplet diameters (~ 140 nm) and PI values < 0.2, lower than those obtained previously and the EE close to 100%. The *in vitro* hemolytic activity of C-AmB was much higher than that observed for the NE-AmB. NE-AmB and C-AMB, both at 1 mg/kg, have reduced significantly the parasite burden in liver and spleen compared to the control, but differences between the two formulations were only observed when the NE-AmB was administered at 2 mg/kg without any sign of acute toxicity, unlike the C-AmB which proved to be lethal at this dose. BUN values, creatinine and histopathological analysis revealed no changes in kidney function. In metabolomics analysis, it was observed that the most affected metabolic pathways were the polyunsaturated fatty acids and arachidonic acid in the group treated with C-AmB, suggesting that this formulation may be associated with

inflammatory processes, oxidative stress and renal ischemia. These data showed that the NE-AmB was able to reduce the toxic effects *in vitro* and *in vivo* and to show efficacy *in vivo*, revealing, therefore, to be a promising alternative for the treatment of VL.

Keywords: nanoemulsions, amphotericin B, visceral leishmaniasis, metabolomics, toxic effects, activity in animal models.

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LISTA DE ABREVIATURAS E SIGLAS

3IS	3-indoxyl sulfate
9-HETE	9-Hydroxy-5E,7Z,11Z,14Z-eicosatetraenoate
ABLE	Emulsão lipídica de AmB
AEA	Arachidonoyl ethanolamide
AFM	Atomic force microscopy
AmB	Anfotericina B
AMDIS	Automated Mass spectral Deconvolution and Identification System
APCI	Atmospheric pressure chemical ionization
API-TOF	Atmospheric pressure inlet-time of flight
AUC	Area under the curve
BGE	Background electrolyte
BHT	Butylated hydroxytoluene
BSTFA	N,O-bis(trimethylsilyl) trifluoroacetamide
BUN	Blood urea nitrogen
C-AmB	Conventional Amphotericin B
CE	Eletroforese capilar
CE-MS	Eletroforese capilar-espectrometria de massas
Chol	Colesterol
Cr	Creatinine
DME_{50}	Dose média efetiva
DMPG	Dimiristoil fosfatidilglicerol
DPMC	Dimiristoil fosfatidilcolina

DSPG	Diestearoilfosfatidilglicerol
EE	Encapsulation efficiency
ESI	Ionização por electrospray
FBS	Fetal bovine serum
FDR	False discovery rate
FT-IR	Espectroscopia no infravermelho por transformada de Fourier
GC	Cromatografia em fase gasosa
GC-MS	Cromatografia gasosa-espectrometria de massas
GFR	Glomerular filtration rate
H&E	Hematoxylin and eosin
HMDB	Human Metabolome database
HPH	High-pressure homogenizer
HPLC	High performance liquid chromatography
HSPC	Fosfatidilcolina de soja hidrogenada
IP	Índice de polidispersão
IS	Internal standard
IV	Intravenosa
L.	Leishmania
LC	Leishmaniose cutânea
LCL	Leishmaniose cutânea localizada
LC-MS	Cromatografia líquida-espectrometria de massas
LMC	Leishmaniose mucocutânea
LPC	Lysoglycerophosphocholines
LPE	Lysoglycerophosphoethanolamines

LT	Leishmaniose visceral
LV	Leishmaniose visceral
MG	Monoglycerides
MS	Espectrometria de massas
NE	Nanoemulsão(s)
NE-AmB	Amphotericin B-loaded nanoemulsion
NIST	National Institute of Standards and Technology
NMR	Nuclear magnetic resonance
NTA	Nanoparticle tracking analysis
OMS	Organização Mundial da Saúde
Oxy-Hb	Oxygenated hemoglobin
PA	Glycerophosphates
Paho	Organização Panamericana da Saúde
PBS	Phosphate buffered saline
PC	Glycerophosphocholines
PCA	Principal components analysis
PCS	Photon correlation spectroscopy
PE	Glycerophosphoethanolamines
PI	Glycerophosphoinositols
PLA_2	Phospholipase A_2
PLS-DA	Partial Least Squares Discriminant Analysis
PPAR-ɣ	Peroxisome proliferator-activated receptor- ɣ
PS	Glycerophosphoserines
PUFA	Polyunsaturated fatty acids

QCs	Quality controls
QTOF	Quadrupole time-of-flight mass spectrometer
RBCs	Red blood cells
RES	Reticular endothelial system
S1P	C16 sphingosine-1-phosphate
SFM	Sistema fagocítico mononuclear
SPHK1	Sphingosine kinase 1
SUS	Sistema Único de Saúde
SUV	“Small unilamellar vesicals”
TCM / MCT	Medium chain triglycerides
TG	Triglycerides
TMCS	Trimethylchlorosilane
UV	Ultravioleta
WHO	World Health Organization

LISTA DE SÍMBOLOS

®	Marca registrada
µ	Micro
R^2	Coeficiente de determinação
Q^2	Coeficiente de previsibilidade

SUMÁRIO

1 INTRODUÇÃO

A anfotericina B (AmB), um dos fármacos utilizados para o tratamento das leishmanioses, é um antibiótico poliênico heptaeno e possui atividades antifúngica e antiparasitária de amplo espectro (CHATTOPADHYAY; JAFURULLA, 2011). Apresenta eficácia elevada e suas formulações estão sendo cada vez mais utilizadas e consideradas como os melhores tratamentos existentes contra a leishmaniose visceral (LV) (CHAPPUIS *et al.*, 2007; WASAN, 2009). No entanto, sua formulação convencional (C-AmB), a qual a AmB está associada ao desoxicolato de sódio, apresenta elevada toxicidade quando administrada por via endovenosa (DL_{50} = 1.4 mg/kg). Várias reações adversas estão associadas ao uso desta formulação, sejam relacionadas à infusão (formulação), sejam tardias (nefrotoxicidade e anemia) devido à toxicidade celular e tecidual dose dependente (FILIPPIN; SOUZA, 2006; BRIME *et al*., 2002).

Para reduzir essa toxicidade diversas formulações lipídicas foram desenvolvidas. Embora estas formulações apresentem menor incidência de reações adversas em comparação a C-AmB, seu uso clínico é ainda muito limitado devido ao seu alto custo e dificuldade de preparação. Assim, o desenvolvimento de novas formulações menos tóxicas e mais acessíveis à população ainda é uma questão em aberto.

As nanoemulsões (NE) têm sido utilizadas há anos na nutrição parenteral e como carreadora de fármacos lipofílicos devido a sua biocompatibilidade. Esses sistemas apresentam ainda potencial para reduzir a citotoxicidade de fármacos citotóxicos e de protegê-los da hidrólise e degradação enzimática em condições fisiológicas (ARAÚJO *et al*., 2011).

Recentemente, uma nova formulação de NE contendo AmB (NE-AmB) foi desenvolvida e mostrou uma excelente estabilidade (CALDEIRA *et al*., 2015). Além disso, a toxicidade induzida pela NE em macrófagos foi significativamente menor do que aquela observada para a C-AmB. Entretanto, essa formulação ainda não foi avaliada *in vivo*. Diante do exposto, o objetivo deste trabalho foi otimizar e caracterizar as NE-AmB, avaliar seus efeitos tóxicos *in vitro* e *in vivo* e sua atividade em animais experimentalmente infectados com *Leishmania (Leishmania) infantum chagasi*, um modelo murino da LV.

2 OBJETIVOS

2.1 Objetivo geral

Otimizar, caracterizar, avaliar os efeitos tóxicos e a atividade de NE contendo AmB em animais experimentalmente infectados com *Leishmania (L.) infantum chagasi.*

2.2 Objetivos específicos

- Preparar e avaliar as características físico-químicas das NE antes e após incorporação da AmB;
- Avaliar a influência da concentração do surfactante Tween 80 sobre o diâmetro dos glóbulos das NE;
- Avaliar a influência do método de preparo das NE sobre o diâmetro dos glóbulos e índice de polidispersão (IP);
- Avaliar a influência do método de incorporação da AmB sobre o diâmetro dos glóbulos e do IP;
- Avaliar a ação hemolítica *in vitro* das NE contendo AmB;
- Comparar as alterações bioquímicas, ureia (BUN) e creatinina, e histopatológicas entre as NE contendo AmB e C-AmB em modelo animal;
- Comparar as alterações metabólicas associadas à nefrotoxicidade entre as NE contendo AmB e C-AmB em modelo animal;
- Avaliar a atividade das NE contendo com AmB, administradas por via endovenosa, em animais experimentalmente infectados com *Leishmania (L.) infantum chagasi*, em diferentes regimes de administração.

3 REVISÃO DA LITERATURA

3.1 Leishmanioses

As leishmanioses constituem um grupo de doenças tropicais negligenciadas consideradas como um grande problema de saúde pública mundial. Elas são classificadas em duas formas principais, leishmaniose tegumentar (LT) e leishmaniose visceral (LV) ou calazar (BERMAN, 2006; CARNEIRO *et al*., 2012).

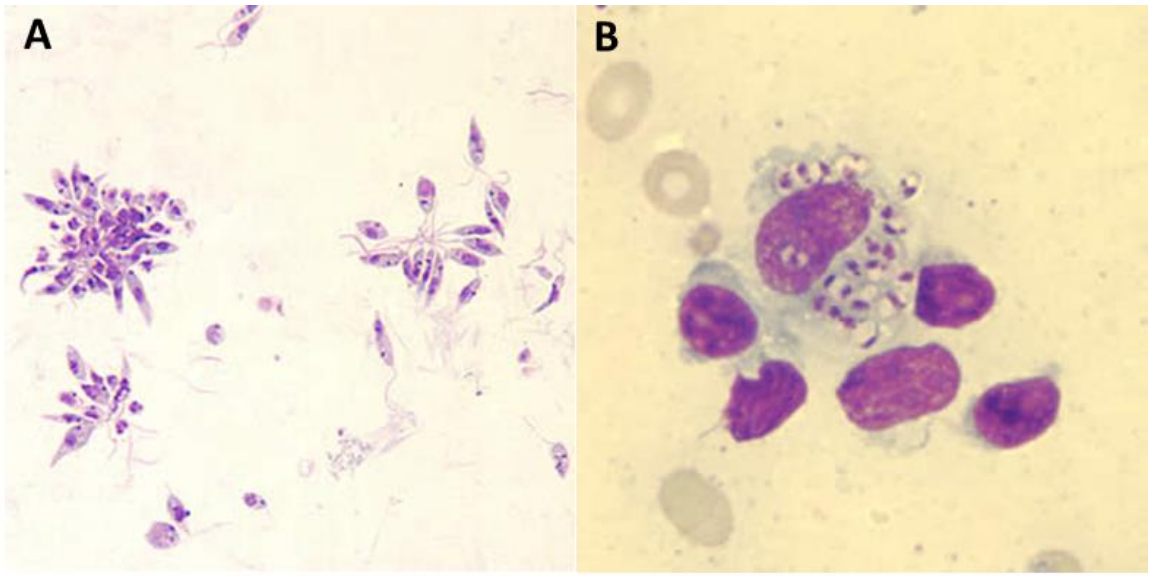
Atualmente, existe uma estimativa de doze milhões de pessoas infectadas em todo o mundo, 350 milhões sofrem risco de infecção e, aproximadamente, dois milhões de novos casos surgem, sendo que 20000 a 30000 mortes ocorrem anualmente devido a LV (ALVAR *et al*., 2012; DESJEUX, 2004; WHO, 2016, MCCALL *et al*., 2013). Sua prevalência se dá principalmente em áreas tropicais e subtropicais e são endêmicas em mais de 98 países do Velho Mundo (África, Ásia e sul da Europa) e do Novo Mundo (América Latina) (AMEEN, 2010; GOTO; LINDOSO, 2010; MOUGNEAU *et al*., 2011; SANTOS *et al*., 2008).

As leishmanioses são doenças causadas por protozoários intracelulares obrigatórios pertencentes à ordem *Kinetoplastida,* família *Trypanosomatidae,* gênero *Leishmania.* Eles parasitam células do sistema fagocitário mononuclear humano (SFM) e de outros mamíferos, especialmente macrófagos (MATLASHEWSK, 2001). A doença é transmitida pela picada de flebotomíneos fêmeas infectadas pertencentes aos gêneros *Phlebotomus spp.* no Velho Mundo e *Lutzomya spp.* no Novo Mundo (STOCKDALE; NEWTON, 2012; SANTOS *et al*., 2008). As leishmanioses podem ser causadas por mais de vinte espécies de *Leishmania* e podem ser transmitidas aos humanos por mais de trinta espécies diferentes de flebotomíneos (AMEEN, 2010; CHAPPUIS *et al*., 2007). Existem, aproximadamente, 70 espécies de animais, dentre elas o homem, que são reservatórios naturais do protozoário (WHO, 2016).

Os parasitas apresentam duas formas evolutivas principais: a promastigota, forma flagelada e a amastigota, forma arredondada com flagelo intracelular (Figura 1). As formas promastigotas são encontradas no intestino do

vetor e diferenciam-se em promastigotas metacíclicas infecciosas, as quais migram para a parte anterior do tubo digestivo do flebotomíneo. Ao picar o hospedeiro, os parasitas são fagocitados pelos macrófagos e transformam em formas amastigotas intracelulares, onde se multiplicam nos vacúolos parasitóforos. As amastigotas podem posteriormente infectar outros macrófagos ou serem ingeridos por outro vetor. No intestino dos flebótomos, as formas amastigotas se diferenciam em promastigotas, completando o ciclo do parasita (Figura 2) (KAYE; SCOTT, 2011).

Figura 1: (A) *Leishmania* - forma promastigota. (B) *Leishmania* - forma amastigota. (Adaptado: http://www.dpd.cdc.gov/dpdx)



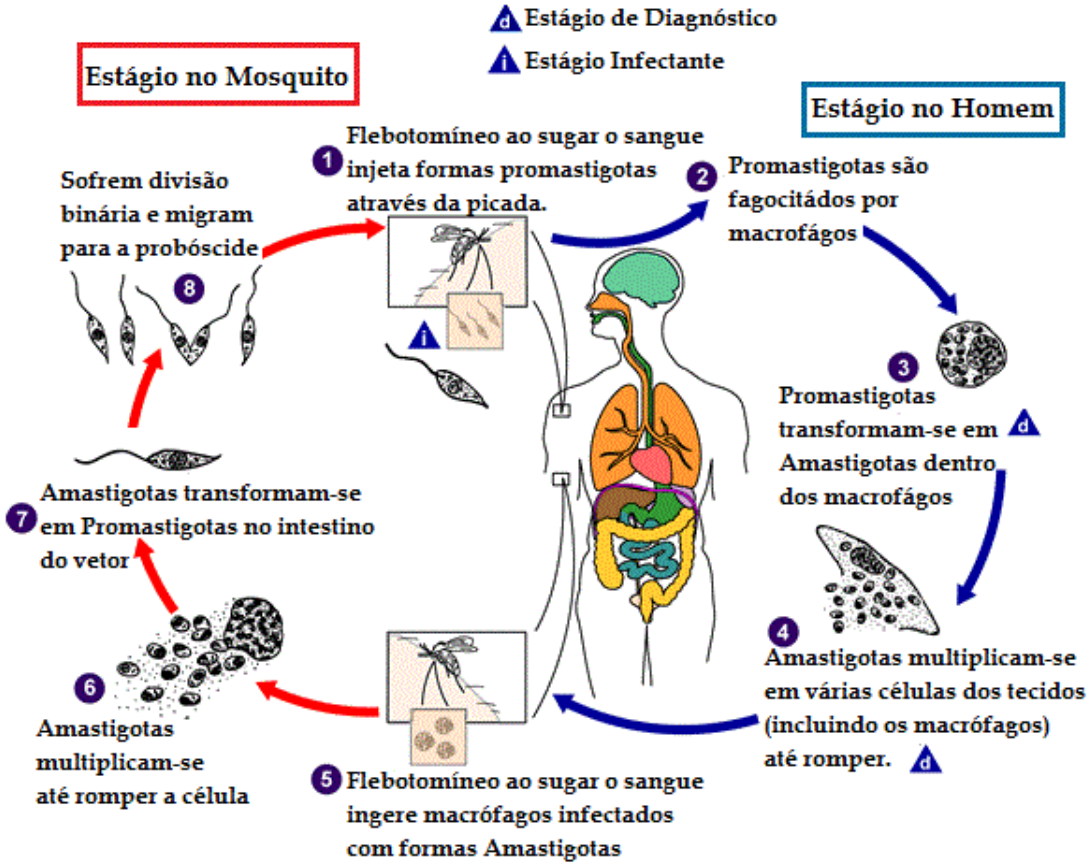
3.1.1 Leishmaniose Tegumentar

Dependendo da espécie infectante de *Leishmania* e da resposta imune mediada por células T dos indivíduos infectados, um espectro de formas clínicas da doença pode se desenvolver (SILVEIRA *et al*., 2009). A leishmaniose tegumentar (LT) em humanos é geralmente dividida em leishmaniose cutânea localizada (LCL) ou leishmaniose cutânea (LC), leishmaniose cutânea difusa (LCD) e leishmaniose mucocutânea (LMC) (REINTHINGER *et al*., 2007).

A incidência mundial da LC varia de 0,7 a 1,2 milhões de novos casos a cada ano, sendo o Afeganistão, Argélia, Colômbia, Brasil, Irã, Síria, Etiópia,

Sudão, Costa Rica e Peru, países responsáveis por 70 a 75 % desses novos casos. Nas Américas, a incidência estimada varia de 187.200 a 307.800 casos, dos quais 38,9% ocorrem no Brasil, embora a notificação oficial não exceda 30.000 casos anuais no país (ALVAR *et al*., 2012; GOTO; LINDOSO, 2010; HEPBURN, 2000; OKWOR; UZONNA, 2016).

Figura 2: Ciclo biológico da Leishmania (Adaptado: http://www.dpd.cdc.gov/dpdx)



As três principais espécies de *Leishmania* responsáveis pela LT no Brasil são a *Leishmania (Viannia) braziliensis*, espécie mais prevalente, seguida pela *Leishmania (Leishmania) amazonensis* e *Leishmania (Viannia) guyanensis* (BARRAL-NETO *et al*., 1995; GOTO; LINDOSO, 2010).

A LC desenvolve uma lesão frequentemente caracterizada como uma úlcera circular e indolor, bem delimitada, com crosta central que, às vezes, é

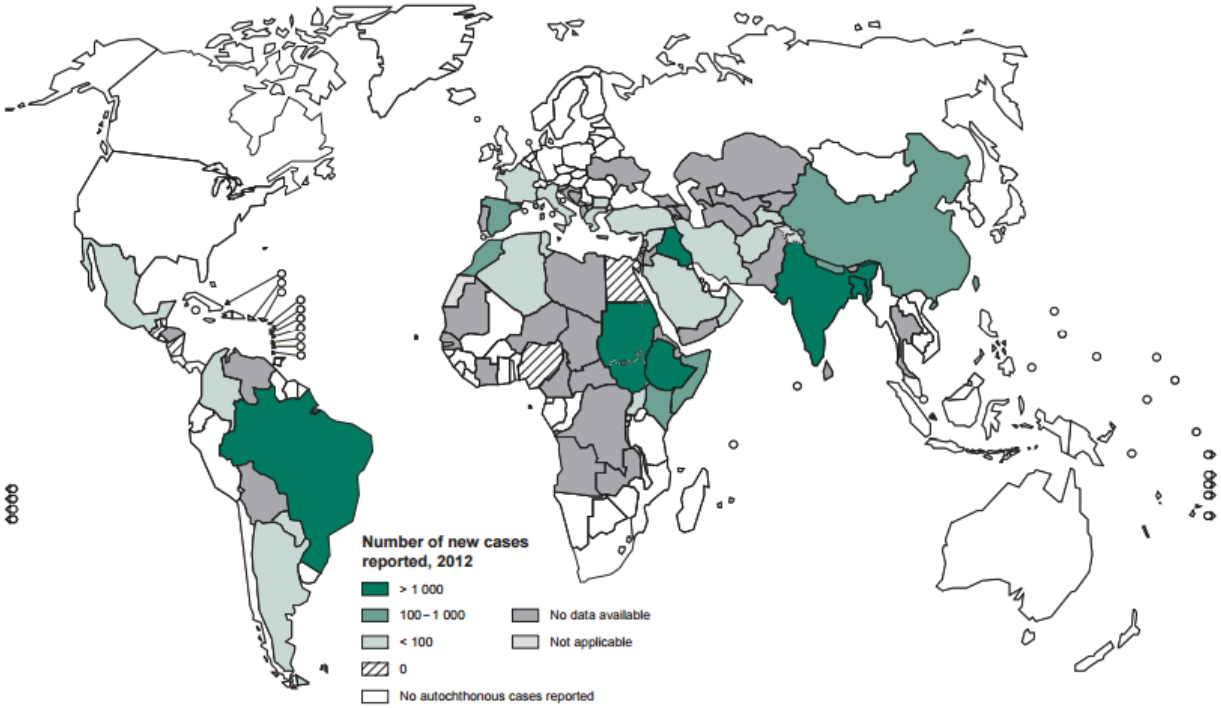
hemorrágica. A LCD é caracterizada pela presença de lesões nodulares, ricas em parasitas, que não ulceram e podem se espalhar por todo o corpo do paciente. A LMC ocorre anos após o início da LC em 5% dos casos e é caracterizada pela destruição das cavidades oral, nasal e faríngea, podendo evoluir para lesões desfigurantes (GOTO; LINDOSO, 2010).

3.1.2 Leishmaniose Visceral

A prevalência mundial da leishmaniose visceral (LV) é estimada em 12 milhões de casos. A cada ano, quinhentos mil deles surgem e cinquenta mil mortes ocorrem por essa forma da doença. Aproximadamente 90 % dos casos ocorrem em zonas rurais e áreas suburbanas de 6 países: Bangladesh, Etiópia, Brasil, Índia, Sudão e Sudão do Sul (ALVAR *et al*., 2012; CHAPPUIS *et al*., 2007; DESJEUX, 2004). A Figura 3 apresenta a distribuição da LV no mundo, sendo que as áreas em verde-escuro são consideradas zonas endêmicas.

A LV é causada por espécies do gênero *Leishmania,* pertencentes ao complexo *Leishmania (Leishmania) donovani* no leste da África e no subcontinente indiano e *Leishmania (Leishmania) infantum* na Europa, norte da África e América Latina. No Brasil, o agente etiológico é a *Leishmania (Leishmania) chagasi*, espécie semelhante à *Leishmania (Leishmania) infantum* (CHAPPUIS *et al*., 2007; GONTIJO; MELO, 2004). Entretanto, com base em diferentes abordagens, as subespécies *L. infantum* e *L. chagasi* não são claramente distinguidas (MAURÍCIO *et al*., 1999). Portanto, torna-se interessante adicionar o nome *L. chagasi*, entre parênteses, após o nome de *L. infantum*, quando nos referirmos ao parasita isolado desta área (DANTAS-TORRES, 2006).

Figura 3: Distribuição da LV no mundo (WHO, 2014).



Dependendo da característica de transmissão, dois tipos de LV são descritos. Na forma zoonótica, causada principalmente pela *L. infantum*, o cão é o principal reservatório. Ela ocorre, principalmente, na Bacia do Mediterrâneo, China, Oriente Médio e América do Sul. A forma antroponótica, a qual ocorre apenas em regiões mais povoadas, é caracterizada pela transmissão entre humanos, sem o animal como reservatório e é causada principalmente pela *L. donovani.* Ela prevalece na África Oriental, Bangladesh, Índia e Nepal (MONGE-MAILLO; LÓPEZ-VÉLEZ, 2013).

A co-infecção com o HIV e *Leishmania* tem sido descrita em mais de 35 países. Inicialmente, a maioria dos casos foi detectada na parte sudoeste da Europa, mas o número de casos está aumentando na África Sub-Sahariana (particularmente na Etiópia), no Brasil e no Sul da Ásia. A co-infecção na Índia ainda não parece ser um problema grave e ocorre em menos de 2% dos doentes com LV (MONGE-MAILLO; LÓPEZ-VÉLEZ, 2013; SUNDAR; CHAKRAVARTY, 2013).

Os pacientes com LV apresentam sinais e sintomas de infecção sistêmica persistente, incluindo febre, fadiga, fraqueza, perda de apetite e perda de peso. Ocorre invasão do parasita no sangue e no SFM, aumentando o tamanho de linfonodos, baço e fígado. À medida que a doença avança, pode ocorrer agravamento da esplenomegalia, causando distensão abdominal e, algumas vezes, agravamento concomitante da hepatomegalia (CHAPPUIS *et al*., 2007).

3.1.3 Tratamento das leishmanioses

O tratamento das leishmanioses depende de vários fatores, como a forma da doença, da espécie e da localização geográfica. Os tratamentos são possíveis e garantem a cura, se forem bem executados. Todos os pacientes diagnosticados com LV requerem a administração imediata de um tratamento completo (WHO, 2016).

A terapia convencional inclui a administração parenteral de antimoniais pentavalentes como o estibogluconato de sódio (Pentostam®; Wellcome Foundation, Londres) e o antimoniato de N-metilglucamina (Glucantime®; Aventis, Brasil). A maioria dos estudos relata taxas de cura superiores a 90 %. No entanto, este regime é acompanhado pelo inconveniente de um tratamento parenteral longo (injeção intravenosa ou intramuscular administrada diariamente, num período de 20-40 dias) e o surgimento de resistência significativa, principalmente na Índia, onde seu uso foi indiscriminado (AMEEN, 2010; SUNDAR; CHAKRAVARTY, 2015). Além disso, a toxicidade relativamente frequente é manifestada por mialgias, artralgias, dores de cabeça, inapetência, febre, vômitos, tontura, inchaço no local da aplicação, pancreatite química, hepatoesplenomegalia e diminuição do nível de plaquetas (CROFT, 2003; HEPBURN, 2003; GONTIJO; CARVALHO, 2003). A cardio, nefro e hepatoxicidade dos antimoniais constituem uma importante limitação à sua segurança. Idealmente os pacientes, em especial os mais idosos, devem ser submetidos à avaliação cardiológica prévia. Por serem abortivos, os antimoniais não podem ser administrados a gestantes (GONTIJO; CARVALHO, 2003).

Nos casos em que o tratamento preconizado com antimoniais pentavalentes não é efetivo ou não pode ser empregado, agentes de segunda

escolha podem ser empregados, tais como anfotericina B (AmB), pentamidina e miltefosina (SANTOS *et al*., 2008).

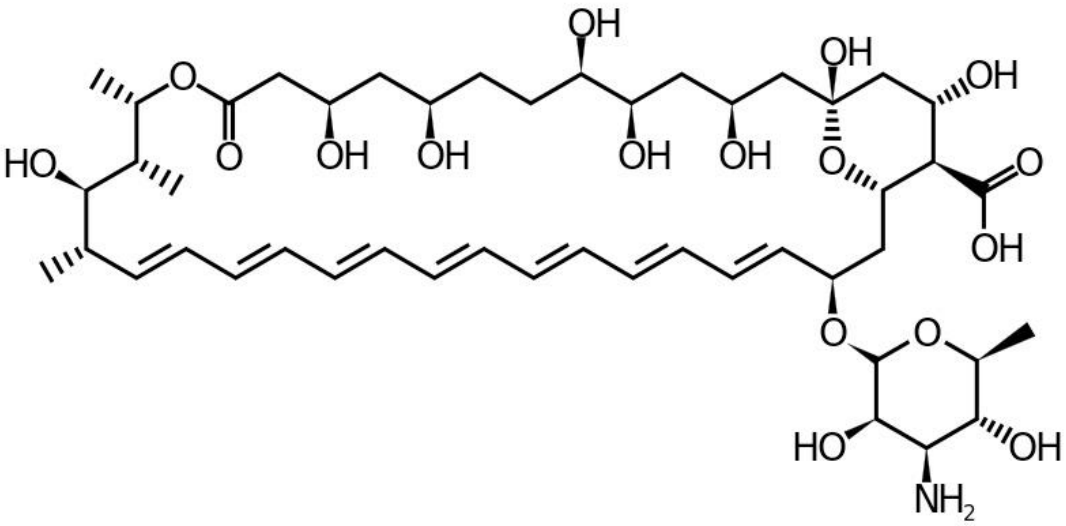
A Organização Panamericana da Saúde recomenda, como tratamento de primeira escolha para a LT, a utilização de antimoniais pentavalentes. Em relação à LV, a recomendação é que o tratamento seja realizado com o uso dos antimoniais ou da AmB (lipossomal ou C-AmB) (PAHO, 2013). Em 2013, o Ministério da Saúde ampliou a indicação do uso da AmB lipossomal para o tratamento de pacientes diagnosticados com LV no Brasil, segundo alguns critérios. Esta medida, além de diminuir os efeitos adversos causados por outros tratamentos, garante o acesso de um maior número de pessoas ao medicamento, que é ofertado gratuitamente pelo Sistema Único de Saúde (SUS).

3.2 Anfotericina B (AmB)

A AmB (Figura 4) é um antibiótico poliênico heptaeno, isolado primeiramente em 1955 a partir de culturas de *Streptomyces nodosus* da Venezuela. Possui atividades antifúngica e antiparasitária de amplo espectro (CHATTOPADHYAY; JAFURULLA, 2011). A AmB e suas formulações são cada vez mais utilizadas, sendo consideradas como um dos melhores tratamentos existentes para a LV, apresentam uma taxa de cura de cerca de 97%, sem relatos de resistência (CHAPPUIS *et al.*, 2007; WASAN, 2009).

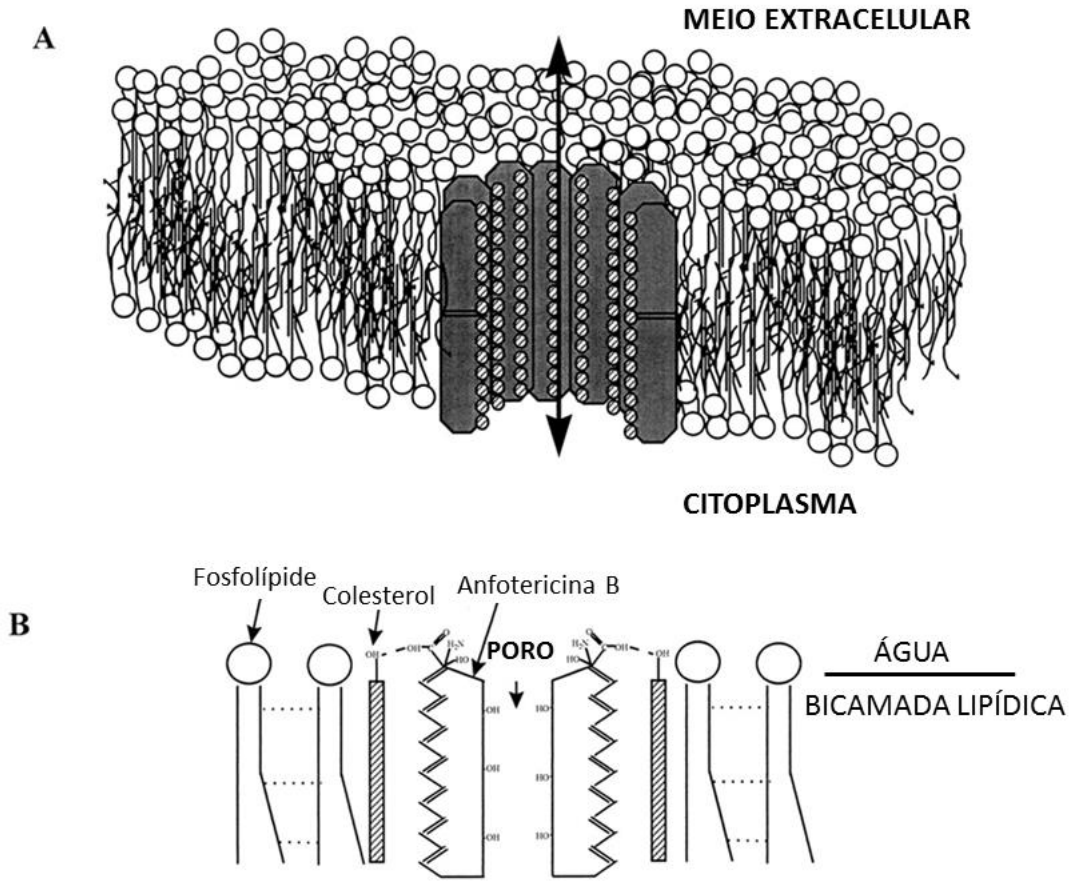
A AmB apresenta características hidrofóbicas, em decorrência da parte apolar de sua molécula, caracterizada pela presença de sete duplas conjugadas carbono-carbono; e características hidrofílicas, decorrentes da presença de inúmeros grupos hidroxila. Além disso, ela possui os grupos carboxílico e amino, que lhe conferem propriedades anfifílicas (DAMASCENO, 2010), sendo solúvel em água em pH abaixo de 2 ou acima de 11. A solubilidade da AmB em água em pH fisiológico é muito baixa (TORRADO *et al*., 2008). Essa insolubilidade da AmB em água leva à formação de agregados em concentrações definidas (monômeros, oligômeros e agregados de oligômeros) (EGITO *et al*., 2002; TORRADO *et al*., 2008).

Figura 4: Fórmula estrutural da AmB (Adaptado: CHATTOPADHYAY; JAFURULLA, 2011).



A atividade leishmanicida da AmB é devida a sua capacidade de interagir com o ergosterol, constituinte da membrana celular de fungos ou espécies de *Leishmania*. Esta interação produz poros nas membranas e altera a permeabilidade celular seletiva com consequente extravasamento de íons (principalmente íons potássio) e substâncias vitais (Figura 5A e B). Distúrbios na atividade enzimática das membranas também ocorrem, provocando a morte celular (GOLENSER; DOMB, 2006). A AmB também se liga ao colesterol, presente na membrana celular dos mamíferos, causando os principais efeitos adversos (LABORÍN; VARGAS, 2009; PAILA *et al.*, 2010).

Figura 5: Mecanismo de ação da AmB (Adaptado: GHANNOUM; RICE, 1999)



Dentre as reações adversas, incluem-se: febre, calafrios, tremores, náuseas, vômitos, cefaléia, hipotensão, hipertensão, arritmias cardíacas, hipocalemia, hipernatremia, diurese aumentada, hipomagnesemia, efeitos tóxicos sobre a medula óssea, hemólise e disfunção renal (FILIPPIN; SOUZA, 2006). A ação hemolítica e a nefrotoxicidade podem ser extremas em alguns casos e podem determinar, muitas vezes, a interrupção do tratamento (JAIN *et al*., 2012).

A insolubilidade da AmB em água limita sua utilização por via oral, devido à baixa biodisponibilidade. Portanto, a administração deve ser realizada por via endovenosa. Este fármaco tornou-se comercialmente disponível como Fungizone® (Bristol-Myers Squibb, EUA) e Anforicin® (Cristália, Brasil), na forma de uma dispersão coloidal na qual o sal biliar desoxicolato de sódio é utilizado

como agente solubilizante, formando um complexo micelar com a AmB (VYAS; GUPTA, 2006; LABORÍN; VARGAS, 2009). Entretanto, a administração desta formulação convencional está associada com elevada incidência de reações adversas (TORRADO *et al*., 2008; VYAS; GUPTA, 2006).

O comportamento desta formulação convencional em solução aquosa é importante para a determinação da interação da AmB com as membranas celulares e, consequentemente, para a compreensão dos seus efeitos adversos. Em água, a AmB se dissocia parcialmente do desoxicolato, e a AmB “livre” forma uma mistura de monômeros solúveis em água e oligômeros insolúveis em vários estágios de agregação. As várias espécies químicas podem interagir com os esteróis da membrana de diferentes maneiras provocando alterações em sua permeabilidade. Estas alterações, em seguida, levam à disfunção celular e, eventualmente, à destruição e morte celular (BRAJTBURG; BOLARD, 1996).

O mais provável é que as células fúngicas e das Leishmanias sejam mais sensíveis à forma monomérica da AmB em relação as células dos mamíferos. O que explica o maior dano a estas células em relação às células de mamíferos refere-se ao tipo de esterol incorporado nas suas membranas. O ergosterol é encontrado em membranas de células fúngicas e de parasitas, e o colesterol é encontrado em células de mamíferos, sendo que a AmB apresenta maior afinidade pelo ergosterol (BRAJTBURG; BOLARD, 1996). Já, a forma agregada da AmB é a principal responsável pelos efeitos adversos, interagindo sem distinção com o ergosterol e com o colesterol, propiciando maior citotoxicidade em mamíferos, principalmente nos rins e hemácias (JAIN *et al.*, 2012). Acredita-se que a AmB também possa atuar como um pró-oxidante nas membranas celulares, causando stress oxidativo associado com a formação de intermediários de radicais livres (OSAKA *et al.*, 1997).

A nefrotoxicidade da AmB é caracterizada por disfunção renal, incluindo redução da filtração plasmática e aumento da atividade da lactato desidrogenase urinária, acompanhada por dano tubular renal. Histopatologicamente, isto é associado à necrose tubular na parte ascendente espessa da alça de Henle, como principal característica da nefrotoxicidade da AmB em camundongos (KONDO *et al.*, 2012; DERAY, 2002). Os túbulos são especialmente susceptíveis a lesão devido às seguintes razões: primeiramente, elevadas concentrações de substâncias tóxicas filtradas estão presentes no fluido tubular, devido à sua

função na reabsorção de água e solutos. Em segundo lugar, as células epiteliais tubulares têm um grande número de transportadores, resultando em elevadas concentrações intracelulares de substâncias tóxicas. E, em terceiro lugar, estas células são energeticamente requisitadas no transporte e metabolismo de solutos (BOUDONCK *et al.*, 2009).

PAHO "guidelines" (Organização Panamericana da Saúde) estabeleceram a AmB desoxicolato (1 mg/kg/dia IV, durante 14 dias dose total de 800 mg) como fármaco de primeira linha para o tratamento da LV nas Américas (PAHO, 2013). Já Organização Mundial da Saúde (OMS) considera a AmB desoxicolato (0,75 - 1 mg/kg/dia IV) como fármaco de terceira escolha para LV (WHO, 2016).

3.2.1 Sistemas lipídicos contendo AmB

As formulações lipídicas de AmB (Ambisome®, Amphocil® ou Amphotec®, Abelcet® e ABLE®) (Tabela 1) constituem uma alternativa atraente em relação a formulação convencional (AmB desoxicolato), já que esses sistemas apresentam uma maior afinidade pelas células do sistema fagocítico mononuclear (SFM), tendendo a se acumularem nos vacúolos parasitóforos das células do parasita. Além disso, apresentam eficácia similar e menor toxicidade, conforme será discutido abaixo (BEKERSKY *et al.* 2002a; GOTO; LINDOSO, 2010; YARDLEY; CROFT, 1997).

Tabela 1: Preparações comerciais de AmB

Formulação	**Estrutura**	**Tamanho das partículas (μm)**	**Composição**
AmBisome®	Lipossomas SUV	0.08	HSPC-Chol-DSPG-AmB (2:1:0,8:0,4)
Amphotec® (Amphocil®)	Dispersão coloidal (forma de disco)	≈ 100 nm (diâmetro) x < 10 nm (espessura)	Colesterilsulfato de sódio- AmB (1:1)
Abelcet®	Complexo lipídico (forma de fita)	1 – 10	DPMC- DMPG-AmB (7:3:3)
ABLE®	Emulsão lipídica	<1	Óleo de soja-lecitina de ovo-AmB 20:1.2:0.5

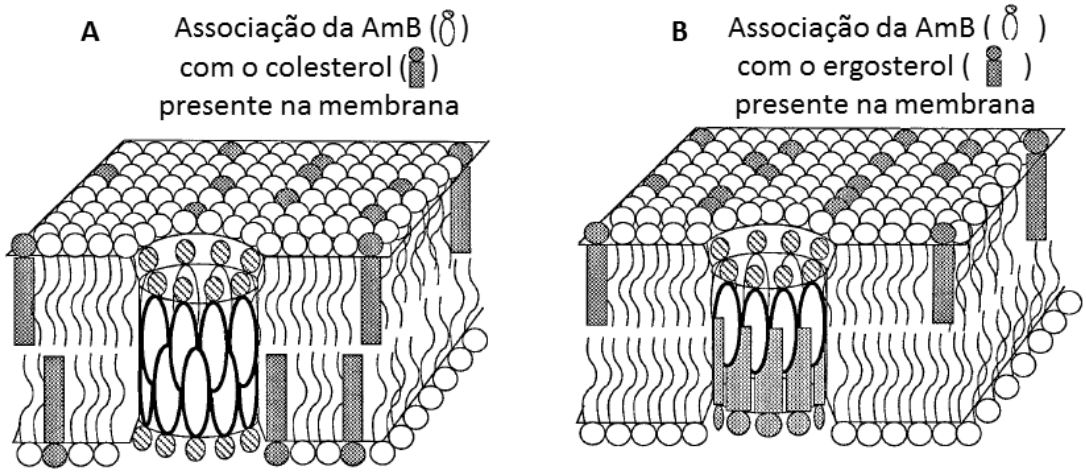
Atualmente, o Ambisome® é a formulação lipídica mais frequentemente usada para o tratamento das Leishmanioses. O Ambisome®, um sistema lipídico

lipossomal, é capaz de interagir fortemente com as moléculas de AmB ancorando-a fortemente na bicamada lipídica devido a interações favoráveis com os lípides. Essa forte associação é responsável pela liberação lenta da AmB para a corrente sanguínea, modificando sua biodistribuição. Isto possibilita a redução da sua captação por células renais e, consequentemente, ocorre diminuição da nefrotoxicidade (IMAN *et al*., 2011; ROMERO; MORILLA, 2008; VYAS; GUPTA, 2006).

PAHO “guidelines” estabeleceram a AmB liposomal (3-5 mg/kg/dia IV por 3-6 dias, dose total dose de 20 mg) como uma das opções de tratamento para LV de primeira linha nas Américas (MONGE-MAILLO; LÓPEZ-VÉLEZ, 2013; PAHO, 2013).

A redução da toxicidade das formulações lipídicas contendo AmB é baseada no fato de que apenas a forma livre danifica as células (Figura 6). Neste modelo, a formação de canais que atravessam as membranas requer mais moléculas de AmB quando o colesterol está presente na membrana (Figura 6A) em comparação com membranas contendo ergosterol (Figura 6B). Em ensaios utilizando membranas celulares intactas, apenas moléculas livres da AmB danificaram membranas de mamíferos, as quais possuem o colesterol como componente. Por outro lado, as formas livres e monoméricas da AmB podem danificar as células fúngicas ou parasitárias, cujas membranas são constituídas por ergosterol ou seus precursores (BRAJTBURG; BOLARD, 1996).

Figura 6: Modelos ilustrativos das possibilidades de formação de canais pela interação da AmB com os esteróis de membranas. A) Membrana contendo colesterol; B) Membrana contendo ergosterol (Adaptado: BRAJTBURG; BOLARD, 1996).



As fortes interações entre AmB e os lípides previne a liberação da AmB na forma agregada e, então, reduz a toxicidade para células de mamíferos. Além disso, o reduzido tamanho das partículas que contém o fármaco diminui o seu nível de absorção por fagocitose. Provavelmente, esses dois fatores se combinam para propiciar baixos níveis de toxicidade às formulações lipídicas (LARABI *et al.,* 2003).

O desenvolvimento de emulsões injetáveis de baixo custo e menor toxicidade do que a formulação convencional constitui uma alternativa atraente para reduzir os efeitos tóxicos da AmB. A mistura do Fungizone® com emulsões lipídicas de uso parenteral (Lipofundin® e Intralipid®) merece ser citada como um dos exemplos desta modalidade (ARAÚJO *et al*., 2005; EGITO *et al*., 2002; SAHDKHAN *et al*., 1997). Este procedimento, comum na prática hospitalar, tem seus méritos, no entanto, a falta de padronização da técnica constitui uma desvantagem. Estudos de estabilidade física demonstraram separação de fases destas emulsões, indicando que a AmB não foi adequadamente inserida na interface da emulsão (WALKER *et al*., 1998). A diminuição na toxicidade pode ser explicada pela inserção das moléculas de AmB na interface da emulsão e posterior liberação lenta dos monômeros do fármaco (EGITO *et al*., 2002).

Considerando a diminuição da toxicidade da mistura AmB com emulsões lipídicas de uso parenteral, uma emulsão lipídica de AmB (ABLE) foi desenvolvida na Índia, cujo tamanho das partículas é inferior a 1 μm. Nos ensaios clínicos diferentes doses foram administradas intravenosamente, resultando numa redução da taxa de reações adversas relacionadas à infusão e vômitos. Além disso, nenhuma nefrotoxicidade ou toxicidade em outro órgão foi observada. No final do tratamento, todos os pacientes foram parasitologicamente e clinicamente curados, entretanto, quatro deles apresentaram parasitas no baço seis meses após o término do tratamento (SUNDAR *et al.*, 2008). Quando ABLE foi comparada à formulação lipossomal de AmB, utilizando um regime de dose única (15 mg/kg), tanto as taxas de cura, quanto a taxa de reações adversas relacionadas à infusão, foram similares. Nenhum paciente apresentou sinais e sintomas de nefrotoxicidade e a hepatotoxicidade. Assim, os resultados de eficácia e de segurança indicam que o tratamento com ABLE é seguro e eficaz (SUNDAR *et al*., 2014).

O Ambisome®, como formulação lipídica mais utilizada para o tratamento das leishmanioses, oferece inúmeras vantagens. Entretanto, sua utilização ainda é muito restrita devido ao seu elevado custo e a sua complexa fabricação em larga escala, já que requer o uso de solventes orgânicos. Portanto, o desenvolvimento de novas formulações lipídicas contendo AmB é ainda uma questão em aberto. Vale ressaltar que as nanoemulsões (NE) apresentam enorme potencial para substituir os lipossomas notadamente pelo fato de serem sistemas que podem ser fabricados sem o uso de solventes orgânicos, o que impacta na diminuição de toxicidade.

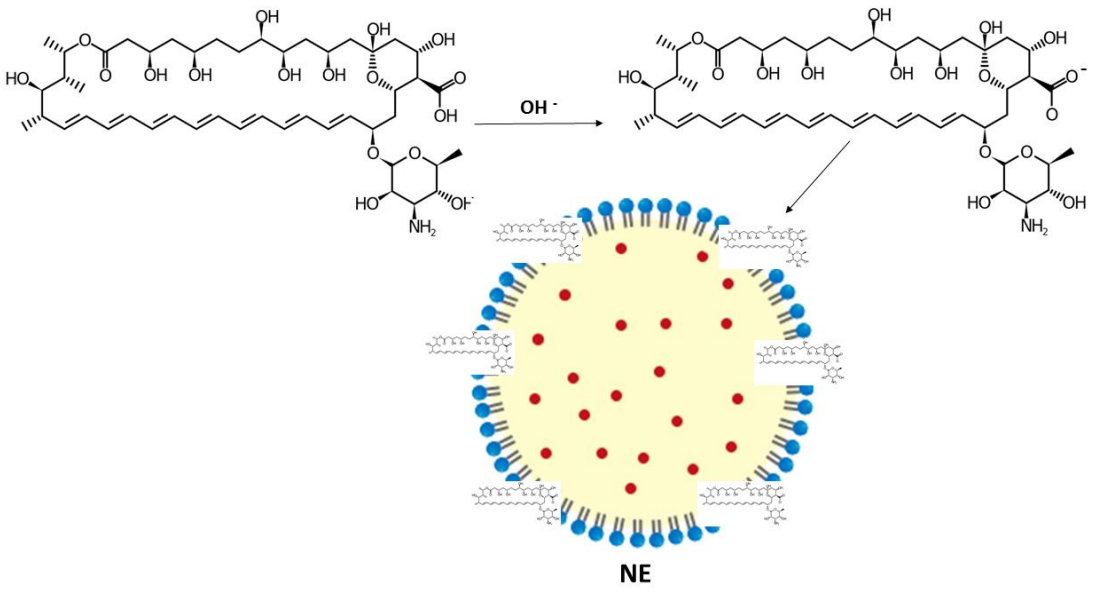
3.2.2 Nanoemulsões (NE)

A incorporação de AmB em NE constitui uma alternativa interessante para a administração endovenosa do fármaco. NE são sistemas heterogêneos de óleo em água (O/A), estabilizados por tensoativos contendo glóbulos entre 100 - 600 nm. A longo prazo, as NE apresentam estabilidade física, ou seja, sem aparente floculação ou coalescência (BENITA; LEVI, 1993; BOUCHEMAL *et al*., 2004). As NE têm sido utilizadas há anos para a nutrição parenteral e como carreadora de fármacos devido à sua biocompatibilidade, capacidade de solubilizar altas

quantidades de compostos hidrofóbicos, capacidade para reduzir a toxicidade de fármacos citotóxicos e de protegê-los a partir da hidrólise e degradação enzimática em condições fisiológicas (ARAÚJO *et al*., 2011).

Lance e colaboradores desenvolveram um procedimento inédito para a incorporação da AmB em emulsões lipídicas que pode ser conduzido de forma satisfatória com uso de uma solução alcalina (NaOH 0,1 N) do fármaco. Esse método é baseado no perfil de pH-solubilidade. A Figura 7 ilustra este procedimento e, após a incorporação da solução alcalina da AmB na NE branco, o pH é imediatamente ajustado para próximo de 7. Evidências da incorporação da AmB na interface foram apresentadas e estudos de toxicidade mostraram que tais formulações são menos tóxicas em comparação com a preparação convencional (LANCE *et al*., 1995; WASHINGTON *et al*., 1991). Entretanto, o potencial destas formulações para o tratamento das leishmanioses ainda não foi investigado. Por outro lado, a estabilidade das emulsões carregadas com AmB foi baixa, o que pode ser atribuído à hidrólise das lecitinas, tensoativos usados no preparo das formulações (LANCE *et al*., 1995).

Figura 7: Representação esquemática do procedimento de incorporação da AmB na interface da NE

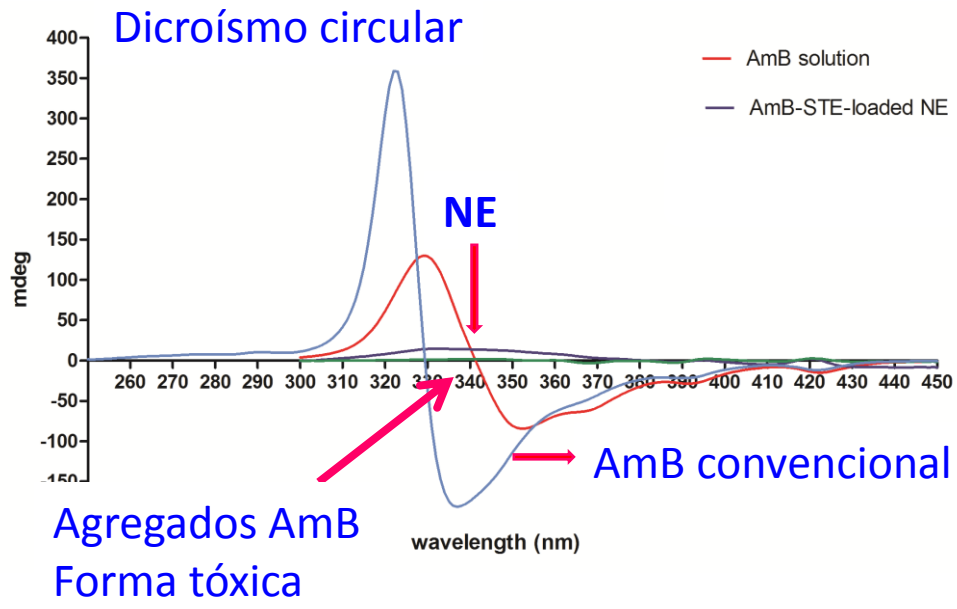


Santos e colaboradores desenvolveram NE carregadas com AmB com elevado teor de encapsulação (95 ± 1%) usando a estratégia do perfil pH-

solubilidade descrita previamente por Lance e colaboradores (1995). Apesar desta formulação não ter sido capaz de aumentar a penetração da AmB na pele, ela apresentou uma excelente estabilidade por até 90 dias, a qual foi atribuída a presença da estearilamina, lípide de caráter catiônico, na interface da NE. Desta forma, esta NE representa uma alternativa interessante em relação às emulsões lipídicas citadas previamente (SANTOS *et al.,* 2012).

Mais recentemente, Caldeira e colaboradores avaliaram a influência da estearilamina na estabilidade de NE carregadas com AmB. O teor de encapsulação/associação da AmB na NE foi alto (aprox. 100%) e uma estabilidade de 180 dias foi observada, independente da presença estearilamina. Extensa caracterização por dicroísmo circular e espectro no ultravioleta mostrou ausência de sinal da forma agregada livre da AmB, a mais tóxica para as células hospedeiras (Figura 8). Além disso, a toxicidade induzida pela NE em macrófagos foi significativamente menor do que aquela observada para a AmB convencional. Portanto, NE carregadas com AmB constituem uma alternativa atrativa para o tratamento das leishmanioses (CALDEIRA *et al*., 2015). Para confirmar ou não o potencial dessa nova formulação estudos *in vivo* de atividade e de toxicidade devem ser conduzidos.

Figura 8: Dicroísmo circular de NE contendo AmB e de uma solução contendo AmB (Adaptado: CALDEIRA *et al.*, 2015).



3.2.3 Atividade leishmanicida *in vivo* de formulações lipídicas contendo AmB

Diversos estudos avaliando eficácia comparativa das formulações convencional e lipídica foram conduzidos em animais experimentalmente infectados com espécies de LV. A maioria deles revelou uma eficácia similar ou maior para as lipídicas em comparação com a AmB convencional, além da possibilidade de serem administradas doses mais elevadas a partir das novas formulações, devido a menor toxicidade.

Gangneux e colaboradores (1996) compararam a eficácia entre o Fungizone® e formulações lipídicas contendo AmB num modelo murino de LV induzida por *L. (L.) infantum*. No primeiro estudo, a eficácia do Fungizone® foi comparada à do Ambisome®. Observou-se que nas doses de 5 a 50 mg/kg de AmB lipossomal houve a erradicação completa de parasitas no fígado, baço e pulmões. A eficácia do Ambisome® na dose de 0,8 mg/kg foi maior do que aquela observada para a AmB convencional (GANGNEUX *et al*., 1996a). No segundo estudo, comparou-se a eficácia do Fungizone® e três formulações lipídicas (mistura AmB + Intralipid®, Abelcet® e Ambisome®). Abelcet® e o Ambisome® (a 12 mg/kg) erradicaram completamente os parasitas dos tecidos. Além disso, estas duas formulações possibilitaram a administração de doses maiores da AmB e foram notadamente mais eficazes em relação ao Fungizone® (dose máxima 0,8 mg/kg) (GANGNEUX *et al*., 1996b)

Outro estudo avaliou a atividade leishmanicida de quatro formulações comerciais (Fungizone®, Ambisome®, Abelcet® e Amphocil®) em camundongos BALB/c infectados com *L. (L.) donovani*. Todas as formulações propiciaram uma significativa redução da carga parasitária no fígado e no baço quando comparadas ao grupo controle. O Fungizone® foi omitido dos estudos de doses múltiplas, devido à sua toxicidade na dose utilizada com outras formulações (2,5 mg/kg/dia) (MULLEN *et al*., 1997).

Paul e colaboradores (1997) compararam a eficácia entre AmB lipossomal e o Fungizone® num modelo murino de LV, utilizando duas diferentes cepas de *L. (L.) infantum*. A AmB lipossomal foi cerca de três vezes mais eficaz do que a AmB convencional para ambas as cepas. Os camundongos não foram tratados

com o Fungizone® a 3 mg/kg já que doses superiores a 1 mg/kg demonstraram ser muito tóxicas ou letais.

Yardley e Croft (2000) compararam a atividade do Fungizone® e das três formulações lipídicas comercialmente disponíveis em camundongos BALB/c infectados por *L.* (*L.) donovani*. Os resultados revelaram que a dose média efetiva (DME_{50}) para o Fungizone®, Ambisome®, Amphocil® e Abelcet®, quando administrados via endovenosa, foi 0,21; 0,73; 0,32 e 2,70 mg/kg, respectivamente. Portanto, a AmB convencional para mais eficaz em comparação com demais formulações, mas diferenças com AmB lipossomal e Amphocil® não foram significativas. Além disso, o Ambisome®e o Amphocil® foram mais eficazes que o Abelcet®. Neste modelo de infecção, a dose máxima tolerada da AmB convencional foi de 1 mg/kg, enquanto para as formulações lipídicas as doses administradas podem ser maiores do que 25 mg/kg sem sinais de toxicidade (YARDLEY; CROFT, 2000).

O conjunto destes dados mostra que as formulações lipídicas constituem sistemas para carrear a AmB com vantagens evidentes em comparação com AmB convencional. Os trabalhos previamente publicados com sistemas lipídicos carreando a AmB não investigaram a eficácia das NE no tratamento da LV em comparação com a AmB convencional. Portanto, em continuidade com estudos prévios, os quais mostraram excelente estabilidade de uma nova formulação de NE carregada com AmB, este trabalho tem por objetivo avaliar a sua eficácia *in vivo* em animais experimentalmente infectados com *Leishmania spp*. Além disso, estudos visando avaliar a toxicidade *in vivo* seriam também interessantes para confirmar ou não o potencial desta NE em diminuir os efeitos tóxicos induzidos por C-AmB.

3.3 Metabolômica

Metabolômica é uma abordagem de sistemas analíticos para estudar os perfis metabólicos em organismo vivo, que promete fornecer informações sobre toxicidade de drogas, processos de doença e função do gene em várias fases de um processo de desenvolvimento (NICHOLSON *et al.*, 2002). Seu principal objetivo é descobrir novos biomarcadores e as vias celulares que auxiliam no

diagnóstico precoce, especialmente antes do início dos sintomas, para permitir intervenções terapêuticas em tempo hábil. A análise metabolômica de fluidos biológicos acessíveis fornece informações sobre todos os tecidos dos quais fornecem e obtém metabólitos daqueles fluidos (ISSAQ *et al*., 2008).

O metaboloma tem sido definido como uma coleção qualitativa e quantitativa de moléculas de baixo peso molecular (< 1500 Da), denominadas metabólitos, presentes numa célula, as quais participam de reações metabólicas gerais e que são necessárias para a manutenção, crescimento e função normal de uma célula. Geralmente, estas moléculas incluem espécies orgânicas e inorgânicas, embora espécies elementares também podem ser estudadas (DUNN; ELLIS, 2005). Isto inclui uma variedade de diferentes classes de compostos, tais como aminoácidos, peptídeos, ácidos orgânicos, lípides, nucleotídeos, açúcares, glicoproteínas, etc (GIOVANE *et al.*, 2008).

A metabolômica apresenta inúmeras vantagens em relação a outras estratégias "-ômicas", sendo a maior delas a sua proximidade ao fenótipo biológico do sistema e, portanto, a rápida observação de perturbações do sistema no metaboloma. As análises clássicas, tanto a análise direcionada ("target analysis") quanto a não-direcionada ("metabolic profiling") são desenvolvidas com a expectativa de que uma medida ou uma combinação de diversas medidas de compostos relacionados revelem algo sobre algum problema ou situação específica (BARBAS *et al.*, 2011). Por outro lado, as técnicas analíticas são caras, o que seria a principal desvantagem. Entretanto, como o custo por amostra é baixo e os tempos de análise inferiores a 30 min, a análise de grandes conjuntos de amostras pode ser facilmente conduzida a um baixo custo total em relação a outras plataformas "ômicas" (ex: proteômica ou transcriptômica) (DUNN *et al*., 2011).

Um perfil metabólico pode ser definido empiricamente como o conjunto de todos os metabólitos ou derivados (identificados ou desconhecidos) detectados após a análise de uma amostra utilizando uma técnica analítica particular. Geralmente, isto inclui eficientes separações cromatográficas, acopladas poderosos detectores [ex: ressonância nuclear magnética (NMR), espectroscopia no infravermelho por transformada de Fourier (FT-IR) e espectrometria de massas (MS)]. Estas técnicas são muitas vezes usadas para

classificar as amostras rapidamente por análise quimiométrica, antes da identificação dos compostos (VILLAS-BÔAS *et al.*, 2005).

Espectrometria de massa (MS) é a tecnologia de detecção mais amplamente aplicada na análise metabolômica, utilizada para identificar os metabólitos após a separação, quer por cromatografia em fase gasosa (GC), cromatografia líquida de alta eficiência (HPLC), ou eletroforese capilar (CE). A combinação destas técnicas é bem adequada para a sondagem de uma grande parte do metaboloma de biofluidos (urina, soro, plasma), uma vez que é capaz de detectar metabólitos lipofílicos e hidrofílicos (DUNN; ELLIS, 2005; HANNA *et al.*, 2013). A análise metabolômica compreende além da separação e detecção dos analitos, o alinhamento dos picos, normalização usando uma diversidade de "softwares" estatísticos comerciais e/ou públicos e análise multivariada dos dados para identificar um metabólito ou metabólitos que podem distinguir um grupo de amostra do outro (DUNN *et al.*, 2011).

3.3.1 Cromatografia gasosa acoplada à espectrometria de massas (GC-MS)

GC-MS é um sistema analítico onde compostos voláteis, de baixa massa molecular e termicamente estáveis são primeiramente separados por cromatografia gasosa e, em seguida, detectados por espectrometria de massas por impacto eletrônico ou por ionização química (DUNN; ELLIS, 2005). Trata-se de uma técnica amplamente utilizada em análises metabolômicas devido a sua elevada capacidade de separação, robustez instrumental e análise reprodutível (ZHANG *et al.*, 2012). A identificação do composto é baseada no tempo de retenção e no seu espectro de massa (padrão de fragmentação específico de um composto). Compostos produzem padrões de fragmentação reprodutíveis quando ionizados por uma voltagem eletrônica fixa (geralmente -70 eV). Assim, a fragmentação do espectro obtido por GC-MS é independente do instrumento e permite a criação de bases de dados e o compartilhamento de dados entre utilizadores, tornando a técnica particularmente valiosa (GARCIA; BARBAS, 2011).

No entanto, vários metabólitos requerem derivatização química para proporcionar volatilidade e estabilidade térmica para a análise (DUNN; ELLIS,

2005; FIEHN *et al.*, 2000). Esta é a principal desvantagem da GC-MS, já que alguns metabólitos podem conter um número de prótons permutáveis e, portanto, uma variedade de compostos de derivatização são formados (TEUL *et al*., 2009). As classes de metabólitos detectáveis incluem carboidratos, aminas, amidas, ácidos orgânicos e aminoácidos.

3.3.2 Cromatografia líquida de alta eficiência acoplada à espectrometria de massas (LC-MS)

LC-MS é uma técnica mais recente para as ciências analíticas em relação à GC-MS e é considerada uma ferramenta poderosa para estudos metabolômicos, a qual permite a separação e caracterização da maioria dos metabólitos. Embora sua resolução seja inferior àquela fornecida por GC, LC não requer volatilidade da amostra e permite a separação de uma faixa mais ampla de pesos moleculares, abrangendo espécies de baixo e alto peso molecular (maior do que 600 Da) e termo lábeis, incluindo fosfolípides, ácidos biliares conjugados, glicosídeos e açúcares (DUNN, 2008). Além disso, LC-MS pode detectar compostos em uma ampla faixa de polaridade e geralmente requerem um pré-tratamento simples da amostra (precipitação de proteínas) (DUNN *et al.*, 2011).

A separação de cada grupo é ditada pelas propriedades do soluto, o qual determina qual o tipo de coluna (fase estacionária) e fase móvel a serem usadas para garantir uma separação bem-sucedida (ISSAQ *et al.*, 2008). As interações entre a amostra, fases móvel e estacionária irão determinar a extensão da retenção e separação dos compostos presentes nas amostras. Os dados coletados incluem o tempo de retenção, abundância relativa, a massa molecular do pico e a carga iônica para cada analito detectado (DUNN, 2008).

LC-MS permite a separação do metabólito por LC seguido por ionização por electrospray (ESI) ou, menos frequentemente, por ionização química à pressão atmosférica (APCI), dentre outras. ESI opera em modo positivo e negativo e detecta apenas aqueles metabólitos que podem ser ionizados por adição ou remoção de prótons ou por adição de outras espécies iônicas (DUNN; ELLIS, 2005). Portanto, a análise em ambos os modos permite uma ampla detecção de diferentes metabólitos numa amostra (THEODORIDIS *et al.*, 2012).

Em geral, as separações de LC-MS são realizadas utilizando colunas C_{18} de fase reversa e solventes que são compatíveis com a técnica de ESI para a análise de compostos de média e baixa polaridade (LENZ; WILSON, 2007).

3.3.3 Eletroforese capilar-espectrometria de massas (CE-MS)

CE-MS é uma técnica de separação poderosa e promissora para metabólitos carregados, oferecendo alta resolução da análise, proporcionando informações, principalmente, sobre compostos polares ou iônicos nos fluidos biológicos (ZHANG *et al.*, 2012). É capaz de integrar algumas vantagens entre eletroforese de alta voltagem e cromatografia líquida de alta eficiência (HPLC) (ex: alta velocidade e tempo de análise curto, baixo consumo de solvente e amostras, excelente eficiência de separação e fácil automatização) (LAO *et al*., 2009). Como CE é baseada num mecanismo de separação diferente em relação a HPLC, esta técnica também revela seletividade diferente e, por isso, tem sido considerada complementar à cromatografia (BARBAS *et al.*, 2011).

CE-MS tem revelado contribuições consideráveis em metabolômica, uma vez que a maioria dos metabólitos primários são intrinsecamente polares (BRITZ-MCKIBBIN, 2011). Os metabólitos são primeiramente separados por CE baseando-se na carga e tamanho e, então, seletivamente detectados usando MS (ESI) para a monitorização de íons ao longo de um grande intervalo de valores de massa/carga (m/z). CE-MS oferece inúmeras vantagens importantes sobre outras técnicas de separação. Dentre as vantagens de CE-MS incluem: curto tempo de análise, pequena quantidade requerida de amostra e preparo simples, injeção de pequenos volumes que variam de 1 a 20 nL e elevada eficiência/resolução. A baixa robustez e sensibilidade destacam-se como as desvantagens da técnica. Assim, CE-MS tem sido utilizada na análise de íons inorgânicos, ácidos orgânicos, aminoácidos, nucleotídeos e nucleosídeos, vitaminas, tióis, carboidratos e peptídeos (SIMÓ *et al*., 2011; BARBAS *et al.*, 2011).

3.3.4 Metabolômica e nefrotoxicidade

A aplicação da metabolômica no estudo da nefrotoxicidade permite a medição simultânea de diversos metabólitos em várias vias de interação, refletindo alterações na fisiologia renal. Vários estudos vêm demonstrando que os biomarcadores metabólicos oferecem um grande potencial para a identificação de alterações bioquímicas precoces associadas à nefrotoxicidade em relação aos biomarcadores tradicionais (BOUDONCK *et al*., 2009; RUSSMANN *et al*., 2009).

Apesar da sua complexidade anatômica, o rim é altamente passível de análise metabolômica, possibilitando a detecção da lesão renal precoce e efeitos específicos de cada região, bem como determinar os mecanismos de toxicidade (BOUDONCK *et al*., 2009). Nos últimos anos, a metabolômica foi introduzida para estudar alterações no metabolismo em lesão renal aguda, especialmente em modelos nefrotóxicos provocados por gentamicina (SUN *et al.*, 2012; HANNA *et al.*, 2013), cisplatina (PORTILLA *et al.*, 2006; PORTILLA *et al.*, 2007), melamina e ácido cianúrico (SCHNACKENBERG *et al.*, 2012).

A toxicidade da AmB já foi investigada pela expressão genômica. Kondo *et al.* (2012) estabeleceu genes de biomarcadores para a detecção precoce de nefrotoxicidade causada pela AmB em camundongos. Estes genes foram mais sensíveis para detectar nefrotoxicidade do que os parâmetros químicos e histopatológicos clínicos tradicionais. No entanto, não há estudos relatados na literatura sobre a toxicidade da AmB usando metabolômica. Através da aplicação de metabolômica para analisar os efeitos tóxicos de novas formulações contendo AmB espera-se contribuir não só para opções alternativas de tratamento para a leishmaniose, mas também para a identificação de novos biomarcadores de nefrotoxicidade.

4 PARTE EXPERIMENTAL, RESULTADOS E DISCUSSÃO

CAPÍTULO 1

Nanoemulsions loaded with amphotericin B reduces the *in vitro* hemolytic toxicity and improves the *in vivo* antileishmanial activity

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Abstract

This work aimed to optimize and characterize a nanoemulsion (NE) loaded with Amphotericin B (AmB) evaluating the *in vivo* activity for the treatment of experimental visceral leishmaniasis (VL) and its *in vitro* hemolytic toxicity. The NE was characterized by the size, polydispersity index (PI), zeta potential and drug encapsulation efficiency (EE). For studies of antileishmanial activity, AmB-NE at 1 and 2 mg/kg was administered intravenously on 3 alternate days in BALB-c mice infected by *Leishmania (Leishmania) infantum chagasi*. When the NE was submitted to gradual increases in pressure, using a high-pressure homogenizer, the PI values and droplets diameter decreased. The droplet diameter (~145 nm), evaluated by photon correlation spectroscopy, nanoparticle tracking analysis or atomic force microscopy, was lower than that obtained in previous studies. The zeta potential was negative and the EE was almost 100%. The *in vitro* hemolytic toxicity for AmB-loaded NE was lower than that observed for the conventional AmB (C-AmB). The *in vivo* studies showed that the AmB-loaded NE and the C-AmB, both at 1 mg/kg, were active against *L*. (*L*.) *infantum chagasi*. C-AmB at 2 mg/kg was very toxic. In contrast, administration of the AmB-loaded NE at 2 mg/kg did not result any sign of acute toxicity promoting a significant reduction in parasite loads in spleen and liver as compared to C-AmB or vehicle. These findings suggest that the AmB-loaded NE constitutes an attractive alternative for the treatment of VL caused by the *L. (L.) infantum chagasi* due to improved efficacy and lower toxicity.

Key words Nanoemulsion; amphotericin B; visceral leishmaniasis; hemolytic toxicity; efficacy evaluation; animal model.

1 Introduction

Visceral leishmaniasis (VL) is a systemic protozoan parasite disease that is transmitted by the phlebotomine sand flies.[1] In the Old world, VL is caused by *Leishmania donovani* (in regions of India, Pakistan, China and Africa) and *Leishmania infantum* (in the Mediterranean region). In the New World, VL is also caused by *L. infantum* (also known as *Leishmania chagasi* or *L. infantum chagasi*), which is highly prevalent in Brazil.[2, 3]

Since the late 1940s, the traditional treatment for VL has been the use of pentavalent antimonials. However, the adverse reactions induced by them are important drawbacks and the development of parasite resistance increases the ineffectiveness.[3,4]Alternative therapies for the treatment of all forms of leishmaniasis include azoles, paromomycin, imiquimod, miltefosine, pentamidine and amphotericin B (AmB).[2, 3]

AmB, a highly hydrophobic drug, is an useful second-line therapy for treatment of the leishmaniasis. This drug and its formulations are considered the best existing treatment alternatives against VL, presenting 97% cure rate and no reported resistance.[5, 6] However, medical application of the conventional formulation (C-AmB), which is composed of mixed micelles of AmB and sodium deoxycholate, is limited by its toxicity, mainly hemolysis and nephrotoxicity, required hospitalization, constant monitoring of patients and prolonged duration of treatment. The lipid formulations such as liposomal AmB (Ambisome®), Abelcet® and Amphocil® can reduce AmB-related toxicity, but the high cost and difficulty of manufacturing represent limitations for their widespread use.[7, 8] Consequently, the development of nanocarriers loaded with AmB remains a promising alternative.

Nanoemulsions (NE) are attractive lipid nanocarriers of AmB, since these delivery systems are able to reduce drug-related toxicity and the manufacture is easy and inexpensive.[9] Recently, we developed AmB-loaded NE with high encapsulation efficiency, high stability and lower concentration of the free form of AmB compared to a conventional AmB formulation, which is the more toxic form of AmB. Moreover, the toxicity induced by the NE in macrophages was significantly lower than that observed for the conventional AmB.[10] Therefore, this

AmB-loaded NE may constitute an alternative for the treatment of leishmaniasis, with less toxic effects. However, the efficacy of this formulation in treatment of experimentally infected animals is not known yet.

In the present study, the potential of these NE for the treatment of experimental VL was investigated in mice infected by *L. (L.) infantum chagasi*. Considering that the droplet size significantly influences the biodistribution of the NE, after intravenous administration,[11, 12] we have studied various process and formulation parameters, which can affect the particle size. *In vitro* hemolytic toxicity was selected as a tool to investigate further the toxicity of the NE loaded with AmB in comparison with C-AmB.

2 Materials and methods

2.1 Materials

AmB and conventional AmB (C-AmB; Anforicin®), a complex of AmB and sodium deoxycholate, were kindly provided by Cristalia Produtos Químicos Farmacêuticos Ltda (São Paulo, Brazil). Medium chain triglycerides (MCT) and polysorbate 80 (Tween 80®) were kindly provided by Croda Brazil Ltda (São Paulo, Brazil). NaOH, HCl, cholesterol and hidroxibutiltolueno (BHT) were purchased from Sigma-Aldrich (Missouri, USA). The glycerol was obtained from the House of Chemical Industry & Trade Ltda. (São Paulo Brazil). HPLC grade acetonitrile and tetrahydrofuran were purchased from Tedia (Rio Janeiro, Brazil). HPLC grade methanol was purchased from J.T. Baker (EUA). Ultra-pure water was obtained from a Milli-Q Water System. All other chemicals were of analytical reagent grade.

2.2 Preparation of the AmB-loaded NE

Composition of the AmB-loaded NE (% w/v) was previously described.[10] Briefly, the oily phase (OP) was composed of MCT (5.0 %), Tween 80 (1.5 %; hydrophilic surfactant), cholesterol (0.5 %), and BHT (0.01 %; antioxidant). The aqueous phase (AP) was composed of glycerol (2.25 %; isotonizer) and purified

water. The NE was prepared by the hot homogenization method. Both oil and aqueous phases were heated at 70 °C and, then, the AP was slowly added to the oil phase and homogenized using an Ultra-Turrax T25 at 8000 rpm for 2 min. The resulting dispersion was further homogenized in a nine cycle homogenizing regime, using a high-pressure homogenizer (HPH) APV-1000 (Invensys, Denmark). The NE was cooled down to room temperature and the AmB was incorporated into the NE at a concentration of 2 μg/mL.[13]The influence of the method used for incorporation of AmB on the blank NE (without AmB) was evaluated. AmB (40 μg for a batch of 20 mL) was either solubilized in 1 mL of a 0.2M sodium hydroxide (NaOH) solution or added (as a powder) to the alkaline blank NE (NaOH was previously added to the NE) followed by magnetic stirring at 600 rpm for 1 min, at room temperature. Next, the pH was adjusted to 7.0-7.5 with hydrochloric acid and finally the final volume was completed with sterile water. A blank NE (without AmB) was also prepared for comparison purpose.

To investigate the influence of the concentration of Polysorbate 80 (0.5, 1.0 or 1.5 %), previous studies using a high intensity ultrasonic processor (Microprocessor controlled, 750 Watt model; Sonics Materials Inc., USA) instead of HPH were performed. Samples were homogenized in an ultrasonic processor after homogenization with the Ultra-Turrax, as described above.

2.3 Characterization of the NE

The NE formulation was characterized for particle size (mean hydrodynamic diameter), polydispersity index (PI), zeta potential, pH, AmB content, and drug encapsulation efficiency (EE). Nanoparticle tracking analysis (NTA) and images of atomic force microscopy (AFM) were also obtained.

2.3.1 Determination of the droplet size, the polydispersity index and the zeta potential

The droplet size distribution and the PI were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK) at a fixed angle of 90 and at 25 °C. Before the measurements, the NE was diluted with distilled water. All measurements were performed in

triplicate. Zeta potential was evaluated by determining the electrophoretic mobility using the Zetasizer. Before measurements, the NE was diluted in water. All measurements were performed in triplicate.

2.3.2 Nanoparticle Tracking Analysis (NTA)

Nanoparticle tracking analysis (NTA) experiments were performed using a NanoSight LM 10 & NTA 2.2 Analytical Software (Nanosight Ltd., Amesbury, UK). After appropriate dilution of the NE (with and without AmB) in ultrapure water, the sample was introduced into the Nanosight sample chamber with a disposable syringe. The samples were measured at room temperature for 60 s with automatic detection. The particles suspended in a fluid were irradiated by a laser source, and light scattering and the image was captured by a charge-coupled device camera.

2.3.3 Atomic force microscopy (AFM)

AFM observation of the NE (with and without AmB) was performed in air at room temperature, on a Dimension 3000 apparatus, as well as on Multimode Equipment, both monitored by a Nanoscope Illa controller from Digital Instruments (Santa Barbara, CA, USA). A droplet (10 µL) of the sample was deposited on a freshly cleaved mica surface, spread and dried at room temperature. The images were obtained in tapping mode, using commercial silicon probes from Nanosensors^{TM}, with cantilevers having a length of 228 nm, resonance frequencies of 75–98 kHz, spring constants of 29–61 N/m and a nominal tip curvature radius of 5-10 nm. The scan rate used was 1 Hz. Dimensional analyses were performed using the “section of analyses” program of the system. A minimum of ten images from each sample was analyzed to assure reproducible results.[14, 15]

2.4 Assay of AmB by high performance liquid chromatography (HPLC)

The AmB content in the NE was measured by the HPLC method previously developed and validated.[10] The AmB concentration in the NE was evaluated by

the reverse phase HPLC method. A Waters liquid chromatograph coupled with a 515 model Waters pump, 717 plus automatic injector, and a 2487 model UV detector at 405 nm were used. A ACE® RP-8 column (5 nm; 250 x 4.6 mm) was used with a mixture of 20 mM sodium edetate/acetonitrile pH 5 (65:35) as mobile phase at a flow rate of 1.2 mL min^{-1} and 20 μL as injection volume.

The concentration of the AmB in the NE was obtained by comparing the area under the curve (AUC) of the AmB absorption peaks, obtained from the solutions prepared from the NE, and the AUC of AmB absorption peaks obtained from standard solutions of the drug. AUC represents the total amount of a substance in a sample.

2.5 AmB content and drug encapsulation efficiency (EE)

The EE of the AmB in the NE was evaluated based on the determination of the AmB concentration before and after filtration (polyvinylidene fluoride membrane, 0.45 μm pore size, Millipore®, USA) as previously described.[13] The AmB that remained insoluble, and hence not properly incorporated into the nanocarrier, was removed by filtration. The drug concentration in the external aqueous phase of the formulations was obtained by the ultrafiltration method using centrifugal devices (Amicon®-Ultra 4 100 k, Millipore, USA). In fact, the AmB concentration in aqueous phase was found to be negligible. The AmB concentration in the NE (before and after filtration) was determined by the validated HPLC method.[10]

The EE of the drug was calculated by the following equation:

EE (%) = [(AmB before filtration - AmB after filtration) / AmB before filtration] x 100

2.6 *In vitro* hemolytic activity

Human blood was collected in heparinized microcentrifuge tubes and subjected to centrifugation at 3000 g for 5 min at 4 °C to separate red blood cells (RBCs). The supernatant along with the buffy clot was discarded and RBCs were washed thrice with phosphate buffered saline (PBS), pH 7.4. The stock of RBCs was prepared by mixing two volumes of RBCs with 11 parts of the PBS.

Subsequently, 1 mL of the RBCs suspension was mixed in 1 mL of PBS containing 10, 20 and 40 μg/mL AmB equivalent formulation (C-AmB and AmB-loaded NE). RBCs mixed with distilled water and PBS was used as positive and negative control, respectively. The samples were incubated at 37 °C for 3 h in a shaker bath and, then, centrifuged at 3000 g for 5 min to separate supernatant, which was allowed to stand at room temperature for 30 min to oxidize hemoglobin (Hb). The absorbance of oxygenated hemoglobin (Oxy-Hb) was measured spectrophotometrically at 540 nm, and percentage hemolysis was calculated by using the following equation:

% hemolysis = AB_s/AB_{100} x 100, where AB_s is the absorbance of the sample and AB_{100} is the absorbance of the positive control.[16, 17]

2.7 *In vivo* anti-leishmanial activity

2.7.1 Parasites and infection of animals

L. (L.) infantum chagasi (MHOM/BR/74/PP75) promastigotes were maintained at 23 ºC in Schineider's medium (Merck, Germany), supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany), 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma-Aldrich, EUA). BALB/c mice (females, 5-6 weeks old) were inoculated with $2x10^7$/0.2 mL stationary growth phase promastigotes of *L. (L.) infantum chagasi* through intravenous injections (lateral tail vein). The Ethics Committee for Animal Experimentation of the Federal University of Minas Gerais approved this study (CEUA/UFMG: 326/2014).

2.7.2 Treatment of infected animals

Treatment of infected BALB/c mice (20-22 g) was initiated 7 days after infection. Animals were divided into four groups (n = 7 per group): AmB-loaded NE (1 mg AmB/kg), AmB-loaded NE (2 mg AmB/kg), C-AmB (Anforicin®) (1 mg/kg) and untreated control group (5% glucose solution). Mice were treated intravenously (lateral tail vein) over the course of 5 alternative days (five administrations/100 μL). Three days after the end of the treatment, all animals

were euthanized under CO_2 atmosphere and viable parasites in liver and spleen were determined by limiting-dilution assay.[18]

2.7.3 Limiting-dilution assay

For quantification of viable parasites by limiting-dilution assay, the liver, and spleen were removed, weighed and homogenized with an Ultra-Turrax in Schineider's medium supplemented with 10 % bovine fetal serum, 1 mL of a 100 U/mL penicillin and 100 μg/mL streptomycin solution. 2 mL of supernatant of homogenate of liver were transferred to a new sterile tube containing 6 mL of medium. Next, the tissue was centrifuged at 52 g (liver) or 45 g (spleen) for two minutes for sedimentation. The supernatants were separated and centrifuged at 1900 rcf for 10 minutes (liver) or 1620 g for 2 minutes (spleen). The formed pellets were resuspended in 1 mL of Schneider's medium containing 20 % fetal bovine serum (FBS), penicillin (100 UI/mL) and streptomycin (100 μg/mL). The dilutions were performed in triplicate (1:10) in successive cultivation plate, 96 wells, sterile, then, they were incubated at 26 ºC for 7 days. Each well was examined in inversion microscope for the presence of parasites, and the parasite load was determined from the highest dilution at which growth was observed.

2.8 Statistical analysis

The data were processed using Graph Prism 4 software. Analyses for the *in vitro* toxicity and *in vivo* anti-leishmanial activity were evaluated using the one-way analysis of variance (ANOVA) test followed by the Tukey's test. The difference was considered significant when the p value was less than 0.05.

3 Results

3.1 NE characterization

Table 1 shows the influence of the surfactant (Polysorbate 80) concentration (0.5, 1.0, 1.5 % w/v) on droplet size, PI, and zeta potential of AmB-loaded NE. The droplet size was 239 ± 13, 214 ± 16, and 163 ± 20 nm for NE

containing Polyborbate 80 at 0.5, 1.0 and 1.5 % respectively. Therefore, the droplet diameter decreased with the Polysorbate 80 concentration. This can be explained by the ability of Polysorbate 80 (a nonionic surfactant) to localize at the oil/water interface and reduce the interfacial tension between the two phases, therefore, providing a reduction in the droplet size of NE. The PI values and zeta potential showed no change. Accordingly, the NE containing Polysorbate 80 at 1.5 % was selected for subsequent studies. These NE were prepared using a high intensity ultrasonic processor and, in order to further decrease the droplets diameter, additional studies were conducted using a HPH.

Table 1: Influence of the concentration of polysorbate 80 (Tween® 80) on the droplet size, the PI and the zeta potential of AmB-loaded NE

Parameters*	Tween® 80 (% w/v)		
	0.5	1.0	1.5
Droplet size (nm)	239 ± 13	214 ± 16	163 ± 20
PI	0.20 ± 0.03	0.19 ± 0.04	0.20 ± 0.05
Zeta Potencial (mV)	-41 ± 1	-36 ± 3	-33 ± 1

* Data represented as mean ± dp, n = 3.
The NE was prepared by the hot homogenization method followed by high intensity ultrasonic processor as described in Section 2. Composition of NE was: 5 % TCM, polysorbate 80 (0.5; 1 or 1.5 %), 0.5 % cholesterol, 0.01 % BHT, 2.25 % glycerol and water q.s. to 100 %.
PI: polydispersity index

The influence of the number of cycles and pressure on the droplet diameter and the PI values of the blank NE were investigated. Firstly, a previous study evaluating the influence of the number cycles (3, 6 or 9) at two pressures (500 or 1000 bar) was performed. The data are described in Table 2. It was observed that the droplet diameter decreased when the number of cycles increased. However, the PI values increased. When the NE was submitted to gradual increases in pressure (3 cycles of 400 bar, 3 cycles of 600 bar and 3 cycles of 800 bar), the PI values and droplets diameter decreased. This procedure was, then, adopted for the preparation of the blank NE.

Table 2: Characterization of blank NE (without AmB) for droplet size and PI after 0, 3, 6, 9 cycles using 400, 500, 600, 800 and 1000 bar using a high-pressure homogenizer

Number of cycles	Pressure (bar)	Droplet size (nm)	PI
0	-	139 ± 6	0.355 ± 0.50
3		131 ± 3	0.344 ± 0.09
6	500	117 ± 5	0.434 ± 0.03
9		99 ± 7	0.395 ± 0.40
3		109 ± 4	0.343 ± 0.25
6	1000	100 ± 9	0.515 ± 0.10
9		89 ± 2	0.394 ± 0.05
3	400		
3	600	136 ± 5	0.160 ± 0.02
3	800		

PI: polydispersity index

Next, the influence of the method used for the incorporation of AmB on the blank NE (as a powder or in NaOH solution) on the droplet diameter and PI was evaluated. Data are showed in Table 3. The incorporation of AmB promoted an insignificant increase in the droplets diameter from 136 ± 5 nm (blank NE) to 148 ± 2 nm (AmB powder) and 145 ± 1 nm (AmB solution in NaOH). The PI values also increased with AmB incorporation, but also significant difference was not observed. The PI was 0.16 ± 0.02, 0.22 ± 0.03, and 0.17 ± 0.04 for NE blank, NE + AmB powder and NE + AmB solution, respectively. It was observed that incorporation of AmB on the NE as a NaOH solution resulted in a PI value similar to the observed for blank NE. Therefore, NE + AmB solution was selected for subsequent studies. The zeta potential for blank NE and AmB-loaded NE was -8 mV and -33 mV, respectively. This negative value can be explained by the presence of free fatty acids, which are ionized in the NE containing AmB due to addition of NaOH, as previously described.[10] There was no difference in the total content and in the EE between the two AmB-loaded NE. The total content was

97 and 99 %, and the EE was 93 and 95 % for NE + AmB powder and NE + AmB solution, respectively.

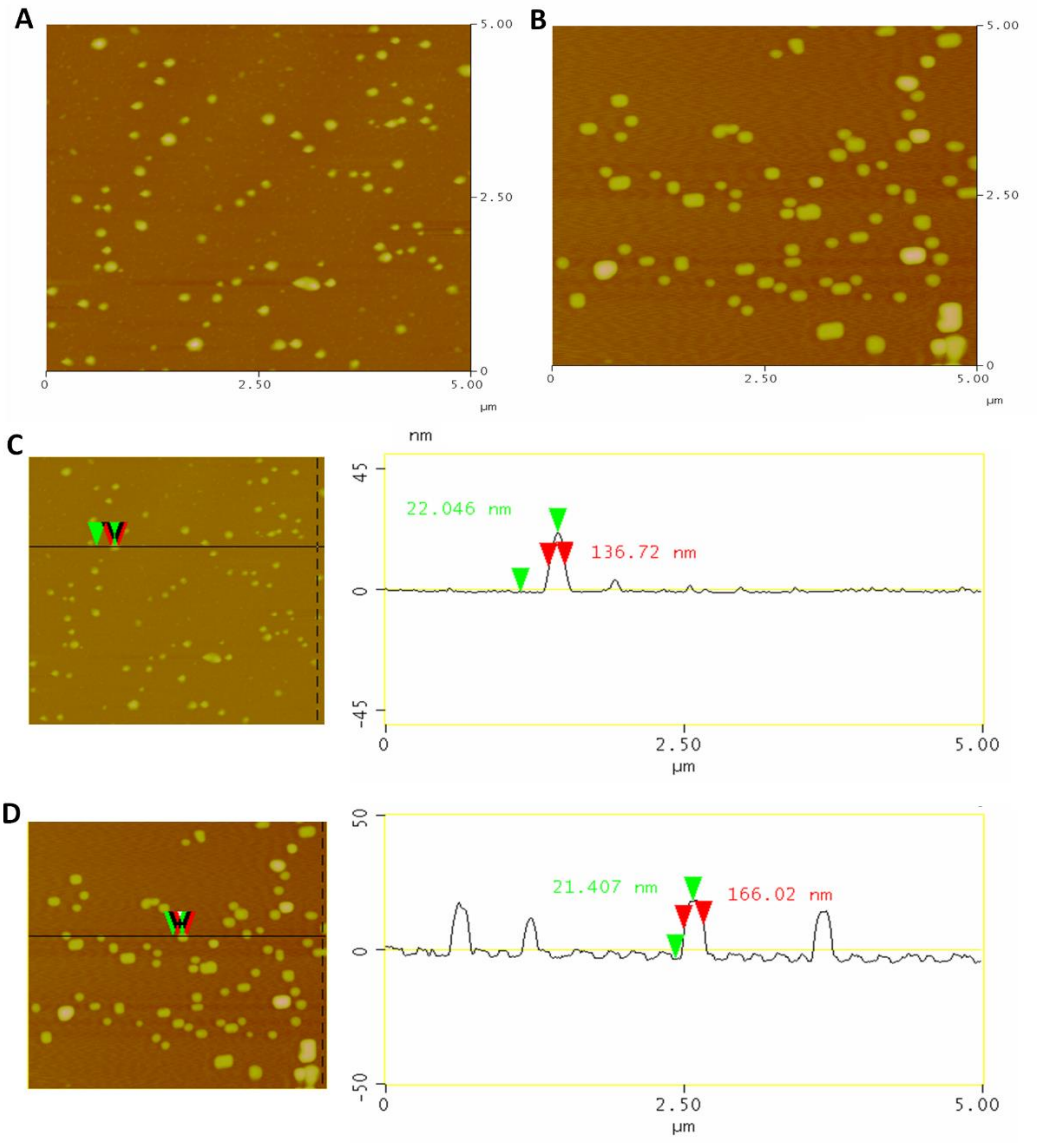
Table 3: Influence of the method used for the incorporation of AmB on the blank NE (as a powder or in NaOH solution) on droplet size, PI, zeta potential, EE and total content

Parameters*	Formulation**		
	Blank NE	NE + AmB powder	NE + AmB solution
Droplet size (nm)	136 ± 5	148 ± 2	145 ± 1
PI	0.16 ± 0.02	0.22 ± 0.03	0.17 ± 0.04
Potencial Zeta (mV)	-8 ± 4	-33 ± 2	-33 ± 3
Total content (%)	-	97 ± 3	99 ± 4
EE (%)	-	93 ± 5	95 ± 3

* Data represented as mean ± dp, n = 3.
** The NE was prepared by the hot homogenization method followed by HPH as described in Section 2. Composition of NE was: 5 % TCM, 1.5 % polysorbate 80, 0.5 % cholesterol, 0.01 % BHT, 2.25 % glycerol and water q.s. to 100 %.
PI: polydispersity index; EE: encapsulation efficiency; HPH: high-pressure homogenizer

The images produced by the AFM technique are three-dimensional, with a high resolution in the nanometrical scale (Figs. 1A and 1B).In this technique, the preparation of the sample is very simple. NE is deposited in a partially dried state onto freshly cleaved mica plates, a fact that allows simultaneous characterization of particle shape, structure and interparticle organization. The mean droplets diameter obtained by AFM measurements were ~136 nm (blank NE) and ~166 nm (AmB-loaded NE) and both NE types presented a flattened form indicated by the height of the droplets (~22 nm) in the AFM images (Figs. 1C and 1D).

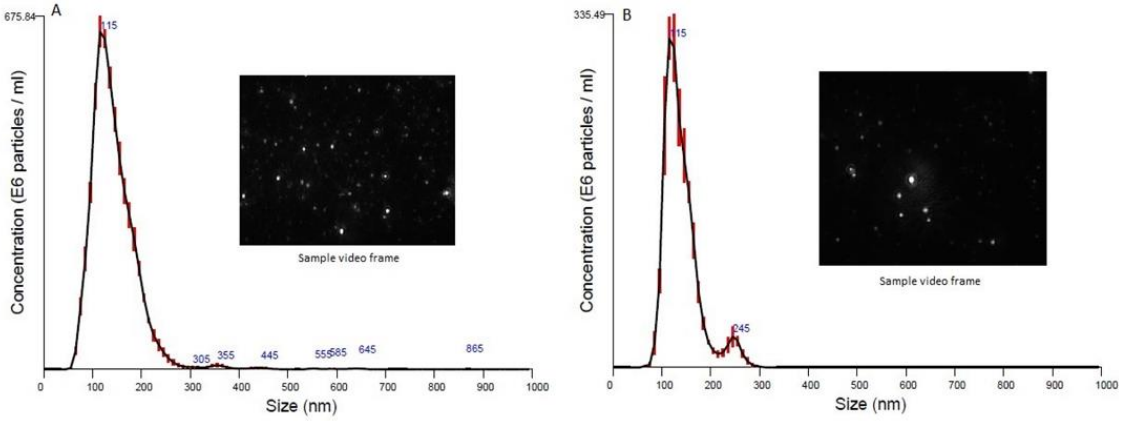
Figure 1: AFM images of blank NE (A), AmB-loaded NE (B) and topographical profile (C) given by the ‘‘section analysis’’ software showing measurements of blank NE diameter (red arrows) and height (green arrows) and AmB-loaded NE (D) Scan size: 2.5 × 2.5 µm.



The nanoparticle size distribution and the three-dimensional images (size vs. intensity vs. concentration graphs) for blank NE and AmB-loaded NE are shown in Figures 2A and 2B, respectively. It can be noted that the average particle size values found for blank NE (139 nm) and AmB-loaded NE (135 nm) are consistent with those obtained by Zetasizer. The difference between blank NE and AmB-loaded NE regarding the concentration of particles/frame and

concentration of particles/mL was evaluated by NTA. The concentration of particles/frame was 280.4 and 99.5, while the concentration of particles/mL was 5.52 x 10^9 and 1.96 x 10^9 for NE blank and AmB-loaded NE, respectively. The data showed that the presence of AmB in the NE reduced the amount of particles/frame. These findings can be attributed to higher potential values for the AmB-loaded NE compared to those obtained for the blank NE. Therefore, the increased repulsion of the particles in the AmB-loaded NE confirms the lower particles/frame.

Figure 2: Size distribution from NTA (Nanoparticle Tracking Analysis) measurements with the corresponding video and 3D (graph size vs. intensity vs. Concentration) obtained for (A) NE blank and (B) AmB-loaded NE.

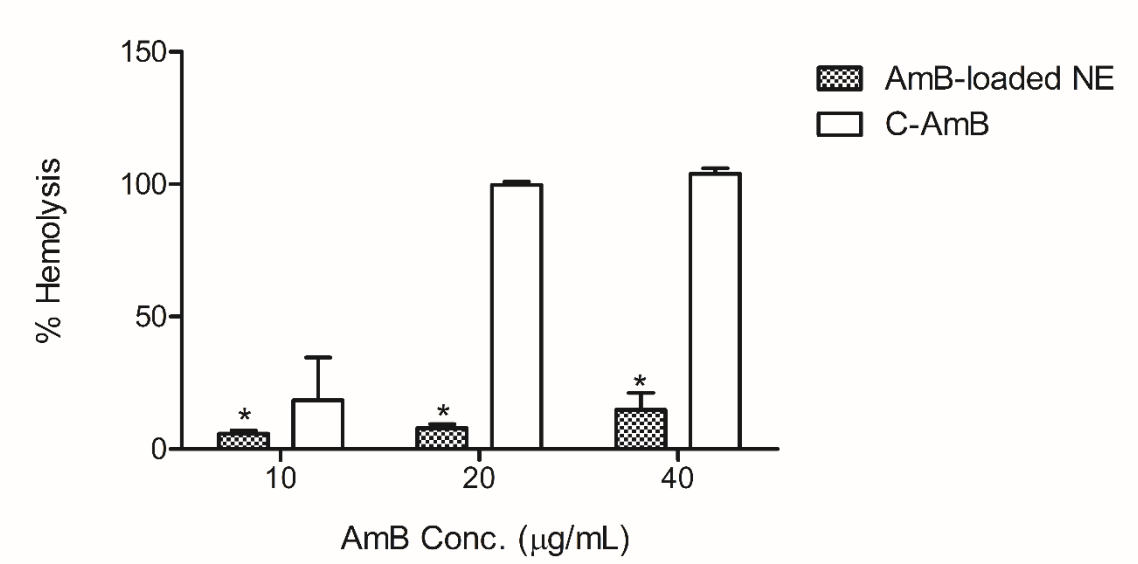


3.2 *In vitro* hemolytic toxicity

Figure 3 shows the hemolytic activity of AmB formulations on human RBC expressed as % hemolysis, as compared to that of positive control. The RBCs were completely lysed in positive control (distilled water) and was considered as 100% hemolysis, while, in the case of negative control (PBS, pH 7.4), no hemolysis was observed, as expected. Incubation of RBCs with AmB at 10, 20 and 40 μg/mL led to hemolysis values of 18.4 ± 16.2%, 99.7 ± 1.2% and 103.9 ± 2.1%, respectively for the C-AmB, while for AmB-loaded NE, at the same concentrations, the values were 5.8 ± 1.3%, 7.9 ± 1.5%, 14.8 ± 6.4%,

respectively. Therefore, hemolytic activity detected for the conventional AmB (C-AmB) was significantly higher than that observed for the AmB-loaded NE ($p < 0.05$).

Figure 3: *In vitro* hemolytic activity caused by AmB formulations. Red blood cells were incubated at 37 °C for 3 h with conventional AmB (C-AmB) and AmB-loaded NE at concentration of 10, 20 and 40 µg/mL in phosphate buffered saline (* $P < 0.05$ when compared with C-AmB group. All values reported are mean ± S.D., n=3).



3.3 *In vivo* anti-leishmanial activity

The *in vivo* anti-leishmanial activity was carried out to evaluate the efficacy of AmB-loaded NE in comparison with C-AmB. The parasite burdens in the spleen and liver were assessed three days after the end of treatment and these data are presented in Figures 4 and 5, respectively. The parasite burden in the spleen was significantly reduced in animals treated with AmB-loaded NE or C-AmB, both at 1 mg/kg/day, as compared to those that received vehicle (5% glucose solution; control group). When the parasite burden in the liver was evaluated, a similar profile was observed, with significant reduction in animals treated with AmB-loaded NE or C-AmB, both at 1 mg/kg, as compared to the control group. Treatment with AmB-loaded NE at 2 mg/kg provided significant

reduction in parasite burden in the spleen and liver in comparison to other groups. C-AmB at 2 mg/kg was very toxic and, therefore, the data concerning this group were not shown.

Figure 4: *In vivo* efficacy of AmB in *L. (L.) chagasi*-infected mice. Female BALB/c mice (n=7) were infected intravenously with *L. (L.) chagasi* promastigotes. Seven days after inoculation, the animals were treated with 5% glucose solution (Control), AmB-loaded NE at 1 or 2 mg/kg (NE 1 mg/kg and NE 2 mg/kg), and Anforicin® (C-AmB at 1 mg/kg) for 5 alternate days. Three days after interruption of treatment, parasite numbers recovered from spleen were evaluated by limiting dilution assay. *P < 0.05 when compared with control group.

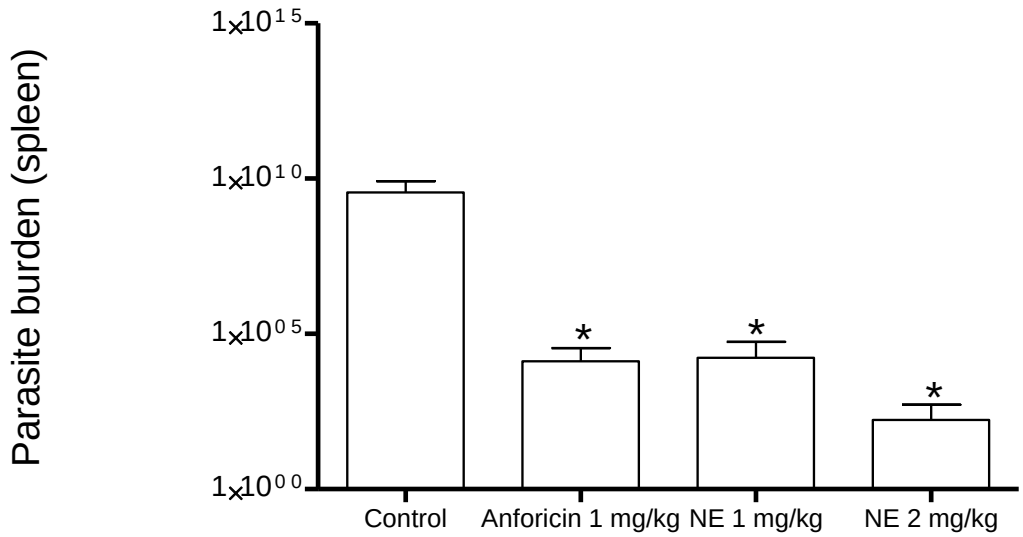
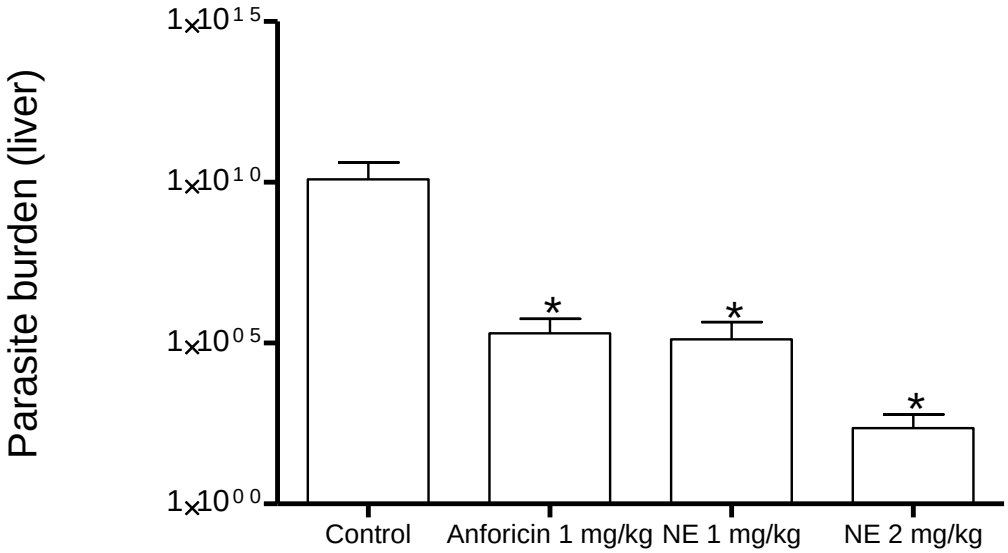


Figure 5: *In vivo* efficacy of AmB in L. (L.) chagasi-infected mice. Female BALB/c mice (n=7) were infected intravenously with L. (L.) chagasi promastigotes. Seven days after inoculation, the animals were treated with glucose solution 5 % (Control), AmB-loaded NE at 1 or 2 mg/kg (NE 1 mg/kg and NE 2 mg/kg, respectively) and Anforicin® (C-AmB 1 mg/kg) for 5 alternate days. Three days after interruption of treatment, parasite numbers recovered from liver were evaluated by limiting dilution assay. *P < 0.05 when compared with control group.



4 Discussion

The clinical use of AmB is limited by its frequent toxicity mainly related to the parenteral administration of the conventional formulation. The lipid formulations appear as alternatives to overcome this drawback mainly due to reduction of self-associated free AmB, the most toxic form to the host cells. Recently, we developed highly stable AmB-loaded NE and the NE-induced toxicity toward macrophages was significantly lower than that observed for the conventional AmB.[10] In present study, the *in vitro* hemolytic toxicity and *in vivo* antileishmanial activity of the AmB-loaded NE were evaluated.

In order to conduct *in vivo* experiments, it was necessary to optimize the NE developed in our previous study, aiming to reduce the droplet size and PI. In fact, the size of oil droplets is a prerequisite for intravenous administration and

the biodistribution of NE strongly depends on this feature.[11, 12] In the present study, the influence of the pressure of the HPH on the size and the PI were investigated. Increasing the pressure in HPH provided reduction of size but PI also increased.

In a HPH, the oil and water mixture is subjected to intense turbulent and shear flow fields. Turbulence is said to be the predominant mechanism even through laminar shear and cavitation may also play an important role. Turbulence leads to the break up of the dispersed phase into small droplets.[19] Our results demonstrated that a gradual increase in pressure provided small droplets (~140 nm) with alow PI (PI < 0.2). These values are lower than those found in our previous study. A plausible explanation for this finding is that the lowest average diameters of the droplets are obtained along a diagonal running from low pressure and high temperature (the beginning of the process) to high pressure and low temperature (the end of the process). The following pressure and temperature combinations lie along this diagonal and constitute the optimum manufacturing range and the lowest diameter values of the droplets are reached after 9-10 homogenization cycles.[20] In a previous study, Noack et al. (2011) used a nine cycles homogenizing regimen, using a two-stage high-pressure homogenizer. They found NE containing small droplets size and a good distribution.[21]

It is important to note that average size of the droplets was similar when evaluated by the three different techniques (PCS, AFM and NTA). Moreover, it was possible to observe an increase in the size of the particles when AmB was incorporated into NE. This suggests the drug incorporation into the NE droplets.[22], which was accompanied by the visual shift of the colour from milky white to yellow.[23] However, analyzes performed by AFM demonstrated a flattening of the droplets, which was detected by the difference between their diameter and height of the particle. This phenomenon was also observed in other studies in which the the internal phase of the nanoparticles is filled with liquid lipid which is surrounded by a flexible membrane wrapped in water (external phase).[14, 24, 25] In addition, NTA analysis provides direct visual information about scattering particles.[26] In this study, it was possible to confirm the absence of insoluble crystals of AmB in AmB-loaded NE samples.

In vitro hemolytic toxicity was selected as a tool to investigate further the toxicity of NE containing AmB in comparison to the conventional formulation. It is well known that free AmB induces hemolysis by pore formation and changing electrolyte balance in erythrocytes.[16]Our data clearly shown that hemolytic activity provided by conventional AmB (C-AmB) was very higher than that observed for the AmB-loaded NE. This can be attributed to an encapsulation of AmB in the NE droplets and the lower exposure of RBCs to the free AmB, since the drug release from the carrier system is low.[17, 27]Another explanation for this finding could be the fact that the conventional AmB formulation is a micellar dispersion of AmB with sodium deoxycholate. Sodium deoxycholate is a surfactant and can induce hemolysis itself in addition to the hemolysis caused by AmB.[16, 28] These results are in agreement with those obtained previously[10], which have shown that AmB-loaded NE was less cytotoxic than C-AmB. The interactions between AmB and surfactant or cholesterol in the droplet interface of the nanoemulsion probably contribute to reduce drug release from the nanocarrier, which causes a decrease in concentration of free AmB. In contrast, the release of AmB from C-AmB is high, probably due to the weak interaction between the AmB and deoxycholate.[10, 29] The data therefore, indicates the protective role of NP in preventing the RBC lysis.

Our *in vivo* studies showed that the AmB-loaded NE and C-AmB, both at 1 mg/kg, were active against *L*. (*L*.) *infantum chagasi* in BALB/c mice but differences between these two formulations were not significant. Previous studies have demonstrated the efficacy of lipids formulations in treatment of *in vivo* model of VL, mainly in animals infected by *L*. *donovani*, at similar doses.[30-32] Factors such as species and strains variability of the *Leishmania*, which display specific biochemical and molecular characteristics, may reflect in a significant variability to drugs' sensitivity.[33, 34] Mice were not treated with higher doses of C-AmB, since doses > 1 mg/kg were reported to be very toxic or lethal.[32, 35, 36] In contrast, administration of the AmB-loaded NE at 2 mg/kg did not resulted any sign of acute toxicity (weight loss and death) in mice, and these findings are consistent with our *in vitro* studies, which showed a marked reduction in toxicity of NE formulation. At this dose, the AmB-loaded NE led to significant reduction in parasite burden in the liver and spleen, as compared to the control group ($P < 0.05$).

Lipid-AmB formulations have been established as a useful treatment for VL. The main restriction against the widespread use of the current approved lipid-AmB formulations for leishmaniasis is their high cost. The search remains for a low cost formulation, which could have good activity against leishmaniasis.[37] Therefore, the AmB-loaded NE formulation emerges as a good alternative for the treatment of VL due to the lower cost compared to liposomal formulations, lower haemolytic toxicity and improved efficiency compared to the conventional formulation.

5 Conclusion

The preparation of the NE loaded with AmB was optimized. We obtained droplet size and PI values lower than those reported previously. The hemolytic activity provided by C-AmB was very higher than that observed for the AmB-loaded NE. AmB-loaded NE (2 mg/kg) led to significant reduction in parasite burden in the liver and spleen, as compared to the control group and did not resulted any sign of acute toxicity. These findings suggest that AmB-loaded NE formulation is a promising alternative for the treatment of VL caused by *L*. (*L*.) *infantum chagasi.*

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CAPÍTULO 2

Metabolomics as a tool to evaluate the toxicity of formulations containing amphotericin B, an antileishmanial drug

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Abstract

Amphotericin B (AmB) is a drug of choice against life-threatening systemic fungal infections and an alternative therapy for the treatment of all forms of leishmaniasis. It is known that AmB and its conventional formulation cause renal damages; however, the lipid formulations can reduce these effects. The aim of the present study was to identify metabolic changes in mice treated with two different AmB formulations, a nanoemulsion (NE) (lipid system carrier) loaded with AmB and conventional formulation (C-AmB). For this purpose, metabolic fingerprinting represents a valuable strategy to monitor, in a non-targeted manner, the changes that are at the base of the toxicity mechanism of AmB. Plasma samples of BALB-c mice were collected after treatment with 3 alternate doses of AmB at 1 mg/kg administered intravenously and analysed with CE, LC and GC coupled to MS. Blood urea nitrogen (BUN) and plasma creatinine levels were also analysed. Kidney tissue specimens were collected and evaluated. It was not observed any alteration in BUN and creatinine levels as well as in histopathological analysis. Approximately 30 metabolites were identified as potentially related to early C-AmB-induced nephrotoxicity. Disturbances in the arachidonic acid, glycerophospholipids, acylcarnitine and polyunsaturated fatty acids (PUFA) pathways were observed in C-AmB-treated mice. In AmB-loaded NE group, it was observed fewer metabolic changes, including changes in plasma levels of cortisol and pyranose. The candidate biomarkers revealed in this study could be useful in the detection of the onset and severity of kidney injury induced by AmB formulations.

Key words: Amphotericin B, metabolomics, biomarkers, plasma, toxicity

1 Introduction

Amphotericin B (AmB), a highly hydrophobic drug, is a natural antibiotic belonging to the polyene group used as a drug of choice against life-threatening systemic fungal infections.[1,2] It is also an alternative therapy for the treatment of all forms of leishmaniasis, replacing antimonials as the first line treatment of visceral leishmaniasis (VL), in India.[3,4] This drug and its formulations are also increasingly being used in other countries against VL, reaching 97 % cure rates, and no reported resistance.[5,6]

AmB leishmanicidal and antifungal activity is due to its ability to bind ergosterol in *Leishmania* and fungal cell membranes. This interaction produces pores on the membrane changing the selective cell permeability, culminating in ion (mainly potassium ions) and vital substances leakage. AmB also binds to cholesterol in the mammalian cell membrane causing the main side effects.[2,7] Considering that AmB has a very little solubility in aqueous solutions at physiological pH, sodium deoxycholate is required for the formation of a complex with the drug.[2] However, it is known that deoxycholate and free AmB present in the conventional formulation (C-AmB) are both responsible for toxicity, mainly hemolysis and nephrotoxicity. AmB is believed to act as a prooxidant in membranes, causing oxidative stress possibly associated with the formation of free radical intermediates.[8] AmB-induced nephrotoxicity is marked by renal dysfunction, including reduction in plasma filtration and increase in urinary lactate dehydrogenase activity, accompanied by renal tubular damage. In histopathology, the main characteristic of AmB nephrotoxicity in mice is tubular necrosis in the thick ascending limb of Henle's loop.[9,10] The tubules are especially susceptible to toxic injury for the following reasons: First, high concentrations of filtered toxicants are present in the tubular fluid, due to their function in solute and water reabsorption. Second, tubular epithelial cells have a large number of transporters, resulting in high intracellular concentrations of toxicants. Third, tubular epithelial cells have high-energy requirements to support metabolism and solute transport.[11]

The lack of an efficient and accurate biomarker has become a leading cause of severe nephrotoxicity due to AmB treatment or other drugs.[12] However,

current biomarkers (blood urea nitrogen, serum creatinine and hematoxylin-eosin staining) for nephrotoxicity have remained unchanged for several decades and are not sensitive enough to early detect renal injury. Increases in serum concentrations of these biomarkers occur only after substantial renal injury, which generally occurs after two-thirds or greater loss of nephron functional capacity.[13]

Metabolomics is the global measurement of metabolome in biological matrices, which provides a systematic view of the change of all metabolites in the physiological or pathological conditions.[14]It has shown a great potential for identification of early biochemical changes associated with nephrotoxicity, as well as, the discovery of potential biomarkers, and underlying disease mechanisms.[15] Metabolomics, as a system approach, could provide extensive information on the dynamic process of nephrotoxicity due to drug toxicity or environmental pollutants.[16] Despite its anatomical complexity, the kidney is highly amenable to metabolomics analysis, providing a unique opportunity to detect early renal injury and region-specific effects as well as determine mechanisms of toxicity.[11] Metabolomics has been introduced to study the changes in metabolism in acute kidney injury within the last few years, especially in nephrotoxic models including those induced by gentamycin,[17,18] aristolochic acid,[19] cisplatin,[20,21] melamine and cyanuric acid.[22] Interestingly, metabolomics may enable the simultaneous measurement of many metabolites in interacting pathways, reflecting alterations in renal physiology due to drug nephrotoxicity.

Mass spectrometry (MS) is the most widely applied technology in metabolomics, used to identify metabolites after separation either by gas chromatography (GC), high-performance liquid chromatography (HPLC), or capillary electrophoresis (CE). The combination of these techniques is well suited for probing a very large part of the plasma metabolome, as they are capable of detecting both lipophilic and hydrophilic metabolites.[18,23]

The lipid formulations, such as liposomal AmB, can reduce AmB-related toxicity, but the high cost and difficulty of manufacturing represent limitations for their widespread use.[1] Nanoemulsions (NE) are attractive lipid systems carriers of AmB, since this delivery systems are able to reduce drug-related toxicity, easy to manufacture and inexpensive.[24] Recently, we developed AmB-loaded NE with high encapsulation efficiency, highly stable and that significantly aggregates less as compared to a conventional AmB formulation, which is the more toxic form of

AmB. Moreover, the toxicity induced by NE in macrophages was significantly lower than that observed for the conventional AmB.[25] Therefore, this AmB-loaded NE may constitute an attractive alternative for the treatment of leishmaniasis, with less toxic effects.

In this study, a multi-platform metabolomic approach was applied to identify metabolic changes in mice treated with two different AmB formulations, AmB-loaded NE and C-AmB, which could be potentially related to early toxicity events induced by AmB. The results found in this study may provide relevant information for clinical use of AmB, and lay the foundation for further research on development of AMB formulations and mechanism of AmB-induced toxicity.

2 Materials and methods

2.1 Materials

AmB and conventional AmB (C-AmB; Anforicin B®), a complex of AmB and sodium desoxycholate, were kindly provided by Cristalia Produtos Químicos Farmacêuticos Ltda (São Paulo, Brazil). Medium chain triglycerides (MCT) and polysorbate 80 (Tween 80®) were kindly provided by Croda Brazil Ltda (São Paulo, Brazil). Cholesterol and hidroxibutiltolueno (BHT) were purchased from Sigma-Aldrich (Missouri, USA). The glycerol was obtained from the House of Chemical Industry & Trade Ltda. (São Paulo Brazil). HPLC grade acetonitrile and tetrahydrofuran were purchased from Tedia (Rio Janeiro, Brazil). HPLC grade methanol was purchased from J.T. Baker (EUA). All other chemicals were of analytical reagent grade.

For studies involving metabolomics, the reference compounds were purchased from Sigma (St. Louis, MO) and Aldrich (Steinhein, Germany). Methanol (LC-MS grade), heptane (GC-MS grade), chloroform (MS grade), acetonitrile (LC-MS grade), isopropanol (LC-MS grade), formic acid (MS grade), pyridine (silylation grade), C18:0 methyl ester (IS), methionine sulfone (IS) and O-methoxyamine hydrochloride were purchased from Sigma-Aldrich (Taufkirchen, Germany). Reagents for derivatization N,O-bis(trimethylsilyl) trifluoroacetamide (BSTFA) plus 1% trimethylchlorosilane (TMCS) was purchased from Pierce Chemical Co (Rockford, IL, USA) and reference masses

purine and HP-0921 (hexakis-(1H,1H,3H-tetrafluoropentoxy)-phosphazene) were from Agilent (atmospheric pressure inlet-time of flight (API-TOF) reference mass solution kit).

2.2 Preparation of the AmB-loaded NE

Composition of the AmB-loaded NE was previously described.[25] Briefly, the oily phase (OP) was composed of MCT (5.0% w/v), Tween 80 (1.5% w/v; hydrophilic surfactant), cholesterol (0.5% w/v), and BHT (0.01% w/v; antioxidant). The aqueous phase (AP) was composed of glycerol (2.25%; isotonizer) and purified water. The NE was prepared by the hot homogenization method. Both oil and aqueous phases were heated at 70 °C and then the AP was slowly added to the oil phase and homogenized using an Ultra-Turrax T25 at 8000 rpm for 2 min. The resulting dispersion was further homogenized in a nine cycle homogenizing regime, using a high-pressure homogenizer APV-1000 (Invensys, Denmark). For the first three cycles, the main pressure was set at 400 bar. The pressure was increased every three cycles to 600 bar and 800 bar, respectively.[26] The formulation was cooled down to room temperature and the AmB was incorporated into the NE at a concentration of 2 µg/mL.[27] AmB (40 µg for a batch 20 mL) was solubilised in 1 mL of a 0.2 M sodium hydroxide (NaOH) solution and it was added to the blank NE (without AmB) using a magnetic stirrer at 600 rpm for 1 min, at room temperature. Next, the pH was adjusted to 7.0-7.5, with hydrochloric acid and the final volume was completed with sterile water. A blank NE (without AmB) was also prepared for comparison purpose.

2.3 Characterization of AmB-loaded NE

Formulations were characterized for particle size (mean hydrodynamic diameter), polydispersity index (PI) and zeta potential. The droplet size distribution and the PI were determined by photon correlation spectroscopy (PCS) using a Zetasizer Nano ZS90 (Malvern Instruments, UK) at a fixed angle of 90° and at 25 °C. Before the measurements, the formulations were diluted with distilled water. All measurements were performed in triplicate. Zeta potential was evaluated by determining the electrophoretic mobility using the Zetasizer. Before

the measurements, the NE was diluted in water. All measurements were performed in triplicate.

2.4 *In vivo* nephrotoxicity studies

2.4.1 Animal handling and sampling

Animal care and handling protocols were in compliance with national animal treatment guidelines and approved by the Animal Committee of Universidad San Pablo, Madrid, Spain. All animal studies were performed in the animal center of San Pablo CEU University. BALB/c female mice aged 6-8wk (19-22g) were acclimated in temperature 25 °C and humidity 60 % with regular day/dark light cycle, starting from one week before the experiment to reduce the stress of adjusting to new environment for animals. Same conditions were used throughout the experiments.

Mice were divided into three groups of 5 to 8 animals each; the control group received a vehicle (5% glucose solution); the AmB-loaded NE group and the C-AmB group received a dose of 1 mg AmB/kg. All groups received, intravenous (IV), the injections over the course of 3 alternative days (three administrations). The weight of each animal was recorded before each dosing. After this procedure, the animals were euthanized 24 h after the last administration. The blood was obtained by cardiac puncture under deep CO_2 narcosis, and collected in tubes impregnated with anticoagulant (EDTA). After extraction, before they extinguished the effects of anaesthesia, it was applied humane euthanasia techniques (cervical dislocation). Plasma was separated from cellular components by centrifugation at approximately 2000 *xg* for 30 min at 4 °C. Immediately after, the plasma was divided into aliquots in 0.1 mL fractions (100 µL) and frozen at -80 °C, where it was remained until the day of analysis.[28]

2.4.2 Blood chemistry measurements

Blood urea nitrogen (BUN) and plasma creatinine levels were used as biochemical markers to evaluate the nephrotoxicity of the formulations. The

analyses were performed using commercial available kits (Gold Analisa Diagnótica Ltda, Brazil).

2.4.3 Statistical analysis

The data were processed using Graph Prism 4 software. Analyses for the blood chemistry measurements and the urinary volume were evaluated using the one-way analysis of variance (ANOVA) test followed by Tukey's test, after log transformation. The difference was considered significant when the p value was less than 0.05.

2.4.4 Renal Histology

Kidney tissue specimens were also collected and fixed in 10 % neutral buffered formalin and subjected to tissue processing followed by embedding in paraffin. The sections were further sliced into layers with 5 µm thickness (Leica, Wetzlar, Germany) and stained with hematoxylin and eosin (H&E) for microscopic examination (Olympus, Tokyo, Japan).

2.5 *In vivo* toxicity using Metabolomics

2.5.1 Sample treatment and analysis

2.5.1.1 LC-MS

Sample treatment and analysis were performed following a previously described approach.[29] In the initial step, a frozen plasma sample was thawed on ice, 3 volumes of ice-cold methanol:ethanol (1:1, v/v) was added to 1 volume of plasma, the mixture vortexed for 1 min, placed on ice for 5 min and then centrifuged at 16700 *xg* for 20 min at 4°C. The supernatant was filtered through a 0.22 µm nylon filter.Samples were randomized previous to their injection in the equipment for analysis.

All chromatographic separations were performed using a 1200 series HPLC (Agilent Technologies, Waldbronn, Germany), Supelco Discovery HS C18

analytical column (15 cm x 2.1 mm, 3 µm) and guard column (2 cm x 2.1 mm, 3 µm; Sigma Aldrich, Steinheim, Germany). The autosampler and columns were maintained at 4 and 40 °C, respectively, the injection volume was set to 10 µL, and the solvent flow was 0.6 $mLmin^{-1}$ (solvent A: water with 0.1 % formic acid, and solvent B: acetonitrile with 0.1% formic acid). The gradient elution started from 25 % B to 95 % B in 35 min, and returned to starting conditions in 1 min, keeping the re-equilibration at 25 % B for 9 min.[29] Data were collected in positive and negative electrospray ionization (ESI) mode in separate runs on a quadrupole time-of-flight mass spectrometer QTOF (Agilent 6520) operated in full scan mode from 50 to 1000 m/z with a scan rate of 1 scan per second. During the analysis, reference masses: 121.0509 m/z ($C_5H_4N_4$) and 922.0098 m/z ($C_{18}H_{18}O_6N_3P_3F_{24}4$) in positive ESI mode; and 119.0363 m/z (proton abstracted purine) and 966.0007 m/z (formate adduct of HP-921) in negative ion mode were continuously measured to allow constant mass correction. The capillary voltage was 3000 V for positive and 4000 V for negative ionization mode, and the nebulizer gas flow rate was 10.5 $Lmin^{-1}$. Samples were analysed in two randomized runs (first for positive and second for negative ion mode).

2.5.1.2 CE-MS

Each plasma sample (100 µL) was defrosted on ice and mixed with 100 µL of 0.2 M formic acid (with 5 % Acetonitrile and 0.4 mM methionine sulfone, internal standard (IS)). Each sample was vortex mixed for approximately 1 min, transferred to a centrifree centrifugation device (Millipore Ireland, Eire) for deprotenization and then centrifugation (2000 *xg*, 70 min, 4 °C). The filtrate was then transferred directly to a vial for analysis.[30]Samples were randomized previous to their injection in the equipment for analysis.

The experiments were performed by use of an Agilent 7100 capillary electrophoresis system coupled to an Agilent Technologies (Wilmington, USA) 6224 Accurate-Mass Time of Flight mass spectrometer system. The coupling was equipped with an electrospray source. A 1200 series ISO Pump from Agilent Technologies was used to supply sheath liquid. The capillary for separation, of diameter 50 µm and length 96 cm, was from Agilent Technologies (Germany). The new capillary was conditioned for 30 min with 1 $molL^{-1}$ sodium hydroxide, 30

min with 2 molL^{-1} aqueous ammonia and 30 min with background electrolyte (BGE) (0.8 molL^{-1} formic acid in 10 % methanol). Before each analysis the capillary was flushed with BGE for 5 min at 950 mbar pressure. Sample injections were done for 35 s at 50 mbar pressure. After injection of each sample the BGE was injected for 10 s at 100 mbar pressure, to improve repeatability. Separation conditions were 25 mbar pressure and 30 kV voltage; the current observed under these conditions was 25 µA. The instrument automatically replaced the BGE before each analysis.

The MS was operated in positive polarity, and other conditions were: drying gas 10 Lmin^{-1}, nebulizer 10 psi, voltage 3500 V, fragmentor 100 V, gas temperature 200 °C, and skimmer 65 V. The sheath liquid used for detection consisted of 50 % methanol, 50 % water, 4 µL formic acid, and reference standards (10 µL of 5 mmolL^{-1} purine and 30 µL of 2.5 mmolL^{-1} HP-921), at a flow rate of 0.6 mL min^{-1} (1:100 split). BGE and sheath liquid were freshly prepared and degassed by sonication for 5 min before use, to ensure proper and reproducible ionization. Data acquisition in CE was performed with a ChemStation B.04.02; in MS it was performed with a MassHunter Work-Station B.05.00 (Agilent Technologies).[30]Samples were analysed in a randomized order.

2.5.1.3 GC-MS

The analytical procedure for metabolic characterization was carried out following a methodology previously described.[31] Briefly, an aliquot (50 µL) of plasma sample was vortex-mixed with 150 µL of cold acetonitrile, vortex-mixed for 2 min, let stand for 5 min, and centrifuged at 16000 *xg* for 10 min at 4 °C. After the deproteinization step, supernatant (100 µL) was transferred to a GC vial with insert and evaporated to dryness in a Speedvac Concentrator (Thermo Fisher Scientific, Waltham, MA, USA). QC samples were prepared by pooling equal volumes of each supernatant and split into vials. 10 µL of O-methoxyamine hydrochloride in pyridine (15 mg/mL) was added to each GC vial, and the resultant mixture was vigorously vortex-mixed for 5 min. Methoxymation was carried out in the dark, at room temperature for 16 h. For derivatization, the solution was vortex-mixed again for 5 min after 10 µL of BSTFA with 1 % TMCS was added as catalyst. Samples were heated in an oven for 1 h at 70 °C to

achieve silylation. Finally 100 µL of heptane containing 10 mg/L of C18:0 methyl ester (IS) was added to each GC vial and vortex-mixed for 2 min before GC analysis. Samples were randomized previous to their injection in the equipment for analysis.

Samples were analysed using a GC instrument (Agilent 7890A) coupled to mass spectrometer with triple-Axic detector (Agilent 5975C). 2 µL of previously derivatized sample was injected in split mode using an Agilent 7693 autosampler. Separation was achieved using a 10 m J&W precolumn (Agilent Technologies®) integrated with a 122-5332G column DB5-MS: 30m length, 0.25 mm i.d. and 0.25 µm film consisted of 95 % dimethyl/5 % diphenyl polysiloxane (Agilent Technologies®). The injector port was held at 250°C, and the helium carrier gas flow rate was set at 1.0 $mLmin^{-1}$. The split ratio was 1:10. The column was initially maintained at 60 °C for 1 min after injection, then temperature was increased at the rate of 10 $°Cmin^{-1}$ to reach a final temperature of 325 °C, and cool down after analysis for 10 min. Temperatures of the injector, transfer line, filament source and quadruple were maintained at 250 °C, 280 °C, 230 °C and 150 °C, respectively. The quadrupole detector (5975 inert MSD, Agilent) was controlled by ChemStation software. The electron ionization source was operated at −70 eV. MS detection was achieved in scan mode over a mass range m/z 50−600 at a rate of 2.7 scan/s. All these conditions were optimized previously.[31]For both MS techniques, the samples from the application were analysed in one randomized run.

2.5.1.4 Data Treatment and Statistical Analysis

The resulting LC-MS and CE-MS data files were cleaned of background noise and unrelated ions by the Molecular Feature Extraction tool in the MassHunter Qualitative Analysis software (B.05.00, Agilent Technologies). The Molecular Feature Extraction, then, creates a listing of all possible components (features) as represented by the full mass spectral data. Each compound is described by mass, retention time, and abundance. Primary data treatment (filtering and alignment) was performed with Mass Profiler Professional B.02.00 software (Agilent technologies), correcting the retention time and mass error. All

electropherograms and chromatograms (samples and QCs) for positive and negative mode and were aligned separately.

The treatment of the data obtained by GC-MS analysis consisted of compound identification, using the Fiehn RTL Library (FiehnLib) and the National Institute of Standards and Technology mass spectra library (NIST MS, 2.0 g, 2014), in the ChemStation software PBM algorithm (G1701EA E.02.00.493, Agilent), with a correct assignment based on the coincidence of the retention time and the spectrum profile. The GC-MS data were normalized with respect to the internal standard (C18:0 methyl ester). Subsequently, deconvolution was performed using Automated Mass spectral Deconvolution and Identification System (AMDIS 2.69) software to identify co-eluting compounds followed by statistical analysis.

From the three analytical platforms selected, compounds with statistically significant differences were obtained by the comparisons: AmB-loaded NE vs. Control, C-AmB vs. Control and AmB-loaded NE vs. C-AmB. Features and compounds from all platforms were filtered by selection of masses present in at least 67 % of the samples in one of two groups (AmB-loaded NE vs. Control, C-AmB vs. Control or AmB-loaded NE vs. C-AmB). Differences among samples were evaluated by using nonparametric Mann-Whitney U test p-value < 0.05 (Microsoft excel). The Benjamini-Hochberg test was used to control for false discovery rate (FDR) and generate adjusted p values ($p < 0.05$).

2.5.2 Compound identification

The identification of compounds (LC-MS or CE-MS), which have presented significant values in class separation, was performed by searching accurate masses against online databases available (HMDB, METLIN, LIPIDMAPS, KEGG). The identity of compounds obtained by LC-MS, which were found to be significant in class separation, was confirmed by LC-MS/MS in the same equipment. Experiments were repeated with identical chromatographic conditions as in the primary analysis. Ions were targeted for CID fragmentation on the fly based on the previously determined accurate mass and retention time. Comparison of the structure of the proposed compound with the fragments obtained was done to confirm the metabolite identity. Accurate mass data and

isotopic distributions for the precursor and product ions were studied and compared to spectral data of reference compounds, if available, obtained under identical conditions for final confirmation, with Agilent Technologies MassHunter Molecular Structure Correlator B.05.00, which correlated accurate mass MS/MS fragment ions for a compound of interest with one or more proposed molecular structures for that compound. In the case of CE-MS, the identification of compounds was possible by the use of standards spiking to sample for confirmation. In the absence of standards, identification of the compounds is considered putative.

2.5.3 Quality control of the methodology

Either for LC-MS, CE-MS or GC-MS fingerprinting experiments, quality control (QC) samples were prepared by pooling equal volumes of plasma. The pool was split into aliquots and each aliquot processed as a sample. QC samples were regularly analysed throughout the run, after every group of six samples, to provide a measurement of the system's stability and performance.[32] The CV of the features in the set of QC samples also provided a parameter to evaluate their reliability.

3 Results

3.1 Preparation and characterization of the NE

AmB-loaded NE was prepared by the hot homogenization followed by high-pressure homogenization method and the physicochemical characteristics were evaluated. The mean size of NE obtained was 145 ± 1 nm, which agrees to an intravenous administration.

3.2 *In vivo* nephrotoxicity studies

3.2.1 Blood chemistry measurements

To investigate the renal toxicity, mice were treated with C-AmB or AmB-loaded NE (equivalent to 1 mg/kg) on 3 alternate days. On day 6, plasma from euthanized mice was collected and analysed for BUN and serum creatinine concentrations. No significant alterations were observed in these parameters regardless the treatment mice were submitted, as compared to control group (Table 1). The levels for BUN and serum creatinine were quite similar among the C-AmB (59.0 ± 6.9 mg/dL, 0.17 ± 0.08 mg/dL), AmB-loaded NE (57.5 ± 2.9 mg/dL, 0.08 ± 0.06 mg/dL) and control group (51.1 ± 6.2 mg/dL, 0.17 ± 0.06 mg/dL). The urine volume was also investigated and the results showed significant differences between the control group (85 ± 75 µL) and each treated groups: AmB-loaded NE (381 ± 130 µL) and C-AmB (1000 ± 460 µL). Differences were also significant between the treated groups.

Table 1: Blood chemistry and Urine volume in AMB experiment

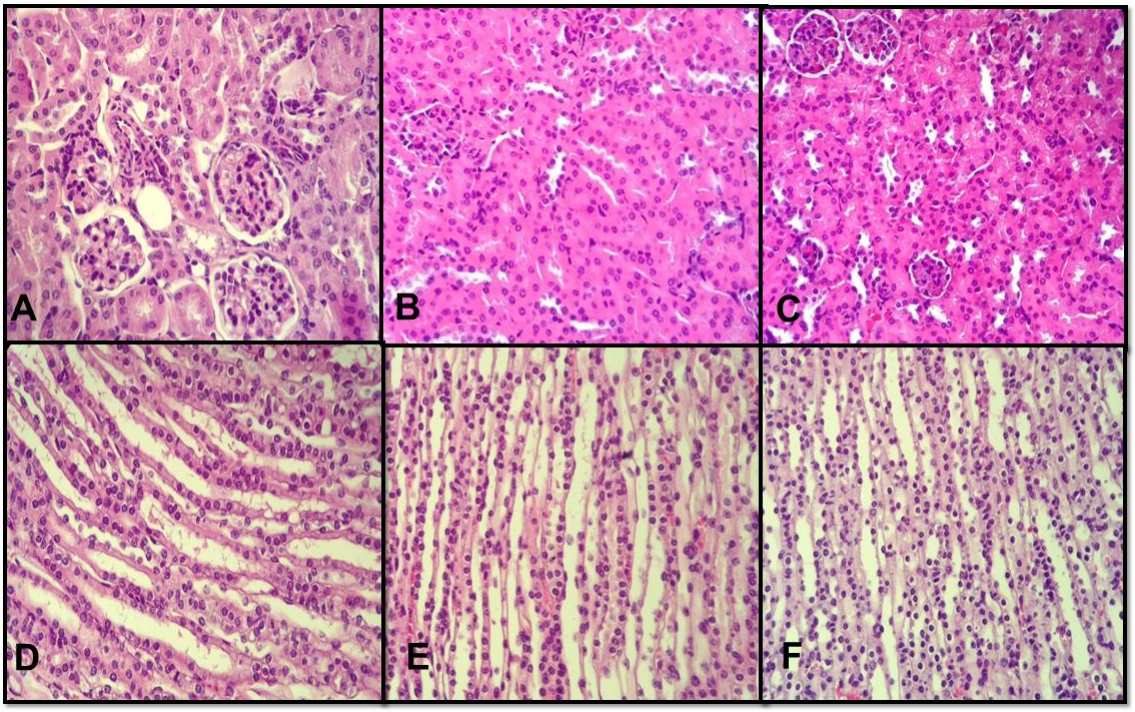
	Cr (mg/dL)	*BUN (mg/dL)*	*Urine volume (µL)*
Control	0.17 ± 0.06	51.1 ± 6.2	85 ± 75
AmB-loaded NE	0.08 ± 0.06	57.5 ± 2.9	381 ± 130
C-AmB	0.17 ± 0.08	59.0 ± 6.9	1000 ± 460

Data are expressed as means ± S.D. The statistical significance of differences between treated and control groups were determined with Tukey's multiple comparison test ($P < 0.05$).

3.2.2 Renal Histology

The effect of AmB on kidney histopathology was analysed by H&E staining of paraffin sections of mice kidneys, after treatment. The histopathological evaluations of kidney sections of NE-loaded or conventional AmB-treated mice revealed no tubular necrosis in Henle's loop of the medullary outer layer for all groups at doses of 1 mg/kg given, administered on three alternate days (Figure 1). Additionally, congestion in the medulla, hyaline cast, dilatation and tubular necrosis in the cortex were not observed.

Figure 1: Light microphotographs of renal cortex and medulla of mice. (A) Renal cortex of a control mouse treated with vehicle only. (B) Renal cortex of a mouse treated with AmB-loaded NE. (C) Renal cortex of a mouse treated with C-AmB. (D) Renal medulla of a control mouse treated with vehicle only. (E) Renal medulla of a mouse treated with AmB-loaded NE. (F) Renal medulla of a mouse treated with C-AmB. Mice were treated with a 3 alternate doses of AMB and sacrificed 24 h later. Histological sections of the cortex and renal medulla were stained with hematoxylin and eosin (H&E; magnification ×400).



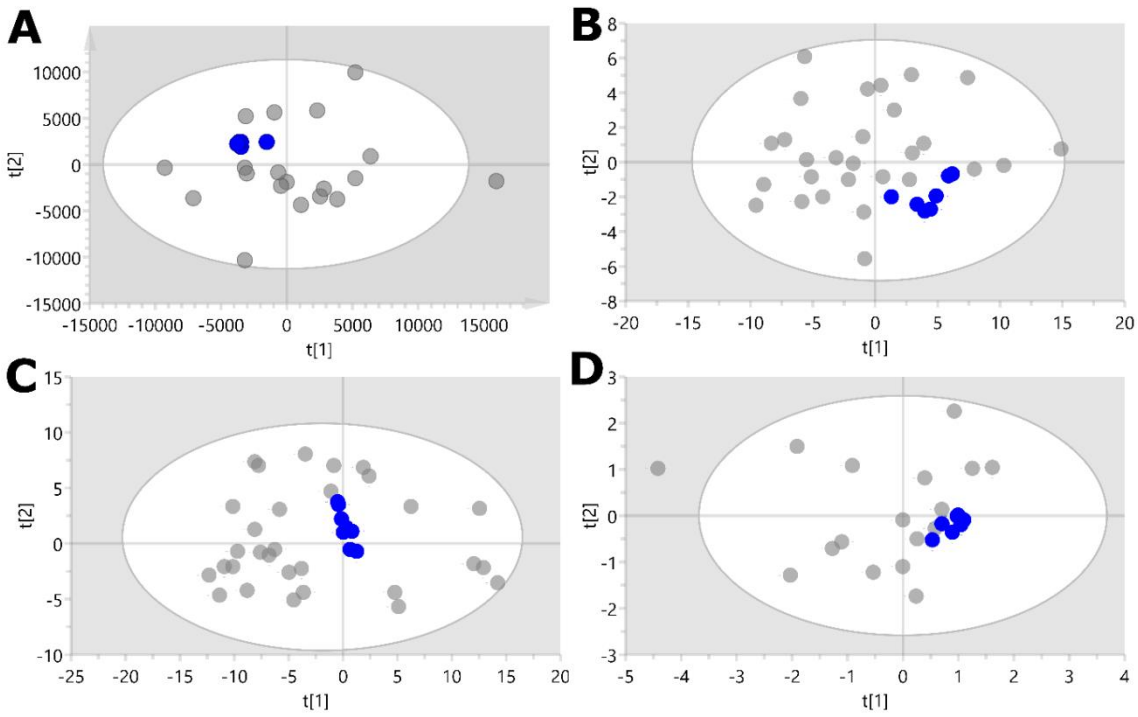
3.3 *In vivo* toxicity using Metabolomics

3.3.1 Samples classification

Chromatograms from the analysis of the plasma samples obtained from each technique were multi-aligned and data were filtered to remove noise. Initially, a principal components analysis (PCA) plot was generated from data of each technique to ensure grouping of QC samples that is indicative of stability in the analyses. PCA is a mathematical model that first analyse data without considering the classes. Later, samples are given a class to check the quality of the model. Figure S1 shows the results from this for LC-MS, CE-MS and GC-MS,

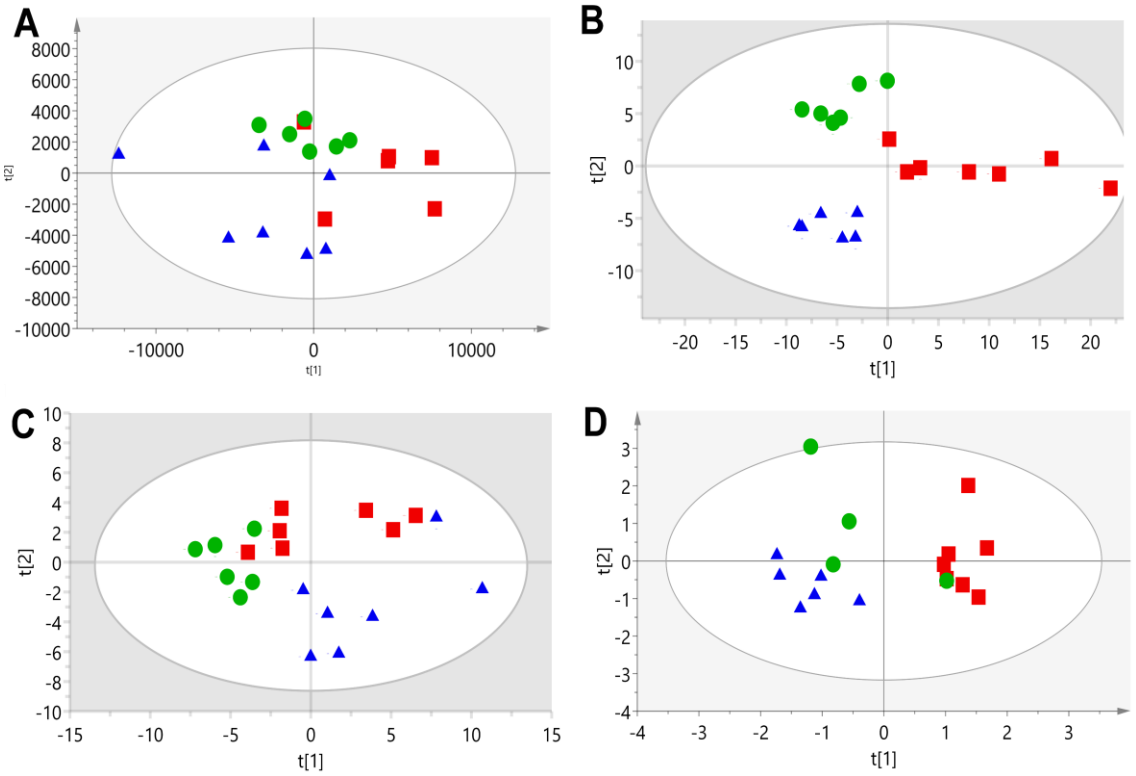
and indicates that the QCs clustered tightly, suggesting that the dataset could be used for further statistical analysis. To identify differences on the abundance of metabolites related to AmB-induced toxicity in plasma samples, the data were filtered according to presence in at least 67% of samples in at least one group, and with coefficient of variation lower than 30% in QC samples, and then, PLS-DA were performed. Filtration of 67% was selected considering that each sample represents 17% in the group (n= 6=100%) and this was based on the relation presence/restriction. Metabolites present in 67% of the sample group were kept because they were present in more than 50% of their samples, which excludes the possibility of a false positive, for example. Furthermore, this filter was not as restrictive; therefore important metabolites that appear at low levels in the blood can be maintained.

Figure S1: PCA model of all platforms showing the clustering of QCs of plasma sample. (A) LC-MS Positive. B) LC-MS Negative. C) CE-MS. D) GC-MS. Filtering was performed by eliminating features present in the QC samples with a coefficient of variation above 30%. QCs (blue circles).



The results of the PLS-DA models (Figure 2) showed the separation of groups and allowed to predict which variable belongs to each group in all platforms (CE-MS, LC-MS and GC-MS). In addition, control and AmB-loaded NE groups tended to be clustered in the same component, and opposite to C-AmB group. R^2 and Q^2 parameters for these models showed high-quality sample classification and good predictive power, except for the model obtained by LC-MS positive (Q^2:0.02).The separation of the three groups by LC-MS positive mode was not suitable. Since the model lacks prediction capability, the metabolites measured with this technique (Table S1) are not the best classifiers and predictors, and should be used to compare the metabolome alterations induced by treatment with the two AmB formulations. On the other hand, models obtained with GC-MS, CE-MS or LC-MS negative presented higher predictive values and, therefore, the corresponding metabolites were considered relevant for discrimination among groups. Both PCA and PLS-DA analysis was performed by SIMCA-P+software (12.0.1 versions, Umetrics).

Figure 2: Scores plot for PLS-DA model built with the filtered data set (66% of samples in at least one group; compounds with a coefficient of variation higher than 30% in the QC samples were also filtered out) obtained from plasma samples analyzes, showing separation between groups. (A) LC-MS (positive) [R^2 = 0.46; Q2 = 0.02], (B) LC-MS (negative) [R^2 = 0.99; Q^2 = 0.93], (C) CE-MS [R^2 = 0.99; Q2 = 0.77], (D) GC-MS [R^2 = 0.57; Q^2 = 0.30]. C (blue triangles), AmB-loaded NE (green circles), C-AmB (red squares).



3.3.2 Metabolites Detected in Plasma

The metabolomics analysis was performed on 24 mice plasma samples, collected from three treatment groups (Control, AmB-loaded NE and C-AmB). The analytical signatures of the chromatography peaks present in the samples were matched against a database (http://ceumass.eps.uspceu.es/mediator/), amediator between KEGG, Metlin and Lipid Maps, containing thousands of metabolites. Table 2 shows the final number of compounds obtained after each step of data treatment. A significant reduction in the number of compounds is seen at each step, demonstrating the importance of data treatment to select the significant metabolites. Statistically significant and identified features

(compounds with $p < 0.05$) are summarized in tables S1 (LC-MS), S2 (CE-MS) and S3 (GC-MS), in addition to the identification provided by the databases. As previously explained, the LC-MS confirmation was performed by comparing the LC-MS/MS fragments with those in databases. For CE-MS, when it was available the standard, the already prepared sample, and the sample spiked with the standards were analysed in the same conditions in order to confirm the identity. Confirmation was performed by comparison of the migration time and isotopic distribution.

Table 2: Number of features obtained after referred steps in data treatment in each analytical technique for plasma samples.

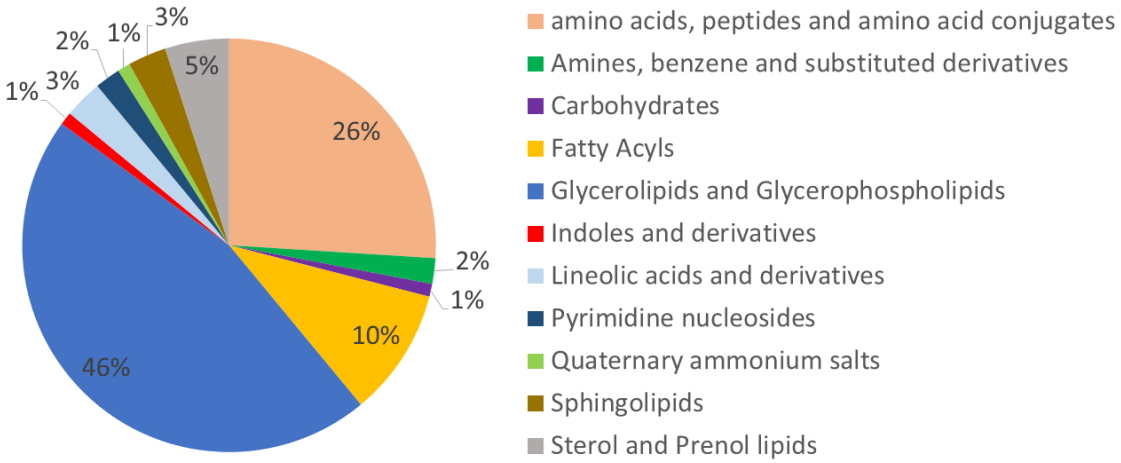
Features	*LC-MS (+)*	*LC-MS(-)*	*CE-MS*	*GC-MS*
Alignment	7960	2853	4631	42
Filter 66%	1044	510	495	23
Significant masses	84	26	45	3
Putatively identified	24	17	27	1
Confirmed compounds	17	15	2	2

For MS-based results, the combination of all analytical platforms revealed 85 metabolites altered in plasma of C-AmB-treated group and 32 metabolites altered in plasma of AmB-loaded NE-treated group. The plasma abundance of AmB was high for both treatment groups, however the concentration for AmB-loaded NE-treated group was about two times higher as compared to C-AmB-treated group. The majority of metabolites decreased in plasma of AmB-treated samples compared to vehicle samples, i.e. the fold changes between the treated group and the control group were negative.

The metabolites were then classified into chemical categories: amines, benzene and substituted derivatives; amino acids, peptides and amino acids conjugates; carbohydrates; fatty acids; glycerolipids and glycerophospholipidis; indoles and derivatives; lineolic acids and derivative, pyrimidine nucleosides; quaternary ammonium salts; sphingolipids; and sterol and prenol lipids, according to the Human Metabolome database (HMDB) or Lipid Maps Pathway

for lipids.[33] The major classes of compounds altered in both AmB-treated mice are represented in Figure 3.

Figure 3: Distribution of metabolites found after a multiplatform metabolomic approach in biochemical categories.



The AmB-treated groups displayed different biochemical trends, which are described in supplementary material (Tables S1, S2 and S3). Some up-regulated metabolites induced by C-AmB includeL-pipecolate, cytidine, L-hexanoylcarnitine and peptides. The metabolites that were up-regulated by AmB-loaded NE include N8-acetylspermidine, deoxycytidine, thyroxine, peptides and some glycerophospholipids. The down-regulated metabolites in C-AmB group included glycerophosphocholine, arachidonic acid, 9-HETE, docosahexaenoic acid, dihydroxypregnenolone, methionine, L-methionine S-oxide, N8-acetylspermidine, thyroxine, 2-hydroxybutyric acid, L-glutamic acid, peptides, lysophospholipids and glycerophospholipids. The down-regulated metabolites in AmB-loaded NE group included docosahexaenoic acid, cortisol, glycerophospholipids and pyranose.

Table S1 Identification of metabolites that were significantly differentiating plasma profiles of treated groups from controls in LC-MS analysis ($p < 0,05$)

Name	Formula	MW (DB)	RT (min)	DET	Mass error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE	MS/MS fragments
Phosphocholine*	$C_5H_{14}NO_4P$	183.0660	18.01	Pos	-0.22	12.99	-15.6	-	-23.1	184.0720, 124.9969, 86.0965, 60.0811
Oleic acid	$C_{18}H_{34}O_2$	282.2559	31.29	Neg	-0.35	19.99	-40.9	-	-	Putative
Sphingosine*	$C_{18}H_{37}NO_2$	299.2824	16.03	Pos	-1.04	18.63	-60.7	-	-	300.2809, 282.2757, 264.2645, 252.2713, 139.1139, 95.0819, 57.0678
Eicosapentaenoic acid	$C_{20}H_{30}O_2$	302.2246	25.48	Neg	-1.98	10.20	-48.8	-	-	Putative
Arachidonic Acid	$C_{20}H_{32}O_2$	304.2402	27.87	Neg	1.97	11.51	-46.3	-	-29.3	303.2315, 285.2254, 259.2400, 59.0135
Dihomo-ɣ-linoleic acid	$C_{20}H_{34}O_2$	306.2559	29.60	Neg	-3.26	16.99	-50.9	-	-39.7	Putative
15-deoxy-Δ12,14-Prostaglandin J2	$C_{20}H_{28}O_3$	316.2039	12.04	Neg	-2.53	11.71	-66.0	-	-	Putative
9-HETE	$C_{20}H_{32}O_3$	320.2351	20.24	Neg	-0.94	7.57	-85.4	-	-	319.2242, 301.2166, 257.2269, 179.1049, 135.1135, 107.0820, 81.0739, 69.0333
Anandamide*	$C_{20}H_{35}NO_2$	321.2668	27.79	Pos	-0.56	6.01	-25.5	-	-16.9	322.1735, 304.2363, 292.1269, 242.0940, 175.1356, 160.1376, 147.1134, 127.0886, 120.0558, 95.0914, 89.0652, 79.0628, 60.0782
Docosahexaenoic acid*	$C_{22}H_{32}O_2$	328.2402	28.05	Pos	3.03	20.15	-37.3	-	-23.2	329.2449, 311.2371, 293.2188, 207.1281, 173.1299, 109.0979, 93.0686, 81.0682, 67.0532
Docosahexaenoic acid	$C_{22}H_{32}O_2$	328.2402	29.03	Neg	0.91	12.53	-	-43.2	-28.9	327.2274, 283.2419, 229.1926, 177.1631, 121.1019, 59.0148
Docosapentaenoic acid	$C_{22}H_{34}O_2$	330.2559	29.55	Neg	-1.82	13.79	-41.2	-	-	Putative
Docosatetranoic acid	$C_{22}H_{36}O_2$	332.2715	30.79	Neg	-3.01	18.50	-38.1	-	-	Putative
Dihydroxypregnenolone	$C_{21}H_{32}O_4$	348.2301	28.36	Neg	-8.62	6.80	-31.5	-	-	347.2316, 280.2365, 279.2316
C16 Sphingosine-1-phosphate*	$C_{16}H_{34}NO_5P$	351.2175	11.37	Pos	-0.48	2.52	-72.8	-	-	Putative

Name	Formula	MW (DB)	RT (min)	DET	Mass error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE	MS/MS fragments
MG(18:3)*	$C_{21}H_{36}O_4$	352.2614	23.18	Pos	1.14	12.70	-	-	-74.9	Putative
MG(18:2)*	$C_{21}H_{38}O_4$	354.2770	25.80	Pos	2.82	17.67	-	-	-60.4	Putative
MG(18:1)*	$C_{21}H_{40}O_4$	356.2927	28.26	Pos	-3.36	16.01	-	-	-68.3	Putative
Calcitroic acid*	$C_{23}H_{36}O_3$	360.2664	19.43	Pos	-4.44	15.30	-	-	-72.0	Putative
Cortisol	$C_{21}H_{30}O_5$	362.2093	5.35	Neg	-2.21	7.58	-	-81.2	-	361.2014, 304.3312, 184.9345, 64.5371
Cholacalcioic acid*	$C_{24}H_{36}O_3$	372.2664	7.90	Pos	1.11	19.31	-	-86.3	-	Putative
PA(15:0)	$C_{18}H_{37}O_7P$	396.2277	27.28	Neg	-1.77	7.13	-39.1	-	-	395.2275, 327.2356, 283.2446, 59.0146
Asn Tyr Leu*	$C_{19}H_{28}N_4O_6$	408.2009	25.80	Pos	-7.59	15.21	-	-	-60.8	Putative
Dihydroxyvitamin D3*	$C_{27}H_{44}O_3$	416.3290	22.23	Pos	0.22	17.16	44.4	-31.2	-	Putative
PE(16:0)*	$C_{21}H_{44}NO_7P$	453.2855	18.58	Pos	1.12	15.80	-23.1	-	-	313.2730, 282.2744, 155.0075, 62.0605
PC(O-12:0/O-2:0)*	$C_{22}H_{48}NO_6P$	453.3219	16.50	Pos	-1.83	1.76	-34.6	-15.8	-	Putative
PC(14:0)*	$C_{22}H_{46}NO_7P$	467.3012	15.52	Pos	0.26	12.68	-34.8	-	-	468.3025, 184.0720, 104.1067, 86.0945
PC(O-14:0/O-1:0)	$C_{23}H_{50}NO_6P$	467.3376	24.77	Neg	-2.57	24.53	-35.1	-	-	466.3358, 405.2826, 140.0111, 78.9594
LPE(18:3)*	$C_{23}H_{42}NO_7P$	475.2699	15.78	Pos	5.90	24.80	-80.3	-	-	Putative
LPE(18:2)	$C_{23}H_{44}NO_7P$	477.2856	1.88	Neg	0.84	9.46	-54.6	-	-46.7	476.2804, 279.2347, 196.0370, 140.0115, 78.9592
PE(18:1)	$C_{23}H_{46}NO_7P$	479.3011	20.38	Neg	-1.88	8.58	-	-36.9	-	478.3007, 281.2499, 255.2328, 196.0368, 140.0108, 78.95588
PE(18:1)*	$C_{23}H_{46}NO_7P$	479.3011	20.30	Pos	0.94	16.82	-35.2	-	-25.8	480.2823, 339.2898, 308.2969, 265.2477, 155.0085, 62.0601
PC(15:0)*	$C_{23}H_{48}NO_7P$	481.3168	17.57	Pos	-0.01	17.33	-33.8	-	-18.4	482.3301, 429.3081, 369.1566, 248.1679, 184.0703, 166.0630, 146.9808, 104.1029, 57.1029
PC(16:1)*	$C_{24}H_{48}NO_7P$	493.3168	17.72	Pos	-1.01	10.93	-	-	-53.6	Putative

Name	Formula	MW (DB)	RT (min)	DET	Mass error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE	MS/MS fragments
PC(16:0)*	$C_{24}H_{50}NO_7P$	495.3325	18.82	Pos	5.68	18.01	-80.2	-	-	496.3369, 184.0736, 104.1067, 86.0964
LPE(20:5)*	$C_{25}H_{42}NO_7P$	499.2699	15.98	Pos	-1.40	19.61	-	-	-66.6	Putative
PE(20:4)	$C_{25}H_{44}NO_7P$	501.2856	17.95	Neg	1.00	7.71	-53.7	-	-33.7	500.2772, 303.2313, 279.2319, 259.2383, 196.0367, 78.9560
LPE(20:3)	$C_{25}H_{46}NO_7P$	503.3012	17.87	Pos/ Neg	0.44	17.97	-43.2	-	-50.6	Putative
LPE(20:2)	$C_{25}H_{48}NO_7P$	505.3168	17.41	Neg	-2.37	1.74	-	-	-24.0	Putative
PC(17:1)*	$C_{25}H_{50}NO_7P$	507.3325	18.58	Pos	-0.18	20.68	-32.4	-	-17.8	508.3303, 258.7617, 184.0708, 124.0633, 104.1073, 86.0932
PC(17:0)*	$C_{25}H_{52}NO_7P$	509.3481	20.87	Pos	0.00	13.43	-	-	-20.3	Putative
LPC (18:2)*	$C_{26}H_{50}NO_7P$	519.3325	17.37	Pos	4.78	14.90	-33.8	-	-23.3	520.3410, 502.3298, 443.2556, 337.2736, 258.1094, 184.0737, 124.9993, 104.1075, 86.0965, 60.0810
LPC(18:1)*	$C_{26}H_{52}NO_7P$	521.3481	20.56	Pos	5.48	15.65	-28.8	-		522.3549, 339.2872, 258.1082, 184.0733, 166.0612, 124.9995, 104.1073, 86.0969, 60.0813
LPE(22:6)	$C_{27}H_{44}NO_7P$	525.2855	17.89	Neg	0.76	7.66	-49.0	-	-34.1	524.2777, 327.2294, 283.2427, 196.0363, 140.0093, 78.9587
LPE(22:5)	$C_{27}H_{46}NO_7P$	527.3012	18.79	Neg	-0.02	18.14	-90.7	-	-61.1	Putative
PC(19:3)*	$C_{27}H_{50}NO_7P$	531.3325	20.92	Pos	2.97	8.52	-77.4	-	-	532.3370, 473.2613, 104.1065
PC(20:5)*	$C_{28}H_{48}NO_7P$	541.3168	16.14	Pos	-0.62	17.00	-40.7	-	-	542.322, 184.072, 104.096, 86.095
LPC(20:4)*	$C_{28}H_{50}NO_7P$	543.3325	17.50	Pos	3.46	15.10	-24.9	-	-	544.3387, 184.0734, 104.1073, 86.0962, 60.0812
PS(20:4)	$C_{26}H_{44}NO_9P$	545.2754	17.22	Neg	-7.34	6.60	-	-	-83.9	Putative
LPC(20:2)*	$C_{28}H_{54}NO_7P$	547.3638	21.61	Pos	-0.31	18.18	-29.4	-	-	548.3737, 184.0723, 104.1064, 86.0959
PS(20:0)	$C_{26}H_{52}NO_9P$	553.338	18.63	Neg	-4.70	8.80	-	-	-31.9	Putative

Name	Formula	MW (DB)	RT (min)	DET	Mass error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE	MS/MS fragments
PS(22:6)	$C_{28}H_{44}NO_9P$	569.2754	17.95	Neg	-4.22	8.87	-47.5	-	-36.1	508.3307, 500.2690, 303.2319, 281.2358, 259.2420, 214.0511, 95.7203, 78.9568
PI (16:0)	$C_{25}H_{49}O_{12}P$	572.2961	22.17	Neg	-2.62	18.34	-39.5	-	-	571.2878, 391.2222, 315.0458, 255.2325, 241.0091, 152.9944, 78.9579
PC(16:0/5:0(CHO))	$C_{29}H_{56}NO_9P$	593.3693	17.89	Neg	-5.73	7.37	-	-	-38.6	Putative
PI(18:2)	$C_{27}H_{49}O_{12}P$	596.2961	18.93	Neg	-3.52	10.29	-	-	-69.9	Putative
PC(16:0/5:0(COOH))	$C_{29}H_{56}NO_{10}P$	609.3642	17.42	Neg	-3.61	12.46	-	-	-24.6	Putative
PI(20:4)	$C_{29}H_{49}O_{12}P$	620.2961	20.14	Neg	-1.128	4.25	-45.3	-	-	619.2872, 439.2218, 315.0489, 303.2318, 241.0113, 152.9946, 78.9599
PI(20:3)*	$C_{29}H_{51}O_{12}P$	622.3118	28.46	Pos	5.10	4.06	-	223.5	-	Putative
PA(P-16:0\/16:1)	$C_{35}H_{67}O_7P$	630.4625	27.87	Neg	-4.92	17.73	-	-	-45.1	Putative
PI(22:6)	$C_{31}H_{49}O_{12}P$	644.2961	20.08	Neg	-2.95	8.10	-	-	-59.2	Putative
PS(P-16:0/13:0)*	$C_{35}H_{68}NO_9P$	677.4632	30.21	Pos	1.48	17.20	-	-90.4	-	Putative
PC(38:7)*	$C_{46}H_{78}NO_8P$	803.5465	30.72	Pos	-0.26	14.14	-33.1	-	-	Putative
PS(42:10)*	$C_{48}H_{74}NO_{10}P$	855.505	20.57	Pos	7.13	20.57	-	-	-32.1	Putative
Amphotericin B	$C_{47}H_{73}NO_{17}$	923.4879	8.63	Neg	-6.17	26.98	669.7	1123.0	-	Putative
TG(44:3)*	$C_{65}H_{114}O_6$	990.8615	19.58	Pos	0.40	3.34	-	-	-47.4	Putative
TG(65:2)*	$C_{68}H_{128}O_6$	1040.971	18.02	Pos	-3.27	15.03	-	-	-27.32	Putative

Note: A, C-AmB group; NE, AmB-loaded NE group; C, Control group; FC, fold change; FC was calculated as follows: (Average [Treated] – Average [Control])/Average[control] × 100); +/–, increase/decrease in treated when compared with controls; RT, retention time; CV, coefficient of variation

*Metabolites obtained from the LC positive, which the prediction capability (Q2:0.02) was low.

Table S2 Putative identification of metabolites that were significantly differentiating plasma profiles of treated groups from controls in CE-MS analysis ($p < 0,05$)

Name	Formula	MW (DB)	MT (min)	error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE
L-Pipecolate*	$C_6H_{11}NO_2$	129.0790	13.23	0.04	29.86	42.4	-	-
Methionine*	$C_5H_{11}NO_2S$	149.0510	13.85	0.03	4.36	-27.6	-	-
Gly Ala	$C_5H_{10}N_2O_3$	146.0691	13.24	1.11	12.66	-	138.8	-
L-Methionine S-oxide	$C_5H_{11}NO_3S$	165.0460	14.85	2.67	10.14	-12.9	-	-13.4
N8-Acetylspermidine	$C_9H_{21}N_3O$	187.1685	9.56	-4.29	13.76	-	47.2	-55.9
Ala Ile	$C_9H_{18}N_2O_3$	202.1317	13.11	-0.22	6.81	-	30.1	-
Deoxycytidine	$C_9H_{13}N_3O_4$	227.0906	12.59	1.29	8.54	-	11.1	-
Ile Pro	$C_{11}H_{20}N_2O_3$	228.1474	12.69	-2.63	14.49	-	26.6	-
Glu Ser	$C_8H_{14}N_2O_6$	234.0852	15.48	-7.65	28.58	76.5	-	-
Cytidine	$C_9H1_3N_3O_5$	243.0855	12.83	-0.52	6.07	43.2	-	-
L-Hexanoylcarnitine	$C_{13}H_{25}NO_4$	259.1783	13.07	-0.17	10.04	48.7	-	-
Phe Val	$C_{14}H_{20}N_2O_3$	264.1474	13.62	-7.19	10.78	95.9	97.1	-
Ile Ala Ala	$C_{12}H_{23}N_3O_4$	273.1689	13.84	4.34	4.48	27.8	46.4	-
Met Phe	$C_{14}H_{20}N_2O_3S$	296.1195	13.68	-3.30	8.76	-	-92.1	913.9
Val Trp	$C_{16}H_{21}N_3O_3$	303.1583	12.04	-9.89	7.43	-	-	-13.4
Ala Ile Leu	$C_{15}H_{29}N_3O_5$	315.2158	14.11	-0.70	9.98	-	55.6	-
Phe Ala Val	$C_{17}H_{25}N_3O_4$	335.1845	14.15	-0.33	7.34	-	64.4	-24.2
Ile Val Ile	$C_{17}H_{33}N_3O_4$	343.2471	14.33	-0.88	8.00	-	43.1	-
Ile Ala Phe	$C_{18}H_{27}N_3O_4$	349.2002	14.25	-0.76	9.71	-	27.0	-
Gly Phe Phe	$C_{20}H_{23}N_3O_4$	369.1689	14.12	0.40	15.38	-	146.7	-
Ile Ile Lys	$C_{18}H_{36}N_4O_4$	372.2737	11.38	1.74	4.74	-	91.5	-36.4
Ser Leu Arg	$C_{15}H_{30}N_6O_5$	374.2278	11.31	3.25	6.57	73.3	43.8	-
Leu Ile Tyr	$C_{21}H_{33}N_3O_5$	407.2420	14.65	-2.05	15.50	45.2	46.9	-
Arg Lys Lys	$C_{18}H_{38}N_8O_4$	430.3016	14.69	3.24	13.78	-	706.3	-
Glu Tyr Gln	$C_{19}H_{26}N_4O_8$	438.1751	14.64	0.05	11.03	326.4	223.3	-

Name	Formula	MW (DB)	RT (min)	error (ppm)	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE
Arg Arg Leu	$C_{18}H_{37}N_9O_4$	443.2968	16.24	5.31	9.58	-	143.0	-
Trp Lys Met	$C_{22}H_{33}N_5O_4S$	463.2253	14.90	4.96	10.98	-	-	820.9
N-(2'-(4-benzenesulfonamide)-ethyl) arachidonoyl amine	$C_{28}H_{42}N_2O_3S$	486.2916	11.80	0.42	5.66	-	54.4	-
Thyroxine	$C_{15}H_{11}I_4NO_4$	776.6867	13.14	5.27	9.91	-	40.5	-22.3

Note: A, C-AmB group; NE, AmB-loaded NE group; C, Control group; FC, fold change; FC was calculated as follows: (Average [Treated] – Average [Control])/Average [Control] × 100). +/–, increase/decrease in treated when compared with controls. MT, migration time. CV, coefficient of variation
* Compounds identified with standards

Table S3 Identification of metabolites that were significantly differentiating plasma profiles of treated groups from controls in GC-MS analysis ($p < 0,05$)

Name	T (target ion)	Q (qualifier ion)	RT (min)	*p*-value	CV for QC (%)	FC (%) A / C	FC (%) NE / C	FC (%) A / NE
2-hydroxybutyric acid	131	205, 147, 73	7.77	0.008	29.94	-100	-	-100
PYRANOSE (Glucose/Altrose/Galactose/Talose)	73	73, 319, 205, 147	17.55	0.019	29.14	-	-36.9	-
L-glutamic acid	156	156, 73, 147, 258	13.16	0.042	13.99	-	-	-100

Note: A, C-AmB group; NE, AmB-loaded NE group; C, Control group; FC, fold change; FC was calculated as follows: (Average [Treated] – Average [Control])/Average [Control] × 100). +/–, increase/decrease in treated when compared with controls. RT, retention time. CV, coefficient of variation

3.3.3 Disturbed metabolic pathways

The webtools MetPA and MBrole were applied for evaluation of the metabolic pathway's the identified metabolites were associated. As detected by MBrole analysis, the top two metabolism disturbed in mice of the C-AmB group were arachidonic acid and glycerophospholipid metabolism. In MetPA analysis, five metabolic pathways (glycerophospholipid, sphingolipid, arachidonic acid, linoleic acid and biosynthesis of polyunsaturated fatty acids (PUFA) were disturbed (Figure 4). In AmB-loaded NE group, the alterations in the metabolic pathways were not considered statistically significant for both analyses. Omega 3 and omega 6 PUFA metabolic pathway and some of its metabolites (dihomo-ɣ-linoleic acid, arachidonic acid, docosatetraenoic acid, docosapentaenoic acid, eicosapentaenoic acid, docosahexanoic acid) significantly decreased in the plasma from C-AmB group compared to the control group. To better synthesize the alterations seen in this pathway in mice treated C-AmB, the metabolic reactions leading to PUFA synthesis were represented in Figure 5. Increases in abundance of one acylcarnitine (l-hexanoylcarnitine) in plasma of C-AmB-treated mice compared to the control group were observed, as well.

Figure 4: Pathway analysis of selected biomarkers by MetPA for C-AmB group ($p < 0.1$).

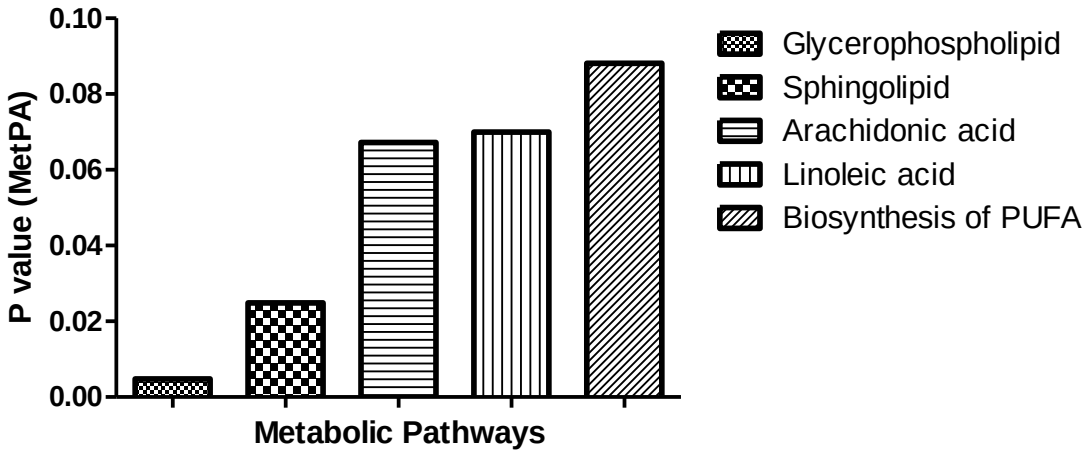
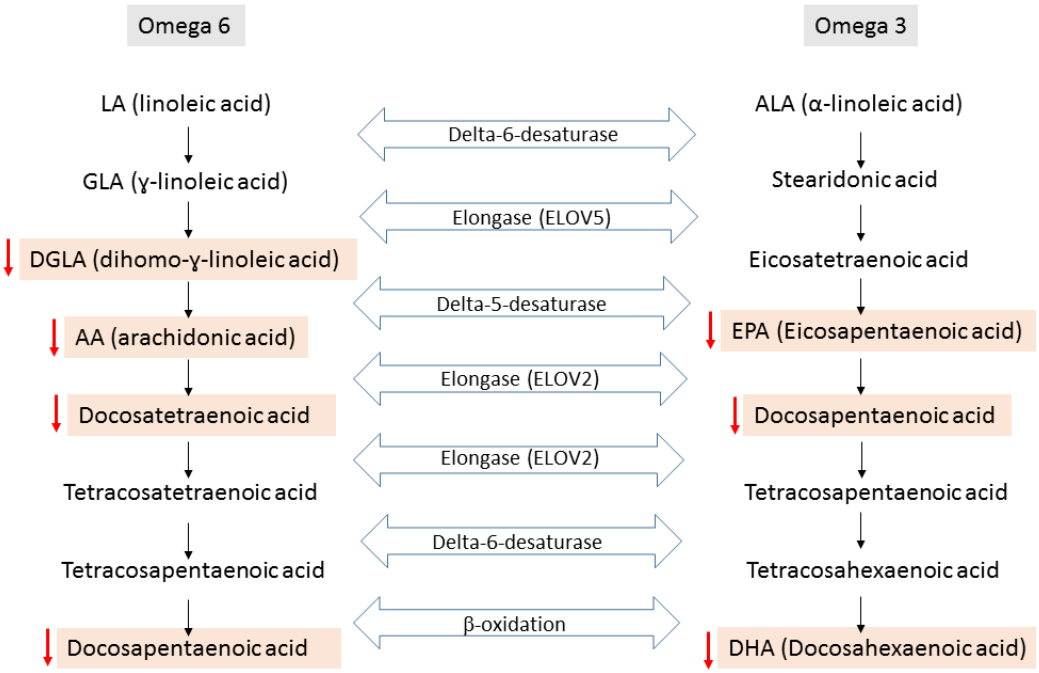


Figure 5: Metabolic pathway of omega 3 and omega 6 PUFA and the metabolites decreased in the C-AmB group compared to the control group.



4 Discussion

AmB has potent antifungal and antiparasitic activities, but C-AmB induces toxic damage to the kidneys, mainly through the induction of pores in membranes, which causes renal tubular dysfunction. Severe vasoconstriction, which reduces renal blood flow and glomerular filtration rate (GFR), has been also associated to AmB-renal injury.[2,10] Although liposomal AmB formulations have significantly circumvented AmB-related nephrotoxicity, they are expensive and not always affordable for treatment. Therefore, improvements of AmB formulations, turning them less toxic and expensive, are still worthwhile. Moreover, the lack of an efficient and accurate biomarker to early detect renal injury has become a leading cause of severe nephrotoxicity due to AmB treatment or other drugs.[12] Therefore, better biomarkers for kidney injury are required. Early detection of kidney injury will influence therapy and potentially morbidity and mortality. In addition, the availability of biomarkers for the early detection of nephrotoxicity during preclinical drug development programs would provide significant savings in cost, time, and animal experiments.[11]

Metabolomics has emerged over a decade as a tool to both “predict” toxicity in animals as well as better understanding the mechanisms of drug toxicity.[34] Therefore, in this study, the metabolic profiles changes induced in plasma samples of mice by treatment with C-AmB and AmB-loaded NE, a new lipid formulation, were analysed through a multiplatform metabolomic approach.

The doses of AmB administered and treatment regimen that were selected for this study were based on previous efficacy studies.[35,36] Mice were not treated with higher doses of C-AmB per kg, since doses > 1 to 2 mg/kg were reported to be very toxic or lethal to normal mice.[37,38] After treatment, we first assessed the levels of serum creatinine and BUN and analysed kidney sections through histopathology, to determine if treatment with AmB formulations has induced renal injury in mice. The urinary volume was the single significantly altered parameter among all groups. The C-AmB treatment induced the formation of the highest urine volume, when compared to the other treatments. This may be related to a higher concentration of the free form of AmB in the plasma of these animals.[8] It is known that AmB induces ADH-resistant polyuria, and it has been suggested that this symptom results from inhibition of the AVP/V2R signalling pathway, which is important for reabsorption of water in the collecting duct of the kidney via the water channel aquaporin-2.[39] Functional and morphological renal changes were not observed following the dosing regimen of 1 mg/kg of AmB in 3 alternate days. These findings are in agreement with those found by Tonomura et al. (2009), which reported that BUN and creatinine levels did not change significantly and there were no tubular necrosis, when animals were treated with a dose of 1 mg/kg of AmB for 7 consecutive days. On the other hand, these changes typically appear when AmB is administered in larger and/or longer administration regimen doses.[39] Although these indices are valid indicators of renal function, they have several limitations including: inability to differentiate specific site of renal injury; the unreliability of creatinine measurement with increasing degree of renal injury; and the long lag time between initial renal injury and creatinine rise, generally after a loss of two thirds or greater of nephron functional capacity.[11,18]Consequently, our data suggest that the further changes in the plasma metabolomic profile in mice treated with the two AmB formulations may be potentially associated with early toxic effects of AmB.

Of note is the fact that the plasma abundance of AmB in NE-treated mice was two times higher than that in the group treated with C-AmB. One previous study had reported that plasma concentrations of AmB in liposomal-treated mice were approximately 5 times higher than in the group treated with conventional formulation, after 1 mg/kg intravenous infusion.[40] It is known that lipid formulations are mainly delivered to the RES of the liver and the spleen.[41]AmB-loaded NE was probably delivered to the RES but it might have produced higher plasma AmB concentrations than did C-AmB due to its small particle size, which are restrained for a long period in the blood circulation.[42]Importantly, the metabolomic data strongly indicated that C-AmB administration cause more metabolic derangements in plasma of treated mice than did AmB-loaded NE, in different pathways.

Interestingly, many of the significantly changed metabolites detected in plasma of mice after C-AmB administration are involved in the arachidonic acid and glycerophospholipidis metabolism. These included omega 6 (dihomo-ɣ-linoleic acid, arachidonic acid, docosatetranoic acid, docosapentaenoic acid) and omega 3 PUFA metabolites (eicosapentaenoic acid, docosapentaenoic acid, docosahexaenoic acid), which were decreased in plasma of C-AmB-treated mice, as compared to the control group. In a previous work, the accumulation of free fatty acids and toxic long chain fatty acid metabolites in freshly isolated proximal tubules subjected to hypoxic injury, and in kidney tissue of animals subjected to ischemia/reperfusion injury were described.[43] It was also documented the activation of intracellular calcium-independent phospholipase A_2 (PLA_2), and the inhibition of mitochondrial fatty acid oxidation as potential mechanism(s) responsible for the accumulation of free fatty acids in ischemic acute renal failure.[20]

The decrease in plasma levels of PUFA coincided with increases in abundance of L-hexanoylcarnitine in plasma of C-AmB-treated mice compared to the control group. This result supports the notion of a reduced activity of fatty acid oxidation enzymes during AmB-induced nephrotoxicity. This metabolic abnormality was previously described in kidney tissue obtained from animal models of acute kidney injury.[44,45] Increase in acylcarnitine levels has been proposed as a biomarker for many metabolic diseases such as diabetes, encephalopathy, nephropathy and cardiomyopathy, which are related to

mitochondrial dysfunction.[46] The carnitine carrier system transports fatty acids through the membrane of renal epithelial cells and they are used for energy production through fatty acid β-oxidation primarily in mitochondria and peroxisomes. During drug-induced kidney injury there is a significant reduction on the expression and activity of mitochondrial and peroxisomal fatty acid oxidation enzymes in kidney tissue.[47,48] Interestingly, in the current study, both reduction in PUFA and increases in carnitines's plasma levels were detected in C-AmB treated mice. Since increases in acylcarnitines abundance lead to decreases in PUFA,[44] these two pathways may be well associated, as early metabolic alterations induced by C-AmB.

Our results revealed a decrease in plasma concentration in C-AmB-treated mice compared to the control group in the arachidonic acid pathway (oleic acid, arachidonic acid, dihomo-ɣ-linoleic acid, docosatetranoic acid, docosapentaenoic acid, 15-deoxy-Δ12,14-prostaglandin J2, 9-HETE). Previous studies have demonstrated that the arachidonic acid, an essential PUFA, mediates inflammation and the functioning of several organs and systems either directly or upon its conversion into eicosanoids.[49] Furthermore, this PUFA may be a physiological inducer of apoptosis and this cytotoxic action may be another role played by PUFA *in vivo*. Nevertheless, its pro-inflammatory and oxidative stress-inducing features are characteristic of necrosis and other pathological conditions.[50]

There is also evidence that AmB activates the synthesis of arachidonic acid metabolites. *In vivo*, AmB administration to rats caused a marked increase in prostaglandin synthetase activity in the kidney. AmB, by depolarizing vascular smooth muscle cells, activates voltage-dependent calcium channels; the resultant surge in intracellular calcium concentrations activates PLA_2, which is the primary enzyme regulating arachidonic acid release. Oxidative cell injury caused by AmB could also stimulate the release of arachidonic acid metabolites.[51]15-deoxy-Δ12,14-prostaglandin J2, a product of prostaglandin D2 metabolism, is known endogenous PPAR-ɣ ligand. This PPAR-ɣ ligand inhibits pro-inflammatory cytokines production in acute and chronic inflammation and prevents ROS generation induced by oxidative stress.[52] Therefore, probably the reduction in plasma concentration of this prostaglandin in C-AmB group can be caused by their increased recruitment to the injured kidney tissue.

Plasma levels of some lysophosphatidylethanolamines (LPEs) were also decreased in C-AmB treated mice, as compared to the control group. Mammalian plasma lysophospholipid levels are postulated to be an useful marker of certain pathological states.[53] The most likely source of plasma LPE is fatty acid release, mainly PUFA such as arachidonic acid, from PE.[54] Since a decrease in plasma concentrations of arachidonic acid was observed and also in some of its precursors and metabolites, it would not be incongruent a concomitant reduction in plasma concentration of LPEs. The importance of plasma lipid abnormalities in renal damage is well recognized, but little attention has been paid to the study of some plasma lipid fractions. As previously suggested, the change in LPEs content may result from the AmB-induced kidney injury. In the current study, the significantly decreased plasma levels of LPEs may be associated to inhibition of phospholipase A activity towards phosphatidylethanolamine, in mice tissues such as the kidney.[56]

Enhanced excretion of amino acids (aminoaciduria) is one of strong indicators of proximal tubule injury in general.[20] In this study, there was a decrease in plasma concentrations of methionine in the C-AmB-treated group compared to the control group. Methionine is an essential amino acid, which cannot be synthesized by the body. It is either incorporated into proteins or broken down for energy and metabolic intermediates. Change of this amino acid and its oxidation product (L-Methionine S-oxide) in C-AmB-treated mice can be explained due to its impaired renal tubular reabsorption.[16] Other studies also found methionine as a biomarker for drug-induced nephrotoxicity.[20,21,55] In addition, it was also found a reduction in plasma concentration of 2-hydroxybutyric acid (alpha-hydroxybutyrate) in the C-AmB-treated group compared to the control group. It is noteworthy that this metabolite is an organic acid derived from alpha-ketobutyrate, which is produced by amino acid catabolism (threonine and methionine) and glutathione anabolism (cysteine formation pathway).[57] Recently, it has been found that 2-hydroxybutyric acid is often found in the urine of patients suffering from lactic acidosis and ketoacidosis.[58] Metabolic acidosis can result from the inability of the kidneys to excrete sufficient amount of acid. It is known that AmB causes renal tubular acidosis[59] and it has been suggested that renal tubular acidification is due to AmB

insertion into the cell membrane; thereby inducing the formation of pores and an intracellular H-ion back flux.[60,61]

There was also an increase in the plasma concentration of cytidine in the group treated with C-AmB compared to the control group. Cytidine is a nucleoside that is part of pyrimidine metabolism. This event may reflect changes in the activity of the renal carriers, in the synthesis/degradation of nucleotides or in the renal filtration rates, resulting from the nephrotoxic drug action.[11,16]

Another change observed may not be related to nephrotoxicity, but to another less common adverse effect associated with the conventional formulation of AmB, which can also cause abnormalities in liver function. It was found an increase in the plasma concentration of L-pipecolate in C-AmB-treated mice group. L-pipecolate is a cyclic imino acid produced during degradation of the lysine. It accumulates in body fluids in patients with generalized peroxisomal genetic disorders and has high levels in patients with chronic liver disease.[62]

Changes in AmB-loaded NE-treated group were also found. It was observed the decrease in plasma concentration of pyranose (glucose / maltose / galactose / talose), suggesting an increase in its elimination through the urine. This condition, known as glycosuria, is considered a potential biomarker of nephrotoxicity and a strong indicator of proximal tubule damage in general, likely caused by impaired tubular reabsorption in the proximal tubule via sodium-dependent glucose transport.[21,63]

It was also observed a decrease in cortisol levels in AmB-loaded NE-treated group. A previous study demonstrated that mice with systemic candidiasis treated with free AmB, cortisol content decreased throughout 28 days in the experimental period.[64]

Many studies suggest that the conventional formulation of AmB is more toxic than lipid formulations.[2,39] As expected, AmB-loaded NE induced less metabolic alterations than treatment with C-AmB, in our *in vivo* model. One factor that could influence the toxicity of AmB-loaded NE is the rate at which it releases AmB (lower amount of the agregated form) to body fluids.[65,66]

5 Conclusion

In the present study, the metabolomic alterations induced by treating mice with AmB-loaded NE, a lipid formulation, and C-AmB were compared. Since traditional toxicity studies (BUN, creatinine and the histopathological analysis) did not reveal any changes in kidney function, the metabolomic alterations observed may be associated to early toxic events of c-AMB treatment, occurring before that a significant renal injury has been established. Approximately 30 metabolites were identified as potentially related to early C-AmB-induced nephrotoxicity. More importantly, a fingerprint metabolomic approach revealed significant metabolic differences between the two AmB formulations tested that could be related to their different patterns of AmB aggregation status, rates of release to body fluids and the lack of deoxycholate. Disturbances in the arachidonic acid, lysophospholipids and fatty acids pathways suggest that treatment with C-AmB may be associated with early alterations in inflammatory process, oxidative stress and ischemia injury in the kidneys. Future investigations of these metabolic alterations may provide additional tools in the detection of the onset and severity of kidney injury induced by AmB and its conventional formulation. They may become also useful to investigate toxicity associated to new drug formulations and regimens, avoiding more serious damage to vital organs. To conclude, metabolomics was shown to be an important tool to detect early metabolic alterations potentially associated to toxicity, since even at low drug doses, significant differences were observed for several metabolites by comparing the two AmB formulations.

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5. DISCUSSÃO GERAL

Atualmente, a AmB tem sido um dos fármacos mais utilizados para o tratamento das leishmanioses. Entretanto, a administração de sua formulação convencional (C-AmB) é frequentemente acompanhada por uma toxicidade que se manifesta através de graves efeitos adversos, principalmente nefrotoxicidade e hemólise (TORRADO *et al*., 2008; VYAS; GUPTA, 2006). As formulações lipídicas (ex: Ambisome®) têm sido consideradas alternativas para superar estas desvantagens, já que as interações entre a AmB e os lípides previnem a liberação da forma agregada do fármaco, possibilitando uma redução da sua toxicidade para células de mamíferos (LARABI *et al.,* 2003).

Recentemente, uma NE carregada com AmB (NE-AmB) apresentando estabilidade por 180 dias, sem sinal degradação do fármaco, e alto teor de encapsulação/associação foi desenvolvida. Observou-se também, por dicroísmo circular, ausência de sinal da forma agregada livre da AmB, a mais tóxica para as células hospedeiras (CALDEIRA *et al*., 2015). Em consonância com esses achados, a NE-AmB mostrou-se significativamente menos tóxica em macrófagos em comparação com a C-AmB. Portanto, a avaliação da toxicidade e atividade leishmanicida *in vivo* tornou-se imperativa. Assim sendo, este trabalho teve como objetivo otimizar, caracterizar, avaliar os efeitos tóxicos *in vitro* e *in vivo* e a atividade da NE-AmB em animais experimentalmente infectados com *Leishmania (L.) infantum chagasi.*

A fim de se realizar estudos de toxicidade e eficácia *in vivo*, tornou-se necessária a otimização desta formulação, levando-se em consideração a redução do diâmetro dos glóbulos e IP, fatores importantes para uma administração intravenosa (HIPPALGAONKAR *et al.*, 2010).

Na primeira parte do presente trabalho, foi realizada a otimização da NE-AmB previamente desenvolvida por Caldeira e colaboradores (2015). Considerando-se que o diâmetro dos glóbulos influencia a biodistribuição das NE (ICHIKAWA *et al*., 2007; HIPPALGAONKAR *et al.*, 2010; HÖEMANN; ZIMMER, 2015), foram conduzidos estudos visando avaliar a influência de diversos parâmetros sobre o tamanho dos glóbulos da NE, destacando-se, neste contexto, a influência método de preparo.

A substituição do ultrassom pela homogeneização em alta pressão (HAP) foi adotada a fim de reduzir o tamanho das partículas e o IP. Após estudos preliminares avaliando a influência do número de ciclos e pressão, optou-se por

submeter a NE branca a aumentos gradativos na pressão (3 ciclos a 400 bar, 3 a 600 bar e 3 a 800 bar). Foi possível obter glóbulos com valores de diâmetro (~ 140 nm) e IP (IP < 0,2) menores do que aqueles obtidos previamente (CALDEIRA *et al*., 2015). Isto pode ser atribuído a associação entre a baixa pressão e a alta temperatura (início do processo) e entre a alta pressão e a baixa temperatura (final do processo), cuja combinação constitui a faixa de fabricação ótima, proporcionando valores mais baixos de diâmetro dos glóbulos (JAHNKE *et al*., 1998).

Foi interessante observar que os valores de diâmetro médio dos glóbulos foram similares quando três métodos diferentes foram usados para essa avaliação (AFM, NTA e PCS). Vale a pena destacar que as análises por NTA mostraram que não foram observados cristais insolúveis de AmB (AmB livre) na NE, confirmando dados previamente relatados por Caldeira e colaboradores (2015). Todas as avaliações de tamanho foram conduzidas após diluição da NE e, no caso das medidas por PCS, observou-se diferenças quando as amostras foram ou não diluídas. Para as amostras diluídas observou-se uma distribuição monomodal e homogênea dos glóbulos (Apêndice A), enquanto que para as não diluídas observou-se uma distribuição multimodal e heterogênea com a presença significante de partículas na ordem de 10 nm (Apêndice B). Isto pode ser atribuído à presença de micelas devido ao excesso de tensoativo (Polisorbato 80/Tween 80).

A presença das micelas nas amostras da NE sugere que parte da AmB pode estar associada à essas estruturas, além da localização na interface óleo/água conforme descrito previamente (CALDEIRA *et al*., 2015; LANCE *et al*., 1995). A fim de investigar a influência das micelas sobre a encapsulação da AmB, uma solução aquosa do polisorbato 80/tween 80 foi preparada e a AmB foi incorporada de forma similar ao descrito para a NE seguido da avaliação do teor de encapsulação. Os dados mostraram claramente que o teor de encapsulação da AmB em micelas do polisorbato 80 foi menor (mas não desprezível) do que aquele observado para a NE sugerindo que parte da AmB pode estar localizada nas micelas. Estudos adicionais de estabilidade e caracterização por dicroísmo circular devem ser conduzidos a fim de aprofundar esses aspectos

Em seguida avaliações da atividade hemolítica *in vitro* e leishmanicida *in vivo* foram conduzidas. A atividade hemolítica induzida pela C-AmB foi

significativamente maior do que aquela observada para a NE-AmB. Isto pode ser atribuído à fraca interação entre a AmB e o surfactante desoxicolato de sódio, permitindo uma liberação rápida da AmB para o meio seguida da interação com os eritrócitos. Além disso, o surfactante desoxicolato de sódio contribui para o efeito hemolítico (ITALIA *et al*., 2009; JAIN; KUMAR, 2010). Por outro lado, é bem provável que a interação da AmB com o colesterol na interface dos glóbulos da NE possibilite a liberação gradativa do fármaco do sistema carreador, causando, por conseguinte, menores concentrações da sua forma livre no meio receptor (CALDEIRA *et al*., 2015; EGITO *et al*., 2002). Portanto, estes resultados indicam um papel protetor dos glóbulos da NE na prevenção da lise dos eritrócitos.

Estudos *in vivo* em camundongos BALB-c experimentalmente infectados com *L. (L.) infantum chagasi* (modelo murino de LV) foram conduzidos para avaliação da atividade da NE em comparação com a AmB convencional (C-AmB). Dois estudos foram realizados: o primeiro com tratamento dos animais durante 3 e o segundo durante 5 dias alternados, em ambos, a AmB foi administrada, por via intravenosa, nas doses de 1 e 2 mg/kg.

O primeiro estudo (tratamento em 3 dias alternados) mostrou que a NE-AmB e C-AmB, ambos a 1 mg/kg, não foram eficazes no tratamento dos camundongos infectados com *L. (L.) infantum chagasi* (Apêndice C e D). Esses dados não estão em consonância com estudos prévios, em que a C-AmB e as formulações lipídicas foram eficazes no tratamento de modelo murino de LV, principalmente em animais infectados com *L. infantum* (GANGNEUX *et al.*, 1996; PAUL *et al.*, 1997). Fatores como a variabilidade de espécies e cepas de *Leishmania*, as quais apresentam características moleculares e bioquímicas específicas, diferenças entre protocolos de tratamento e métodos para avaliação da eficácia podem refletir em uma diferença de atividade (DEY *et al*., 2002; ESCOBAR *et al.*, 2002).

Os animais não foram tratados com C-AmB a 2 mg/kg, uma vez que nessa dose a formulação foi muito tóxica ou letal (DL_{50}=2.9 mg/kg) (GONDAL *et al*., 1989; OLSEN *et al*., 1991; PAUL *et al*., 1997). Em contraste, administração da NE-AmB a 2 mg/kg não foi observado efeitos adversos, como perda de peso ou morte, o que está em consonância com os dados mencionados acima de

toxicidade hemolítica, resultando, ademais, em redução significativa da carga parasitária no fígado e baço (Apêndice C e D).

Para o segundo estudo (5 dias alternados), os grupos que receberam NE-AmB e C-AmB, ambos a 1 mg/kg, apresentaram redução significativa da carga parasitária no fígado e baço em comparação com o controle (Figuras 4 e 5, capítulo 2). Entretanto, não houve diferença entre os dois grupos. Estudo anterior mostrou que formulação lipídica (Ambisome®) foi mais eficaz do que a AmB convencional no tratamento da LV em animais infectados com *L. infantum* (GANGNEUX *et al.*, 1996a). Isto tem sido atribuído à maior captação da AmB pelo fígado e baço a partir dos nanocarreadores (GANGNEUX *et al.*, 1996b; PAUL *et al.*, 1997), o que está em consonância com achados sobejamente conhecidos, os quais mostram que esses sistemas tendem a se acumular no fígado e baço após administração intravenosa (VYAS; GUPTA, 2006). Em contraste, nem todos os trabalhos mostraram vantagens das formulações lipídicas em comparação com a AmB convencional (YARDLEY; CROFT, 2000). Finalmente, a NE-AmB, administrada a 2 mg/kg, promoveu uma redução significativa da carga parasitária no fígado e baço em comparação com o controle e a C-AmB (1 mg/kg) sem apresentar sinal de toxicidade comumente observado com C-AmB na mesma dose.

Dois cenários podem ser visualizados para explicar a ausência de diferença entre a NE-AmB e a C-AmB. A liberação da AmB a partir da NE seria lenta não permitindo tornar o fármaco biodisponível. A fim de investigar essa hipótese, estudos de liberação e biodistribuição podem ser conduzidos futuramente. O segundo cenário poderia estar relacionado à sensibilidade da espécie de *Leishmania* usada no presente trabalho. A despeito do provável acúmulo e liberação do fármaco nos tecidos de interesse isso não seria suficiente para garantir uma eficácia farmacológica.

Na segunda parte do trabalho, Capítulo 2, avaliou-se as alterações metabólicas em camundongos tratados com NE-AmB e C-AmB, ambas a 1 mg/kg, com o objetivo de verificar diferenças de efeitos tóxicos entre as formulações. Doses da C-AmB > 1 mg/kg não foram utilizadas, já que propiciaram a morte dos animais. Foram também realizadas análises bioquímicas tradicionais e histopatológicas dos rins. Nesta avaliação, os biomarcadores convencionais de nefrotoxicidade, ureia nitrogenada (BUN) e creatinina, não apresentaram alterações significativas

nos seus níveis entre os grupos controle e os dois tratados. As imagens obtidas dos túbulos renais também não revelaram quaisquer sinais de alterações. Mesmo sendo considerados indicadores da função renal, esses biomarcadores não diferenciam uma lesão renal específica e apenas apresentam alterações após um maior acometimento do órgão e diminuição da capacidade funcional do néfron (BOUDONCK *et al*., 2009; HANNA *et al*., 2013). Entretanto, houve diferença estatisticamente significativa no volume urinário entre os 3 grupos. Os animais tratados com C-AmB apresentaram maior volume urinário em relação aos outros dois grupos. Isto pode estar relacionado com uma maior concentração da forma livre de AmB no plasma destes animais (Osaka *et al*., 1997).

Portanto, torna-se interessante e necessária a descoberta de biomarcadores mais preditivos para detectar alterações precoces nos perfis plasmáticos. Deste modo, a metabolômica surge como uma boa alternativa para a identificação dessas perturbações após administração das formulações. Sabe-se que a metabolômica tem demonstrado grande potencial para identificação de alterações metabólicas associadas à toxicidade renal, assim como para a descoberta de biomarcadores potenciais e mecanismos patológicos subjacentes (RUSSMANN *et al.*, 2009; FUCHS; HEWITT, 2011).

Os dados encontrados no presente trabalho indicam uma maior alteração no metaboloma dos animais tratados com C-AmB em relação aos tratados com NE-AmB. A via dos ácidos graxos poliinsaturados (PUFA) apresentou uma redução nas concentrações plasmáticas dos seus metabólitos (PUFA ômega 3 e 6), a qual coincidiu com o aumento dos níveis das acilcarnitinas. Isto pode ser atribuído à isquemia renal aguda promovendo redução da atividade das enzimas responsáveis pela oxidação dos ácidos graxos a acilcarnitinas nos néfrons (LI *et al*., 2009; PORTILLA *et al*., 2000).

Coincidentemente, o ácido araquidônico (PUFA ômega 6) e alguns dos seus metabólitos também apresentaram suas concentrações plasmáticas reduzidas no grupo tratado com C-AmB. Estas alterações podem explicar a presença dos mesmos na região renal. Sabendo-se que existem evidências de que a AmB ativa a síntese de metabólitos do ácido araquidônico (SAWAYA *et al*., 1995), os quais podem mediar a inflamação e a função de vários órgãos e sistemas, estas características podem estar associadas à nefrotoxicidade induzida pela AmB (POMPEIA *et al*., 2003; SHOEB *et al*., 2011).

É interessante observar a ocorrência de outras alterações lipídicas no plasma dos animais tratados com C-AmB. Houve redução dos níveis de lisofosfatidiletanolaminas (LPEs), lisofosfatidilcolinas (LPCs), triglicerídeos (TG), esfingosina e anandamida (AEA). As anormalidades lipídicas têm sido bem reconhecidas e consideradas de fundamental importância nos casos de danos renais (SUN *et al*., 2012).

O metaboloma dos camundongos tratados com NE-AmB revelou alterações menos significativas, o que poderia ser esperado de acordo com os resultados obtidos previamente por Caldeira e colaboradores (2015) e pela avaliação da atividade hemolítica *in vitro* descrita no Capítulo 1. A explicação mais provável para estes achados seria a liberação mais lenta e gradativa da AmB à partir das NE, propiciando menores concentrações da sua forma livre (tóxica) na corrente sanguínea.

Finalmente, foi interessante observar, a princípio, que a concentração plasmática da AmB no grupo tratado com NE-AmB foi cerca de duas vezes maior do que aquela obtida para o grupo tratado com C-AmB (Tabela S1 – Capítulo 2). Isto pode ser explicado pelo tamanho reduzido dos glóbulos da NE, permitindo um maior tempo de circulação desta formulação na corrente sanguínea (BEKERSKY *et al.*, 2002b).

O Capítulo 2, além de ter contribuído para a comparação entre as alterações metabólicas entre duas formulações de AmB (NE-AmB e C-AmB), também foi de extrema relevância no que se refere à descoberta de novos biomarcadores associados à administração da AmB, já que não existem estudos prévios relacionados ao fármaco. Entretanto, seria interessante uma validação destes biomarcadores como possíveis indicadores de nefrotoxicidade para facilitar estudos posteriores. Isto possibilitaria o diagnóstico precoce deste grave efeito adverso da AmB e evitaria maiores danos a órgãos vitais, como os rins.

6. CONCLUSÃO GERAL

O objetivo do trabalho foi otimizar, caracterizar, avaliar a toxicidade *in vitro* e *in vivo* e a atividade da NE-AmB em animais experimentalmente infectados com *Leishmania (L.) infantum chagasi*

A preparação da NE-AmB desenvolvida por Caldeira e colaboradores (2015) foi otimizada, sendo obtidos diâmetro dos glóbulos e valores de IP menores do que aqueles obtidos previamente. A atividade leishmanicida da NE-AmB foi similar àquela observada para a C-AmB em modelo animal de LV quando ambas foram administradas na mesma dose. Entretanto, a NE conduziu a uma redução significativa da carga parasitária no fígado e no baço no modelo murino de LV após a administração a 2 mg/kg sem qualquer sinal de efeito tóxico, ao contrário da C-AmB que se revelou letal nessa dose. A atividade hemolítica *in vitro* da NE-AmB foi muito menor do que aquela induzida pela formulação da AmB convencional. Foram observadas alterações metabólicas, as quais poderiam estar associadas à toxicidade precoce dos tratamentos, detectadas antes mesmo que uma lesão renal fosse estabelecida. É interessante considerar que a metabolômica foi capaz de revelar diferenças significativas entre os dois grupos de tratamento, sendo que o grupo tratado com C-AmB revelou maiores alterações no seu metaboloma. Vias metabólicas, como as dos PUFA e do ácido araquidônico, foram as mais afetadas, sugerindo que a C-AmB pode estar associada a processos inflamatórios, stress oxidativo e isquemia renal.

Assim sendo, as NE-AmB constituem uma alternativa interessante para tratamento da LV com alta eficácia e baixa toxicidade. A fácil fabricação e transposição para a escala industrial, além de economicamente viável, torna essa formulação uma alternativa atraente em comparação com as outras formulações lipídicas para o tratamento intravenoso da LV.

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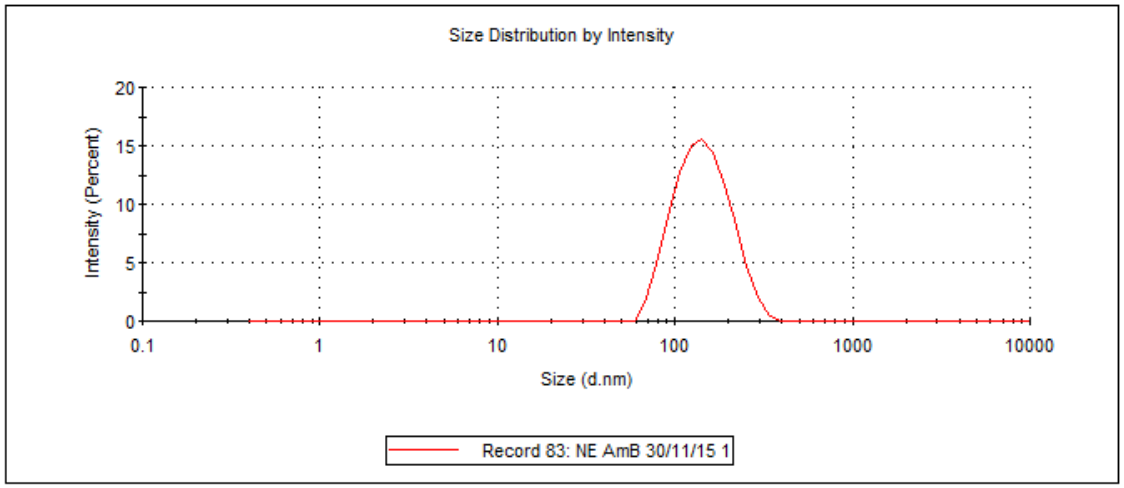
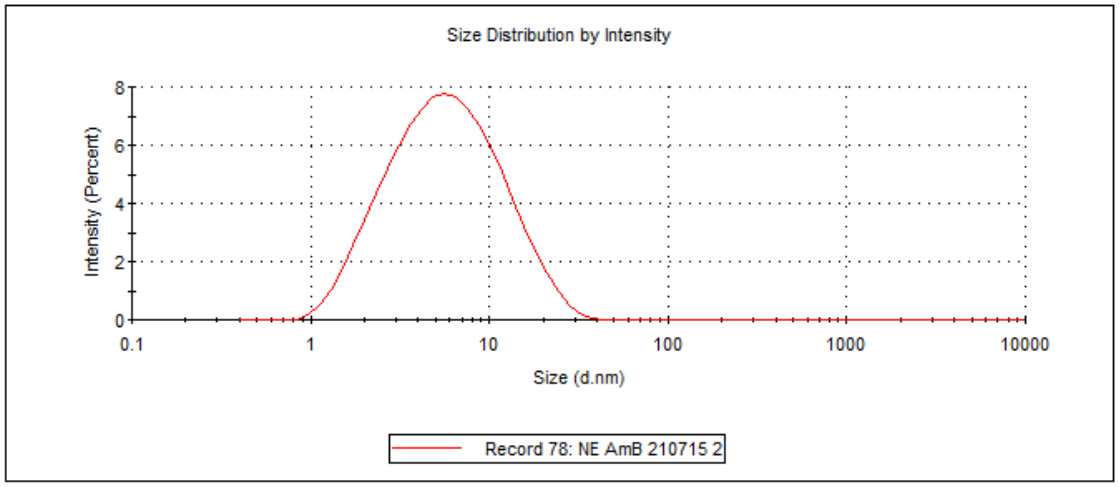
APÊNDICE A

Distribuição monomodal e homogênea dos glóbulos em amostras diluídas de NE-AmB.



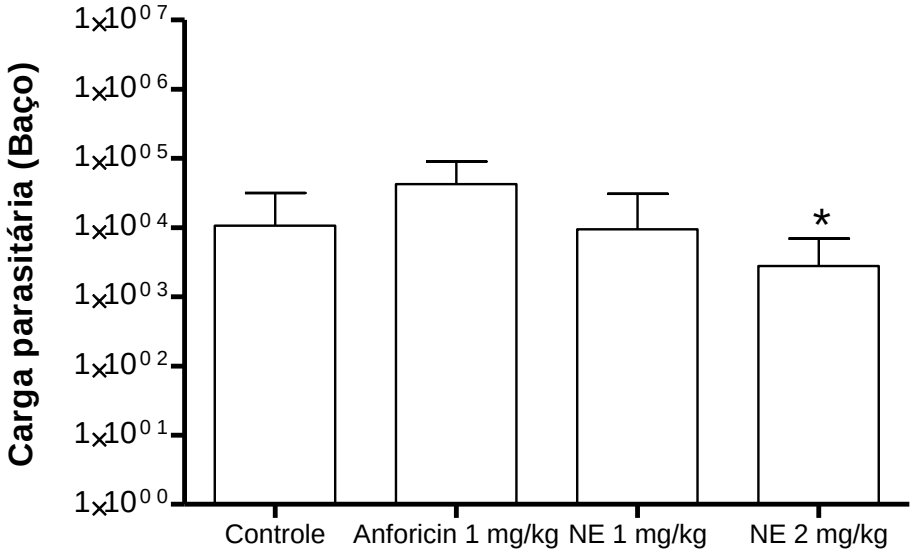
APÊNDICE B

Distribuição multimodal e heterogênea dos glóbulos em amostras não diluídas de NE-AmB



APÊNDICE C

Eficácia *in vivo* da AmB em animais experimentalmente infectados com *L. (L.) chagasi*. Camundongos BALB/c fêmeas (n=7) foram infectados por via intravenosa com promastigotas de *L. (L.) chagasi*. Sete dias após a inoculação, os animais foram tratados com uma solução de glicose a 5% (Controle), NE contendo AmB a 1 ou a 2 mg/kg (NE 1 mg/kg e NE 2 mg/kg), e Anforicin® (C-AmB a 1 mg/kg) por 3 dias alternados. Três dias após a interrupção do tratamento, os parasitas recuperados do baço foram avaliados pelo método de diluição limitante. **P* < 0.05 quando comparado com o grupo controle.



APÊNDICE D

Eficácia *in vivo* da AmB em animais experimentalmente infectados com *L. (L.) chagasi*. Camundongos BALB/c fêmeas (n=7) foram infectados por via intravenosa com promastigotas de *L. (L.) chagasi*. Sete dias após a inoculação, os animais foram tratados com uma solução de glicose a 5% (Controle), NE contendo AmB a 1 ou a 2 mg/kg (NE 1 mg/kg e NE 2 mg/kg), e Anforicin® (C-AmB a 1 mg/kg) por 3 dias alternados. Três dias após a interrupção do tratamento, os parasitas recuperados do fígado foram avaliados pelo método de diluição limitante. **P* < 0.05 quando comparado com o grupo controle e C-AmB.