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Infectologia e Medicina Tropical

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AVALIAÇÃO DE UMA MOLÉCULA COM ATIVIDADE ANTILEISHMANIAL PARA EMPREGO NO TRATAMENTO CONTRA AS LEISHMANIOSES

Belo Horizonte

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Débora Vasconcelos Costa Medonça

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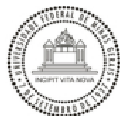

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"AVALIAÇÃO DE UMA MOLÉCULA COM ATIVIDADE ANTILEISHMANIAL PARA EMPREGO NO TRATAMENTO CONTRA AS LEISHMANIOSES"

DÉBORA VASCONCELOS COSTA MENDONÇA

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“A mente que se abre a uma nova ideia
jamais voltará ao tamanho original.”
Albert Einstein

RESUMO

As estratégias terapêuticas contra as leishmanioses devem ser melhoradas, haja vista que os fármacos disponíveis apresentam problemas como toxicidade, alto custo e/ou crescente resistência dos parasitos. Assim, a pesquisa e descoberta de novos compostos antileishmaniais que apresentem atividade contra *Leishmania* spp., mas baixa toxicidade para os hospedeiros mamíferos, tornam-se desejáveis. Neste trabalho, um derivado da naftoquinona denominado Flau-A ou 2-(2,3,4-tri-O-acetil-6-desoxi-β-L-galactopiranosiloxi)-1,4-naftoquinona foi avaliado contra promastigotas e amastigotas de *Leishmania amazonensis* e *L. infantum*, espécies causadoras de leishmanioses tegumentar e visceral, respectivamente, nas Américas. Os resultados mostraram que Flau-A foi eficaz contra as formas dos parasitos, e apresentou baixa toxicidade para células de camundongos e humanos, com melhores resultados que a anfotericina B, usada como controle. O mecanismo de ação mostrou que a molécula atuou na mitocôndria dos parasitos causando sua morte, e o tratamento de macrófagos infectados e a inibição da infecção usando parasitos pré-incubados mostraram o potencial terapêutico *in vitro* da mesma. Então, a eficácia *in vivo* da molécula foi avaliada em modelo murino infectado com *L. amazonensis* ou *L infantum*, sob a forma livre ou incorporada em um sistema de micelas formadas de Poloxâmero P407 (Flau-A/M). Os resultados mostraram que o tratamento com Flau-A ou Flau-A/M reduziu o diâmetro médio das lesões causadas por *L. amazonensis* e a carga parasitária na lesão (*L. amazonensis*), fígado, baço, linfonodos drenantes e medula óssea; quando comparados aos demais grupos. Tais animais desenvolveram uma resposta imune do tipo Th1 específica aos parasitos, com níveis elevados de IFN-gama, IL-12, TNF-α, GM-CSF, além de anticorpos IgG2a, associados a baixos níveis IL-4, IL-10 e anticorpos IgG1. Esses animais também apresentaram baixa toxicidade, quando comparado aos demais grupos. Flau-A/M foi mais efetiva que a molécula livre na resposta terapêutica contra as duas espécies de *Leishmania*. Desta forma, a composição micelar poderia ser considerada como candidata terapêutica para o tratamento contra as leishmanioses.

Palavras-chave: flau-a; tratamento; toxicidade; anfotericina b; micelas.

ABSTRACT

Therapeutic strategies against leishmaniasis must be improved, given that the drugs present problems such as toxicity, high cost and/or increasing of parasites resistance. Thus, the research and discovery of new antileishmanial compounds that have activity against *Leishmania spp*., but low toxicity to mammalian hosts, become desirable. In this study, a naphthoquinone derivative called Flau-A or 2- (2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy) -1,4-naphthoquinone was evaluated against promastigotes and amastigotes of *Leishmania amazonensis* and *L. infantum*, species that cause cutaneous and visceral leishmaniasis, respectively, in Americas. The results showed that Flau-A was effective against parasites forms and presented low toxicity for mice and humans cells, with better results than amphotericin B, used as a control. The mechanism of action showed that the molecule acted in parasites mitochondria causing its death, and the treatment of infected macrophages and the inhibition of infection using pre-incubated parasites showed its therapeutic potential *in vitro*. Then, the *in vivo* efficacy of molecule was evaluated in a murine model infected with *L. amazonensis* or *L. infantum*, either in the free form or incorporated in a system of micelles formed from Poloxamer P407 (Flau-A / M). The results showed that treatment with Flau-A or Flau-A/M reduced the average diameter of the lesions caused by *L. amazonensis* and the parasitic load on the lesion (*L. amazonensis*), liver, spleen, draining lymph nodes and bone marrow; when compared to the other groups. Such animals developed a specific Th1 immune response to parasites, with high levels of IFN-gamma, IL-12, TNF-α, GM-CSF, in addition to IgG2a antibodies, associated with low levels IL-4, IL-10 and IgG1 antibodies. These animals also showed low toxicity, when compared to the other groups. Flau-A/M was more effective than the free molecule in the therapeutic response against the two species of *Leishmania*. Thus, the micellar formulation could be considered as a therapeutic candidate for the treatment against leishmaniasis.

Keywords: flau-a; treatment; toxicity; amphotericin b; micelles.

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LISTA DE SIGLAS E ABREVIATURAS

®	Marca registrada
°C	Graus Celsius
µg	Micrograma
µL	Microlitro
Abs	Absorbância
ALT	Alanina aminotransferase
AmpB	Anfotericina B
AmpB/M	Anfotericina B micelar
ANOVA	Análise de Variância
AST	Aspartato aminotransferase
CC50	Concentração da substância necessária para inibir a viabilidade de 50% das células
CO2	Dióxido de carbono
COEP	Comitê de Ética em Pesquisa
COLTEC	Colégio técnico da Universidade Federal de Minas Gerais
DAT	Teste de aglutinação direta
dLN	Linfonodo drenante
DMSO	Dimetilsulfóxido
DO	Densidade óptica
EDTA	Ácido Etilenodiamino Tetra-Acético
ELISA	Análise de imunoadsorção por ligação enzimática
FDA	Food and Drug Administration
Flau-A	(2-((7-chloroquinolin-4- yl)oxy)-3-(3-methylbut-2- en- 1-yl)naphthalene-1,4- diona)
Flau-A/M	(2-((7-chloroquinolin-4- yl)oxy)-3-(3-methylbut-2- en- 1-yl)naphthalene-1,4- diona) micelar
g	Grama
GM-CSF	Fator estimulante de colônia macrófago-granulócito
h	Hora
IC_{50}	Concentração da substância necessária para inibir a viabilidade de 50% dos parasitas
ICT	Teste imunocromatográfico
IFAT	Ensaio de imunofluorescência indireta
IFN-γ	Interferon-gama
Ig	Imunoglobulina
IL-10	Interleucina 10
IL-12	Interleucina 12
IL-2	Interleucina 2
IL-4	Interleucina 4
iNOS	Óxido nítrico sintase induzível
IS	Índice de seletividade

L-AmpB	Anfotericina B lipossomal
LC	Leishmaniose cutânea
LCD	Leishmaniose cutâneo-difusa
LCL	Leishmaniose cutânea localizada
LD	Leishmaniose disseminada
LMC	Leishmaniose muco-cutânea
LT	Leishmaniose tegumentar
LV	Leishmaniose visceral
M	Molar
MET	Microscopia eletrônica de transmissão
mg	Miligrama
min	Minutos
mL	Mililitro
mM	Milimolar
mmol	Milimol
MTT	Brometo de 3-(4,5-dimetialtiazol-2-il)-2,5- digenil- 2H- tetrazólico
n	Número amostral
nm	Nanômetro
NO	Óxido nítrico
NP´s	Nanopartículas
OMS	Organização Mundial da Saúde
p/p	Peso por peso
p/v	Peso por volume
PA	Para análise
PBS	Tampão fosfato salina
PCR	Reação em cadeia da polimerase
PF127	Pluronic F127
pH	Potencial hidrogeniônico
PI	Iodeto de propídeo
RBC50	Concentração da substância necessária para inibir a viabilidade de 50% das hemácias
RPMI	Roswell Park Memorial Institute
Sb^{v}	Antimoniais pentavalentes
SFB	Soro fetal bovino
SLA	Extrato solúvel de *Leishmania*
Th1	Resposta de linfócitos helper tipo 1
Th2	Resposta de linfócitos helper tipo 2
TNF	Fator de necrose tumoral
v/v	Volume por volume
WHO	World Heath Organization
µM	Micromolar

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1. INTRODUÇÃO

As leishmanioses são doenças causadas por parasitos protozoários do gênero *Leishmania*, sendo consideradas como uma das seis doenças tropicais negligenciadas mais importantes na atualidade. Além disso, apresentam alta incidência e um grande número de pessoas está exposto aos riscos de infecção em todo o mundo (WHO, 2010). Por isso, muitos são os desafios para o controle e combate a estas doenças. Neste sentido, o tratamento para as leishmanioses ainda possui limitações que reduzem a adesão e a continuidade por parte dos pacientes, tais como a longa duração, a via de administração dos medicamentos e a toxicidade sistêmica observada pelos fármacos de primeira e segunda escolha (OSORIO *et al.*, 2007). Há de se destacar também o aumento do número de casos de recidiva à doença após o fim do tratamento além de muitos relatos relacionados à falha terapêutica, fatos atualmente observados em pacientes de diversas regiões do mundo (VÉLEZ; COLMENARES; MUÑOZ, 2009).

De forma ideal, o tratamento para as leishmanioses deve curar os pacientes, eliminando os riscos de recidiva e a capacidade de desenvolvimento de parasitos resistentes, além de causar pouco efeito colateral (WHO, 2010). Assim sendo, a busca por novas substâncias com ação antileishmanial, ou a redução da toxicidade dos fármacos atuais, sob a forma de novos sistemas de delivery de tais compostos, seria desejável.

As quinonas representam uma ampla e variada família de metabólitos de distribuição natural. Nos últimos anos intensificou-se o interesse nestas substâncias, não só devido à sua importância nos processos bioquímicos vitais, como também ao destaque cada vez maior que apresentam em variados estudos farmacológicos (SILVA; FERREIRA; SOUZA, 2003). Na natureza, estão envolvidas em etapas importantes do ciclo de vida de seres vivos, principalmente nos níveis da cadeia respiratória e da fotossíntese (GOODWIN; MERCER, 1972). A distribuição dessas substâncias nos variados organismos implica, possivelmente, em funções biológicas múltiplas, agindo de forma conspícua em seus diversos ciclos bioquímicos. Em estudos farmacológicos, as quinonas mostram variadas biodinamicidades, destacando-se, dentre muitas, as propriedades microbicidas, tripanossomicidas, viruscidas, antitumorais e inibidoras de sistemas celulares reparadores, processos

nos quais atuam de diferentes formas (SILVA; FERREIRA; SOUZA, 2003). Desta forma, estas substâncias apresentam potencial ação antileishmanial, que foi analisada através de experimentos *in vitro* e *in vivo*.

Uma das estratégias adotadas para o emprego das novas formas de delivery, é a formulação de sistemas micelares, capazes de transportar os fármacos de interesse aos principais órgãos alvos. Desta forma, para o desenvolvimento de um novo sistema de delivery farmacológico, foi utilizado neste estudo o Poloxâmero P407 que é um copolímero ideal para a formulação de micelas, uma vez que possui baixa toxicidade, excelente compatibilidade com outros produtos químicos e alta solubilidade. Estudos anteriores realizados pelo mesmo grupo que desenvolveu este trabalho demonstrou que micelas contendo AmpB à base de P407 são eficazes no tratamento de camundongos BALB/c infectados com *L. amazonensis* (MENDONÇA et al., 2016), além de avaliarem uma gama de outros compostos incorporados a esse sistema micelar para o tratamento das leishmanioses, como o clioquinol (TAVARES et al., 2020) e a digitoxigenina (FREITAS et al., 2021). Em todos os trabalhos as formulações de sistemas de delivery se mostraram eficazes e efetivas.

Tendo em vista os fatos expostos, o presente estudo objetivou a avaliação de uma molécula derivada da lausona (uma naftoquinona), e sua veiculação em um sistema micelar, em modelos *in vitro* e *in vivo* como agente antileishmanial.

2. REVISÃO DA LITERATURA

2.1. Epidemiologia das leishmanioses

As leishmanioses são um complexo de doenças infecto-parasitárias que apresentam ampla distribuição geográfica, sendo encontradas nos cinco continentes e consideradas endêmicas em 98 países no mundo, principalmente, nas regiões tropicais. Este complexo de doenças pode acometer diversos hospedeiros mamíferos, como o homem e espécies de animais silvestres e domésticos, como o cão (DESJEUX, 2004). Estima-se que ocorra incidência mundial anual de aproximadamente 50.000 a 90.000 casos de leishmaniose visceral (LV), sendo a maioria no Brasil, na África Oriental e na Índia, e 600.000 mil a 1 milhão de novos casos anuais de leishmaniose tegumentar (LT), havendo elevada taxa morbidade e mortalidade (ALVAR *et al.*, 2012; WHO, 2020). Segundo os dados da Organização Mundial de Saúde, em 2018, mais de 90% dos novos casos das doenças ocorreram

nos seguintes países: Afeganistão, Argélia, Bolívia, Brasil, Colômbia, Irã, Iraque, Paquistão, Síria e Tunísia.

É estimado que 380 milhões de pessoas encontram-se expostas ao risco de contrair a infecção pelo parasito *Leishmania*. O Brasil é responsável por aproximadamente 95% dos casos de LV e 40% dos casos de LT no Continente Americano, fato que exalta a doença como um importante problema de Saúde Pública (ALVAR *et al*., 2012). O acometimento é majoritariamente associado à desnutrição, condições precárias de moradia, indivíduos com sistema imune debilitado e com dificuldade de recursos, sendo essas características as de pessoas de situação econômica desfavorável (ASHFORD, 2000).

2.2. Etiologia e ciclo biológico do parasito *Leishmania*

As leishmanioses são doenças causadas por parasitos protozoários pertencentes ao gênero *Leishmania*, Sub-Reino Protozoa, Filo Sarcomastigophora, Ordem Kinetoplastida e Família Trypanosomatidae. O vetor transmissor é um inseto da Ordem Diptera, Família Psychodidae, Sub-Família Phlebotominae, pertencente aos gêneros *Phlebotomus* em países do Velho Mundo e *Lutzomyia* nas Américas, sendo que apenas as fêmeas possuem hábitos hematófagos sendo assim, capazes de transmitir o parasito aos hospedeiros mamíferos (GRIMALDI; TESH, 1993).

O gênero *Leishmania* compreende 22 espécies patogênicas para o homem. O parasito é digenético, ou seja, durante seu ciclo de vida, é encontrado em duas formas ou estágios: uma forma promastigota que é extracelular e alongada, e possui um flagelo que permite a mobilidade no intestino de insetos vetores **(Fig 1a)** e a forma amastigota, que é arredondada, intracelular e se multiplica nas células do sistema fagocítico mononuclear, principalmente macrófagos no hospedeiro mamífero **(Fig 1b)**. Ambas as formas do parasito se reproduzem por divisão binária e possuem uma única mitocôndria modificada conhecida como cinetoplasto (DEBRAY et al., 2015).

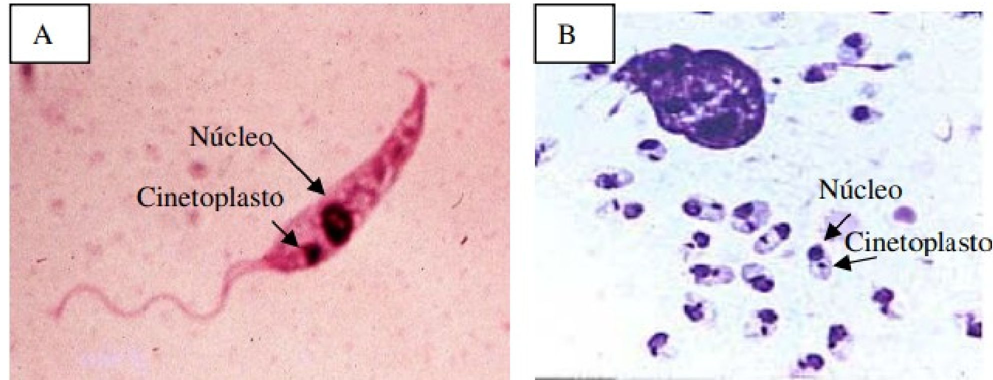

Figura 1: Micrografia das formas morfológicas mais comuns de *Leishmania spp.* **(**A) Promastigota; (B) Amastigotas.
Fonte:(A)<http://www.med.unimarburg.de/stpg/ukm/lt/hygiene/schwarz/projects.html>;
(B)http://www.vet.upenn.edu/schoolresources/communications/publications/bellwether/48/foxhounds.

No Brasil e em outros países das Américas, há a ocorrência de várias espécies do parasito que podem causar LT. Dentre elas, podemos citar *L. (V.) lainsoni, L. (V.) naiffi, L. (V.) lindenberg* e *L. (V.) shawi*, porém, a maioria dos casos é atribuída às espécies *L. (V.) guyanensis, L. (Leishmania) amazonensis* e principalmente *L. (V.) braziliensis*, baseado em achados parasitológicos e moleculares que o apontam como principal agente etiológico (GRIMALDI, G.; TESH; MCMAHON-PRATT, 1989; MARZOCHI; MARZOCHI, 1994; DE ALMEIDA, M. C.; VILHENA, V.; BARRAL, A.; BARRAL-NETTO, 2003; SILVEIRA; LAINSON; CORBETT, 2004). Já a espécie *L. (L.) infantum* é a principal responsável pelos quadros de LV no país (PISCOPO, TV ; AZZOPARDI, 2007).

As fêmeas de flebotomíneo dos gêneros *Phlebotomus* e *Lutzomyia* infectam-se ao sugar o sangue de humanos (antroponoses) ou mamíferos infectados (zoonoses) pelas formas amastigotas. As formas amastigotas se transformam em promastigotas no intestino do inseto e se replicam. Durante o repasto sanguíneo do inseto, os parasitos são inoculados passivamente no hospedeiro vertebrado na forma promastigota metacíclica que é fagocitada por diferentes células do sistema fagocítico mononuclear. Dentro dessas células, as promastigotas se transformam e replicam como amastigotas, que infectam macrófagos adicionais localmente ou em tecidos distantes, após a disseminação e estabelecem a infecção. (BELKAID *et al*., 2000; GOTO; LINDOSO, 2010b) (**Figura 2**).

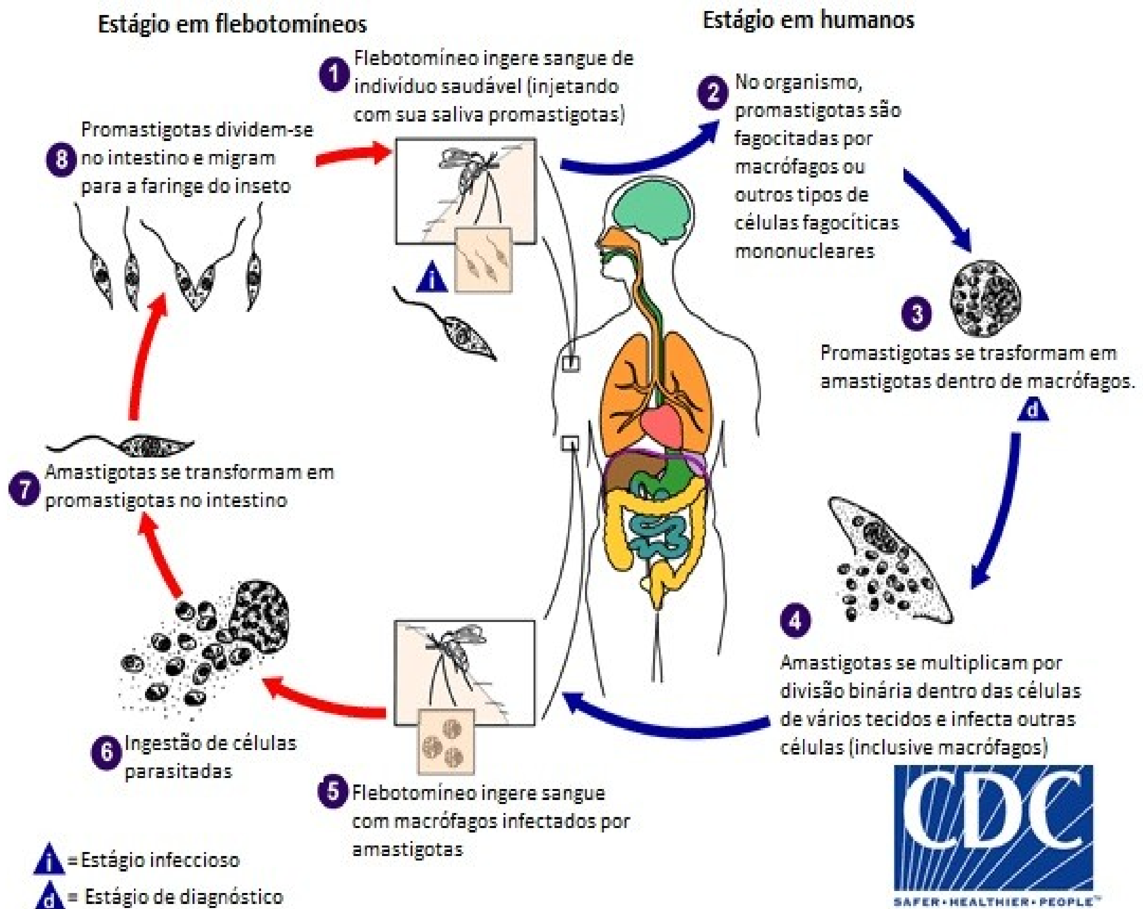

Figura 2 – Ciclo biológico do parasito *Leishmania*. Fonte: adaptado de ttps://www.cdc.gov/dpdx/leishmaniasis/index.html. Acesso em: 10 de janeiro de 2021.

2.3. Manifestações clínicas das leishmanioses

A infecção por *Leishmania* pode causar no homem um conjunto de síndromes clínicas que podem envolver a pele, membranas mucosas das vias aéreas superiores e órgãos internos. Tais síndromes são diversificadas e representam um complexo de doenças: a leishmaniose visceral (LV) que é geralmente fatal se aguda e não tratada e a leishmaniose tegumentar (LT), que pode-se apresentar nas formas cutânea (LC), muco-cutânea (LMC) e cutâneo-difusa (LCD).

A leishmaniose cutânea localizada (LCL) é a forma clínica mais comum de LT e pode ser causada pelas espécies *L. major, L. tropica, L. braziliensis*, dentre outras. Ela pode se apresentar como uma úlcera cutânea indolor, rosa e arredondada com bordas bem delimitadas e levantadas, uma base indurada e um fundo limpo onde uma crosta central, que pode sangrar, pode aparecer. Pode ocorrer a resolução espontânea, deixando uma cicatriz hipopigmentada, lisa e fina, no entanto, alguns

casos evoluem para formas mais graves da doença (GOTO; LINDOSO, 2010b; WHO, 2010). Além disso, a LCL pode apresentar recidivas com reaparecimento da doença nos sítios de lesões previamente cicatrizadas. Isso pode ocorrer décadas após a resolução das lesões primárias e, muitas vezes, formam-se dentro da borda da cicatriz anterior (SCHUBACH *et al*., 1998; MAROVICH *et al*., 2001).

A LCD, uma das possíveis intercorrências da LC, é caracterizada pelo aparecimento de múltiplas lesões pleomórficas em duas ou mais áreas não contíguas do corpo, que são provavelmente causadas por disseminação hematogênica ou linfática e tem sido descritas associação com *L. braziliensis, L. panamensis, L. guyanensis e L. amazonensis.* (GOTO; LINDOSO, 2010), sendo uma forma difícil de curar com os tratamentos atualmente disponíveis. É importante mencionar que nas Américas a LCD tende a ser mais grave do que em regiões do Velho Mundo, principalmente por conta de um maior período de evolução das lesões (OPAS, 2013).

Cerca de 1 a 5% dos pacientes podem desenvolver LMC, pela capacidade que algumas espécies do parasito têm em migrar para as mucosas e causar destruição tecidual (SCHUBACH *et al.*, 1998). A LMC é causada principalmente por *L. braziliensis e L. guyanensis* nas Américas, sendo uma forma desfigurante e mutilante da doença, que pode ser fatal devido às complicações secundárias (MCGWIRE; SATOSKAR, 2014). Geralmente, ocorre meses ou anos após a LCL. A LMC afeta, principalmente, a mucosa nasal, mas a mucosa oral também pode ser atingida. Os sintomas, que são inespecíficos, podem incluir prurido no nariz que progride para formação de crosta e sangramento. Inicialmente, a inflamação nasal e a congestão são observadas no exame da narina; contudo, ulceração e perfuração do septo podem ocorrer lentamente. Este quadro é de difícil diagnóstico parasitológico e apresenta falha terapêutica ao tratamento convencional (BOAVENTURA *et al*., 2006; GOTO; LINDOSO, 2010b).

A LV, também conhecida como kala-azar, resulta da infecção de células fagócitas do sistema reticulo endotelial devido ao espalhamento de parasitos e macrófagos infectados a partir do local inicial da infecção cutânea. Nas Américas, a LV é causada principalmente pela espécie *L. infantum*. A proliferação de parasitos em macrófagos no fígado, baço e medula óssea de pacientes origina hepatoesplenomegalia progressiva e supressão da medula óssea. A menos que sejam tratados, os pacientes desenvolvem pancitopenia e imunossupressão e são

propensos a super-infecções com outros patógenos (MCGWIRE; SATOSKAR, 2014).

A manifestação e evolução clínica da infecção humana dependem da espécie do parasito, do ambiente e da resposta imune do hospedeiro. Se a resposta imune for capaz de combater a infecção, há o desenvolvimento concomitante de resistência à reinfecção ao longo da vida (SACKS; NOBEN-TRAUTH, 2002a). No caso de falha da imunidade, a doença torna-se crônica, com a infecção progredindo para o sistema retículo-endotelial. É importante observar que algumas espécies de parasitos responsáveis por lesões cutâneas da doença também podem causar infecções visceralizantes (CHEUKA *et al.*, [s.d.]; DESJEUX, 2004; WHO, 2020).

2.4. Imunologia das leishmanioses

É geralmente aceito que o controle da infecção por *Leishmania* no hospedeiro mamífero é mediada pela resposta imune inata e adaptativa do mesmo. A interação e a resposta contra *Leishmania* é manifestada não só em termos da evolução clínica ou subclínica da infecção, mas também pela taxa de cura espontânea e recorrência da doença (ADOLFO *et al.*, 2001).

Os neutrófilos são as primeiras células que os parasitos têm contato no local da inoculação durante o repasto sanguíneo do flebotomíneo e as células do sistema imune inato, incluindo células *Natural Killer* têm sido reveladas de fundamental importância no curso da infecção e doença (DA SILVA SANTOS; BRODSKYN, 2014). A manifestação clínica da infecção por *Leishmania* é mediada pela resposta celular, sendo dependente da polarização da resposta auxiliar Th1 ou Th2. Uma resposta do tipo Th1 é mediada por citocinas pró-inflamatórias como interferon-gama (IFN-γ), fator de necrose tumoral alfa (TNF-α), fator estimulador de colônias de granulócitos e macrófagos (GM-CSF) e interleucina 12 (IL-12), que participam do controle e resolução da infecção. Já a resposta do tipo Th2, com a produção de citocinas como interleucina-4 (IL-4) e interleucina-10 (IL-10) induz à susceptibilidade e progressão da doença no hospedeiro infectado. Tais respostas imunes respondem pelo espectro clínico diversificado, principalmente, da LT. As lesões localizadas de auto-cicatrização têm um perfil celular predominantemente do tipo Th1, enquanto que a resposta mediada por células Th2 caracterizam lesões difusas e não cicatrizantes. A LMC demonstra uma resposta mista, mas com predominância do tipo Th1, que pode explicar a atividade inflamatória agressiva e cronicidade da

doença (AFONSO; SCOTT, 1993). Em adição, macrófagos estimulados com IFN-γ e IL-12 tornam-se ativados e expressam níveis elevados de óxido nítrico sintase induzível (iNOS). A síntese de óxido nítrico (NO) por iNOS é essencial para a eliminação de parasitos, uma vez que a ausência de iNOS por deleção genética provoca infecção incontrolável e lesões ulcerativas no local da infecção (DIEFENBACH *et al.*, 1998).

Camundongos têm sido utilizados como modelo de infecção para estudos de candidatos vacinais contra a infecção por várias espécies de *Leishmania spp.*, como *L. infantum* e *L. amazonensis,* auxiliando na elucidação da relação parasito-hospedeiro e avaliação da resposta imune às diversas espécies de *Leishmania*, favorecendo o entendimento das funções das células que compõem esse sistema complexo (LOCKSLEY; LOUIS, 1992; SACKS; NOBEN-TRAUTH, 2002b; WILSON; JERONIMO; PEARSON, 2005). Nesse sentido, camundongos C57BL/6 infectados com *L. major*, por exemplo, desenvolvem uma resposta do tipo Th1 com produção de citocinas pró-inflamatórias como IFN-γ e IL-12, que atuam na ativação de macrófagos parasitados (MOSSER; EDWARDS, 2008). A população de macrófagos com atividade microbicida é ativada e produz ânions superóxido e radicais de oxigênio e nitrogênio. Através do metabolismo do aminoácido L-arginina pela enzima óxido nítrico sintase-indutível (iNOS), há a produção de NO que mata o parasito intracelular, sendo a expressão de iNOS induzida por TNF-α (TRACEY *et al.*, 2008).

Já em camundongos BALB/c infectados com *L. major*, a susceptibilidade à infecção ocorre pelo desenvolvimento de uma resposta do tipo Th2 com níveis elevados de IL-4. Quando os macrófagos entram em contato com essa citocina, paralelamente à ativação de fatores de transcrição, há expressão da enzima Aginase I, a qual converte L-arginina em ornitina e não inibe a proliferação dos parasitos no citoplasma dos macrófagos (KREIDER *et al.*, 2007; LOKE *et al.*, 2017 MOSSER; EDWARDS, 2008). Outra citocina importante na proliferação dos parasitos é a IL-10, que tmbém atua na desativação das células parasitadas contribuindo para o desenvolvimento da infecção (KANE *et al.*, 2017; NOBEN-TRAUTH *et al.*, 2017).

Um indicativo da geração da resposta imune do tipo Th1 ou Th2 em camundongos BALB/c pode ser também mostrada a partir da produção dos isotipos de imunoglobulinas G (IgG) dos subtipos 1 e 2a. Citocinas secretadas por células do sistema imune tais como linfócitos T, atuam sobre linfócitos B induzindo a mudança

dos isotipos dos anticorpos IgGs produzidos por tais células. Nesta linhagem de camundongos, a citocina IL-4, induz, preferencialmente, a produção de IgG1, enquanto que o IFN-γ induz à produção de IgG2a (DOHERTY *et al*., 1993).

Toda essa dinâmica apresentada pela resposta imune evocada pela infecção por *Leishmania,* pode ser resumida para melhor entendimento de forma esquemática, como a apresentada na **Figura 3.**

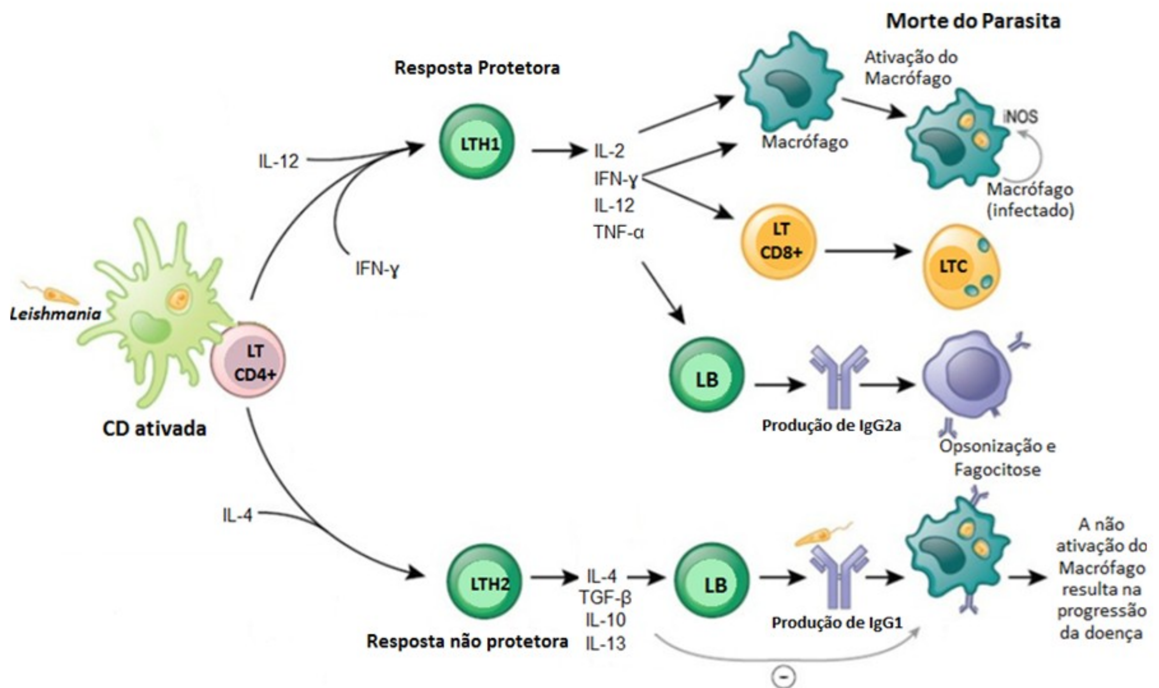

Figura 3: esquema da resposta imunológica induzida pela infecção por *Leishmania* (adaptado de Ezra *et al*, 2010)

2.5. Diagnóstico das leishmanioses

O diagnóstico das leishmanioses é um desafio, uma vez que a doença causa manifestações clínicas também comuns a outras doenças (CHAPPUIS *et al*., 2007). Assim, os sintomas podem ser confundidos com hanseníase, câncer, dentre outras, na LT; e com malária, esquistossomose, tripanossomíase, tuberculose, dentre outras, na LV (SINGH, 2006). Deste modo, o diagnóstico deve ser realizado com base na associação de parâmetros clínicos e exames laboratoriais (TESH, 1995).

O diagnóstico parasitológico é mais conclusivo quando comparado aos demais testes, uma vez que a identificação do parasito realizada por meio de análises microscópicas em amostras biológicas não deixa dúvidas sobre a infecção, porém, apresenta limitações quando a infecção apresenta baixa carga parasitária, além do que a leitura da lâmina exige tempo e treinamento adequado e, além disso,

é um método invasivo em relação à coleta das amostras (ASHFORD, 2000; TAVARES; FERNANDES; MELO, 2003; REITHINGER *et al.*, 2007).

Testes imunológicos sorológicos, como o ensaio imunoenzimático (ELISA), ensaio de imunofluorescência indireta (IFAT), teste de aglutinação direta (DAT), Western-blot e o teste imunocromatográfico (ICT) se baseiam na detecção de anticorpos e/ou antígenos específicos dos parasitos em amostras de soro ou plasma dos pacientes, porém, apresentam também variações em sua sensibilidade e/ou especificidade (MARZOCHI; MARZOCHI, 1994; TAVARES; FERNANDES; MELO, 2003; DE PAIVA-CAVALCANTI *et al.*, 2015).

Usualmente, o diagnóstico é realizado através de testes sorológicos e moleculares. A sensibilidade da pesquisa parasitológica é baixa quando comparada com a do IFAT, que é inferior à dos testes de ELISA e DAT que, geralmente, apresentam alta sensibilidade, mas especificidade variável (ROMERO; BOELAERT; LOUZIR, 2010). Desta forma, o diagnóstico final é realizado com base na avaliação de parâmetros clínicos, inquéritos epidemiológicos e na confirmação por exames laboratoriais. Ao se deparar com um paciente suspeito, a confirmação deve ser via pesquisa direta do parasito, seja por identificação de amastigotas em tecido, de promastigotas em culturas, ou ainda de seus produtos, como pesquisa de anticorpos via aglutinação, imunofluorescência indireta e ELISA. Caso confirmado, inicia-se a investigação epidemiológica e o tratamento preconizado (TESH, 1995; GONTIJO, 2003).

2.6. Tratamento das leishmanioses

Apesar da infecção pelo parasito *Leishmania* e a doença “leishmaniose” terem sido reconhecidas pela ciência médica há mais de um século, ainda há pouca disponibilidade de terapias eficazes e específicas, apesar dos esforços e da enorme incidência da doença nos países em desenvolvimento (KLING; KÖRNER, 2013). Os fármacos de uso corrente para o tratamento têm problemas conhecidos de toxicidade, eficácia, administração, duração do tratamento e, consequentemente, baixa adesão dos pacientes, além de custos elevados. Além disso, não são capazes de eliminar completamente os parasitos nos indivíduos infectados *(*DESJEUX, 2004; EGGER *et al*., 2010).

Os critérios de cura são apenas clínicos, pois nem parâmetros parasitológicos nem laboratoriais satisfazem tal objetivo. Na LC, o critério de cura é a epitelização da lesão e o desaparecimento da induração na base da úlcera, que deve ocorrer até 3 meses após o tratamento, já nos casos de LV, os critérios utilizados são a redução da hepatoesplenomegalia bem como a ausência de sintomas após o tratamento. A escolha farmacológica depende da forma clínica da doença e, nos casos de LT, do tamanho e localização da lesão, do número de lesões e potencial de disseminação do parasito (AMEEN, 2010). Se a cura clínica de acordo com os critérios estabelecidos não for atingida, é considerada como recidiva e deve ser administrado novo tratamento com o mesmo ou novo regime terapêutico (GOTO; LAULETTA LINDOSO, 2012).

Os antimoniais pentavalentes começaram a ser empregados em 1945 e permanecem sendo o tratamento de primeira escolha na maior parte do mundo (TIUMAN *et al.*, 2011). Infelizmente, o aumento na falha terapêutica destes medicamentos tem sido documentado em várias regiões geográficas. Os fármacos se encontram disponíveis em duas formulações: antimoniato de meglumina (GLUCANTIME®) e estibogluconato de sódio (PENTOSTAM®), sendo o primeiro, a formulação utilizada no Brasil. Os principais efeitos adversos são artralgia e mialgia, porém, efeitos relacionados à cardiotoxicidade ou insuficiência renal podem ocorrer, principalmente, em pacientes idosos. O uso deste medicamento não é indicado durante a gravidez. A eficácia dos antimoniais varia de acordo com as espécies de *Leishmania*, a apresentação clínica da doença e o estado nutricional e imunológico dos hospedeiros (GOTO; LINDOSO, 2010a).

Dentre os medicamentos de segunda escolha, encontra-se a anfotericina B (AmpB), que é um antifúngico com atividade antileishmanial eficaz, proém, capaz de causar toxicidade elevada. Para melhorar o índice terapêutico da AmpB e reduzir sua toxicidade, foram desenvolvidas formulações baseadas em lipídios para administração parenteral, como AmBisome® (formulação empregada e disponível no Brasil, produzido pela Gilead Sciences, Inc., Foster City, CA, EUA; Amphocil® (Kadmon Pharmaceuticals, New York, NY, EUA) e Abelcet® (Sigma-Tau Pharmaceuticals, Inc., Gaithersburg, MD, EUA). A Organização Mundial da Saúde recomenda o uso de AmpB lipossomal (L-AmpB) com base em sua alta eficácia e segurança. As formulações lipídicas apresentam vantagens em relação à formulação convencional, incluindo maior concentração nos órgãos primários, tais como baço,

fígado e pulmões e menor concentração nos rins, com redução marcante da nefrotoxicidade, além da diminuição dos efeitos adversos relacionados à infusão (VYAS; GUPTA, 2006). Entretanto, apesar da melhora do índice terapêutico e diminuição da toxicidade para as formulações lipídicas, sua utilização permanece limitada, principalmente, pelo custo elevado dos produtos (EGGER *et al*., 2010; RIBEIRO *et al*., 2014).

A paramomicina é um antibiótico aminoglicosídeo, geralmente administrado por via intramuscular. Foi desenvolvida na década de 1960 como um agente leishmanicida, mas permaneceu negligenciado até a década de 1980, quando formulações tópicas contendo paromomicina (15%) mais cloreto de metilbenzetônio (12%) se mostraram eficazes para o tratamento da LCL (CROFT; COOMBS, 2003). A dor leve no local da injeção é o evento adverso mais comum (55% dos pacientes). A ototoxicidade reversível ocorre em 2% dos pacientes. A toxicidade renal é rara. Alguns doentes podem desenvolver hepatotoxicidade, indicada por concentrações elevadas de enzimas hepáticas (WHO, 2010).

A miltefosine é um alquilfosfolipídeo que foi desenvolvido como medicamento antineoplásico oral e, posteriormente, aplicado no tratamento contra as leishmanioses (SINDERMANN; ENGEL, 2006). A entrada da miltefosina nesse arsenal terapêutico é considerada um marco, sendo o primeiro agente antileishmanial oralmente eficaz identificado. No entanto, a miltefosina tem limitações, incluindo distúrbios gastrointestinais, toxicidade hepática e renal. Uma vez que a miltefosina é teratogênica, é contraindicada na gravidez e para as mulheres em idade fértil. Outro problema potencial é a meia-vida prolongada do fármaco (150 a 200 h), fato que levanta preocupações quanto ao surgimento da resistência dos parasitos (ORGANIZATION, 2012).

Como o tratamento é um problema crescente, o desenvolvimento de novos medicamentos que podem substituir ou complementar as atuais alternativas terapêuticas disponíveis é necessário (CROFT; COOMBS, 2003). Os estudos relacionados ao tratamento das leishmanioses descritos até o momento, e os que ainda estão em desenvolvimento, visam à obtenção de novos fármacos a serem utilizados no tratamento das leishmanioses com pouco efeito tóxico comparando ao tratamento atual, mas mantendo sua eficiência contra os parasitos. É importante ressaltar que os custos devem ser minimizados para permitir sua disseminação e

uso, principalmente, em países mais pobres, onde há incidência elevada de casos da doença (SANTOS *et al.*, 2008).

As investigações atuais para o desenvolvimento de novos medicamentos se baseiam, principalmente, em ensaios usando: a) fármacos comumente utilizados em outras patologias (reposicionamento de fármacos), b) substâncias isoladas de microrganismos e plantas e c) substâncias utilizadas na medicina popular (SANTOS *et al.*, 2008). Um número impactante calculado com base em experiências anteriores de desenvolvimento de fármacos é de que se faz necessário apoiar cerca de 120 projetos em desenvolvimento para garantir um novo fármaco para uma doença parasitária específica (ORGANIZATION, 2012).

2.7. Naftoquinonas

Nos últimos anos, um interesse crescente em novas terapias e no uso de produtos naturais tem sido observado, principalmente, de derivados de plantas como fontes de novos compostos quimioterápicos com maior atividade e menos efeitos adversos. Esses estudos resultam da necessidade urgente de desenvolver novos medicamentos de baixo custo e da descoberta de novas moléculas com potente atividade antiparasitária e melhorea das características farmacológicas. Embora muitos avanços tenham sido feitos no tratamento das leishmanioses, ainda há muito a ser realizado (IWU; JACKSON; SCHUSTER, 1994)

As plantas são uma fonte potencial de novas drogas antiprotozoárias. A atividade biológica dos extratos vegetais foi atribuída a compostos pertencentes a diversos grupos químicos, incluindo quinonas, alcalóides, flavonóides, fenilpropanóides, esteróides e terpenóides (IWU; JACKSON; SCHUSTER, 1994). As quinonas representam uma ampla e variada família de metabólitos de distribuição natural. Nos últimos anos, intensificou-se o interesse nestas substâncias, não só devido a sua importância nos processos bioquímicos, como também ao destaque cada vez maior que apresentam em variados estudos farmacológicos. Na natureza, estão envolvidas em etapas importantes do ciclo de vida de seres vivos, principalmente nos níveis da cadeia respiratória e da fotossíntese. A distribuição dessas substâncias nos variados organismos implica, possivelmente, em funções biológicas múltiplas, agindo de forma conspícua em seus diversos ciclos bioquímicos (SILVA; FERREIRA; SOUZA, 2003)

Em estudos recentes, dentre as variadas ações farmacológicas das quinonas, destacam-se as propriedades anticancerígenas (SU *et al*., 2010), antivirais, tripanocidas, imunomoduladoras e antimicrobianas (RIFFEL *et al.*, 2002). Com base na sua estrutura molecular, as quinonas são divididas em diferentes grupos, utilizando-se como critério o tipo de sistema aromático que sustenta o anel quinonoídico: benzoquinonas - um anel benzênico (**Fig 4a)**; naftoquinonas - um anel naftalénico (**Fig 4b)**; antraquinonas - um anel antracênico linear ou angular (**Fig 4c)** (SILVA; FERREIRA; SOUZA, 2003).

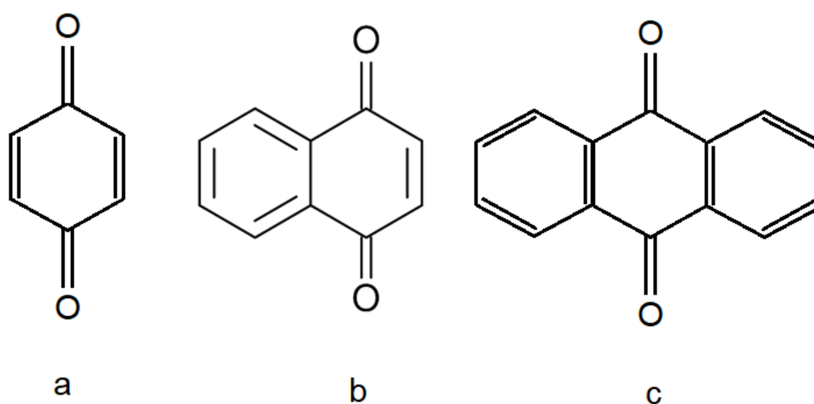

Figura 4: Representação das estruturas químicas das quinonas – Benzoquinona (a); Naftoquinona (b); Antraquinona (c)

As naftoquinonas são amplamente distribuídas na natureza, sendo encontradas em bactérias, fungos e vegetais, participando de diversos processos biológicos. São constituintes da membrana plasmática de eubactérias e possuem elevada importância na cadeia de transporte de elétrons. Quando presentes em fungos podem realizar funções diferenciadas, como gerar espécies reativas de oxigênio no meio extracelular. Nos vegetais, estão envolvidas na fotossíntese, além de serem produzidas para defesa contra agentes infecciosos. Essas moléculas possuem importância farmacológica por suas propriedades microbicidas, antitumorais e inibidoras de sistemas celulares reparadores, podendo atuar pela formação deletéria de espécies reativas de oxigênio, sendo empregadas com ação antifúngica, anti-inflamatória, analgésica e antimalárica (SILVA; FERREIRA; SOUZA, 2003). Por possuírem em seu esqueleto molecular um sistema de anel α, β-bisdienônico, as naftoquinonas têm a capacidade de reatividade química reversível da oxiredução. Essa característica é de fundamental importância em vários processos biológicos (PATAI, 2010).

Os primeiros relatos sobre a utilização de extratos de plantas que possuem naftoquinonas são do Egito antigo. O extrato da folha de *Lawsonia inermis* (popularmente conhecida como Henna) foi empregado como cosmético, sendo utilizado na pintura de cabelos, pele e unhas. Após alguns anos, o extrato passou também a ser aproveitado para o tratamento de micoses e feridas. De certa forma, a partir do século XIX, esses compostos adquiriram importância para a indústria, sendo empregados como corantes de alimentos, indicadores de pH e, posteriormente, como produto de partida para a síntese de cristais líquidos (RAHIMAN *et al.*, 2013). A partir desta espécie, pode ser isolada a molécula conhecida como lausona ((2- hidroxi-1,4-naftoquinona) (**Fig 5).**

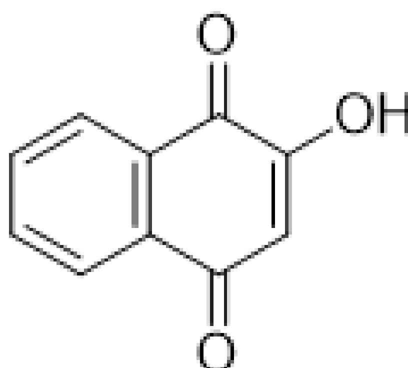

Figura 5: Representação da estrutura química da lausona

Entre os derivados da 1,4-naftoquinona, alguns antimaláricos promissores foram descritos (REZENDE *et al.*, 2013; SCHUCK *et al.*, 2013; SHARMA *et al.,* 2013) e algumas outras séries de derivados da 1,4-naftoquinona também foram amplamente avaliadas contra *Mycobacterium tuberculosis* (FERREIRA *et al*., 2010) e *Plasmodium falciparum* (SHARMA *et al.,* 2013), além de moluscicidas contra *Biomphalaria glabrata* (CAMARA *et al*., 2008). Relatos também mostraram o potencial destes derivados contra *Trypanosoma cruzi e Leishmania (L.) donovani* (CARNEIRO *et al*., 2012; LEZAMA-DÁVILA *et al*., 2012; DE ARAUJO *et al*., 2014).

Tais relatos levaram a um maior interesse quanto a pesquisas relacionando tais substâncias com o tratamento das leishmanioses. Uma vez que são facilmente sintetizadas, fato que favorece o desenvolvimento de novos compostos, o desafio de sintetizar derivados de naftoquinonas para melhorar suas propriedades farmacológicas tem sido aceito por vários institutos de pesquisa e laboratórios (RIFFEL *et al*., 2002). Desta forma, neste projeto, utilizou-se a (2-((7-chloroquinolin-

4- yl)oxy)-3-(3-methylbut-2- en- 1-yl)naphthalene-1,4- diona), denominada Flau-A, (**Figura 6)**, molécula derivada da lausona, com o objetivo de avaliar sua ação leishmanicida *in vitro* e *in vivo* contra LT e LV.

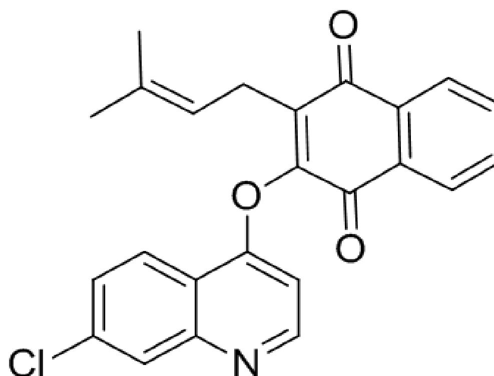

Figura 6: Representação da estrutura química da molécula (2-((7-chloroquinolin-4- yl)oxy)-3-(3-methylbut-2- en- 1-yl)naphthalene-1,4- diona).

2.8. Nanomateriais

Há cada vez mais otimismo de que a nanotecnologia, aplicada à medicina, trará avanços significativos no diagnóstico e tratamento de doenças. As aplicações previstas para os nanomateriais em medicina incluem a administração de fármacos, diagnósticos tanto *in vitro* como *in vivo*, nutracêuticos e produção de materiais biocompatíveis melhorados (DUNCAN, 2003; DE JONG; BORM, 2008). Nos últimos anos, os sistemas de distribuição de medicamentos, que representam uma nova abordagem farmacológica, receberam atenção considerável no campo do desenvolvimento de medicamentos. Técnicas farmacológicas são empregadas para controlar e melhorar as propriedades farmacocinéticas (absorção, distribuição, metabolismo e excreção) de um fármaco (TOMII, 2002).

Sistemas de liberação controlada, tais como lipossomas e nanopartículas (NPs), proporcionam uma maior eficácia e segurança quando os fármacos são adsorvidos ou encapsulados em veículos, reduzindo a dose e as reações adversas das formulações convencionais (PERRONE *et al.,* 2015). A razão pela qual as nanopartículas são atraentes para fins médicos é baseada em suas características importantes e únicas, tais como a sua relação superfície e massa que é muito maior do que a de outras partículas, suas propriedades quânticas e sua capacidade de adsorver e transportar outros compostos. As NPs têm uma superfície (funcional)

relativamente grande que é capaz de ligar, adsorver e transportar outros compostos tais como fármacos, sondas químicas e proteínas (DE JONG; BORM, 2008). A composição das nanopartículas manipuladas pode variar. Os materiais podem ser de origem biológica como fosfolípides, lipídeos, ácido láctico, dextrano e quitosana, ou ter características químicas de vários polímeros, carbono, sílica e metais (DE JONG; BORM, 2008).

2.8.1.1. Micelas

As micelas **(Figura 7)** têm estrutura núcleo-casca em que a parte hidrofóbica é localizada em seu interior, enquanto a parte hidrofílica está no exterior, o que é particularmente adequado para a solubilização de moléculas insolúveis em água ou parcialmente solúveis. Ao mesmo tempo, esta estrutura as protege contra degradação química e metabolismo por agentes biológicos (WEI, H ; ZHANG, XZ ; CHENG, H ; CHEN, WQ ; CHENG, SX ; ZHUO, 2006; HYUN *et al.*, 2008). Elas podem ser obtidas a partir de copolímeros anfifílicos que têm a capacidade de micelização, formando as já citadas estruturas núcleo-casca.

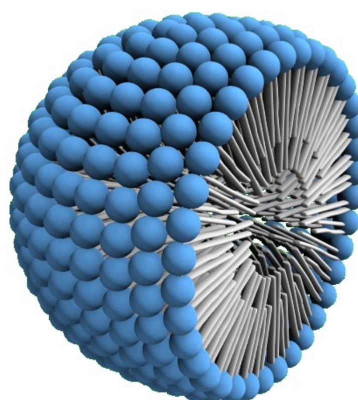

Figura 7: Representação esquemática de uma micela

2.8.1.2. Poloxâmero P407 (Pluronic F-127®)

Algumas abordagens têm sido utilizadas para melhorar as formas de dosagem parentérica existentes para aumentar a adesão do doente. Uma destas abordagens é a utilização de géis de Pluronic F-127 (PF127) **(Figura 8)**. O PF127 é um copolímero de bloco que compreende segmentos poli (oxietileno) e poli (oxipropileno) com massa de aproximadamente 12.500 g/mol (LERCHE W, 1972). Este polímero tem baixa toxicidade, excelente compatibilidade com outros produtos

químicos e boa solubilidade. As concentrações de PF127 de 20% ou mais em solução aquosa exibem a propriedade única de gelificação térmica reversível. Estas preparações transformam-se de solução de baixa viscosidade em géis semi-sólidos, ao aquecer de 4°C a 37°C. Esses géis podem ser localizados perto do local de injeção e a distribuição sistêmica do fármaco pode ser minimizada. Estas propriedades fazem do PF127 um veículo atrativo para liberação controlada (ZHANG *et al.*, 2002).

Dentre os vários estudos já realizados com o PF127, podemos citar um sistema de libertação de fármaco para uso oftálmico (A. BOCHOTA, B. MASHHOUR, F. PUISIEUX, 1998; SUKETU D. DESAI, 1998), parenteral (OKADA F, HOSOKAWA M, 1987; BANGA, 1997; E.A. PEC, Z.G. WOUT, 1992; MORIKAWA K, PAAVOLA A, YLIRUUSI J, KAJIMOTO Y, KALSO E, WAHLSTRÖM T, 1995) e percutâneo (LEE *et al.*, 1994; BARICHELLO *et al.*, 1999; SUH; MATTHEW, 2000).

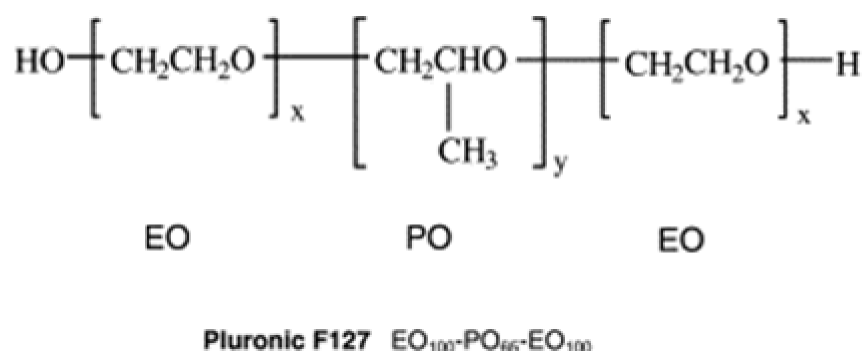

Figura 8: Estrutura química do Poloxâmero 407. Fonte: (CHATTOPADHYAY et al., 2002)

Estudos mostraram que a solubilização micelar com tensioativos tais como Mirj 59 ou copolímeros tais como vários Pluronic® (poli (óxido de etileno) -bloco-poli (N-hexil estearato l-aspartamida), poli (óxido de etileno) -bloco-poli (A-benzil-1-aspartato)) ou monoglicéridos, diminuíram a hemólise e/ou a nefrotoxicidade enquanto se mantinha a atividade da AmpB (FORSTER; WASHINGTON; DAVIS, 1988; LAVASANIFAR A, SAMUEL J, SATTARI S, 2002; ESPOSITO *et al.*, 2003; MONICA L. ADAMS, AFSANEH LAVASANIFAR, 2003; MENDONÇA *et al.*, 2016).

Estas soluções existem na forma de uma dispersão molecular ou como micelas dos copolímeros em bloco. O núcleo é um compartimento incompatível com água que é segregado do exterior aquoso pelas cadeias hidrofílicas do invólucro,

formando desse modo, dentro do núcleo, uma carga para a incorporação de agentes terapêuticos (KABANOV; BATRAKOVA; ALAKHOV, 2002). Desta forma, um sistema micelar formado a partir do PF-127 com o objetivo de transportar Flau-A foi proposto neste trabalho para emprego no tratamento contra as leishmanioses.

3. OBJETIVOS

3.1. Objetivo geral

Avaliar a atividade antileishmanial *in vitro* e *in vivo* da molécula derivada da naftoquinona denominada Flau-A [2-(2,3,4-tri-O-acetil-6-deoxi-β-L-galactopiranosiloxi)-1,4-nanaftoquinona] contra diferentes espécies de *Leishmania*.

3.2. Objetivos específicos

- Avaliar a atividade antileishmanial da Flau-A contra formas promastigotas em fase estacionária e amastigotas de *L. amazonensis* e *L. infantum* e a citotoxicidade em macrófagos peritoneais murinos e hemácias humanas;

- Verificar o uso potencial da Flau-A no tratamento de macrófagos infectados e na inibição da infecção usando células pré-incubadas com a molécula e avaliar o mecanismo de ação em *Leishmania amazonensis*;

- Preparar um sistema de micelas a partir do Poloxâmero P-407 contendo Flau-A e tratar camundongos BALB/c infectados com *L. amazonensis* e *L. infantum* com a molécula pura ou incorporada no sistema micelar (Flau-A/M);

- Avaliar o grau de proteção nos animais infectados e tratados com as formulações, por meio de:

-medições do diâmetro das lesões no dorso dos animais (*L. amazonensis*);
-carga parasitária em fragmentos de lesão (*L. amazonensis*), no baço, fígado e linfonodos drenantes da infecção;
-resposta celular pela produção das citocinas IFN-γ, IL-4, IL-10, IL-12p70, GM-CSF e óxido nítrico pelos esplenócitos dos animais;

-resposta humoral pela determinação dos níveis de IgG1 e IgG2a específicos aos parasitas.

- Avaliar a toxicidade *in vivo* das formulações a partir da dosagem de parâmetros bioquímicos.

4. METODOLOGIA

4.1. Aprovação do projeto pelo Comitê de Ética em Pesquisa da UFMG

O projeto foi aprovado pelo Comitê de Ética em Pesquisa Animal da UFMG (protocolo 085/2017). Foram utilizados camundongos BALB/c fêmeas de 8 semanas de idade. Os animais foram adquiridos junto ao Biotério Central do Instituto de Ciências Biológicas e mantidos no Biotério do Departamento de Patologia Clínica do COLTEC, sob as devidas condições de manejo.

4.2. Parasitos

As espécies *L. infantum* (MHOM/BR/1970/BH46) e *L. amazonensis* (IFLA/BR/1967/PH-8) foram utilizadas. As formas promastigotas em fase estacionária de crescimento foram cultivadas em meio Schneider´s (Schneider´s Insect Medium, Sigma-Aldrich, St. Louis, MO, USA), suplementado com 20% de soro fetal bovino inativado (SFB, Sigma), 20 mM de L-glutamina, 200U/mL de penicilina e 100 µg/mL de estreptomicina, em pH 7,4. Os parasitos foram cultivados a 24°C e repiques das culturas foram efetuados de cinco em cinco dias, período em que os parasitos se apresentavam em fase logarítmica de crescimento. Estoques de parasitos foram mantidos em nitrogênio líquido a -196ºC.

4.3. Cultivo de formas amastigotas

Para a obtenção das formas amastigotas-like, 10^9 formas promastigotas em fase estacionária de crescimento de *L. amazonensis* ou *L. infantum* foram centrifugadas a 2.000 xg por 10 min, e lavadas com PBS 1x estéril. Após a lavagem, o pellet formado foi ressuspendido em 5 mL de soro fetal bovino inativado e incubado por 48 horas em estufa de CO_2 a 37°C. Após o período, as culturas contendo as formas amastigotas-like foram centrifugadas a 2.000 x g por

10 min e, posteriormente, lavadas três vezes com PBS 1x estéril. O pellet foi congelado a -80°C, até o momento do uso. A morfologia dos parasitos foi observada em microscópio ótico binocular.

4.4. Obtenção das substâncias

Poloxâmero P407 (Pluronic® F127) foi adquirido junto a Sigma-Aldrich (número de catálogo 16758; St. Louis, EUA), bem como a anfotericina B (AmpB) (1397-89-3), que foi usada como controle. O Ambisome® foi obtido comercialmente.

4.5. Preparo da Flau-A

Um protocolo técnico desenvolvido recentemente foi usado para a preparação de Flau-A. Resumidamente, 0,30 g (1,72 mmol) de lausona foram dissolvidos em 5 mL de diclorometano PA e transferidos para um balão de fundo redondo de 100 mL. Uma solução contendo carbonato de potássio (10% p/v) foi adicionada, e a mistura foi agitada durante 30 min à temperatura ambiente. Em seguida, Brometo de 2,3,4-tri-O-acetil-6-desoxi-β-L-galactopiranosil (3,44 mmol) e n-Bu4NBr (22% mol) foram adicionados, e a mistura foi novamente agitada por 18 h em temperatura ambiente. Então, ácido clorídrico 6 mol/L foi adicionado até que a solução atingisse pH 3,0, e a mistura foi transferida para um funil de separação para obtenção da fase orgânica. Assim, a solução foi lavada em 100mL de água destilada, seca com sulfato de sódio anidro e concentrada. O produto foi passado em cromatografia de coluna (hexano/acetato de etila, 7: 3 v/v), e a molécula purificada foi obtida com um rendimento de 34%. O produto foi considerado puro de acordo com seu próton e espectros de RMN de 13 carbonos, além da análise por TLC. Para os experimentos *in vivo*, Flau-A foi diluída em solução salina tamponada com fosfato (PBS 1x) pH 7,4.

4.6. Preparo da Flau-A micelar

As micelas poliméricas foram preparadas conforme descrito a seguir: Poloxâmero P407 (18% p/p) foi diluído em PBS 1x pH 7,4 sob agitação magnética durante 18h a 4°C. Em seguida, Flau-A (8.0 mg) foi diluída em 500 µL de diclorometano PA e solubilizada por meio de vórtice. A mistura foi adicionada à solução de Poloxamer previamente preparada sob vigorosa agitação magnética e em banho de gelo, até a formação de uma emulsão viscosa. A solução de

diclorometano foi evaporada usando um evaporador rotativo (Buchi, Flawil, Suíça). O produto foi obtido como um gel amarelo transparente à temperatura ambiente. O conteúdo de Flau-A na solução micelar foi avaliado espectrofotometricamente usando um método ultravioleta: amostras da solução Flau-A/M foram coletadas e diluídas em metanol PA. As absorbâncias foram medidas em um espectrofotômetro UV/Vis (Double beam AJX-6100 PC; Micronal, São Paulo,Brasil), no comprimento de onda de 380nm. A concentração de Flau-A foi calculada usando uma curva padrão (0 a 18 μM) preparada em metanol PA. As análises foram realizadas usando três replicatas. Micelas vazias (18% p/p) foram preparadas usando o mesmo protocolo técnico descrito para elaboração da Flau-A/M.

4.7. Ensaio de viabilidade em *Leishmania*

A atividade anti-promastigota foi avaliada pelo teste colorimétrico do MTT (3-(4,5-dimetiltiazol-2-il)-2-5 difenil tetrazólico; Sigma-Aldrich). A concentração inibitória de 50% de *L. infantum* e *L. amazonensis* (IC_{50}) foi avaliada pela incubação das formas promastigotas estacionários dos parasitos (10^6 células) com Flau-A (0 a 100,0 μg/mL) ou AmpB (0 a 10,0 μg/mL; Sigma-Aldrich) em placas de 96 poços (Nunc, Nunclon, Roskilde, Dinamarca) por 48 h e a 24°C. Os valores de densidade ótica (DO) foram medidos em um espectrofotômetro de microplaca (Molecular Devices, Spectra Max Plus, San Jose, CA, EUA) a 570 nm. Os valores de IC_{50} foram calculados por regressão sigmoidal usando curvas dose-resposta pelo software Microsoft Excel (versão 10.0) (FREITAS et al., 2019).

4.8. Citotoxicidade em macrófagos murinos e hemácias humanas e índice de seletividade

A citotoxicidade foi avaliada *in vitro* em macrófagos murinos e hemácias humanas, para as quais foram determinadas as concentrações que inibem 50% dos macrófagos (CC_{50}) e das hemácias (RBC_{50}). Macrófagos murinos (5×10^5) ou suspensão de hemácias humanas a 5% foram incubados na presença de Flau-A e (0 a 100,0 μg/mL) ou AmpB (0 a 10,0 μg/mL) em meio RPMI 1640 por 48 h (macrófagos murinos) ou 1 h (hemácias) a 37°C em 5% de CO_2. A viabilidade dos macrófagos foi avaliada pelo método MTT. A suspensão de hemácias foi centrifugada a 1.000 × g por 10 min a 4°C, após a qual a porcentagem de lise celular foi avaliada espectrofotometricamente a 570 nm. A ausência (branco) ou 100% de

hemólise foi determinada substituindo a substância por um volume igual de PBS ou água destilada, respectivamente. Os valores de CC_{50} e RBC_{50} foram calculados por regressão sigmoidal usando curvas dose-resposta no software Microsoft Excel (versão 10.0). O índice de seletividade (IS) foi calculado pela razão entre os valores de CC_{50} e IC_{50}.

4.9. Tratamento de macrófagos infectados

Os ensaios foram feitos com macrófagos peritoneais utilizando formas promastigotas de *L. amazonensis* e *L. infantum*. Macrófagos foram obtidos por meio de lavado peritoneal dos camundongos BALB/c previamente estimulados com 3 mL de tioglicolato 3%. Em seguida, os macrófagos foram lavados, contados em câmara de Neubauer e colocados para aderir em placas de 24 poços por 24 horas, com lamínulas de vidro, em meio suplementado com 10% de SBF a 33ºC e 5% de CO_2. Após este tempo, os macrófagos foram incubados com as formas promastigotas de *Leishmania* na relação 1:10 (macrófago/parasito), durante 4 h em meio suplementado com 5% de SFB 33ºC, 5% CO_2. Após lavagem com PBS para retirada das promastigotas que não entraram nas células, os macrófagos foram tratados com Flau-A em diferentes concentrações (0 a 10.0 µg/mL) ou anfotericina B (0 a 1.0 µg/mL). Após 48 h de tratamento, as células foram coradas pelo método Panótico, conforme descrição do fabricante Renylab® e a porcentagem de macrófagos infectados e o número de amastigotas por macrófago foram determinados pela contagem de 200 células, em triplicata (TAVARES *et al.,* 2019).

4.10. Inibição de infecção em macrófagos

Formas promastigotas dos parasitos (5 x 10^6 células) foram incubadas com Flau-A (0, 2.5, 5.0 e 10.0 µg/mL) ou AmpB (0, 0.25, 0.5 e 1.0 µg/mL) por 48 h e a 37^oC. As células foram lavadas e adicionadas à cultura de macrófagos previamente preparados e aderidos a lamínulas (na proporção de 10 parasitos por célula), durante 24 h a 37°C em ambiente de 5% de CO_2. Os parasitos foram lavados três vezes em meio RPMI e corados com Giemsa. A porcentagem de infecção por parasitos pré-tratados e o número de amastigotas recuperados foram determinados pela contagem de 200 células, em triplicata, em microscópio ótico.

4.11. Estudos de mecanismo de ação

4.11.1. Obtenção de amastigotas intracelulares para o estudo do mecanismo de ação

Foi utilizado um protocolo para obtenção dos amastigotas intracelulares baseado na metodologia descrita por Miranda e colaboradores (2017), com algumas modificações. Em resumo, macrófagos peritoneais foram infectados com promastigotas de *L. amazonensis* (10 parasitos: macrófago) e incubados a 33°C for 4 h na atmosfera com 5% de CO_2. Em seguida, macrófagos infectados foram tratados com Flau-A na concentração referente ao IC_{50}. Após 24 h de tratamento, os macrófagos infectados e tratados foram removidos por raspagem mecânica e as células submetidas à centrifugação diferencial a 1.000 rpm por 5 minutos para a separação dos amastigotas dos macrófagos. Os amastigotas foram recolhidos do sobrenadante, contados em câmara de Neubauer e utilizados em diferentes protocolos para avaliação do mecanismo de ação do composto, os quais serão descritos abaixo.

4.11.2. Análises morfológicas em parasitos tratados

Os danos ultraestruturais nos amastigotas intracelulares de *L. amazonensis* tratados ou não (controle) com Flau-A por 24 h foram avaliados por microscopia eletrônica de transmissão (MET). Em resumo, os parasitos previamente tratados foram fixados em glutaraldeído, pós-fixados em solução contendo tetróxido de ósmio e ferrocianeto de potássio, desidratados e embebidos em resina Epon. Seções ultrafinas foram coradas com acetato de uranila e citrato de chumbo e posteriormente, observados em microscópico eletrônico de transmissão Zeiss 900 TEM (RIBEIRO *et al.*, 2014).

4.11.3. Análise do potencial de membrana mitocondrial ($\Delta\Psi$)

Os corantes fluorescentes rodamina 123 (Sigma-Aldrich, USA) e mitotracker (Life Technologies) foram utilizados para a análise do potencial de membrana mitocondrial (RIBEIRO, FRANCA, *et al.,* 2014; ANTINARELLI *et al.*, 2015). Baseando no IC_{50} da Flau-A, amastigotas intracelulares de *L. amazonensis* foram tratadas por 24 h a 24°C e incubadas com rodamina 123 (0.5 µg/mL) durante 15 min no escuro a 37ºC. A aquisição dos dados e análise foi realizada por citometria de fluxo (FACsCanto II, Becton Dickinson, Rutherford, NJ, USA) equipado com o software DIVA (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA),

segundo descrito por Antinarelli et al. (2015). Nas análises com a sonda Mitotracker, os parasitos tratados com Flau-A foram lavados com PBS e incubados com mitotracker a 500 nM por 40 min, no escuro. Após sucessivas lavagens com PBS, a fluorescência foi analisada com o auxílio de um fluorímetro (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) a 540 nm de emissão e 600 nm de excitação (COIMBRA *et al.,* 2016). Carboxicianeto-4-(trifluorometoxi)-fenilhidrazona (FCCP-Sigma Aldrich, USA) foi utilizado como controle positivo.

4.11.4. Detecção de espécies reativas de oxigênio

Formas amastigotas intracelulares de *L. amazonensis* foram tratadas com Flau-A na concentração do seu IC_{50}. Após 24 h, os parasitos foram lavados com PBS, contados em câmara de Neubauer e incubados com a sonda 2′,7′-dichlorodihydrofluorescein diacetate (H2DCFDA, Invitrogen Molecular Probes) na concentração de 20 µM por 30 min à temperatura ambiente. A fluorescência foi monitorada com o auxílio de um fluorímetro (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) a 485nm (excitação) e 528nm (emissão) (ANTINARELLI *et al*. 2015).

4.11.5. Avaliação da integridade da membrana celular

Baseando no IC_{50} do composto, amastigotas intracelulares de *L. amazonensis* (10^7 céls/mL) foram tratadas com Flau-A por 24 h e, posteriormente, marcadas com iodeto de propídeo (Sigma-Aldrich, USA) a 1.0 µg/mL por 15 min, no escuro. A fluorescência foi analisada com o auxílio de um fluorímetro (FLx800, BioTek Instruments, Inc., Winooski, VT, USA) a 540 nm de emissão e 600 nm de excitação. Como controle positivo, as células foram aquecidas a 65°C por 10 min (ANTINARELLI *et al.* 2015).

4.11.6. Avaliação da exposição de fosfatidilserina na membrana celular

Promastigotas de *L. amazonensis* (10^7céls/mL) foram previamente tratadas com Flau-A na concentração do seu IC_{50} por 24 h e a 25°C. Para o ensaio de avaliação da externalização de fosfatidilserina e da integridade da membrana plasmática, os parasitos foram lavados com PBS 1x e uma suspensão de 5 x 10^6 promastigotas foram incubadas com 1 µL de anexina V-FITC (Invitrogen, Molecular Probes) e 1 µg/mL iodeto de propídeo (PI, Sigma-Aldrich, USA) por 20 min no

escuro. As amostras foram analisadas em citômetro de fluxo FACsCanto II (Becton Dickinson, Rutherford, NJ, USA) equipado com software DIVA (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA) (STROPPA *et al.,* 2017). Miltefosina foi utilizada como controle positivo.

4.12. Infecção e regimes terapêuticos

4.12.1. Modelo de leishmaniose tegumentar

Camundongos BALB/c (n=10 por grupo) foram infectados pela via subcutânea na base da cauda com 10^7 formas promastigotas estacionárias de *L. amazonensis*. Após o desenvolvimento das lesões (2 a 3 milímetros), cerca de 50 dias após a infecção, o tratamento foi iniciado. Para tal, os animais foram divididos em grupos distribuídos de acordo com o tamanho das lesões. Os tratamentos consistiram nos seguintes grupos: (a) Salina (PBS): 50 µL de PBS 1x, pela via subcutânea, diariamente, durante 15 dias; (b) Branco da micela: 50 µL de micela não incorporada com Flau-A (10 mg/Kg), pela via subcutânea, a cada dois dias, durante 15 dias; (c) Anfotericina B: 50 µL de anfotericina B (1 mg/Kg), pela via intraperitoneal, a cada 2 dias, por 15 dias; (d) Ambisome®: 50 µL de Ambisome® (2 mg/Kg), por via endovenosa, a cada 5 dias, durante 15 dias; (e) Flau-A: 50 µL de Flau-A (10 mg/Kg), pela via subcutânea, a cada 2 dias, durante 15 dias; (f) Fau-A micelar (Flau-A/M): 50 µL de Flau-A incorporada no sistema micelar (5 mg/Kg), pela via subcutânea, a cada dois dias, durante 15 dias. O acompanhamento do desenvolvimento das lesões foi realizado semanalmente com base na leitura do diâmetro médio das lesões com uso de paquímetro eletrônico. Os animais foram eutanasiados 30 dias após o tratamento.

4.12.2. Modelo de leishmaniose visceral

Camundongos BALB/c (n=12 por grupo) foram infectados pela via subcutânea com 10^7 formas promastigotas estacionárias de *L. infantum*. Quarenta e cinco dias após a infecção, o tratamento foi iniciado. Para tal, os animais foram divididos em grupos homogeneamente distribuídos. Os tratamentos consistiram nos seguintes grupos: (a) Salina (PBS): 50 µL de PBS 1x, pela via subcutânea, a cada 2 dias, durante 10 dias; (b) Branco da micela: 50 µL de micela não incorporada com Flau-A (5 mg/Kg), pela via subcutânea, a cada dois dias, durante 10 dias; (c) Miltefosina: 50 µL de miltefosina (2 mg/Kg), pela via oral, a cada 2 dias, por 10 dias; (d) Flau-A: 50

μL de Flau-A (5 mg/Kg), pela via subcutânea, a cada 2 dias, durante 10 dias; (f) Flau-A micelar (Flau-A/M): 50 μL de Flau-A incorporada no sistema micelar (5 mg/Kg), pela via subcutânea, a cada dois dias, durante 10 dias. Metade dos animais foi eutanasiada um dia após o tratamento e o restante 15 dias após o final do tratamento.

4.13. Carga parasitária

A carga parasitária foi avaliada por meio da técnica de diluição limitante e PCR quantitativo (qPCR), após a coleta das bordas da lesão (na infecção com *L. amazonensis*), do fígado, baço e linfonodos drenantes (dLNs) dos animais. A técnica de diluição limitante desenvolvida por (TITUS, MARCHAND, BOON, & LOUIS, 1985) e modificada por (V. T. MARTINS *et al*., 2013) foi utilizada para avaliar a carga parasitária e os resultados foram expressos como o log negativo do título (diluição correspondente ao último poço positivo) ajustado por miligrama de tecido ou órgão. A carga parasitária no baço dos animais infectados foi também avaliada pela técnica de qPCR, de acordo com protocolo escrito em Tavares et al. (2018).

4.14. Resposta celular

O perfil da resposta celular foi avaliado pela dosagem das citocinas IFN-γ, IL-4, IL-10, IL-12 e GM-CSF nos sobrenadantes de cultura dos esplenócitos, após os estímulos com SLA. As dosagens foram realizadas utilizando os kits Intertest Mouse (Pharmingen), de acordo com as instruções do fabricante. Foi também realizada a dosagem de óxido nítrico (NO) nos sobrenadantes de cultura celular utilizando-se, para tal, o método de Griess (Green et al., 1982). A técnica de citometria de fluxo foi utilizada para avaliar a frequência de células T CD4+ e CD8+ produtoras de IFN-γ TNF-α e IL-10, conforme metodologia descrita em (MENDONÇA *et al*., 2016).

4.15. Resposta humoral

A produção de anticorpos foi avaliada após o término dos tratamentos. Para isso, os níveis de anticorpos IgG1 e IgG2a específicos aos parasitos foram dosados pela técnica de ELISA como descrito (FERRAZ COELHO *et al*., 2003).

4.16. Avaliações toxicológicas

Foram colhidas amostras de soro dos animais infectados e tratados para realização de dosagens bioquímicas. A função hepática foi analisada por dosagem de aspartato aminotransferase (AST) e alanina aminotransferase (ALT) e a função renal a partir das dosagens de ureia e creatinina. As análises foram realizadas usando kits comerciais (Labtest Diagnostica®, Belo Horizonte, Minas Gerais, Brasil), de acordo com as instruções do fabricante.

4.17. Análise estatística

Os resultados foram inseridos em planilhas do Microsoft Excel (versão 10.0) e analisados no GraphPad Prism ™ (versão 6.0 para Windows). As análises estatísticas foram realizadas pela análise de variância unidirecional (ANOVA), seguida pelo pós-teste de Bonferroni para comparações múltiplas entre os grupos. As diferenças foram consideradas significantes quando $P < 0.05$.

5. RESULTADOS E DISCUSSÃO

Os resultados e discussão serão apresentados sob a forma de artigos científicos, conforme as normas gerais que regem o Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, UFMG (Resolução nº 02/2013, de 18 de setembro de 2013).

5.1. Artigo 1

No primeiro artigo a ser apresentado, foi avaliada a atividade antileishmanial da Flau-A contra formas promastigotas e amastigotas-like de *Leishmania*, sua citotoxicidade em macrófagos murinos, sua atividade frente ao tratamento de macrófagos infectados e à inibição de infecção de macrófagos com parasitos pré incubados bem como seu possível mecanismo de ação em formas promastigotas de *Leishmania amazonensis.*

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derivate against promastigote and amastigote stages of Leishmania infantum and Leishmania amazonensis and its mechanism of action against L. amazonensis species. Parasitology Research 2018 Feb;117(2):391-403. doi: 10.1007/s00436-017-5713-6

Title
Antileishmanial activity of a naphthoquinone derivate against promastigote and amastigote stages of *Leishmania infantum* and *Leishmania amazonensis* and its mechanism of action against *L. amazonensis* species.

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Abstract

Leishmaniasis has become a significant public health issue in several countries in the world. New products have been identified to treat against disease; however, toxicity and/or high cost are limitations. The present work evaluated the

antileishmanial activity of a new naphthoquinone derivate, Flau-A [2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone], against promastigote and amastigote-like stages of *Leishmania amazonensis* and *L. infantum*. In addition, the cytotoxicity in murine macrophages and human red cells was also investigated. The mechanism of action of Flau-A was assessed in *L. amazonensis*, as was its efficacy in treating infected macrophages and inhibiting infection of pretreated parasites. Results showed that Flau-A was effective against promastigotes and amastigotes-like of both parasite species, as well as showed low toxicity in mammalian cells. Results also highlighted morphological and biochemical alterations induced by Flau-A in *L. amazonensis*, including a loss of mitochondrial membrane potential, as well as increased reactive oxygen species production, cell shrinkage and alteration of the plasma membrane integrity. The present study demonstrates for the first time the antileishmanial activity of Flau-A against two *Leishmania* species and suggests that the mitochondria of the parasites may be the main target organelle. Data shown here encourages the use of this molecule in new studies concerning treatment against *Leishmania* infection in mammalian hosts.

Keywords: Leishmania spp.; antileishmanial activity; treatment; naphthoquinones; mechanism of action; mitochondrial dysfunction.

Introduction

Leishmaniasis is a major public health problem in the world, where approximately 350 million people are at the risk of contracting the infection, being estimated that 12 million people are clinically affected by the disease (WHO, 2016). The clinical manifestations of leishmaniasis have varied from self-healing cutaneous lesions present at the site of the phlebotomine vector bite to the visceral form of the disease, which can be fatal if untreated (Andrade et al. 2016).

Tegumentary leishmaniasis (TL) usually is found in Afghanistan, Saudi Arabia, Syria, Iran, Algeria, Iraq, Brazil, and Peru, being the disease mainly caused by *Leishmania braziliensis*, *L. panamensis*, *L. guyanensis*, and *L. amazonensis* species (Ullah et al. 2016). Visceral leishmaniasis (VL) leads to nearly 50,000 deaths annually and is caused by *L. donovani* complex parasites, such as *L. donovani* and *L. infantum* (Araújo et al. 2017).

The treatment against leishmaniasis is based on pentavalent antimonials. Other drugs, such as amphotericin B (AmpB), paramomycin, miltefosine, and pentamidine have being used as second-line drugs (Copeland and Aronson 2015). However, these compounds present limitations, such as toxic side effects leading to hepatic, splenic, and renal toxicity; the long duration of treatment, and/or parasite resistance. Due to few current alternatives available on the market, the identification of new compounds to be used to treat against leishmaniasis could be considered desirable (Chakravarty and Sundar 2010; Sundar and Chakravarty 2013; Chávez-Fumagalli et al. 2015).

As a consequence, the investigation of synthetic molecules presenting biological actions other than antileishmanials could allow applying these products against *Leishmania* parasites, being considered as a promising field of research for new pharmaceuticals (Cheuka et al. 2016). In this context, quinones consist of a variety of aromatic metabolites found in plants, fungi, algae, and bacteria. These molecules include anthraquinones, benzoquinones, and naphthoquinones, and can easily be synthesized, allowing obtain a high yield and lower cost of production to be tested as therapeutic candidates against diseases such as leishmaniasis (Pinto et al. 2014).

In this context, naphthoquinones have been used as promising anticancer, antiviral, trypanocidal, and/or antimicrobial compounds (Riffel et al. 2002; Su et al. 2010; Pinto et al. 2014). Among the 1,4-naphthoquinone derivatives, some molecules present also antimalarial activity (Rezende et al. 2013; Schuck et al. 2013), while others show activity against *Mycobacterium tuberculosis* (Ferreira et al. 2010), *Plasmodium falciparum* (Sharma et al. 2013), and *Biomphalaria glabrata* (Camara et al. 2008).

In the present study, aiming to identify new 1,4-naphthoquinone derivates with antileishmanial activity, as well presenting low toxicity in mammalian cells, the 2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone, namely Flau-A, was evaluated as an antiparasitic agent against *L. infantum* and *L. amazonensis* species. Also, aiming to investigate the action in *Leishmania*, the mechanism of action of Flau-A was evaluated in *L. amazonensis*.

Materials and Methods

Synthesis of Flau-A

Lawsone 0.30 g (1.72 mmol) was dissolved in 5 mL of dichloromethane, and transferred to a 100 mL round bottom flask. A solution containing potassium carbonate (10% w/v, 5 mL) was added and the mixture was stirred at room temperature, during 30 min. Next, 2,3,4-tri-*O*-acetyl-6-deoxy-β-L-galactopyranosyl bromide (2.0 g, 3.44 mmol) and n-Bu_4NBr (0.12 g, 22% mol) were added, and the mixture was stirred at room temperature for 18 h. Then, hydrochloric acid 6 mol/L was added to obtain a pH 3.0, and the mixture was transferred to a separatory funnel, aiming to separate the organic phase and extracting the aqueous phase with dichloromethane (3 x 50 mL). The organic phase was washed using distilled water (100 mL), dried over anhydrous sodium sulfate, and concentrated. The crude product was used in a column chromatography (hexane/ethyl acetate, 7:3) to obtain 0.26 g (34% yield) of the purified molecule.

Parasites and mice

L. infantum (MHOM/BR/1970/BH46) and *L. amazonensis* (IFLA/BR/1967/PH-8) were used. Parasites were grown at 24°C in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), consisting of Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA) and 20 mM L-glutamine, pH 7.4. Stationary-phase promastigotes were cultured as described elsewhere (Coelho et al. 2003). To obtain the amastigote-like forms, a technical protocol was developed according described, with few modifications (Duarte et al. 2015). Briefly, 1 x 10^9 stationary-phase promastigotes were washed three times in sterile phosphate buffer saline (PBS) 1x and incubated for 48 or 72 h for *L. amazonensis* or *L. infantum*, respectively, in 3 mL FBS, at 37°C. Then, parasites were washed three times in cold PBS 1x, and their morphology was evaluated after staining by the Giemsa method in an optical microscope. Murine peritoneal macrophages were collected from BALB/c mice (female, 8 weeks old), which were purchased from the Institute of Biological Sciences of the Federal University of Minas Gerais (UFMG). The study was developed in compliance with the National Guidelines of the Institutional Animal Care and approved by the Use Committee for the Ethical

Handling of Research Animals (protocol number 085/2017). Also, this work was approved by Human Research Ethics Committee of UFMG (protocol number CAAE–32343114.9.0000.5149).

Viability assay in *Leishmania* species

A previous titration curve was performed to determine the best time of inhibition of parasite growth. The inhibition of *Leishmania* growth was then evaluated by cultivating *L. infantum* or *L. amazonensis* stationary promastigotes or amastigotes-like (1×10^6 parasites) in the presence of Flau-A (0.1 to 20.0 μg/mL) and in 96-well culture plates (Nunc, Nunclon, Roskilde, Denmark), for 48 h at 24°C. AmpB (0.1 to 2.0 μg/mL, Sigma-Aldrich, USA) was used as a control. Cell viability was assessed by measuring the cleavage of 5 mg/mL of 3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich, USA). The optical density (O.D.) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 570 nm. The product concentration needed to inhibit 50% of the *Leishmania* viability (IC_{50}) was determined by applying a sigmoidal regression of the dose-response curves. Data showed are representative of three independent experiments, performed in triplicate, which presented similar results.

Cytotoxicity in murine cells and selectivity index

To evaluate the toxicity against mammalian cells, Flau-A was incubated with murine macrophages. For this, a previous titration curve was performed to determine the best time of inhibition of macrophage viability. The inhibition of 50% of the macrophage viability (CC_{50}) was calculated by cultivating them (5×10^5 cells, per well) with Flau-A (0.1 to 20.0 μg/mL) or AmpB (0.1 to 2.0 μg/mL), in 96-well plates (Nunc) for 48 h at 37°C. Then, cell viability was evaluated by MTT. Selectivity index (SI) values were calculated by the ratio between the CC_{50} and IC_{50} results. Data showed are representative of three independent experiments, performed in triplicate, which presented similar results.

Cytotoxicity against human cells

To evaluate the toxicity against human cells, the hemolytic activity was investigated by incubating Flau-A (1.0 to 100.0 μg/mL) with a 5% red blood cell (human O type) suspension, which was obtained from three healthy donors, during 1 h at 37°C. Suspension was centrifuged by 1,000 x *g* and 10 min, when the cell lyses percentage was determined spectrophotometrically at 570 nm. The absence (blank) or 100% of hemolysis were determined by replacing Flau-A for an equal volume of PBS 1x or distilled water, respectively. Results were calculated by the hemolysis percentage as compared to the negative (PBS 1x) and positive (distilled water) controls. Data showed are representative of three independent experiments, performed in triplicate, which presented similar results.

Treatment of infected macrophages

To evaluate the efficacy of Flau-A in treating infected macrophages, cells (5 x 10^5 per well) were seeded on round glass coverslips within 24-well plates in RPMI 1640 medium, which was supplemented with 20% FBS and 20 mM L-glutamine, pH 7.4, and incubated during 24 h at 37°C in 5% CO_2. Stationary-phase promastigotes (5 x 10^6) were added to the wells, and cultures were incubated for 48 h at 37°C in 5% CO_2. Free parasites were removed by extensive washing with RPMI 1640 medium, while infected macrophages were treated with Flau-A (0, 2.5, 5.0 and 10.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL), for 48 h at 24°C in 5% CO_2. After fixation with 4% paraformaldehyde, macrophages were stained by the Giemsa method and the percentage of infected cells, as well as the number of intra-macrophage amastigotes, was determined by counting 200 cells in an optical microscope. Data showed are representative of three independent experiments, performed in triplicate, which presented similar results.

Inhibition of macrophage infection

The inhibitory effect of Flau-A in *Leishmania* was evaluated by treat parasites with this molecule, and then use them to infect murine macrophages. For this, parasites (5 x 10^6) were incubated with Flau-A (0, 2.5, 5.0 and 10.0 µg/mL) or AmpB

(0, 0.1, 0.5 and 1.0 μg/mL) for 1 h at 24°C. Cells were washed three times in RPMI 1640 medium, quantified, and incubated with macrophages (5 x 10^5 cells), for 24 h at 37°C in 5% CO_2. After, they were washed and stained by the Giemsa method to evaluate the percentage of infected macrophages, as well as the number of intra-macrophage amastigotes by counting 200 cells in triplicate and in an optical microscope. Data showed are representative of three independent experiments, performed in triplicate, which presented similar results.

Mechanism of action in *L. amazonensis*

Morphological studies in Flau-A-treated parasites

L. amazonensis promastigotes (1 x 10^7 cells) were untreated or treated with Flau-A (0.73 and 1.46 μg/mL, corresponding to one and two times the IC_{50} value obtained after 48 h of the *in vitro* cultures, respectively), for 2 h at 24°C. Next, parasites were fixed with 4% paraformaldehyde for 20 min, washed with PBS 1x, and placed on glass slides. After staining with Giemsa, slides were examined in an optical microscope and photographed. The volume of treated parasites was evaluated by a FACSCanto II flow cytometer (Becton Dickinson, Rutherford, NJ, USA), equipped with DIVA software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). The forward scatter (FSC) parameter was analyzed as presenting a correlation with the cell volume, and a total of 10,000 events were acquired, according described (Ribeiro et al. 2013).

Measurement of the mitochondrial membrane potential ($\Delta\Psi m$)

The evaluation of $\Delta\Psi m$ was performed by using two fluorescent dyes: Mitotracker® Red CM-H2XROS (Life Technologies, USA) and Rh123 (Sigma-Aldrich, USA). For this, *L. amazonensis* promastigotes (1 x 10^7 cells) were untreated or treated with Flau-A (0.73 and 1.46 μg/mL, corresponding to one and two times the IC_{50} value obtained after 48 h of the *in vitro* cultures, respectively), for 2 h at 24°C. The reduction of parasite viability was of 29.42%, when a concentration of 0.73 μg/mL was used and of 37.53%, when the concentration of 1.46 μg/mL was employed in the experiments, in comparison to untreated cells. Then, parasites were

washed with PBS 1x and incubated with 50 nM Mitotracker® for 40 min at 24ºC (Coimbra et al. 2016). The fluorescence intensity was spectrofluorometrically measured (FLx800, BioTek Instruments, Inc., Winooski, VT, USA), at an excitation wavelength of 540 nm and an emission wavelength of 600 nm. Additional assays were performed using Rh123 as described elsewhere (Antinarelli et al. 2015). The data acquisition and interpretation were performed in a FACSCanto II flow cytometer, in a total of 10,000 events acquired using FITC channel. The variation index (VI), which allows quantifying changes in the fluorescence intensity of Rh123, was determined by the (MT-MC)/MC equation, with MT representing the median fluorescence of the treated parasites and the MC the median fluorescence of negative controls. Miltefosine (18.0 µg/mL, Sigma-Aldrich, USA) and carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, 2.5 and 5.0 µg/mL) were used as controls.

Detection of the reactive oxygen species (ROS)

The ROS production was determined by using the fluorescent 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) probe, as described elsewhere (Coimbra et al. 2016). For this, *L. amazonensis* promastigotes (1 x 10^7 cells) were treated or untreated with Flau-A (0.73 and 1.46 µg/mL, corresponding to one and two times the IC_{50} value obtained after 48 h of the *in vitro* cultures, respectively), for 2 h at 24°C. Cells were washed with PBS 1x and incubated with H_2DCFDA. The fluorescence intensity was measured in a spectrofluorometer (FLx800, BioTek Instruments, Inc., Winooski, VT, USA), with excitation and emission wavelengths of 485 and 528 nm, respectively. Miltefosine (18.0 µg/mL) was used as a control, and experiments were performed in triplicate, presenting similar results.

Evaluation of the cell membrane integrity

L. amazonensis promastigotes (1 x 10^7 cells) were untreated or treated with Flau-A (0.73 and 1.46 µg/mL, corresponding to one and two times the IC_{50} value obtained after 48 h of the *in vitro* cultures, respectively), for 2 h at 24°C. Cells were washed with PBS 1x, and incubated with propidium iodide (1.0 µg/mL) during 15 min in the dark at room temperature (Stroppa et al. 2017). The fluorescence intensity was measured using a spectrofluorometer (FLx800, BioTek Instruments, Inc., Winooski,

VT, USA), with excitation and emission wavelengths of 540 and 600 nm, respectively. As a control, cells were heated at 65°C for 10 min. Results were obtained from three independent experiments performed in triplicate, which presented similar results.

Evaluation of phosphatidylserine exposition on the cell membrane

The exposure of phosphatidylserine on the cell surface was evaluated by using annexin V-FITC (Invitrogen, USA) and propidium iodide, as described elsewhere (Stroppa et al. 2017). For this, *L. amazonensis* promastigotes (1×10^7 cells) were untreated or treated with Flau-A (0.73 and 1.46 µg/mL, corresponding to one and two times the IC_{50} value obtained after 48 h of the *in vitro* cultures, respectively), for 2 h at 24°C. After, parasites were washed with PBS 1x and resuspended in Annexin V binding buffer. For labeling, 10 µL propidium iodide (1.0 µg/mL) and 5 µL of Annexin-FITC were added for 15 min at room temperature and in the dark. Data acquisition and interpretation were performed in a FACSCanto II flow cytometer, and a total of 10,000 events were acquired. Miltefosine (18.0 µg/mL) used as a control.

Toxicology studies

A toxicological evaluation was performed in BALB/c mice, like described (Duarte et al. 2016; Mendonça et al. 2016). For this, mice (n=8 per group) were inoculated subcutaneously once a day in their left hind footpad with saline, AmpB (1 mg/kg body weight), or Flau-A (5 mg/kg of body weight), during 15 days. Once a day, variations in the body weight and clinical signals were monitored. One day after the end of treatment, blood samples were collected for biochemical analysis, being the cardiac function analyzed by the dosage of creatine kinase-MB, and the hepatic function analyzed by dosage of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Nephrotoxicity was evaluated by examining the blood urea nitrogen (BUN) and serum creatinine (CRTN) levels. Experiments were performed using commercial kits (Labtest Diagnostica®, Minas Gerais, Brazil), in an auto-analyzer apparatus (Thermo Plate TP analyzer, São Paulo, Brazil).

Statistical analysis

Results were evaluated in Microsoft Excel (version 10.0) and analyzed by the GraphPad PrismTM (version 6.0 for Windows). The IC_{50} and CC_{50} values were calculated from the mean percentage reduction of the promastigotes and amastigotes-like (IC_{50}) or murine macrophages (CC_{50}), respectively, when compared to the untreated controls. The curves were determined by applying sigmoidal regression to logarithm concentration/response data. The one-way analysis of variance (ANOVA), followed by the Dunnett's test, was used for multiple comparisons between the groups. Results were expressed as mean ± standard deviation, and differences in relation to the untreated controls were considered significant when $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Results

Antileishmanial activity of Flau-A

The antileishmanial activity of Flau-A was evaluated against promastigotes and amastigotes-like of *L. infantum* and *L. amazonensis* species. Results using *L. infantum* showed IC_{50} values of 0.73 and 0.07 µg/mL for Flau-A and AmpB against promastigotes, respectively, while against the amastigote-like forms the values were of 1.03 and 0.23 µg/mL, respectively. Results using *L. amazonensis* showed IC_{50} values of 0.73 and 0.10 µg/mL for Flau-A and AmpB against promastigotes, respectively, while against amastigote-like forms the values were of 1.57 and 0.38 µg/mL, respectively.

Cytotoxicity in mammalian cells

Regarding CC_{50} values, results in murine macrophages were of 18.48 and 1.0 µg/mL for Flau-A and AmpB, respectively. With these data, SI values (CC_{50}/IC_{50} ratio) were calculated, and they were of 25.3 and 14.3, when Flau-A and AmpB were tested against *L. infantum* promastigotes, respectively, while against the amastigote-like forms they were of 17.9 and 4.3, respectively. Results against *L. amazonensis* promastigotes were of 25.3 and 10.0, respectively, while against the amastigotes-like they were of 11.8 and 2.6, respectively. The hemolytic activity in human type O-type

red cells was also investigated; however, no hemolysis was found when Flau-A was tested (data not shown).

Flau-A reduces parasite load in *Leishmania*-infected macrophages

The effect of the Flau-A in treat infected macrophages was investigated by counting 200 cells in triplicate. In the results using *L. amazonensis*, infection reduction in treated and infected macrophages was of 81.0% and 91.0%, respectively, when 2.5 and 10.0 µg/mL of this molecule were tested, with a reduction in the number of recovered amastigotes in the order of 92.0% and 98%, respectively (Table 1). Using AmpB (1.0 µg/mL), reductions in the infection degree and in the number of recovered amastigotes were of 89.0% and 88.0%, respectively. When *L. infantum* was used, infection reduction in Flau-A-treated and infected macrophages (2.5 and 10.0 µg/mL) was of 55.0% and 85.0%, respectively, while the reduction in the number of recovered amastigotes was of 78.0% and 97.0%, respectively. Using AmpB (1.0 µg/mL), reductions in the infection degree and in the number of recovered amastigotes were of 79.0% and 89.0%, respectively (Table 1).

Table 1

Leishmania amazonensis

Compounds	**Concentration (µg/mL)**	**Infectiveness reduction in infected and treated macrophages (%)**	**Percentage of reduction of the recovered amastigotes**
Flau-A	10.0	91.0±1.2	98.0±3.2
	5.0	88.0±2.3	95.0±2.8
	2.5	81.0±2.4	92.0±4.3
	0	-	-
Amphotericin B	1.0	89.0±1.1	88.0±3.3
	0.5	78.0±1.9	80.0±4.1
	0.1	69.0±2.5	58.0±4.2
	0	-	-

Leishmania infantum

Compounds	**Concentration (µg/mL)**	**Infectiveness reduction in infected and treated macrophages (%)**	**Percentage of reduction of the recovered amastigotes**
Flau-A	10.0	85.0±5.5	97.0±6.6
	5.0	75.0±4.6	89.0±3.2
	2.5	55.0±3.6	78.0±4.3
	0	-	-
Amphotericin B	1.0	79.0±3.8	89.0±2.9
	0.5	63.0±4.4	81.0±4.3
	0.1	56.0±3.3	59.0±5.5

	0	-	-

Table 1 Treatment of infected macrophages using Flau-A Murine macrophages were infected with L. amazonensis or L. infantum stationary promastigotes (10 parasites per one macrophage) and later treated with Flau-A (2.5, 5.0, and 10.0 µg/mL) or amphotericin B (0.1, 0.5, and 1.0µg/mL) for 48h at 37°C in 5% CO_2. The infection reduction in the treated cells was calculated by applying a sigmoidal regression of concentration-inhibition curves by counting 200 cells, in triplicate. Results were expressed as mean ± standard deviation.

Pre-treatment with Flau-A inhibits macrophage infection

The infectivity of parasites that were first treated with Flau-A and later used to infect macrophages was also evaluated. In the results using *L. amazonensis*, infection reduction in treated and infected macrophages was of 46.0% and 68.0%, respectively, when 2.5 and 10.0 µg/mL of Flau-A were used. In addition, the reduction in the number of recovered amastigotes was of 90.0% and 97%, respectively (Table 2). Using AmpB (1.0 µg/mL), reductions in the infection degree and in the number of recovered amastigotes were of 81.0% and 98.0%, respectively. When *L. infantum* was employed in the tests, infection reduction in Flau-A-treated and infected macrophages (2.5 and 10.0 µg/mL) was of 70.0% and 83.0%, respectively, whereas the reduction in the number of recovered amastigotes was of 86.0% and 96.0%, respectively. Using AmpB (1.0 µg/mL), reductions in the infection degree and in the number of recovered amastigotes were of 89.0% in both cases (Table 2).

Table 2

Leishmania amazonensis

Compounds	Concentration (µg/mL)	Infectiveness reduction using pre-treated parasites	Percentage of reduction of the recovered amastigotes
Flau-A	10.0	68.0±2.7	97.0±2.8
	5.0	49.0±3.9	93.0±2.6
	2.5	46.0±4.4	90.0±3.4
	0	-	-
Amphotericin B	1.0	81.0±4.5	98.0±4.3
	0.5	59.0±6.5	78.0±3.2
	0.1	52.0±5.4	65.0±2.4
	0	-	-

Leishmania infantum

Compounds	Concentration (µg/mL)	Infectiveness reduction using pre-treated parasites	Percentage of reduction of the recovered amastigotes

Flau-A	10.0	83.0±5.4	96.0±2.1
	5.0	75.0±4.8	91.0±3.2
	2.5	70.0±3.7	86.0±2.8
	0	-	-
Amphotericin B	1.0	89.0±5.5	89.0±4.2
	0.5	65.0±4.8	80.0±3.3
	0.1	51.0±5.4	52.0±2.2
	0	-	-

Table 2 Inhibition of infection of murine macrophages using pretreated parasites The inhibition of infection in Flau-A-treated Leishmania parasites was evaluated by incubating these cells (5× 106 cells) with Flau-A (2.5, 5.0, and 10.0 µg/mL) or amphotericin B (0.1, 0.5, and 1.0 µg/mL) for 1 h at 24 °C. Then, parasites were washed, quantified, and incubated with murine macrophages (in a ratio of 10 parasites per one cell) for 24 h at 37 °C in 5% CO2. The infection reduction in the treated cells was calculated by applying a sigmoidal regression of concentration-inhibition curves by counting 200 cells, in triplicate. Results were expressed as mean±standard deviation.

Morphological alterations induced by Flau-A in *L. amazonensis*

Aiming to evaluate the influence of Flau-A in the morphology of the parasites, we noticed the occurrence of morphological changes in *L. amazonensis* promastigotes that were treated with Flau-A. In the results, changes in the shape of the parasites were observed, with a rounded body and loss of cell volume, when compared to untreated controls, which showed an elongated cell body (Fig. 1). To confirm these results, the FSC parameter, which correlates with the cell volume, was also analyzed, and results showed changes in the cell population profile in relation to the parasite size, in a dose-dependent way. In addition, a gradual increase in this cellular population presenting reduced size was also observed (Fig. 2).

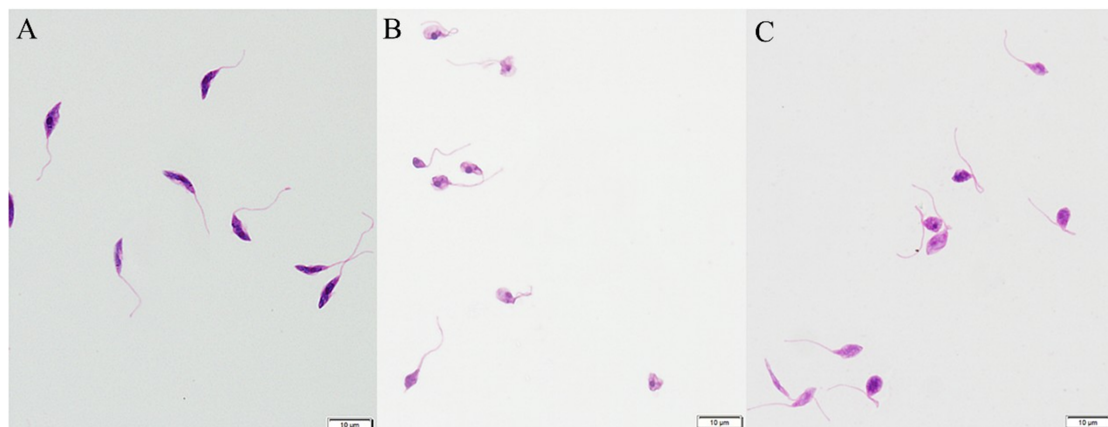


Fig 1. Morphology of L. amazonensis promastigotes after treatment using Flau-A. Parasites (1×107 cells) were untreated (a) or treated with 0.73 or 1.46 µg/mL of Flau-A (b and c, corresponding to 1 and 2 times the IC50 value obtained after 48h of the in vitro cultures, respectively) for 2h at 24 °C. Cells were fixed with paraformaldehyde, stained with Giemsa, and visualized in an optical microscope (Olympus BX53). Scale, 10 µm.

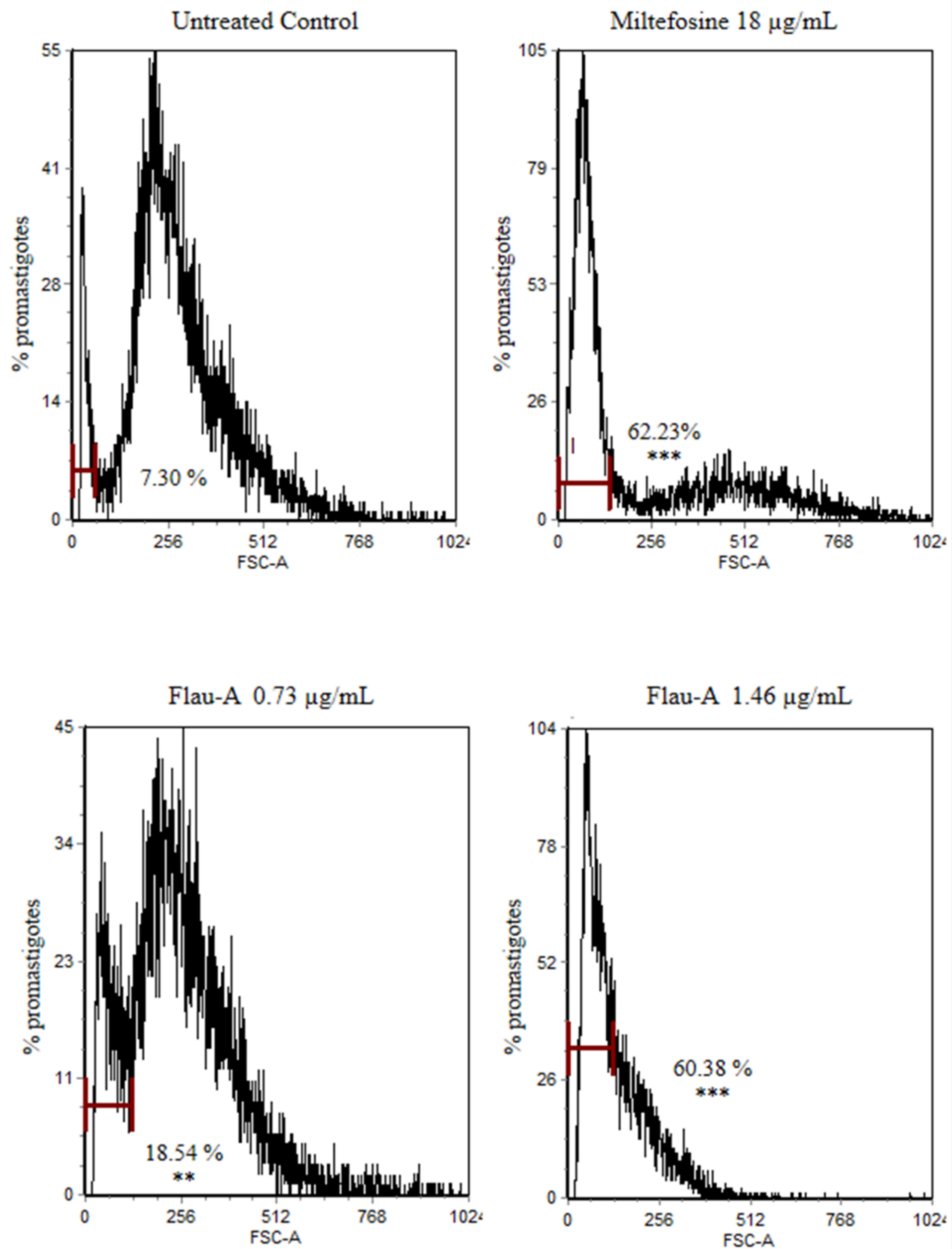
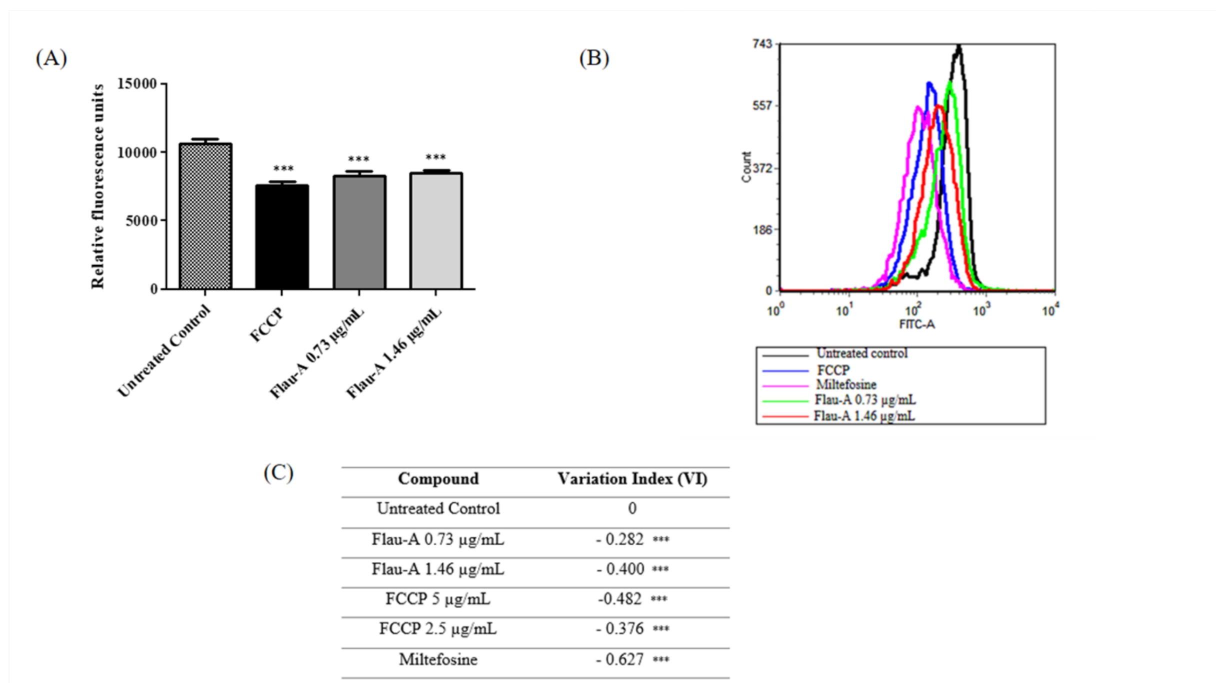


Fig. 2 Representative histograms showing changes in the L. amazonensis volume after treatment using Flau-A. Parasites (1×107 cells) were treated with 0.73 or 1.46 µg/mL of Flau-A (corresponding to 1 and 2 times the IC50 value obtained after 48 h of the in vitro cultures, respectively) for 2 h at 24 °C. Cells were analyzed by flow cytometry, and a total of 10,000 events were acquired. Experiments were performed 3 times and in triplicate. Miltefosine was used as a drug control. (**) and (***) indicate statistical differences in relation to the untreated control (P>0.01and P<0.001, respectively).

Flau-A induces depolarization of ΔΨm, increases the ROS production and induces rupture the *L. amazonensis* plasma membrane, but without phosphatidilserine exposure on the surface of parasite membrane

Promastigotes treated with Flau-A and stained with Mitotracker® showed reduction in their ΔΨm, presenting similar results to those using FCCP, a classic protonophore uncoupler that was used as a positive control (Fig. 3A). The ΔΨm was also evaluated through a flow cytometry assay using the Rh123 probe. The histogram of total Rh123 fluorescence (Fig. 3B) and results of the variation index (Fig. 3C) showed that the fluorescence was reduced. Promastigotes treated with one and two times the IC_{50} value showed a reduction in the fluorescence intensity in the order of 28.2% and 40.0%, respectively, when compared to the untreated controls. Cells treated with miltefosine showed a decrease in their ΔΨm in the order of 62.7%, while FCCP reduced the ΔΨm in 37.6% and 48.2%, when the concentrations of 2.5 and 5.0 μg/mL, respectively, were used. These results corroborated with data obtained using Mitotracker®, indicating a depolarization of the ΔΨm in Flau-A-treated parasites.



Compound	Variation Index (VI)
Untreated Control	0
Flau-A 0.73 μg/mL	- 0.282 ***
Flau-A 1.46 μg/mL	- 0.400 ***
FCCP 5 μg/mL	-0.482 ***
FCCP 2.5 μg/mL	- 0.376 ***
Miltefosine	- 0.627 ***

Fig. 3 Alteration in the ΔΨ of L. amazonensis after treatment using Flau-A. Parasites (1×107 cells) were treated with 0.73 or 1.46 μg/mL of Flau-A (corresponding to 1 and 2 times the IC50 value obtained after 48hoftheinvitrocultures,respectively)for2hat24°C.Then,theywere probed with Mito Tracker® and analyzed spectrofluorometrically). FCCP (5.0μg/mL) was used as controls (a).

Representative histograms showing treated promastigotes stained with Rh123 by flow cytometry are shown. FCCP (5.0 µg/mL) and miltefosine (18.0 µg/mL) were used as controls (b). VI values were obtained by the (MT−MC)/MCequation, where MT corresponds to the median fluorescence of the treate dparasites, and MC is the median fluorescence of the control parasites. FCCP (2.5 and 5.0 µg/ mL) and miltefosine (18.0µg/mL) were used as controls (c). Experiments were performed 3 times and in triplicate. (***) indicates statistical difference in relation to the untreated control (P<0.001).

The oxidative stress is characterized by an increase in ROS production, and mitochondria are one of the major intracellular sources of these reactive species (Chandrasekaran et al. 2013). Thus, we evaluated the ROS production in *L. amazonensis* promastigotes by using the fluorescent H_2DCFDA probe. Results demonstrated that Flau-A-treated promastigotes by using one and two times the IC_{50} value expanded the ROS production in the order of 2.5 and 3.3 times, respectively, when compared to the untreated controls, while using miltefosine the value was increased by 2 times (Fig. 4).

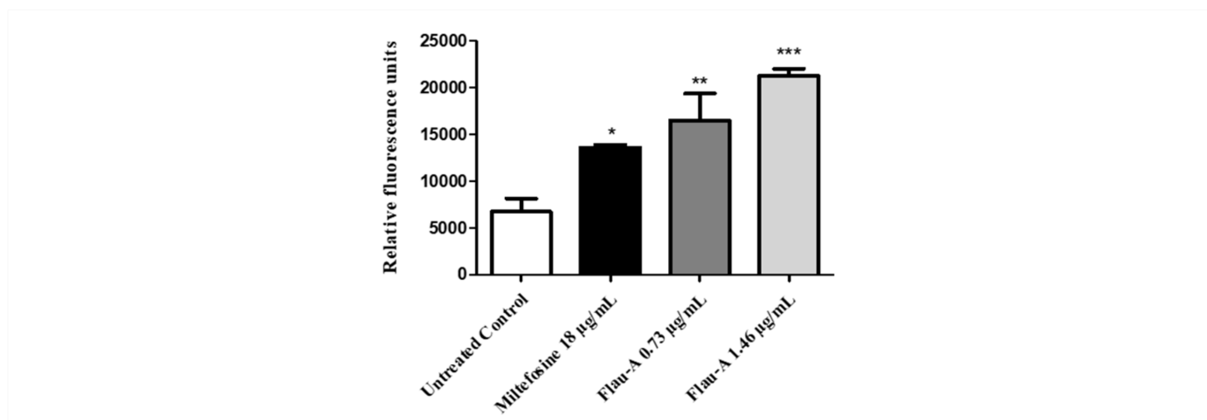


Fig. 4 Production of ROS in Flau-A-treated L. amazonensis. Parasites (1×107 cells) were treated with 0.73 and 1.46 µg/mL of Flau-A (corresponding to 1 and 2 times the IC50 value obtained after 48 h of the in vitro cultures, respectively) for2hat24°C.Cellswerestainedwith H2DCFDA for 30 min at 24 °C, and the fluorescence was spectrofluorometrically measured. Miltefosine (18.0 µg/mL) was used as a drug control. Results were expressed as mean ± standard deviation. (*), (**), and (***) indicate statistical differences in relation to the untreated control (P>0.05,P>0.01,andP<0.001, respectively)

The plasma membrane integrity was also evaluated by labeling it with propidium iodide, a fluorescent dye that is unable to cross the entire plasma membrane, but when it is ruptured, it is allowed to enter and bind to the DNA cell (Scariot et al. 2017). Results showed that Flau-A-treated promastigotes by using one and two times the IC_{50} value induced to an increase in their fluorescence intensity in the order of 3.0 and 4.1 times, when compared to the untreated controls, respectively

(Fig. 5). Parasites heated at 65ºC for 10 min, which were used as positive controls, showed an increase in the fluorescence intensity in the order of 4.3 times.

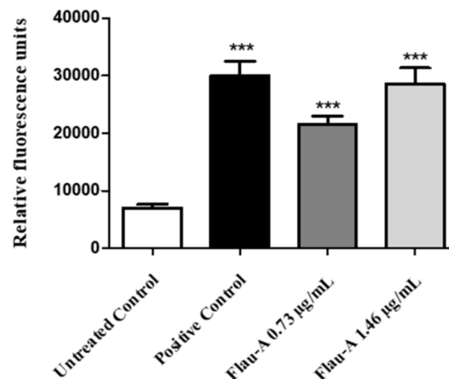


Fig. 5 Integrity of the Flau-A-treated L. amazonensis membrane. Parasites (1×107 cells) were treated with 0.73 and 1.46 µg/mL of FlauA(correspondingto1and2timestheIC50 valueobtainedafter48hofthe in vitro cultures, respectively) for 2 h at 24 °C. Cells were stained with propidium iodide (PI) for 15min at 24°C, and the fluorescence intensity was spectrofluorometrically measured. Cells pre warmed at 65 °C for 10 min were used as positive control. Results were expressed as mean ± standard deviation. Three independent experiments were performed and they presented similar results. (***) indicates statistical difference in relation to the untreated control (P<0.001)

Phosphatidylserine exposure is one characteristic of apoptotic cells (El-Hani et al. 2012). In the present study, the treatment using Flau-A not induced a statistical increase in the percentage of cells stained only with Annexin-FITC (4.99% in untreated control and 6.69% using 1.46 µg/mL of Flau-A; lower right quadrant). The dot plot analysis showed an increase in the percentage of propidium iodide-positive cells (5.13% and 13.05%, by using 0.73 and 1.46 µg/mL of Flau-A, respectively; upper left quadrant), while an increase in the double-stained annexin^{+}/PI^{+} cell percentage was also observed by using both concentrations, probably reflecting an initial rupture of the plasma membrane and subsequent entry of this reagent and Annexin probes into the cell. As a control, miltefosine-treated promastigotes exhibited percentages of 41.71% and 4.72% of annexin- and propidium iodide-positive cells, respectively.

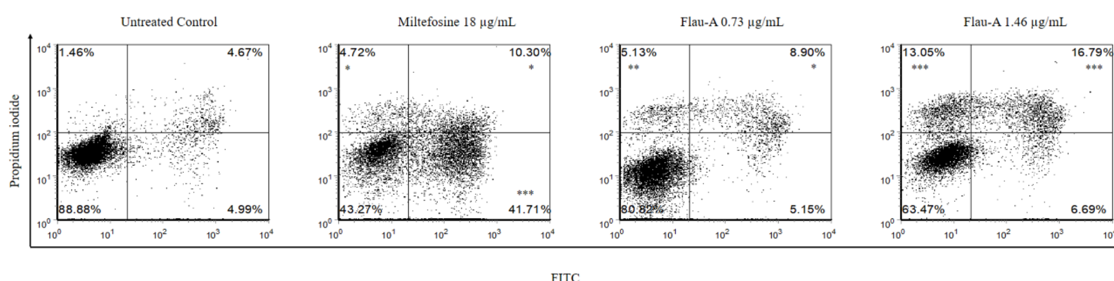


Fig. 6 Representative dot plots of phosphatidylserine exposure on the Flau-A-treated L. amazonensis membrane. Parasites (1×107 cells) were treated with 0.73 and 1.46 µg/mL of Flau-A (corresponding to 1 and 2 times the IC50 value obtained after 48 h of the in vitro cultures, respectively) for 2 h at 24 °C. Cells were stained with fluorescent probes containing Annexin V-FITC and propidium iodide (PI), and they were analyzed by flow cytometry. Miltefosine (18.0µg/mL) was used as a drug control. The Annexin V-positive cell percentage is shown in the lower right quadrant, the PI+ cells are shown in the upper left quadrant, and the Annexin V+/PI+ cells are shown in the upper right quadrant. Dot plots are representative of three independent experiments, which were performed in triplicate, and presented similar results. (*), (**), and (***) indicate statistical differences in relation to the untreated control (P>0.05,P>0.01,andP<0.001, respectively).

Flau-A is not toxic for mammalian models

A clinical and biochemical evaluation was performed in AmpB or Flau-A-treated mice. In the results, no clinical signal or laboratorial abnormality was found in treated animals, which showed also a positive variation in their weight in the order of 7.0%. On the other hand, AmpB-treated mice presented weakness and a negative variation in their weight in the order of 8.0%. These animals showed also higher levels of AST, ALT, BUN, and CRTN, indicating an organic toxicity caused by the administration of this drug.

Discussion

The treatment of leishmaniasis has been based on chemotherapy, although drugs are toxic, difficult to administrate, and parasite resistance has increased (Chávez-Fumagalli et al. 2015). In this context, the search for less toxic, highly effective, and low-cost products are desirable. In the present study, a new naphthoquinone derivative showed an effective antileishmanial activity against *Leishmania* species able to cause tegumentary and visceral leishmaniasis in the world, and this molecule was also found to present a low toxicity in distinct mammalian cells.

The identification of new compounds presenting biological properties remains a challenge. Natural products have served as lead structures for the development of chemotherapeutics against diseases, due to their biological activity, bioavailability, high yield, and low cost of production (Winkler et al. 2007). In this context, naphthoquinones have been considered promising scaffolds against parasites, and some classes of molecules exhibit *in vitro* antileishmanial activity *(Lezama-Dávila et al. 2012; Costa et al. 2014).*

Leishmania parasites present two main morphological forms in their life cycle: promastigote and amastigote. Promastigotes are found in the gut of sand flies and live extracellularly, whereas amastigotes reside inside of macrophages in infected mammalian hosts (Saraiva et al. 2005). The most of the studies aiming to identify new antileishmanial targets have tested molecules against the promastigote forms (Gao et al. 2012; Sazgarnia et al. 2012), although amastigotes should be considered also relevant, since they are responsible for the development of the active disease in the mammalian hosts (Muylder et al. 2011; Fernandes et al. 2012). As a consequence, promastigote assays could be used as prescreening, whereas intra amastigotes assays could be employed to follow the biological trials (Ullah et al. 2016).

In our study, Flau-A was effective against stationary promastigotes, amastigotes-like and intra-macrophage amastigotes of both *L. amazonensis* and *L. infantum* species, besides presents no significant toxicity in two mammalian cells. In addition, a reduction in the percentage of infected macrophages was found when parasitized cells were treated with Flau-A, as well as a reduction in the infection of macrophages was visualized when this molecule was pre-incubated with parasites, then demonstrating an important biological activity of it against *Leishmania*, as well as an efficacy in activates macrophages to kill parasites.

Since this study's purpose was to identify new antileishmanial candidates to be used in the *in vivo* treatment against leishmaniasis, a toxicity study was performed in BALB/c mice by using Flau-A, which was administered in the animals during 15 days. As a control, AmpB was used. Higher levels of AST, ALT, BUN, and CRTN enzymes were found in the AmpB-treated animals, possibly indicating a hepatic and renal toxicity when this product was inoculated in the mice. On the other hand, no toxicity was found when Flau-A was administered in the animals. Our experimental model used a subcutaneous route to administrate the molecules, as well as a pre-defined dose of Flau-A and AmpB. We understand that other routes and doses could eventually induce higher or lower toxicity in the animals; however, due to Ethical limitations limiting the number of animals to perform a dose-response curve, we have adopted routes and doses already described as effective in other studies (Duarte et al. 2016; Mendonça et al. 2016). In addition, similar organic alterations were also found in BALB/c mice, when intravenous route was employed to administer AmpB;

thus demonstrating the toxicity of this compound in the mammalian hosts (Deray 2002; Mishra et al. 2013; Ribeiro et al. 2014; Asad et al. 2015). As a consequence, we can infer that Flau-A is safe to administer in these hosts, which opens the possibility to test it in future studies for the treatment against leishmaniasis.

Works evaluating the mechanism of action of antileishmanial drugs could provide important information for the optimization of hit compounds, since the majority of the studies have showed an antileishmanial effect, but without present the mechanism of action of the tested compounds (FUMAROLA; SPINELLI; BRANDONISIO, 2004; YARDLEY et al., 2005). In the present study, the mechanism of action of Flau-A was investigated in *L. amazonensis*. The modifications induced in Flau-A-treated promastigotes showed changes in parasite morphology, which were reflected by a marked decrease in their mobility and alterations in the flagellum size, possibly resulting from alterations in the cytoskeleton reorganization associated with modifications in their mitochondrial activity.

Undoubtely, mitochondria are relevant target organs in *Leishmania*, and our study showed also that Flau-A is acting in this organelle, like described by others studying different molecules and trypanosomatids (Menna-Barreto and Castro 2014; Lage et al. 2015). Mitochondria are organelles important in parasites, acting in the metabolism, cell differentiation, calcium homeostasis, death cell and in the oxidative phosphorylation (Smith et al. 2012; Menna-Barreto and Castro 2014). Unlike eukaryotic organisms that present several, if not thousands, of mitochondrial; trypanosomatids such as *Leishmania* have a single organelle in their cytoplasm (Souza et al. 2009), fact that can favor the development of drugs acting in this *Leishmania* organelle (Menna-Barreto and Castro 2014, Antinarelli et al. 2015, Stroppa et al. 2017). The present study used two probes, namely Rh123 and Mitotracker®, which are considered to be $\Delta\Psi m$-sensitive dyes, and results showed that the treatment with Flau-A interferes in the electrochemical potential gradient of the parasites´ mitochondrial membrane, by reducing their $\Delta\Psi m$. In addition, results revealed also an increase in the ROS production, indicating that it may well be responsible for mitochondrial dysfunction. During mitochondrial metabolism, ROS is formed by the incomplete reduction of molecular oxygen (Forkink et al. 2010; Smith et al. 2012). Cells can protect this molecule in physiological conditions; however, oxidative stress can occurs if ROS is produced in large amounts, then contributing to

mitochondrial dysfunction and parasite´ irreversible damage, culminating with cell death.

In addition to its effect on parasite mitochondria, the treatment with Flau-A altered also the permeability of the plasma membrane of *L. amazonensis* promastigotes. Using propidium iodide, a probe able to cross the membrane in non-intact cells, it was observed that the treatment with Flau-A affect the *Leishmania* membrane, allowing then to cause the rupture of cell membrane. This fact was observed by both fluorimetry and flow cytometry techniques, being the rupture of plasma membrane usually considered as an event related to cell necrosis (Proto et al. 2013).

On the other hand, when Annexin V-FITC was used, we observed a low population Annexin V-positive/propidium iodide-negative cells (~7.0%), reveling a loss of membrane asymmetry and exposure of phosphatidylserine residues at the outer plasma membrane of the parasites. These cells can not necessarily indicate cell death due to the treatment with Flau-A, but suggest this action in a parasites´ subpopulation, like described by Santos et al. (2013), which shown that stationary-phase promastigotes of *Leishmania*, in opposite to the log-phase cells, present a subpopulation that exposes high frequency of Annexin-V positive cells. Other studies demonstrated also that Annexin-positive parasites can be result of a "apoptotic mimicry", where *Leishmania* exposes phosphatidylserine with the purpose to infect macrophages, although this event seems to be usually found when the amastigote forms are evaluated (Wanderley and Barcinski 2010; El-Hani et al. 2012).

In summary, our data shown that Flau-A presents an effective antileishmanial activity against two important *Leishmania* species worldwide, besides a rapid *in vitro* effect manifesting changes in membrane permeability, mitochondrial functionality, and parasite morphology. In addition, the ability of this molecule in reducing the infection in murine macrophages, as well as its effectiveness in inhibiting the infection of these cells, when pretreated parasites are used, demonstrates that it could well be applied as a new candidate to treat against tegumentary and visceral leishmaniasis in mammalian hosts.

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Conflict of interest

The authors confirm that they have no conflicts of interest in relation to this work.

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5.2. Considerações finais do artigo 1

Os resultados apresentados neste primeiro artigo indicam que a Flau-A apresenta atividade contra formas promastigotas e amastigota-like de *Leishmania amazonensis* e *Leishmania infantum*, bem como é capaz de reduzir a infecção e

número médio de formas amastigotas, em macrófagos parasitados e em seguida tratados e com parasitos pré tratados.

A substância também não apresentou toxicidade quando testada em macrófagos murinos, hemácias humanas e animais sadios.

As alterações na permeabilidade da membrana plasmática, na morfologia, no potencial de membrana mitocondrial e o aumento na produção de ROS em formas promastigotas de *L. amazonensis* indicam um provável mecanismo de ação mitocondrial da molécula estudada.

Desta forma, a molécula denominada Fleu-A se apresenta como potencial candidata a fármaco no tratamento das leishmanioses em modelos de estudo *in vivo.*

5.3. Artigo 2

No próximo artigo, o objetivo foi avaliar o uso da Flau-A para o tratamendo da leishmaniose tegumentar em um modelo murino infectado com *Leishmania amazonensis.* Para tal avaliação a molécula foi administrada em sua forma livre e e

na forma de um sistema micelar, visando uma maior efetividade e eficácia do tratamento.

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Title

In vivo antileishmanial efficacy of a naphthoquinone derivate incorporated into a Pluronic® F127-based polymeric micelle system against *Leishmania amazonensis* infection.

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ABSTRACT

New therapeutic strategies against leishmaniasis are desirable, since the treatment against disease presents problems, such as the toxicity, high cost and/or parasite resistance. As consequence, new antileishmanial compounds are necessary to be identified, as presenting high activity against *Leishmania* parasites, but low toxicity in mammalian hosts. Flau-A is a naphthoquinone derivative recently showed to presents an *in vitro* effective action against *Leishmania amazonensis* and *L. infantum* species. In the present work, the *in vivo* efficacy of Flau-A, which was incorporated into a Poloxamer 407-based micelle system, was evaluated in a murine model against *L. amazonensis* infection. Amphotericin B (AmB) and Ambisome® were used as controls. The animals were infected and later treated with the compounds. Thirty days after the treatment, parasitological and immunological parameters were evaluated. Results showed that AmB, Ambisome®, Flau-A or Flau-A/M-treated animals presented significantly lower average lesion diameter and parasite burden in tissue and organs evaluated, when compared to the control (saline and micelle) groups. Flau-A or Flau-A/M-treated mice were those presenting the most significant reductions in the parasite burden, when compared to the others. These animals developed also a more polarized antileishmanial Th1 immune response, which was based on significantly higher levels of IFN-ɣ, IL-12, TNF-α, GM-CSF, and parasite-specific IgG2a isotype; associated with low levels of IL-4, IL-10, and IgG1 antibody. The absence of toxicity was found in these animals, although mice receiving AmB have showed high levels of renal and hepatic damage markers. In conclusion, results suggested that the Flau-A/M compound may be considered as a possible therapeutic target to be evaluated against human leishmaniasis.

Keywords: Tegumentary leishmaniasis; chemotherapy; toxicity; amphotericin B; Pluronic® F127; 2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone.

Introduction

Leishmaniasis is a neglected disease with approximately 350 million people at risk of infection by *Leishmania* parasite, and with 2 million new cases being reported annually, occurring mainly in developing countries [1]. The clinical manifestations of the disease range from cutaneous leishmaniasis to the visceral disease [2]. Tegumentary leishmaniasis (TL) is considered endemic in Latin America, and the main species able to cause the disease are *Leishmania braziliensis*, *L. amazonensis*, *L. guyanensis*, *L. panamensis,* and *L. mexicana*. The disease can be subclinical or produces self-healing cutaneous lesions, although the uncontrolled parasite replication leading to mutilation and morbidity can be observed in the patients [3,4]. Visceral leishmaniasis (VL), which can be fatal if acute and untreated, is mainly found in India, Bangladesh, Sudan, South Sudan, Ethiopia, and Brazil. The disease is caused by *L. donovani* and *L. infantum* species, and about 20,000 to 40,000 deaths are registered annually [5].

Among the distinct *Leishmania* spp. able to cause human disease, the *L. amazonensis* parasite is an important etiological agent of TL in the South America, being responsible by the cases of diffuse cutaneous leishmaniasis [6]. Regarding to the disease in BALB/c mice, the animals are highly susceptible to this parasite species, showing progressive swelling at the inoculation site followed by metastasis and visceralization [7,8]. This mouse lineage develops a parasite-specific Th2 immune response, which is characterized by production of high levels of IL-4, IL-10, among other anti-inflammatory cytokines; while the resistance is associated with the development of an specific Th1 immune response, primed by the production of IFN-γ, IL-2, GM-CSF, IL-12, among other pro-inflammatory cytokines [9,10].

The treatment against leishmaniasis is based on the use of pentavalent antimonials in Brazil and other developing countries. Other second-line products, such as miltefosine, amphotericin B (AmB), paromomycin, and pentamidine are also used; however, side effects such as hepatic, cardiac, and renal toxicity are also registered [11]. In addition, parasite resistance is increasing, leading to the necessity to use higher doses and longer-term therapy, thus causing higher toxicity in the patients [12]. AmB-containing lipid-based formulations have been employed for the treatment against leishmaniasis. These compounds present higher efficacy and lower toxicity in the patients, when compared to the use of free drug. However, the high cost is an impeditive factor, and limits their use in developing countries [13]. In this context, the search for new, safer, and cheaper drugs to treat against leishmaniasis

is urgently needed, and one strategy could be based on the employ of new synthetic and/or natural products.

Quinones are a known class of molecules exhibiting distinct biological activities, such as antitumor [14], molluscicidal [15], bactericidal [16], fungicidal [17], and trypanocidal [18,19] functions. Studies have showed also the use of this molecules´ class against *Leishmania* parasites [20-22]. However, the further development of these compounds has been hampered, due to the absence of *in vivo* experiments or due to their low efficacy, when they are tested as therapeutics in mammalian models [22].

Since structural modifications in known molecules can be considered a cheaper and faster pathway to develops new antileishmanial agents [23]; in the present study, a quinoline derivate, namely 2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone or Flau-A, was investigated against *L. amazonensis* infection in BALB/c mice. This molecule was previously showed to presents an *in vitro* antileishmanial activity against *L. amazonensis* and *L. infantum* species, as well as low toxicity in murine macrophages and human red cells [24]. Here, Flau-A was incorporated into a Poloxamer 407-based micelle system, and the therapeutic efficacy of this product was evaluated in *L. amazonensis*-infected BALB/c mice, comparing with the use of free AmB and its liposomal formulation, Ambisome®. The efficacy of the products was evaluated 30 days after the treatment, when immune response and parasite burden in the infected tissue and organs were evaluated in the treated and infected animals.

Materials and methods

Chemicals

A technical protocol recently developed [24] was used for the preparation of Flau-A. Briefly, 0.30 g (1.72 mmol) lawsone was dissolved in 5 mL dichloromethane PA and transferred to a 100 mL round bottom flask. A solution containing potassium carbonate (10% w/v) was added, and the mixture was stirred for 30 min at room temperature. Next, 2,3,4-tri-*O*-acetyl-6-deoxy-β-L-galactopyranosyl bromide (3.44

mmol) and n-Bu_4NBr (22% mol) were added, and the mixture was again stirred for 18 h at room temperature. Then, 6 mol/L hydrochloric acid were added to reach at pH 3.0, and the mixture was transferred to a separator funnel to obtain the organic phase. Thus, it was washed in 100 mL distilled water, dried by using anhydrous sodium sulfate and concentrated. The product was passed in a column chromatography (hexane/ethyl acetate, 7:3 v/v), and the purified molecule was obtained in a 34% yield. The product was considered pure according to its proton and 13-carbon NMR spectra, besides TLC analysis. For the *in vivo* experiments, Flau-A was diluted in phosphate-buffered saline (PBS 1x) pH 7.4. Poloxamer 407 (Pluronic® F127) was purchased from Sigma-Aldrich (catalog number 16758; St. Louis, MO, USA). AmB (Cristália, São Paulo, São Paulo, Brazil) was resuspended in methanol/DMSO (9:1 v/v), and maintained at -80º until use. Ambisome® (Gilead Sciences, Inc. San Dimas, USA) was obtained as a lyophilized powder, and resuspended in water to prepare a stock solution.

Preparation of the Flau-A/M

The polymeric micelles were prepared as described previously [25]. Briefly, Poloxamer 407 (18% w/w) was diluted in PBS 1x, pH 7.4, under magnetic agitation for 18 h at 4°C. Then, Flau-A (8 mg) was diluted in 500 μL dichloromethane PA and solubilized by using vortex. The mixture was added to the previously prepared Poloxamer solution under vigorous magnetic agitation and in an ice bath, until a viscous emulsion has been obtained. The dichloromethane solution was evaporated by using a rotary evaporate (Buchi, Flawil, Switzerland), and the product was obtained as a transparent yellow gel at room temperature. The Flau-A content in the micellar solution was evaluated spectrophotometrically using an ultraviolet method, as described previously [26]. Briefly, samples of the Flau-A/M solution were collected and diluted in methanol PA. Absorbances were measured in a UV/Vis spectrophotometer (Double beam AJX-6100 PC; Micronal, São Paulo, Brazil), at a wavelength of 380 nanometers (nm). The concentration of Flau-A was calculated using a standard curve (0 to 18 μM), which was previously prepared in methanol PA. The analyses were carried out using three replicates. Empty micelles (18% w/w) were prepared by using the same technical protocol described for preparation of the Flau-A/M.

Parasite and mice

L. amazonensis (IFLA/BR/1967/PH-8) was used. The parasites were grown in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed by the Schneider's medium plus 20% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, 100 μg/mL streptomycin, and 50 μg/mL gentamicin pH 7.4, at 24°C. Female BALB/c mice (8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences (ICB), Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. The animals were maintained under pathogen-free conditions. The study was approved by Committee for the Ethical Handling of Research Animals from UFMG (protocol number 085/2017).

Infection and therapeutic schedules

Mice (n=10 per group) were infected with 10^7 *L. amazonensis* stationary promastigotes in their base of the tail through subcutaneous injection. Fifty days after infection (with lesions presenting between 2 to 3 millimeters), animals were separated in groups according to lesion size, aiming to ensure similar average lesion diameter among the evaluated groups. Then, they were treated with one of the following regimens:

(i) Saline (control) group: mice received 50 μL of PBS 1x by subcutaneous route, once a day during 15 days;

(b) Empty micelle (micelle) group: mice received 50 μL of non-incorporated micelles (10 mg/kg body weight) by subcutaneous route, every two days during 15 days;

(c) Amphotericin B (AmB) group: mice received 50 μL of AmB (1 mg/kg body weight) by intraperitoneal route, every two days during 15 days;

(d) Ambisome® group: mice received 50 μL of product (2 mg/kg body weight) by intravenous route, every five days during 15 days;

(e) Flau-A group: mice received 50 μL of Flau-A (10 mg/kg body weight) by subcutaneous route, every two days during 15 days;

(f) Flau-A/micelle (Flau-A/M) group: mice received 50 μL of Flau-A-containing micelles (5 mg/kg body weight) by subcutaneous route, every two days during 15 days.

Then, the lesion average diameter was measured weekly by using an electronic caliper (799-6/150 model, Starrett®, Brazil), and 30 days after the treatment, the animals were euthanized, when parasitological and immunological parameters were evaluated.

Cellular response evaluated by capture ELISA and flow cytometry

Aiming to evaluate the antileishmanial cellular response induced after the treatment, the soluble *L. amazonensis* antigenic (SLA) extract was prepared as described previously [27]. Briefly, 10^9 stationary promastigotes were washed three times in cold sterile PBS 1x, pH 7.4. After six cycles of freezing and thawing, followed by ultrasonication (Ultrasonic processor, GEX600), with six cycles of 30 sec at 38 MHz, the suspension was centrifuged at 9,000 x *g* for 30 min at 4°C, and the supernatant containing *L. amazonensis* SLA was collected. The protein concentration was estimated by the Bradford method [28], and aliquots were stored at -80°C until use. Murine splenocytes (n=10 per group) were collected 30 days after the treatment, and cells (5 x 10^6) were plated in 24-well plates (Nunc) and incubated in DMEM plus 20% FBS and 20 mM L-glutamine, at pH 7.4. Then, cells were unstimulated (medium, background control) or stimulated with SLA (25.0 μg/mL), for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the supernatants using commercial kits (BD Pharmingen®, San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was evaluated in the cell supernatant by Griess reaction [29].

To investigate the origin of the IFN-γ production in the treated and infected animals, splenocytes were stimulated with SLA (25.0 μg/mL) and incubated in the presence of 5.0 μg/mL of monoclonal antibodies (mAb) against mouse CD4 (GK 1.5) or CD8 (53-6.7). Appropriate isotype-matched controls – rat IgG2a (R35-95) and rat IgG2b (95-1) – were used. Antibodies (no azide/low endotoxin™) were purchased from BD (Pharmingen®). A flow cytometry assay was also performed to evaluate the IFN-γ, TNF-α and IL-10-producing $CD4^+$ and $CD8^+$ T cell frequency as described previously [30]. Results were expressed as indexes, which were calculated by the

ratio between the cytokine-producing $CD4^+$ and $CD8^+$ T cell percentages versus the values obtained in the unstimulated cultures (SLA/CC ratio).

Humoral response

The antileishmanial IgG1 and IgG2a isotype production was evaluated in serum samples of the treated and infected animals. For this, *L. amazonensis* SLA was used as an antigen (1.0 μg per well), and samples were 1:100 diluted in PBS-T (PBS 1x plus 0.05% Tween 20), with incubation for 1 h at 37°C. After washing plates five times, the anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies were added (in 1:5,000 and 1:10,000 dilutions, respectively, in PBS-T, Sigma-Aldrich), and reactions were developed by incubation with 2 μL H_2O_2, 2 mg ortho-phenylenediamine and 10 mL citrate-phosphate buffer pH 5.0, for 30 min and in the dark, and stopped by the addition of 20 μL H_2SO_4 2 N. The optical density was determined by an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

Parasite burden

The parasite load was evaluated in the infected tissue, draining lymph node (dLN), spleen, and liver of the animals by a limiting dilution technique [31]. For this, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile PBS. Tissue debris were removed by centrifugation at 150 × *g* and cells were concentrated by centrifugation at 2,000 x *g*. The pellet was resuspended in 1 mL of complete Schneider's medium and 220 μL of the resuspension was plated onto 96-well flat-bottom microtiter plates (Nunc), and diluted in log-fold serial dilutions in complete Schneider's medium with a 10^{-1} to 10^{-12} dilution. Each sample was plated in triplicate and read 7 days after the beginning of the culture at 24°C. Results were expressed as the negative log of the titer (*i.e.*, the dilution corresponding to the last positive well), which was adjusted per milligram of tissue or organ.

The parasite load was also evaluated in the infected tissue by a qPCR technique as described previously [30]. Briefly, infected tissue DNA was extracted by using a phenol-chloroform method. Standard curves were obtained from DNA extracted from 10^8 parasites for kDNA and of 10^8 peritoneal macrophages for β-actin, under the same conditions used to extract the other samples. PCR was performed on Step One™ Instrument (48 wells-plate; Life Technologies) using 2x SYBR® Green

PCR Master Mix (5 μL, Applied Biosystems), with 2 mM of each primer (1 μL) and 4 μL of DNA (5 ng/μL). Samples were incubated at 95°C for 10 min, and submitted to 40 cycles of 95°C for 15 s and 60°C for 1 min. Results were converted into number of parasites per nucleated cell (multiplied by one thousand to facilitate visualization).

Toxicology study

To evaluate the toxicity of the treatment, the nephrotoxicity was investigated by the dosage of urea nitrogen and creatinine, while the hepatic function was analyzed by dosage of the alanine transaminase (*ALT*) and aspartate transaminase (*AST*), in the serum samples of the treated and infected animals, by means of commercial kits (Labtest Diagnostica®, Belo Horizonte). As control, serum samples from naive (non-treated and non-infected) mice (n=8) were used.

Statistical analysis

Results were entered into Microsoft Excel (version 10.0) spreadsheets and analyzed by GraphPad Prism™ (version 6.0 for Windows; GraphPad Software, Fay Avenue, La Jolla, CA, USA). The one-way analysis of variance (ANOVA) and Tukey´s post-test were used for comparisons between the groups. Differences were considered significant when $P < 0.05$. The experiments were repeated and results were similar. Data shown in this study are representative of the first experiment.

Results

Efficacy of the therapeutics against L. amazonensis infection

The *in vivo* therapeutic efficacy employing AmB, Ambisome®, Flau-A or Flau-A/M was evaluated in BALB/c mice against *L. amazonensis* infection. For this, the average lesion diameter and parasite burden in the infected tissue, liver, spleen and draining lymph node of the animals were investigated, 30 days after the treatment. Results showed that treated and infected animals presented significant reductions in the edema in the infected tissue, when compared with the controls (saline and micelle). The reduction percentage in the lesion diameter in the AmB, Ambisome®, Flau-A and Flau-A/M groups was of 21.0%, 24.0%, 43.0%, and 51.0%, respectively,

when compared to the saline group; and of 18.0%, 21.0%, 41.0%, and 49.0%, respectively, when compared to the micelle group (Fig. 1A). Flau-A and Flau-A/M-treated mice presented the most significant reductions in the average lesion diameter, in the order of 28.0% and 37.0%, respectively, when compared to the AmB group; and of 25.0% and 35.0%, respectively, when compared with the Ambisome® group. In the evaluation of the parasite load, the AmB, Ambisome®, Flau-A and Flau-A/M-treated mice´ groups showed also significant reductions in the tissue and organic parasitism, when compared to the controls, being the most significant reductions also found in the Flau-A and Flau-A/M groups (Fig. 1B). A qPCR assay was performed in the infected tissue, and results showed also that Flau-A and Flau-A/M groups were those presenting the most significant reductions in the parasitism, when compared to the others (Fig. 2).

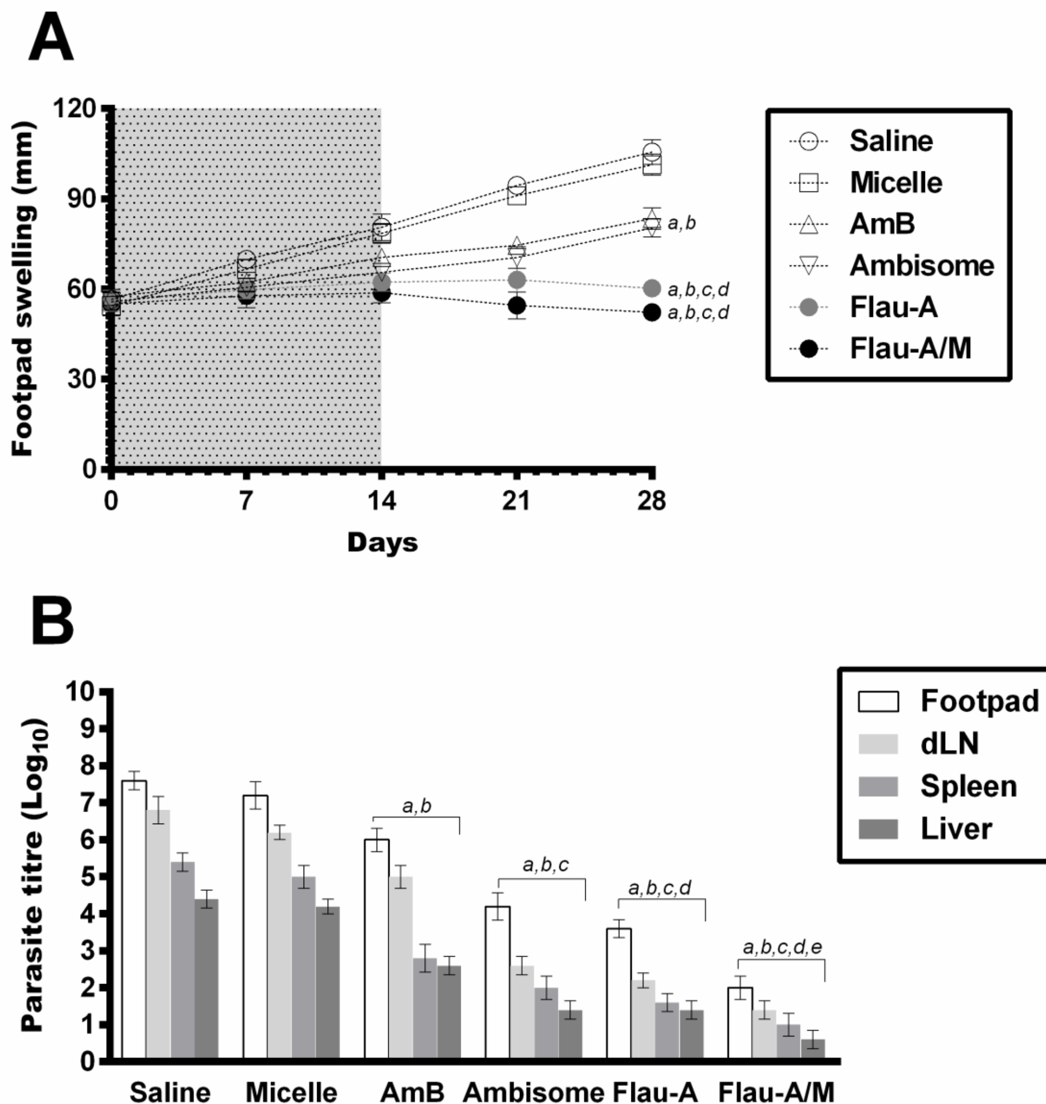

Fig. 1. Average lesion diameter and parasite burden in the treated and infected animals. BALB/c mice were infected with 10^7 *L. amazonensis* stationary promastigotes and, 50 days post-infection, they were separated into groups (n=10 per group), and received saline or were treated with empty micelles, AmB, Ambisome®, Flau-A or Flau-A/M. The evolution of lesion size was monitored weekly, and during four weeks, in the treated and infected mice. Lines represent the lesion average diameter (area) expressed as the mean ± standard deviation of the groups (A). The parasite load was evaluated 30 days after the treatment in the infected tissue, draining lymph node, spleen, and liver by a limiting dilution technique (B). Bars represent the mean ± standard deviation of the groups. The letters *a*, *b, c*, and *d* indicate statistically significant differences in relation to the saline, micelle, AmB, and Ambisome® groups, respectively ($P < 0.001$).

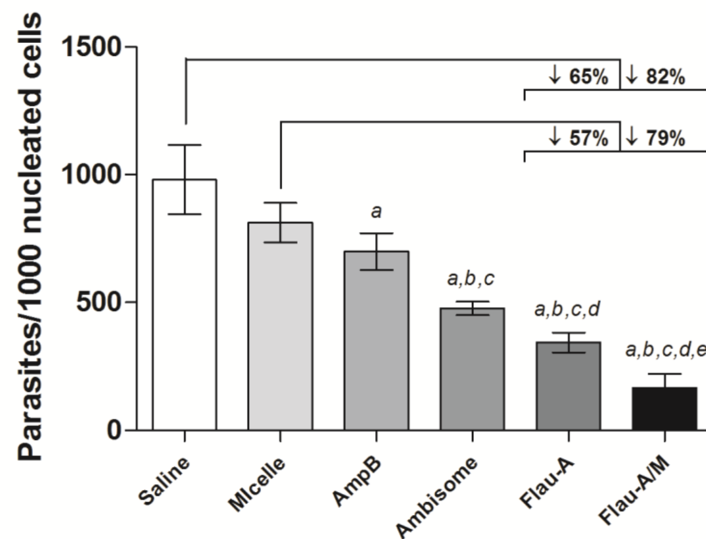

Fig. 2. Parasite burden evaluated by qPCR technique. The parasite load was also evaluated 30 days after the treatment in the infected tissue, by using a qPCR technique. The detection of parasites was expressed as number of parasites per 1,000 nucleated cells in the treated and infected animals. Data are showed in linear scale, and bars represent the mean plus standard deviation of the groups. The letters *a*, *b, c*, *d,* and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.005$).

Immunogenicity generated by the therapeutics against L. amazonensis infection

The profile of Th1 and Th2 cytokines was evaluated in the cellular supernatant of the SLA-stimulated cultures, 30 days after the treatment. Results showed that spleen cells collected from the AmB, Ambisome®, Flau-A, and Flau-A/M groups produced higher IFN-γ and lower IL-4 and IL-10 levels, when compared with the values found in the controls (Fig. 3A). The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was also evaluated, and results showed a significantly lower IFN-γ production when anti-CD4 or anti-CD8 antibody was added in the cell cultures in the treated groups, thus demonstrating the importance of both T cell subtypes in the production of this cytokine (Fig. 3B). A flow cytometry assay was developed to investigate the cytokine-producing T cell frequency, and results showed that Ambisome®, Flau-A and Flau-A/M-treated mice were those presenting higher levels of IFN-γ and TNF-α-producing $CD4^+$ and $CD8^+$ T cells, when compared to the others, as well as lower levels of IL-10-producing T cell (Fig. 4); thus corroborating with the immune profile found in the ELISA experiments.

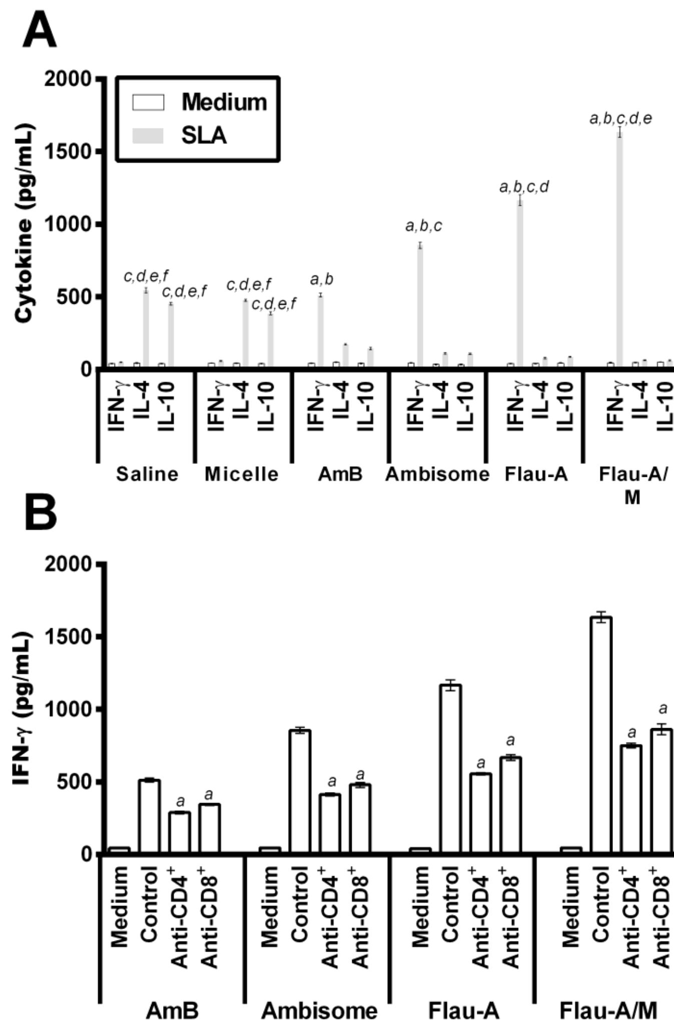

Fig. 3. Cellular response generated after the treatment. Spleen cells of the treated and infected mice (n=10 per group) were obtained 30 days after the treatment, and cells (5 x 10^6) were unstimulated (medium, background control) or stimulated with *L. amazonensis* SLA (25 µg/mL) for 48 h at 37°C in 5% CO_2. IFN-γ, IL-4, and IL-10 levels were measured in the supernatant by capture ELISA (A). The letters *a*, *b, c*, *d,* and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$). The involvement of $CD4^+$ and $CD8^+$ T cells in the IFN-γ production was evaluated by means of the incubation of the splenocytes (5 x 10^6 cells) with SLA (25 µg/mL), for 48 h at 37°C in 5% CO_2, in the absence (control) or presence of monoclonal antibodies (mAb) against mouse $CD4^+$ or $CD8^+$. The supernatant was collected and the IFN-γ production was measured by capture ELISA. Bars represent the mean ± standard deviation of the groups. The letter *a* indicates the existence of statistically significant difference in relation to the control group ($P < 0.0001$).

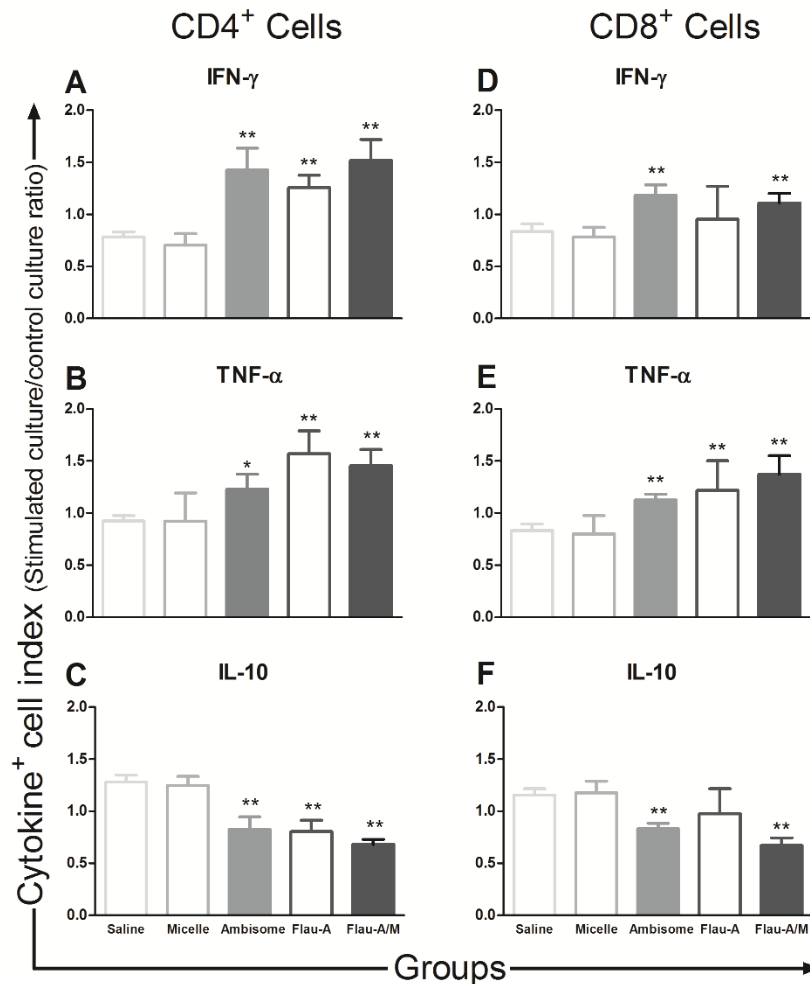

Fig. 4. Frequency of intracytoplasmic cytokine-producing CD4$^+$ and CD8$^+$ T cells. BALB/c mice were infected with 10^7 *L. amazonensis* stationary promastigotes and, 50 days post-infection, they received saline or were treated with empty micelles, Ambisome®, Flau-A or Flau-A/M. The IFN-γ, TNF-α, and IL-10-producing CD4$^+$ and CD8$^+$ T cell frequency was evaluated in the spleen cells (n=10 per group), 30 days after the treatment. Results were reported as cytokine indexes, which were calculated by the ratio of cytokine$^+$-T cell percentage in the stimulated cultures versus the values obtained in the unstimulated cultures (SLA/CC ratio). Bars represent the mean plus standard deviation of the groups, for CD4$^+$ (A, B and C) and CD8$^+$ (D, E and F) T cells. (*) and (**) indicate the existence of statistically significant difference in relation to the saline and micelle groups, respectively ($P < 0.05$).

It is known that macrophages stimulated by IFN-γ are able to activate NO synthesis to destroy intracellular amastigotes. In an attempt to evaluate the parasite-specific activation of macrophages in the treated and infected animals; the nitrite, IL-12 and GM-CSF production was investigated in the cell supernatant. Results showed higher levels of these markers in the AmB, Ambisome®, Flau-A, and Flau-A/M groups; although higher production has been observed in the Flau-A and Flau-A/M groups (Fig. 5). The humoral response was evaluated, and results showed that Flau-A and Flau-A/M-treated mice also presented a predominance of antileishmanial IgG2a isotype, when compared to the IgG1 levels (Fig. 6).

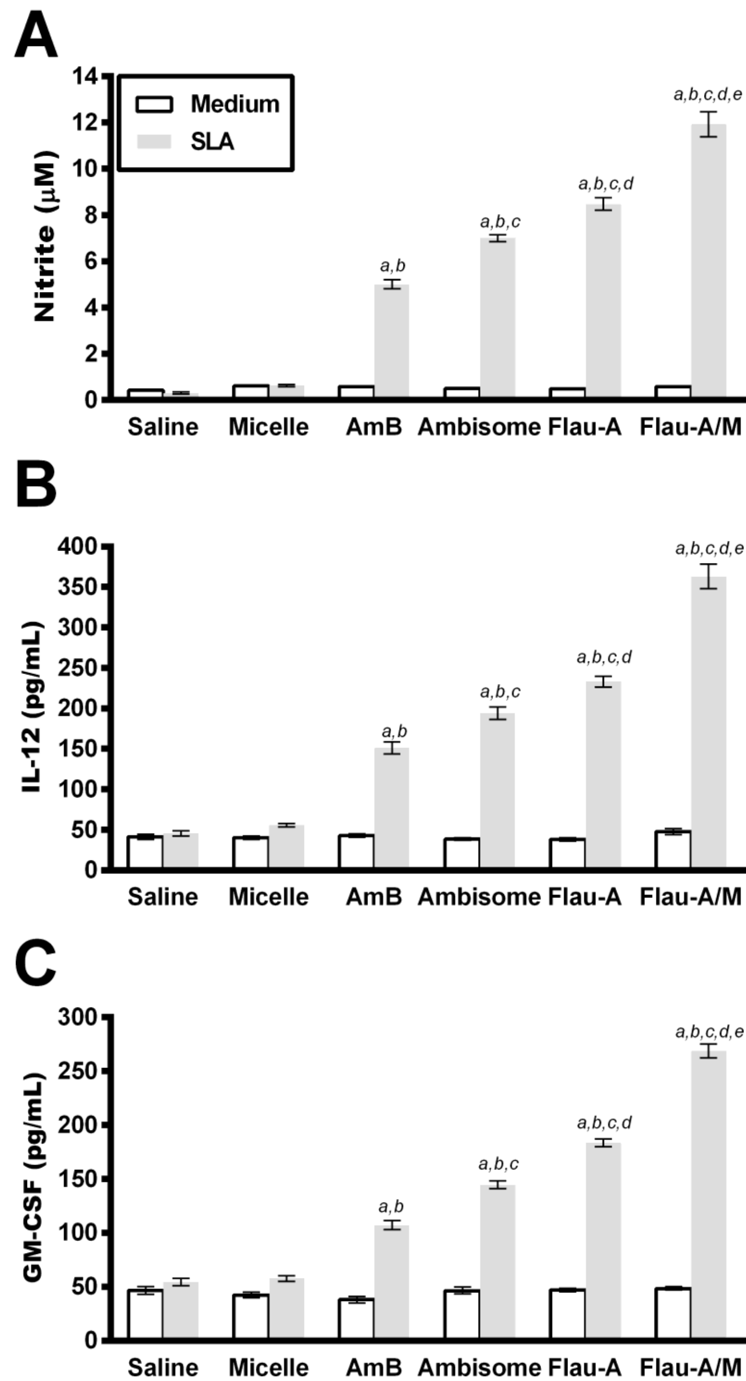

Fig. 5. Nitrite, IL-12 and GM-CSF production. Spleen cells (5 x 10^6) of the treated and infected mice (n=10 per group) were collected 30 days after the treatment, and they were unstimulated (medium, background control) or stimulated with SLA (25 µg/mL), for 48 h at 37°C in 5% CO_2. The nitrite, IL-12 and GM-CSF production was evaluated in the cell supernatant. Bars represent the mean ± standard deviation of the groups. The letters *a*, *b, c*, *d,* and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.001$).

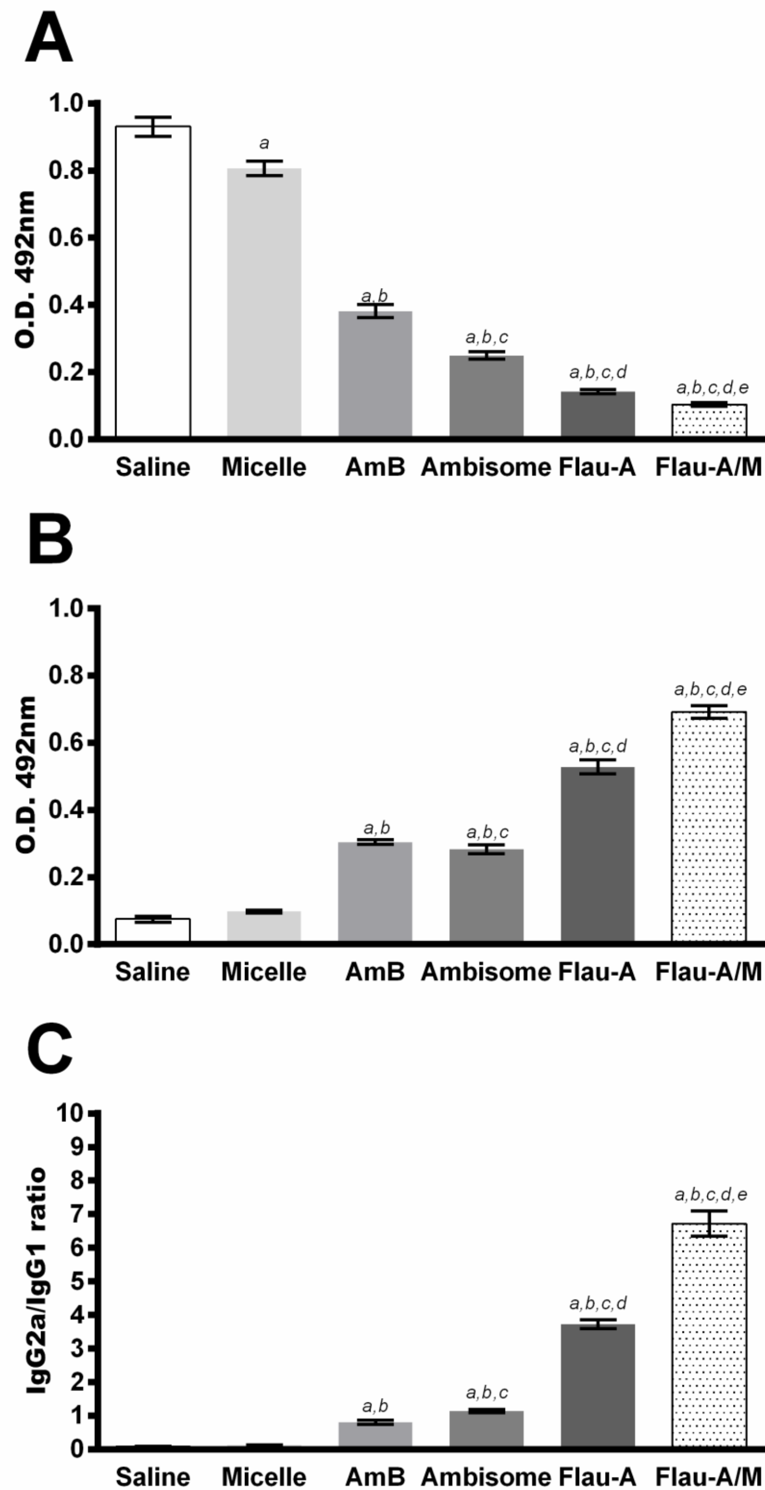

Fig. 6. Antibody production in the treated and infected animals. Sera samples of the treated and infected animals (n=10 per group) were collected 30 days after the treatment, and they were used to evaluate the parasite-specific IgG1 (A) and IgG2a (B) isotype production. Also, ratios between the IgG2a and IgG1 levels were calculated and are showed (C). Bars represent the mean ± standard deviation of the groups. The letters *a*, *b, c*, *d*, and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$).

Evaluation of the in vivo toxicity

The toxicity was analyzed in the treated and infected animals, and results showed significant increase in serum enzymes associated with renal and hepatic damage in free AmB-treated mice, with significantly higher levels of ALT, AST, urea, and creatinine, when compared with the values obtained in the Ambisome®, Flau-A, and Flau-A/M groups (Fig. 7). In addition, clinical signals, such as ataxia and weakness, were found in AmB-treated mice, which presented also a reduction in their body weight in the order of 9.0%, possibly reflecting the toxicity of the drug. On the other hand, Flau-A and Flau-A/M-treated mice presented positive variation in their body weight, in the order of 5.0% and 8.0%, respectively (data not shown).

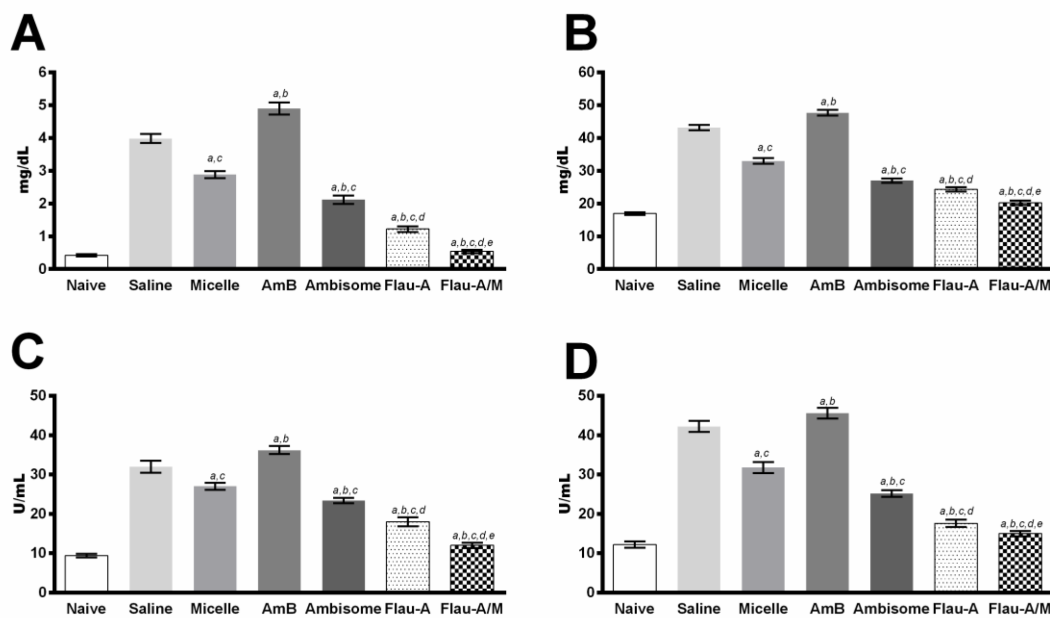

Fig. 7. Toxicity generated in the treated and infected animals. The levels of creatinine (A), urea (B), alanine aminotransferase (C), and aspartate aminotransferase (D) were evaluated in the sera samples of infected and treated mice (n=10 per group), which were collected 30 days after the treatment. Samples of naive (non-infected and non-treated) mice (n=8) were used as control. Bars represent the mean ± standard deviation of the groups. The letters *a*, *b, c*, *d*, and *e* indicate statistically significant differences in relation to the saline, micelle, AmB, Ambisome®, and Flau-A groups, respectively ($P < 0.0001$).

Discussion

Leishmaniasis is a tropical disease affecting populations in developing countries in the world. The treatment against disease can causes toxicity, presents high cost and the parasite resistance is increasing [32]. In this scenario, we antileishmanial compounds, such as those plant-derivates, could be identified and applied for a more effective an non-toxic treatment against the disease [33,34]. A previous study demonstrated for the first time an *in vitro* effective antileishmanial activity of a quinoline derivate, Flau-A, against *L. amazonensis* and *L. infantum* species. Results pointed out also for the parasite mitochondria as being the target organelle of this antileishmanial molecule. As a consequence, in the present study, Flau-A was incorporated into a Poloxamer 407-based polymeric micelle system and tested against *L. amazonensis* infection. Results showed that this product (Flau-A/M) was highly effective against *in vivo* infection, as well as was it was not toxic after application in the mice, thus representing a new candidate to be tested against human disease.

Here, the efficacy of the therapeutics was evaluated by means of immunological and parasitological parameters, which were investigated 30 days after the treatment. Higher periods of time were not possible to be analyzed, due to the severity of the infection in control mice, as well as by the Ethical aspects in our University. Regarding to the cytokine profile, the Flau-A or Flau-A/M-treated mice developed a more polarized Th1 immune profile, which was based on higher levels of IFN-γ, IL-12 and GM-CSF. In addition, these animals produced also higher levels of antileishmanial nitrite, thus demonstrating a specific macrophage activation that can be related with the reduction in the parasite load in the infected tissue and organs of these animals. On the other hand, saline and micelle groups´ mice mounted a polarized Th2 response, which was characterized by high levels of IL-4, IL-10, and parasite-specific IgG1 antibody. Others have also found these immune correlates, when distinct biological candidates against *Leishmania* parasites are evaluated [35-37].

Plant metabolites have been investigated for their antileishmanial and antitrypanosomal activities [38]. Naphthoquinones are present in several plant families, and these products have been employed in traditional medicine as anticancer and microbicidal agents [39,40]. Due to their structural

properties, quinones can participate in multiple oxidative processes. Their fundamental feature is the redox property, which is induced by means of the formation of a fully aromatic system [41]. In spite of the use of synthetic naphthoquinone derivatives, the mechanisms involved in their cytotoxicity are still unknown. The cytotoxic effects of these compounds, such as menadione, might be due to oxidative stress and arylation of cellular thiols [42]. In our model, mice that were infected and later treated with Flau-A or Flau-A/M did not present significant toxicity, when renal and hepatic damage markers were investigated. In addition, Ambisome® group mice showed also low levels of these markers, when compared with the controls; although the values have been higher in comparison to results found in Flau-A or Flau-A/M groups. Otherwise, AmB-treated mice presented significantly higher levels of AST, ALT, urea and creatinine; thus reflecting the toxicity of the drug in this mammalian model, such as showed in other studies [43-45].

Poloxamer 407 (Pluronic F127) was used as a non-ionic surfactant composed of a symmetric tri-block copolymer of propylene oxide and ethylene oxide. The polypropylene oxide block is sandwiched between the more hydrophilic polyethylene oxide blocks [46]. Micelles made of block copolymers, such as Poloxamer 407, have a hydrophobic core and a hydrophilic shell, which can effectively harbor amphiphilic moiety preventing its direct exposure to vital organs [47]. The interface formed by the hydrophilic block prevents the micellar aggregation, the protein recognition and non-specific adherence, thereby sparing body from adverse effects induced by antileishmanial molecules [48]. In addition, Poloxamer 407-based formulations have been successfully used against leishmaniasis, and they can be considered as alternative in comparison to the traditional formulations, in terms of efficacy, target orientation, toxicity, and cost [49].

Usually, these Poloxamer P407-based polymeric micelles are administered by subcutaneous route in murine models, thus turning into a semi-rigid gel when in contact with the local tissue, and creating a reservoir system aiming to maintain the product in the extracellular space to allow its action against parasites in the infection site. In course of time, as the gel matrix is diluted by body fluids and phagocytosis, the product will be gradually released into the circulation, enabling its systemic action in a controlled manner [26]. Here, Flau-A was incorporated to this delivery system, and the formed compound was showed to be highly effective against *L. amazonensis* infection. In addition, parasitological analyses demonstrated the systemic efficacy of

the treatment using Flau-A/M, since significant reductions in the parasite load were also observed in spleen, liver, and dLN of the treated and infected animals. Similar results were also found when other natural or synthetic molecules were associated to delivery systems and administered in *Leishmania*-infected mice [50,51].

As limitation of our work, distinct therapeutic regimens and parasitological and immunological analyses in other periods of time were not evaluated. In addition, the products were administered by different routes in the infected animals, and it can be considered as a variable to interfere in the results obtained in our study. Nevertheless, we believe that the present work can be considered relevant, since the Flau-A-containing micelles showed an *in vivo* effective antileishmanial action, which was associated with low toxicity in our tested murine model. As a consequence, and due to its stability and easily of production, Flau-A/M can be considered in future studies as an antileishmanial agent against human leishmaniasis.

Authors´ contribution

Conceived and designed the experiments: EAFC DVCM GSVT DSD DPL PAFR FL. Performed the experiments: DVCM GSVT DSD TGS LMC DSD FMO LMRA DLV MCD BMR DMS. Analyzed the data: EAFC MACF. Contributed reagents/materials/analysis tools: JMB RJA ESC. Wrote the paper: EAFC.

Conflict of interest

The authors confirm that they have no conflicts of interest in relation to this work.

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5.4. Considerações finais do artigo 2

O tratamento de camundongos Balb/C com leishmaniose tegumentar com a Flau-A na sua forma livre e na sua forma micelar (Flau-A/M) levou a uma redução na

carga parasitária nos órgãos avaliados e no tamanho das lesões quando comparadas aos grupos controle.

Houve um aumento nas dosagens de IFN-Ɣ, nitrito, IL-12, GM-CSF e IgG-2a e uma diminuição nas dosagens de IL-4, IL-10 e IgG-1 nos grupos tratados com Flau-A e Flau-A/M quando comparados aos grupos contole, sugerindo uma polarização da resposta de linfócitos T do tipo 1 (pró inflamatória) em detrimento da do tipo 2 (anti-infamatória).

Os animais tratados com Flau-A e Flau-A/M apresentaram uma diminuíçaõ na concentração sérica de AST, ALT, Ureia e creatinina, indicando que o tratamento leva a uma redução nos danos hepáticos e renais causados pela própria infecção.

Tendo em vista os resultados obtidos, Flau-A e Flau-A/M, demonstraram efetividade no tratamento da leishmaniose tegumentar *in vivo* em um modelo murino.

5.5. ARTIGO 3

Neste trabalho o objetivo foi avaliar a eficácia terapêutica da Flau-A e de Flau-A/M frente a um modelo murino de leishmaniose viceral.

Artigo submetido ao periódico *Experimental Parasitology*

Title: A naphthoquinone derivative, Flau-A, shown to be effective against tegumentary leishmaniasis, is also therapeutic against Leishmania infantum infection and visceral leishmaniasis

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Abstract

Flau-A, a naphthoquinone derivative, has recently been tested against tegumentary leishmaniasis and proved to be a therapeutic agent against the disease. In the present study, Flau-A was tested free or incorporated into Poloxamer 407-based micelles against *Leishmania infantum*. Parasitological and immunological analyses were performed at one and 15 days post-therapy. Results showed that treatment with miltefosine used as a control drug, free Flau-A or its micellar composition (Flau-A/Mic) induced significantly higher levels of antileishmanial IFN-ɣ, IL-12, GM-CSF and nitrite than the corresponding controls. Lower levels of IL-4 and IL-10 were also observed in stimulated spleen cells, both at one and 15 days post-treatment, suggesting a long-term efficacy. Evaluation of the humoral response showed predominance of anti-parasite IgG2a isotype antibody in sera of treated animals, suggesting also development of a Th1-type response. Furthermore, mice treated with either miltefosine, Flau-A or Flau-A/Mic presented significant reductions in the parasite load in their spleens, livers, bone marrows and draining lymph nodes. In addition, organ toxicity was not observed with neither of these compounds. All in all, treatment of *L. infantum*-infected mice with Flau-A/Mic showed the best performance in both parasitological and immunological analyses, and therefore merits further consideration for therapy of visceral leishmaniasis (VL).

Keywords: Visceral leishmaniasis; Flau-A; treatment; miltefosine; delivery system; toxicity.

1. Introduction

Leishmaniases are diseases caused by protozoan parasites of the genus *Leishmania* that affect the poorest populations in developing countries, being endemic in 98 countries with a global incidence estimated of about 0.9 to 1.6 million new cases per year [1]. There are about 20 parasite species able to cause the disease in humans, and tegumentary (TL) and visceral (VL) leishmaniasis are the main clinical forms known [2]. TL can cause a large spectrum of clinical manifestations in humans, ranging from single cutaneous lesions to mucosal tissue destruction [3]. VL is the most severe clinical form of leishmaniasis and it can cause prolonged fever, anemia, hepatosplenomegaly, hypergammaglobulinemia, and pancytopenia, which can lead patients to death if the disease is acute and is left untreated [4,5].

One of the most important aspects of Leishmania infection is the ability of the parasites to evade the infected host´s immune response [6]. If well-successful, they persist within the infection site and, depending on the species, can disseminate to internal organs and establish an acute infection. Typically, the development of a parasite-specific Th2-type response is related to the occurrence of active disease, with high levels of cytokines such as IL-4, IL-10 and IL-13 in the infected hosts [7,8]. On the other hand, the protective response to Leishmania infection is predominantly a Th1-type immune response, which is associated with the production of cytokines such as IFN-ɣ, IL-12, GM-CSF, among others [9,10]. Thus, those therapeutics able to subvert the hosts' immune profile to a protective Th1-type immune response are suitable candidates to guarantee a long-term therapeutic response against re-infection [11,12].

Treatment against leishmaniasis presents some limitations, such as parasite resistance, side effects, prolonged duration and complicated drug administration [13]. Pentavalent antimonials, such as sodium stibogluconate (Pentostam®, GlaxoSmithKline, United Kingdom) and meglumine antimoniate (Glucantime®, Aventis, France), are the first choice to treat leishmaniasis. However, administration of these drugs is unpleasant for the patient and they are highly toxic, normally causing chemical pancreatitis, increased levels of aminotransferases in serum, electro-cardiographic abnormalities, among others [14,15]. Other therapeutic options include pentamidine, paramomycin, miltefosine and free or liposomal amphotericin B, which benefits are similarly limited by their toxicity and/or high cost [16-18].

Therefore, there is the urgent need to identify new, safe and effective antileishmanial agents.

Natural products have been a valuable source for the identification of new pharmaceuticals [19-21]. Numerous metabolites from different chemical classes isolated from plant, microbial and marine sources such as alkaloids, phenolic compounds, quinones, terpenes, among others, have demonstrated similar and, in some cases, superior potency associated with low toxicity in comparison to commercial drugs. Naphthoquinones have been used as promising scaffolds for drug design, since these compounds present with diverse biological activities [22-24], such as antileishmanial action [25,26].

We recently tested a naphthoquinone derivative called 2-(2,3,4-tri-O-acetyl-6-deoxy-β-L-galactopyranosyloxy)-1,4-naphthoquinone (Flau-A) against *L. amazonensis* infection in BALB/c mice, when administered free or incorporated into Poloxamer 407-based polymeric micelles [27]. In that study, the micellar composition (Flau-A/M) induced the highest reductions in the lesion average diameter and parasite burden in the infected tissue, spleen, liver and draining lymph nodes of mice, which were associated with a polarized antileishmanial Th1-type immune response characterized by the production of IFN-ɣ, IL-12, TNF-α and GM-CSF in the stimulated spleen cells. In the present study, and following the rationale to identify new and/or repurpose broad-spectrum antileishmanial agents, the efficacy of free Flau-A or within the micellar system was evaluated against *L. infantum*, the main parasite species responsible for the majority of VL cases in the Americas. The efficacy of the therapeutics was compared to the use of miltefosine, a very well characterized antileishmanial oral drug [12,28,29]. Immunological, parasitological and toxicological parameters were evaluated one and 15 days post-treatment.

2. Materials and Methods

2.1. Chemicals and preparation of Flau-A/Mic composition

Poloxamer 407 and miltefosine were purchased from Sigma-Aldrich (USA), with catalog numbers 16758 and 58066-85-6, respectively. The technical protocol described by Mendonça et al. [27] was used for the production of Flau-A, as well as

for preparing the micelles. Briefly, Poloxamer 407 (18% w/w) was diluted in phosphate buffered saline (PBS) at pH 7.4 under magnetic agitation, for 18 h at 4°C. Then, Flau-A (8.0 mg) was diluted in 500 μL dichloromethane and solubilized by vortexing. The mixture was added to the previously prepared Poloxamer solution under vigorous magnetic agitation in an ice bath, until a viscous emulsion was obtained. The dichloromethane solution was evaporated by using a rotary evaporator (Buchi, Flawil, Switzerland), and the final product was obtained as a transparent yellow gel at room temperature. The molecule content incorporated into the micelles was evaluated spectrophotometrically by using an ultraviolet method as described previously [30]. For this, the Flau-A/Mic-containing solution was mixed with methanol PA and the absorbance was measured in a spectrophotometer (Double beam AJX-6100 PC; Micronal, São Paulo, Brazil) at 380 nm. The concentration of Flau-A was calculated using a standard curve (0 to 18.0 μM), which was previously prepared in methanol PA. The analyses were carried out in triplicate. Empty micelles (Poloxamer 407 18% w/w) were prepared using the same protocol described above.

2.2. Mice

Female BALB/c mice (8 weeks old) were purchased from the Institute of Biological Sciences of Federal University of Minas Gerais (UFMG; Belo Horizonte, Minas Gerais, Brazil), and were kept under pathogen-free conditions. The study was approved by the Committee for the Ethical Handling of Research Animals of UFMG (protocol number 085/2017).

2.3. Parasite

Stationary promastigotes of *L. infantum* parasite (MHOM/BR/1970/BH46) were grown at 24ºC in complete Schneider's medium (Sigma-Aldrich, USA) supplemented with 20% (v/v) heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin and 100 μg/mL streptomycin, at pH 7.4. Soluble Leishmania antigenic extract (SLA) was prepared as described previously [31]. Briefly, parasites (10^{9} stationary promastigotes) were washed three times in sterile PBS at pH 7.4, frozen and thawed five times and ultrasonicated (Ultrasonic processor, GEX600) using five cycles of 30 sec at 38 MHz. The suspension was centrifuged at 9,000 x g for 30 min at 4°C and

the supernatant containing SLA was collected. The protein concentration was estimated by the Bradford method [32].

2.4. Infection and treatment schedules

Mice (n=12 per group) were infected subcutaneously in their right hand footpad with 10^7 *L. infantum* stationary promastigotes. Forty-five days post-infection, infected animals were administered with saline alone (50 µL, PBS); empty micelles (50 µL B/Mic, 5 mg/kg body weight); miltefosine (50 µL, 2 mg/kg body weight); free Flau-A (50 µL, 5 mg/kg body weight); or Flau-A-containing micelles (50 µL Flau-A/Mic, 5 mg/kg body weight). Except for miltefosine which was administered by oral route, all other compounds were administered subcutaneously, every two days for a period of 10 days. Half of the animals were euthanized one and 15 days post-treatment, after which parasitological and immunological evaluations were performed. Experiments were performed in duplicate.

2.5. Cellular response

Spleens of the infected and treated mice were collected one and 15 days after treatment. Spleen cells (5 x 10^6 per mL) were plated in duplicate in 24-well plates (Nunc) and incubated in DMEM supplemented with 20% (v/v) FBS and 20 mM L-glutamine at pH 7.4 (medium) or stimulated with *L. infantum* SLA (50.0 µg/mL) for 48 h at 37°C in 5% (v/v) CO2. IFN-ɣ, IL-4, IL-10, IL-12p70, and GM-CSF levels were measured in the cell supernatant by capture ELISA using commercial kits (BD Pharmingen®, San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was evaluated in the same cell supernatant by Griess reaction [33]. A flow cytometry assay was also performed in order to evaluate the IFN-γ, TNF-α and IL-10-producing CD4+ and CD8+ T-cell frequency after treatment [12]. Briefly, cells (5 x 10^6 per mL) were incubated in RPMI 1640 medium in polypropylene tubes (Pharmingen®) and were left untreated (medium) or stimulated with SLA (50.0 µg/mL) for 48 h at 37°C in 5% (v/v) CO2. IFN-ɣ, TNF-α and IL-10-producing CD4+ and CD8+ T-cell frequencies were analyzed based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC). After selection of the region R1 of interest containing FSCLow and SSCLow phenotype

cells, density plots for the distribution of CD4/FL1 or CD8/FL1 versus IFN-γ/FL2+, TNF-α/FL2+ and IL-10/FL2+ cells were performed to determine the IFN-γ+, TNF-α+, and IL-10+ T cell frequencies in the cultures. Results were expressed as indexes, which were calculated as the ratio between the cytokine-producing T cell percentages versus the corresponding values in the unstimulated cultures.

2.6. Humoral response

Parasite-specific IgG1 and IgG2a antibody levels were measured in sera samples collected from the infected and treated mice, one and 15 days post-treatment. *L. infantum* SLA was used as the antigen (1.0 µg per well) and samples were diluted 1:100 in PBS-T (PBS plus 0.05% (v/v) Tween 20). Anti-mouse IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich, USA) were used both at a 1:10,000 dilution in PBS-T. Reactions were developed by adding a substrate solution composed by H_2O_2, ortho-phenylenediamine and citrate-phosphate buffer (pH 5.0) and incubation for 30 min in the dark. Next, reactions were stopped by addition of 2 N H_2SO_4 and the optical density (OD) values were immediately read in a spectrophotometer (Molecular Devices, Spectra Max Plus, Canada) at 492 nm.

2.7. Parasite burden

The parasite load was evaluated in the spleen, liver, bone marrow (BM) and draining lymph nodes (dLNs) of treated mice by limiting dilution technique, one and 15 days post-treatment [12]. Briefly, organs were collected and macerated in a glass tissue grinder using sterile PBS, and tissue debris were removed by centrifugation at 150 × g. Cells were concentrated by centrifugation at 2,000 x g and pellets were resuspended in 1 mL of complete Schneider's medium and then serially diluted (10-1 to 10-12) in the same medium. Cells were cultured in triplicate at 24°C for 7 days before being analyzed. Results were expressed as the negative log of the titer (the dilution corresponding to the last positive well) adjusted per milligram of organ. Parasitism in the spleen of treated animals was evaluated also by quantitative Polymerase Chain Reaction technique (qPCR) [27]. Briefly, standard curves for kDNA and β-actin were obtained using DNA extracted from 10^8 parasites and 10^8 peritoneal macrophages, respectively, under the same conditions used to extract the samples. Reactions were developed in an ABI Prism 7500 Sequence Detection

System (96 wells-plate; Applied Biosystems) using 2x SYBRTM Select Master Mix (5 µL; Applied Biosystems), with each primer at 2 mM (1 µL) and 4 µL DNA (25 ng/µL). Samples were incubated at 95°C for 10 min and subjected to 40 cycles of 95°C for 15 s and 60°C for 1 min. After each cycle, fluorescence data were recorded. Parasite load was quantified by interpolation of the standard curve, which was performed in duplicate, and expressed as the number of parasites per nucleated cell (multiplied by one thousand to facilitate visualization).

2.8. Toxicity in vivo

Toxicity in vivo was evaluated one and 15 days after therapy. Urea, creatinine, alanine transaminase (ALT) and aspartate transaminase (AST) levels were measured in murine sera using commercial kits (Labtest Diagnostica®, Belo Horizonte), according to the manufacturer's instructions. As a control, sera samples collected form uninfected and untreated mice (n=6) were used.

2.9. Statistical analysis

Data analyses were performed in Microsoft Excel software (version 10.0) and GraphPad PrismTM (version 6.0 for Windows; GraphPad Software, Fay Avenue, La Jolla, CA, USA). One-way analysis of variance (ANOVA) followed by Bonferroni´s post-test were used for comparisons between the groups. Differences were considered significant when $P < 0.05$. All experiments were performed in duplicates, with similar outcomes.

3. Results

3.1. Cellular response after treatment

The immunological profile of the infected mice was evaluated one and 15 days post-treatment. Results showed that, one day after therapy, spleen cells collected from the miltefosine, Flau-A or Flau-A/Mic treated mice produced significantly higher levels of IFN-ɣ, IL-12, and GM-CSF than the corresponding controls, with associated lower levels of IL-4 and IL-10 (Fig. 1A). Importantly, this Th1-type immune response was maintained 15 days post-treatment (Fig. 1B). In contrast, control mice receiving saline or B/Mic produced significantly higher levels of IL-4 and IL-10, both one and 15 days after therapy (Fig. 1). Nitrite levels were evaluated in the cell supernatants as an indicator of nitric oxide (NO) production. Both one (Fig. 2A) and 15 (Fig. 2B) days

post-treatment, levels of this molecule were found significantly higher in miltefosine, Flau-A or Flau-A/Mic treated mice, when compared to the saline and B/Mic mice groups. A flow cytometry assay showed also that mice groups receiving miltefosine, Flau-A or Flau-A/Mic presented higher levels of IFN-ɣ and TNF-α-producing CD4+ and CD8+ T cells, when compared to the control groups, which showed higher IL-10-producing T-cell presence (Fig. 3). Overall, by comparing all groups, Flau-A/Mic-treated mice were those that developed the most polarized Th1-type cellular response.

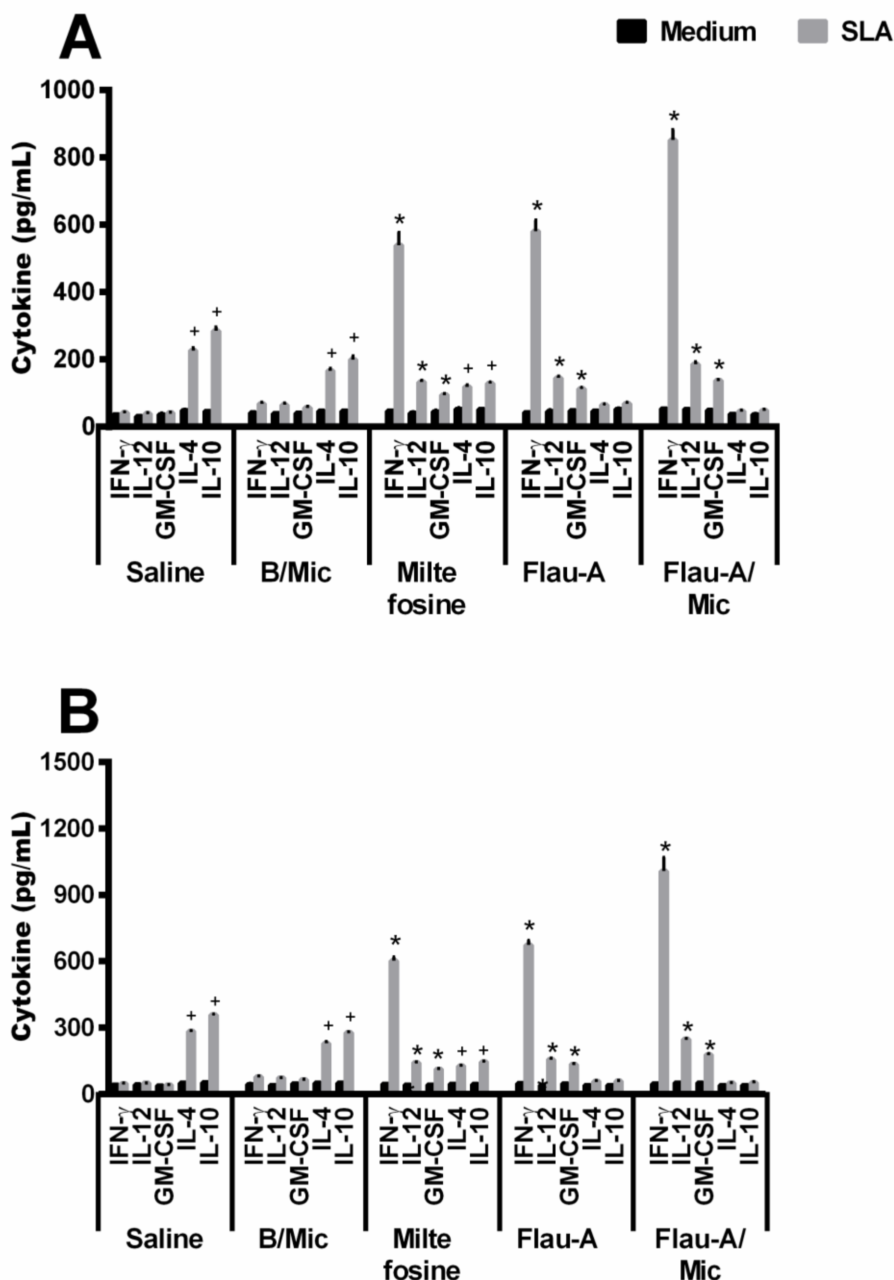

Fig. 1. Cellular response developed after treatment. Splenocytes from the infected mice (n=12 per group) were collected one and 15 days post-treatment, when cells (5 x 10^6 per mL) were left untreated (medium) or stimulated with *L. infantum* SLA (50.0 μg/mL) for 48 h at 37°C in 5% (v/v) CO_2. IFN-γ, IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the cell supernatant by capture ELISA, one (A) and 15 days (B) post-treatment. Bars indicate the mean plus standard deviation of the groups. (*) indicate statistically significant difference in relation to the saline and Mic/B control groups (P < 0.0001). (+) indicate statistically significant difference in relation to the miltefosine, Flau-A, and Flau-A/Mic groups (P < 0.001).

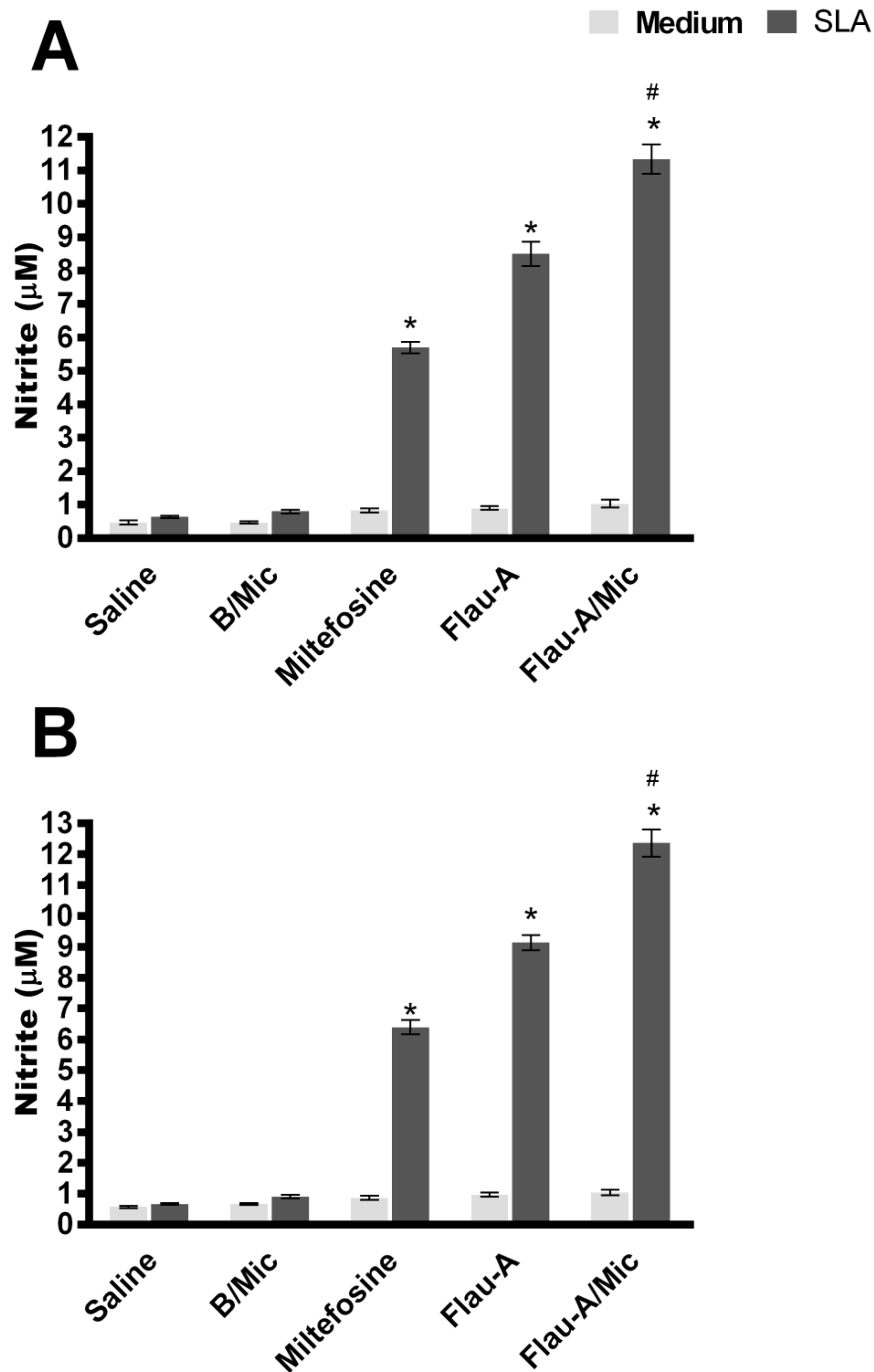

Fig. 2. Nitrite secretion. Spleen cells collected from the infected mice (n=12 per group) one (A) and 15 days (B) post-treatment were left untreated (medium) or stimulated with *L. infantum* SLA (50.0 µg/mL) for 48 h at 37°C in 5% (v/v) CO_2. Nitrite levels were measured in the cell supernatants by Griess reaction. Bars indicate the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the saline and Mic/B control groups (P < 0.0001). (#) indicate statistically significant difference in relation to the miltefosine, Flau-A, and Flau-A/Mic groups (P < 0.0001).

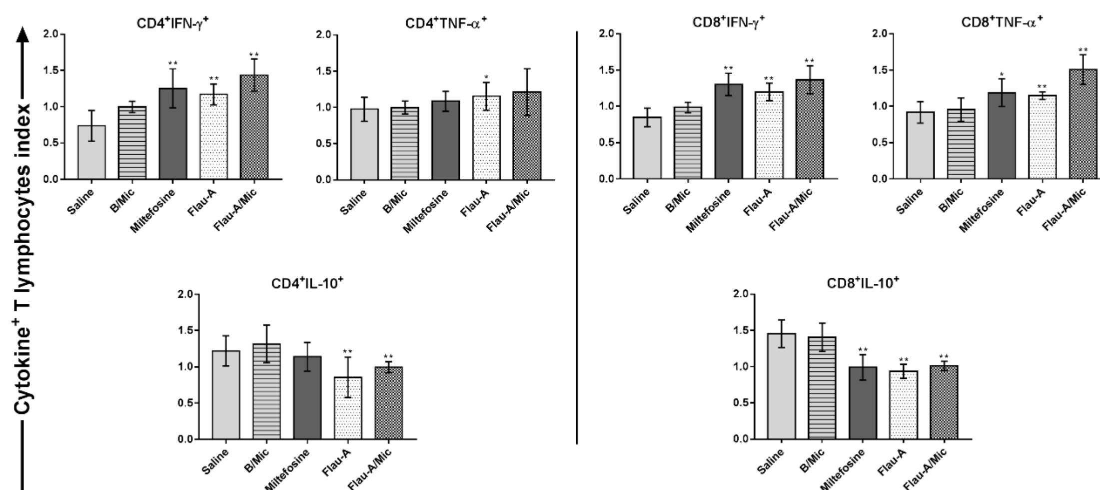

Fig. 3. Intracytoplasmic cytokine-producing CD4$^+$ and CD8$^+$ T-cell subtype profile. BALB/c mice (n=12 per group) were infected and then treated with empty micelles (B/Mic), miltefosine, Flau-A or Flau-A/Mic, or left untreated (saline). Fifteen days post-treatment, animals were euthanized and their splenocytes were collected. Cells were left untreated (medium) or stimulated *in vitro* with *L. infantum* SLA (50.0 µg/mL) for 48 h at 37°C in 5% (v/v) CO_2. IFN-γ-, TNF-α- and IL-10-producing CD4$^+$ and CD8$^+$ T-cell frequency was evaluated by flow cytometry assay and data was expressed as cytokine indexes, corresponding to the ratio between the stimulated culture and the control culture. Bars indicate the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the saline control group (P < 0.05). (**) indicate statistically significant difference in relation to the B/Mic control group (P < 0.05).

3.2. Humoral response developed post-treatment

Antileishmanial IgG1 and IgG2a isotype levels were evaluated in the infected mice, one and 15 days after treatment. One day after therapy, miltefosine, Flau-A or Flau-A/Mic treated mice produced significantly higher IgG2a levels in comparison to IgG1 values (Fig. 4A). This humoral response was maintained 15 days post-treatment (Fig. 4B). In contrast, saline and B/Mic mice groups produced significantly higher anti-parasite IgG1 levels both one and 15 days post-treatment (Fig. 4). In agreement with the observed cellular response, antibody isotype production indicated that Flau-A/Mic-treated mice developed a more polarized Th1-type humoral response

than all other treated groups, based on the induction of higher IgG2a levels in response to treatment.

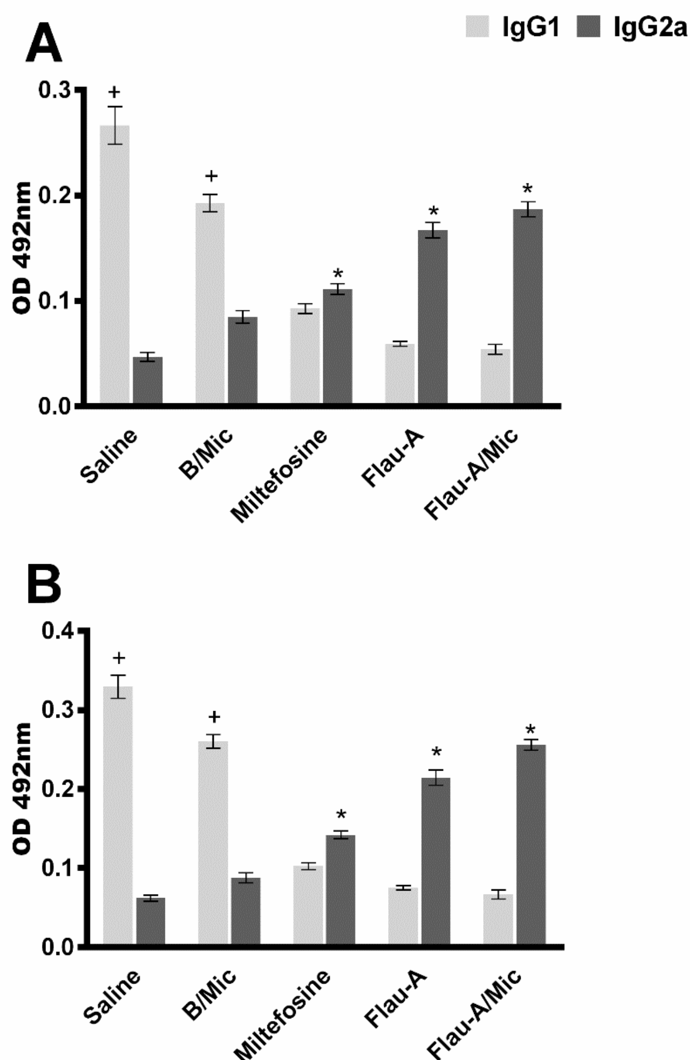

Fig. 4. Humoral response generated after treatment. Levels of anti-parasite IgG1 and IgG2a isotype antibodies measured on sera samples collected from infected mice (n=12 per group), one (A) and 15 (B) days after treatment. Bars indicate the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the saline and B/Mic control groups (P < 0.0001). (+) indicate statistically significant difference in relation to the miltefosine, Flau-A, and Flau-A/Mic groups (P < 0.0001).

3.3. Parasite load evaluated in the treated animals

The parasite load was evaluated in distinct organs of the infected animals, one and 15 days post-treatment, by limiting dilution technique. Miltefosine, Flau-A or Flau-A/Mic treated mice presented significant reductions in the parasitism in their liver, spleen, BM and dLNs, when compared to the saline and B/Mic control groups, at both time points after therapy (Fig. 5). One day post-treatment, the parasite burden

in the miltefosine, Flau-A or Flau-A/Mic treated mice was significantly reduced at 54.5%, 72.7% and 90.0%, respectively, in their livers (Fig. 5A); 57.1%, 64.3% and 85.7%, respectively, in their spleens (Fig. 5B); 56.3%, 68.7% and 87.5%, respectively, in their dLNs (Fig. 5C); and 50.0%, 62.5% and 87.5%, respectively, in their BMs (Fig. 5D), when compared to the saline group. Fifteen days post-treatment, reductions in the parasite load in miltefosine-, Flau-A- and Flau-A/Mic-treated mice were of 42.9%, 57.1% and 85.7%, respectively, in their livers (Fig. 5A); 62.5%, 68.8% and 87.6%, respectively, in their spleens (Fig. 5B); 57.9%, 68.4% and 84.2%, respectively, in their dLNs (Fig. 5C); and 36.4%, 54.6% and 81.8%, respectively, in their BMs (Fig. 5D), when compared to the saline group. The splenic parasitism was also evaluated by qPCR technique, with similar results showing parasite load reductions of 39.4%, 75.8% and 97.0% after one day of treatment with miltefosine, Flau-A and Flau-A/Mic, respectively, (Fig. 6), and of 38.5%, 66.7% and 92.3%, respectively, 15 days after therapy. Notably, Flau-A/Mic-treated mice group showed the highest reduction in the parasite load in all of the organs analyzed, when compared to all other groups, with both parasitological techniques.

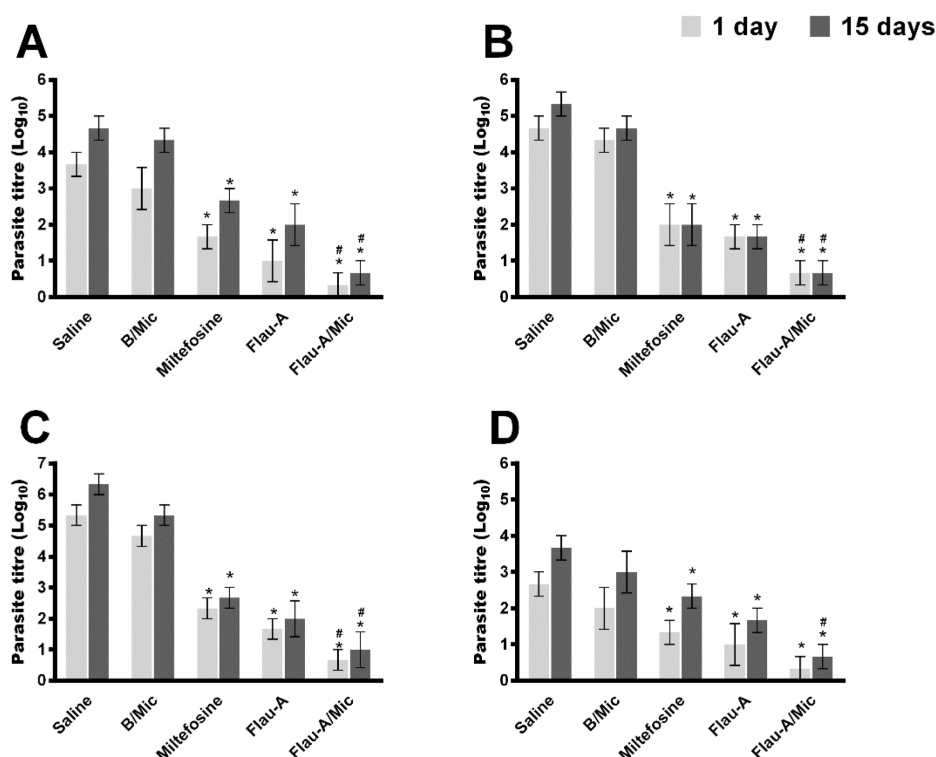

Fig. 5. Parasite load. The parasite load was evaluated by limiting dilution technique in the livers (A), spleens (B), draining lymph nodes (dLN) (C) and bone marrows (BM) (D) collected from the infected mice (n=12 per group), one and 15 days post-treatment. Bars indicate the mean ± standard deviation

of the groups. (*) indicate statistically significant difference in relation to the saline and B/Mic control groups (P < 0.0001). (#) indicate statistically significant difference in relation to the miltefosine and Flau-A groups (P < 0.005).

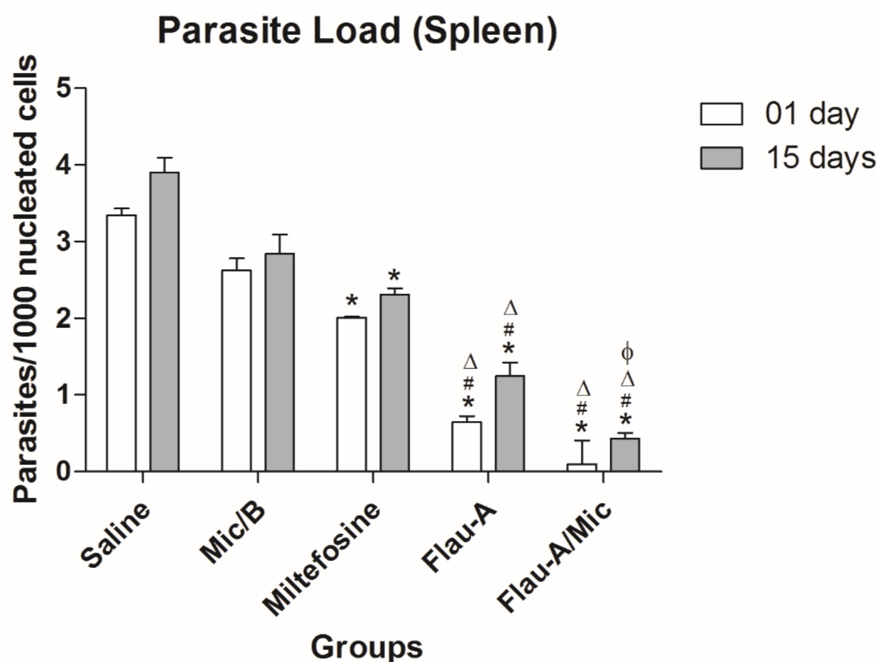

Fig. 6. **Splenic parasite load.** Splenic parasitism was also evaluated by qPCR technique on the infected mice (n=12 per group), one and 15 days post-treatment. Bars indicate the mean plus standard deviation of the groups. (*) indicate statistically significant differences in relation to the saline control group (P < 0.05), (#) indicate statistically significant differences in relation to the B/Mic control group (P < 0.05), (Δ) indicate statistically significant differences in relation to the miltefosine group (P < 0.05) and (ϕ) indicate statistically significant differences in relation to the Flau-A group (P < 0.05).

3.4. Toxicity in vivo

Toxicity of the therapeutics was evaluated in vivo using sera samples obtained from the infected and treated animals. Results showed that saline and B/Mic control groups presented significantly higher levels of creatinine (Fig. 7A), urea (Fig. 7B), ALT (Fig. 7C) and AST (Fig. 7D), when compared to miltefosine, Flau-A and Flau-A/Mic treated mice. Among the three therapeutics tested, Flau-A/Mic-treatment was the one resulting in the lowest levels of these enzymatic markers of organ damage, suggesting this micellar composition could be suitable for administration to other mammalian models of infection.

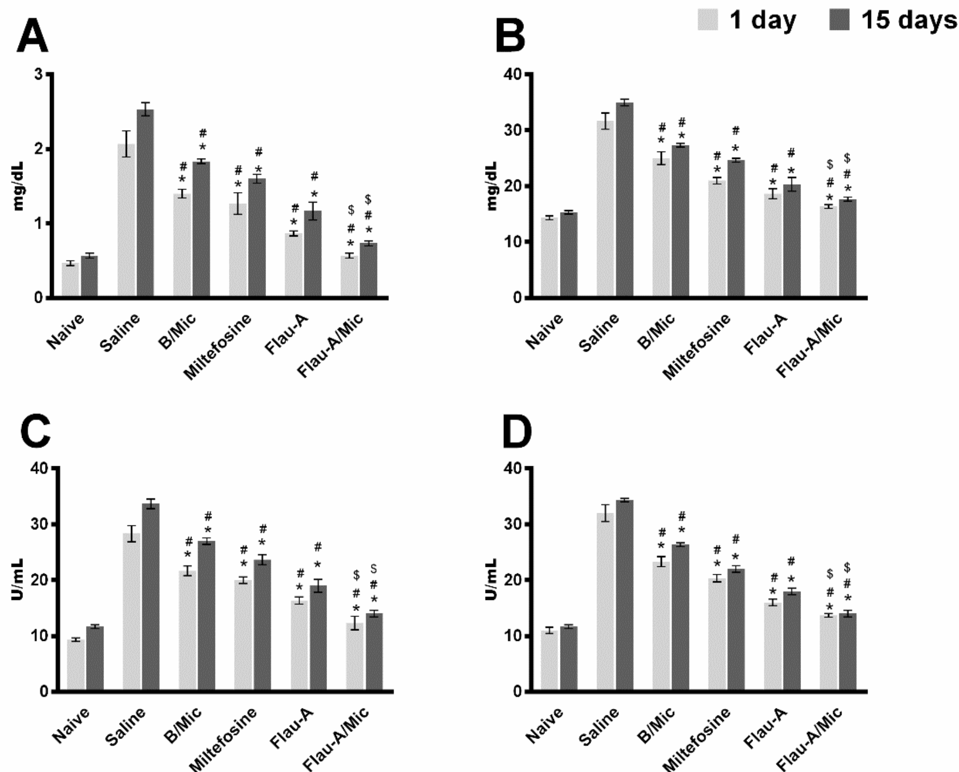

Fig. 7. Determination of compound toxicity *in vivo*. Levels of creatinine (A), urea (B), alanine aminotransferase (C) and aspartate aminotransferase (D) were measured in sera samples obtained from infected mice (n=12 per group), one and 15 days post-treatment. Samples collected from uninfected and untreated mice (n=6) were used as control (naive). Bars indicate the mean ± standard deviation of the groups. (*) indicate statistically significant difference in relation to the uninfected and untreated mice group (P < 0.05). (#) indicate statistically significant difference in relation to the saline group (P < 0.05). ($) indicate statistically significant difference in relation to the B/Mic, miltefosine and Flau-A groups (P < 0.05).

4. Discussion

Drugs normally used to treat VL are toxic and/or expensive [13]. With an urgent need to improve the quality of the treatment, a search for new, less toxic and affordable antileishmanial agents is now a priority. Natural compounds have long been a valuable source of new medicinal agents against leishmaniasis [21,34]. Previous studies of naphthoquinone derivatives have shown that these molecules present medically relevant biological activities, such as antileishmanial action [25,35,36]. In a recent study, we tested Flau-A, a naphthoquinone derivative, in a BALB/c mice infection model with *L. amazonensis*, a species reported to cause TL in the Americas [27]. In that study, we showed that when Flau-A was incorporated into a Poloxamer 407-based polymeric system, it was able to reduce significantly the

levels of the parasite load in the infected murine tissues and organs after treatment, as well as to induce development of an antileishmanial Th1-type immune response.

With the aim to identify new and broad-spectrum antileishmanial agents that could exert their function against different Leishmania species, in the present study, free Flau-A and incorporated into a micellar system were evaluated against L. infantum infection for the treatment of VL. Two time points post-treatment were evaluated aiming to verify the long-term therapeutic action of the compound, using miltefosine as a control drug. A significant antileishmanial action of this naphthoquinone derivative was observed in vivo when used for treating the infected mice. Significant reductions in the parasite load in their spleens, livers, BMs and dLNs were found, in agreement with the development of a specific Th1-type immune response. In addition, no significant toxicity was observed due to the treatment. As expected, miltefosine also showed therapeutic action against infection, with significant reductions in the organ parasitism and development of a Th1-type response. However, treatment with the Flau-A/Mic composition was the most effective in terms of its parasitological and immunological performance.

Miltefosine is used as an oral antileishmanial drug worldwide [37,38], and it has been proved effective to protect against infections with distinct parasite species [39-41]. However, several reports have shown also disease relapse and the occurrence of post-kala-azar dermal leishmaniasis in miltefosine-treated patients, apart from negative side effects related to its use [42,43]. In the present study, a toxicological analysis performed in murine sera samples indicated a slight degree of hepatic and renal damage in miltefosine-treated mice, when compared to the treatment with either Flau-A in its free form or Flau-A/Mic. Lower toxicity of Flau-A (especially in the form of a micellar composition) in comparison to miltefosine suggests this novel compound could be considered also in future studies using other mammalian models for Leishmania infection, and probably testing alternative delivery systems. Low toxicity for other naphthoquinone derivatives has been similarly reported for the treatment of mammalian hosts with *Leishmania* infections [44-46].

Development of a Th1-type immune response, characterized by IFN-ɣ production, is key to fight Leishmania infections in mammalian hosts. Progression of a Th2-type response, identified by the production of anti-inflammatory cytokines such as IL-4 and IL-10, is instead detrimental and is in fact associated to the occurrence of active disease [47-49]. In our study, SLA-stimulated spleen cells collected from

miltefosine-, Flau-A- and Flau-A/Mic-treated mice produced significantly higher levels of IFN-γ, IL-12 and GM-CSF, with concomitant lower levels of IL-4 and IL-10, both one and 15 days post-therapy. These observations suggest long re-stimulation of T cells after treatment. The presence of nitrite in the stimulated spleen cells was evaluated as an indicator of NO production, a key effector molecule for parasite killing inside macrophages [50,51]. Results showed that miltefosine, Flau-A and Flau-A/Mic treated mice cells produced higher levels of this molecule, implying this protective mechanism of action against parasites was taking place in vivo. In all cases, results suggested that the Flau-A/Mic composition was the most effective immunotherapeutic agent tested against *L. infantum* infection.

Apart from the identification of new antileishmanial targets, the use of delivery systems has improved the quality of immune response and allowed to reduce the toxicity of traditional treatments with old drugs, as lower concentrations of active compound are required when incorporated into delivery systems [52]. Nanoparticles, microspheres and micelles have been applied with such therapeutic purposes [53-55]. The use of Poloxamer-based polymeric micelles for the delivery of antileishmanial agents has improved the drug efficacy against distinct Leishmania spp. For instance, Espuelas et al. [56] showed that AmpB-incorporated Poloxamer 188 (P188) micelles were effective in stimulating murine macrophages to kill *L. donovani* parasites. The authors demonstrated a synergic action between micelles and AmpB and suggested their interaction with the parasite membrane, which promoted the insertion of AmpB into the parasite. Singh et al. [57] developed a micellar formulation composed by AmpB-loaded Pluronic F127 micelles. The product was used to treat *L. donovani*-infected hamsters, and results showed that it stimulated the development of antileishmanial Th1-type response in the treated animals without causing toxicity. That study reported also significant reduction in the organ parasitism after treatment.

The lack of comparison between different dose schedules can be considered a limitation of this study. Nevertheless, the data here presented strongly suggest an antileishmanial action in vivo of Flau-A against *L. infantum* species, mainly when administered within Pluronic® F127-based polymeric micelles. Results clearly showed specific production of Th1-type cytokines with resulting significant reduction of parasitism in distinct organs. In sum, Flau-A/Mic is a strong antileishmanial

therapeutic candidate which merits serious consideration in future studies of TL and VL treatments in other mammalian hosts.

Conflict of interest

The authors confirm that they have no conflicts of interest in relation to this work.

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5.6. Considerações finais do artigo 3

Os resultados obtidos a partir de parâmetros celulares e imunológicos que são a diminuição da carga parasitária, aumento na produção de IFN-ɣ, IL-12, nitrito, TNF-α e IgG-2a, indicam uma polarização da resposta de linfócitos T para o tipo 1, de resistência á infecção, no grupo de animais tratados com Flau-A e Flau-A/M e de polarização para a resposta de linfócitos T do tipo 2, de suscetibilidade, para os animais dos grupos controle, demonstrada pelo aumento da carga parasitária nos órgãos avaliados, e aumento na produção de IL-10, IL-4 e IgG-1.

Além disso, a substância pura ou veiculada no sistema micelar foi capaz de reduzir a concencentração sérica de marcadores de danos hepáticos e renais (AST, ALT, ureia e creatinina), indicando ausência de toxicidade das mesmas nos referidos órgãos.

Assim, Flau-A e Flau-A incorporada em um sistema micelar de delivery se mostraram eficazes para o tratamento de leishmaniose viceral causada por *Leishmania infantum* em camundongos Balb/C.

6. CONCLUSÃO FINAL

Com base nos resultados apresentados nos artigos científicos, pode-se concluir que a molécula Flau-A e sua formulação micelar pode ser considerada para estudos futuros com vistas ao tratamento contra as leishmanioses tegumentar e visceral.

7. PERSPECTIVAS

- Avaliar a composição micelar contendo Flau-A em outros modelos de mamíferos, como hamster, para proteção contra as leishmanisoes tegumentar e visceral.

- Realizar a associação da Flau-A micelar com fármacos já empregados para o tratamento das leishmanioses e testar a maior ou menor eficácia da associação em comparação ao uso das drogas livres.

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ANEXOS

AUTORIZAÇÃO DO COMITÊ DE ÉTICA PARA EXPERIMENTAÇÃO ANIMAL

UNIVERSIDADE FEDERAL DE MINAS GERAIS

CEUA
COMISSÃO DE ÉTICA NO USO DE ANIMAIS

CERTIFICADO
Certificamos que o projeto intitulado "Emprego de moléculas sintéticas no tratamento in vitro e in vivo das leishmanioses.", protocolo do CEUA: 85/2017 sob a responsabilidade de Eduardo Antonio Ferraz Coelho que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem) para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da Lei nº 11.794, de 8 de outubro de 2008, do Decreto nº 6.899 de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA) DA UNIVERSIDADE FEDERAL DE MINAS GERAIS, em reunião de 05/06/2017.

Vigência do Projeto (requerida pelo pesquisador)	20/05/2017 a 10/01/2021
Finalidade	Pesquisa
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)

Sexo	feminino
Origem	Biotério do ICB/UFMG
***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
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***Espécie/linhagem**	Camundongo isogênico / BALB/c
Nº de animais	8
Peso/Idade	20g / 8(semanas)
Sexo	feminino
Origem	Biotério do ICB/UFMG

Considerações posteriores:

05/06/2017	Aprovado na reunião do dia 05/06/2017. Validade: 05/06/2017 à 04/06/2022

Belo Horizonte, 23/09/2018.

Atenciosamente,

Sistema Solicite CEUA UFMG
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DEPÓSITO DE PATENTE PARA UTILIZAÇÃO DE [2- (2,3,4-TRI-O-ACETIL-6-DESOXI-B-L-GALACTOPIRANOSILOXI) -1,4-NAFTOQUINONA] EM COMPOSIÇÕES FARMACÊUTICAS LEISHMANICIDAS

03/11/2017

Pedido nacional de Invenção, Modelo de Utilidade, Certificado de Adição de Invenção e entrada na fase nacional do PCT

Número do Processo: BR 10 2017 023735 4

Dados do Depositante (71)

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PETICIONAMENTO ELETRÔNICO Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 03/11/2017 às 15:13, Petição 870170084816

Petição 870170084816, de 03/11/2017, pág. 1/27

Dados do Pedido

Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de Utilidade (54): "COMPOSIÇÕES FARMACÊUTICAS LEISHMANICIDAS CONTENDO FUCOSÍDEO PERACETILADO DERIVADO DA LAUSONA E USO"

Resumo: A presente invenção trata de composições farmacêuticas contendo um derivado da lausona, um fucosídeo peracetilado, denominado Flau-A [2-(2,3,4-tri-O-acetil-6-deoxi-ß-L-galactopiranosiloxi)-1,4-naftoquinona], as quais possuem atividade leishmanicida seletiva contra formas promastigotas e amastigotas de Leishmania. As composições descritas na presente tecnologia podem ser usadas no tratamento da leishmaniose tegumentar (formas clínicas cutânea, mucosa e cutâneo-difusa) e visceral em mamíferos, incluindo homem e cão.

Figura a publicar: 1

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Documentos anexados

Tipo Anexo	Nome
Comprovante de pagamento de GRU 200	Comprovante de pagamento GRU.PDF
Portaria	Port. Comprov. Poderes - Professor Ado.pdf
Relatório Descritivo	Relatório Descritivo - Pasta 864.pdf
Reivindicação	Reivindicações - Pasta 864.pdf
Desenho	Desenhos - Pasta 864.pdf
Resumo	Resumo - Pasta 864.pdf