

**UNIVERSIDADE FEDERAL DE MINAS GERAIS**  
**Faculdade de Medicina**  
**Programa de Pós-Graduação em Ciências da Saúde:**  
**Infectologia e Medicina Tropical**

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**AVALIAÇÃO DO CLIOQUINOL PARA O  
TRATAMENTO DAS LEISHMANIOSES  
TEGUMENTAR E VISCERAL**

Belo Horizonte

2019

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# **AVALIAÇÃO DO CLIOQUINOL PARA O TRATAMENTO DAS LEISHMANIOSES TEGUMENTAR E VISCERAL**

Tese apresentada ao Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG, como requisito parcial para a obtenção do título de Doutor junto ao referido Programa.

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Belo Horizonte

2019

Tavares, Grasielle de Sousa Vieira.

T231a Avaliação do clioquinol para o tratamento das leishmanioses tegumentar e visceral [manuscrito]: subtítulo. / Grasielle de Sousa VieiraTavares. - - Belo Horizonte: 2019.  
84 f.: il.

Orientador (a): Eduardo Antonio Ferraz Coelho.  
Área de concentração: Infectologia e Medicina Tropical. Tese (doutorado): Universidade Federal de Minas Gerais,  
Faculdade de Medicina.

1. Leishmaniose. 2. Quinolinas. 3. Terapêutica. 4. Toxicidade. 5. Poloxâmero. 6. Clioquinol. 7. Dissertação Acadêmica. I. Coelho, Eduardo Antonio Ferraz. II. Universidade Federal de Minas Gerais, Faculdade de Medicina. III. Título.

NLM: WR 350



UNIVERSIDADE FEDERAL DE MINAS GERAIS

FACULDADE DE MEDICINA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS DA SAÚDE INFECTOLOGIA E MEDICINA TROPICAL

FOLHA DE APROVAÇÃO

“AVALIAÇÃO DO CLIOQUINOL PARA O TRATAMENTO DAS LEISHMANIOSES TEGUMENTAR E VISCERAL”

## GRASIELE DE SOUSA VIEIRA TAVARES

Tese de Doutorado defendida e aprovada, no dia 18 de dezembro de 2019, pela Banca Examinadora designada pelo Colegiado, como requisito para obtenção do grau de Doutora em Ciências da Saúde pelo Programa de Pós-Graduação em CIÊNCIAS DA SAÚDE - INFECTOLOGIA E MEDICINA TROPICAL, da Universidade Federal de Minas Gerais.

Esclarecemos que a banca examinadora, no uso de suas atribuições solicitou a mudança do título da tese, diante disso, a tese anteriormente intitulada “Pesquisa por Novos Compostos com Atividade Antileishmanial Baseada em uma Molécula Derivada de Quinolina para a Melhoria do Tratamento das Leishmanioses”, foi alterada para “Avaliação do clioquinol para o tratamento das leishmanioses tegumentar e visceral”.

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Documento assinado eletronicamente por Eduardo Antonio Ferraz Coelho, Coordenador(a) de curso de pós-graduação, em 23/08/2024, às 10:24, conforme horário oficial de Brasília, com fundamento no art. 5º do [Decreto nº 10.543, de 13 de novembro de 2020](#).



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*Dedico:*

*À Deus, fonte de vida e amor. À minha mãe por tudo, sem você não teria conseguido alcançar meus sonhos. Ao Renato pelo seu amor e companheirismo. À minha filha Sarah por existir, meu amor por você é infinito.*

## AGRADECIMENTOS

À Deus, pelo dom da vida e pelas bênçãos depositadas em meu caminho.

À minha mãe pelo seu amor incondicional, por estar sempre ao meu lado, te amo muito.

À minha filha Sarah, o meu grande amor e orgulho. O maior presente que Deus me deu.

Ao meu marido, pelo seu amor e apoio. Obrigada por toda ajuda e incentivo para superar os momentos difíceis.

Ao meu orientador Eduardo Antonio Ferraz Coelho, que me acolheu em seu laboratório com os braços abertos, me dando todo o suporte necessário para concretização deste trabalho. Sendo não somente meu mestre, mas também muitas vezes meu amigo.

Ao meu tio de alma Carlos Alberto Pereira Tavares, pela confiança e carinho. O senhor é o meu exemplo de pesquisador.

Aos amigos de laboratório, pela aceitação, ajuda e compreensão nos momentos difíceis. E pelos momentos de alegria e descontração.

A Lourena, Vivian, Miguel e Daniela Pagliara pelos ensinamentos. Ao Daniel e Patricia por toda ajuda e companheirismo. A Debora minha amiga-irmã por estar sempre ao meu lado e tornar o dia-a-dia no laboratório muito mais feliz e animado. Ao Guilherme amigo leal que irei levar para toda vida. Ao João meu primeiro “aluninho” que na realidade é um grande professor.

A todos os professores que contribuíram transmitindo conhecimento e também foram parceiros de experimentos, em especial a Mariana Duarte.

Agradeço à CAPES, ao CNPq, à FAPEMIG, ao INCT e ao COLTEC/UFMG pelo apoio financeiro para a realização desta tese. Agradeço também ao Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical, e novamente à FAPEMIG e à CAPES pela oportunidade de realizar esta pesquisa.

Enfim: a todos que contribuíram diretamente ou indiretamente, o meu muito obrigado. Vocês fizeram parte desta realização!

*“Crê em ti mesmo, age e verás os resultados. Quando te  
esforças, a vida também se esforça para te ajudar.”*

*Chico Xavier*

## RESUMO

As leishmanioses podem causar diferentes quadros clínicos no hospedeiro mamífero que dependem, principalmente, da espécie infectante e do estado imunológico do hospedeiro infectado. Novas terapias contra as leishmanioses são desejáveis, uma vez que os fármacos atuais aplicados contra esse complexo de doenças apresentam problemas, como a toxicidade, alto custo e/ou resistência parasitária. Em um estudo realizado recentemente pelo nosso grupo de pesquisa, um novo derivado de quinolina, clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol), foi avaliado quanto à sua ação antileishmanial *in vitro* contra as espécies *Leishmania infantum* e *L. amazonensis*. Como resultado, o ICHQ foi efetivo contra as duas espécies do parasito, sendo eficaz no tratamento de macrófagos infectados, induzindo alterações no potencial de membrana mitocondrial, na integridade celular dos parasitos e aumento na produção de espécies reativas de oxigênio. O ICHQ foi incorporado em um sistema de micelas poliméricas baseadas em Poloxâmero P407 (ICHQ/M), e sua atividade antileishmanial foi avaliada *in vivo* em camundongos BALB/c infectados por *L. amazonensis*. A anfotericina B (AmpB) e sua formulação lipossomal (Ambisome®) foram utilizados como controle. Avaliações parasitológicas e imunológicas foram realizadas 30 dias após o tratamento. Na avaliação dos resultados, pode-se observar que camundongos infectados com *L. amazonensis* e tratados com ICHQ/M mostraram redução significativa da carga parasitária e o desenvolvimento de uma resposta imune Th1, que foi caracterizada por níveis elevados de IFN- $\gamma$ , IL-12 e GM-CSF, além de baixos níveis de IL-4 e IL-10. Dessa forma, ICHQ e seu sistema micelar podem ser considerados como uma alternativa para o tratamento futuro contra as leishmanioses.

**Palavras-chave:** Leishmanioses; Quinolina; Tratamento; Toxicidade; Poloxâmero P407; clioquinol.

## ABSTRACT

Leishmaniasis can cause different clinical manifestations in the mammalian hosts, which depend mainly on the infecting species and the immune status of the infected hosts. New therapies against disease are desirable, since the current drugs present problems, such as toxicity, high cost and/or parasitic resistance. In a recent study by our research group, a new quinoline-derivative, namely clioquinol (ICHQ, 5-chloro-7-iodo-8-quinolinol), was evaluated for its *in vitro* antileishmanial action against *Leishmania infantum* and *L. amazonensis* species. As result, ICHQ was highly effective against the two parasite species, as well as in the treatment of infected macrophages, causing alterations in the mitochondrial membrane potential, cellular integrity and increase in the production of reactive oxygen species by the parasites. ICHQ was incorporated into a polymeric micelle system based on Poloxamer P407 (ICHQ/M), and the *in vivo* antileishmanial activity was evaluated against *L. amazonensis* infection in BALB/c mice. Amphotericin B (AmpB) and its liposomal formulation (Ambisome®) were used as controls. Parasitological and immunological analyses performed 30 days after treatment showed that ICHQ/M-treated and *L. amazonensis*-infected mice presented a significant reduction of the parasite load, as well as the development of a Th1 immune response, which was characterized by high levels of IFN- $\gamma$ , IL-12 and GM-CSF, as well as low production of IL-4 and IL-10. In conclusion, ICHQ and its micellar system can be considered as an alternative for the future treatment against leishmaniasis.

**Keywords:** Leishmaniasis; Quinoline; Treatment; Toxicity; Poloxamer P407; Clioquinol.

## LISTA DE ABREVIATURAS E SIGLAS

<b>®</b>	Marca Registrada
<b>AmpB</b>	Anfotericina B
<b>B-ICHQ/M</b>	Micela sem 5-chloro-7-iodoquinolin-8-orto
<b>BSA</b>	Albumina sérica bovina
<b>CEUA</b>	Comitê de Ética de Uso de Animais
<b>CO<sub>2</sub></b>	Dióxido de Carbono
<b>dLN</b>	Linfonodo drenante
<b>DMSO</b>	Dimetilsulfóxido
<b>EDTA</b>	Ácido etilenodiaminotetracético
<b>FBS</b>	Soro fetal bovino
<b>GM-CSF</b>	Fator estimulador de colônias granulócito-monocítico
<b>ICB</b>	Instituto de Ciências Biológicas
<b>ICHQ</b>	5-chloro-7-iodoquinolin-8-ol
<b>ICHQ/M</b>	5-chloro-7-iodoquinolin-8-ol micelar
<b>IFN-<math>\gamma</math></b>	Interferon-gama
<b>IgG</b>	Imunoglobulina G
<b>IL</b>	Interleucina
<b>iNOS</b>	Enzima óxido nítrico sintase induzível
<b>KCl</b>	Cloreto de potássio
<b>KH<sub>2</sub>PO<sub>4</sub></b>	Fosfato de potássio monobásico
<b>LC</b>	Leishmaniose cutânea
<b>LCD</b>	Leishmaniose cutâneo-difusa
<b>LM</b>	Leishmaniose mucosa
<b>LT</b>	Leishmaniose tegumentar
<b>LV</b>	Leishmaniose visceral
<b>LVC</b>	Leishmaniose visceral canina
<b>LVH</b>	Leishmaniose visceral humana
<b>mg</b>	Miligrama
<b>MHC</b>	Complexo principal de histocompatibilidade
<b>mL</b>	Micro litro
<b>mM</b>	Milimolar
<b>MTT</b>	3-(4,5-dimetiltiazol-2yl)-2,5-difenil brometo de tetrazolina

<b>Na<sub>2</sub>HPO<sub>4</sub></b>	Fosfato de sódio dibásico
<b>NO</b>	Óxido nítrico
<b>PCR</b>	Reação em Cadeia da Polimerase
<b>RIFI</b>	Reação de Imunofluorescência Indireta
<b>RPMI</b>	Meio de cultura Roswell Park Memorial Institute
<b>SLA</b>	Extrato solúvel antigênico de <i>Leishmania</i>
<b>RT-PCR</b>	PCR em tempo real
<b>TGF-β</b>	Fator de transformação de crescimento beta
<b>Th</b>	T helper
<b>TNF-α</b>	Fator de necrose tumoral alfa
<b>WHO</b>	Organização Mundial de Saúde

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## ***CONSIDERAÇÕES INICIAIS***

## CONSIDERAÇÕES INICIAIS

Esta tese de doutorado teve como objetivo geral avaliar a atividade antileishmanial *in vitro* e *in vivo* de uma molécula derivada de quinolina, o clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol, 5-cloro-8-hidroxi-7-iodoquinolina) contra diferentes espécies de *Leishmania*, no caso, *L. amazonensis*, e *L. infantum*, para o desenvolvimento de um tratamento a ser empregado contra as leishmanioses.

A escolha do tema desta tese ocorreu devido ao fato da necessidade do desenvolvimento de novas terapias contra as leishmanioses, uma vez que os fármacos atuais aplicados contra esse complexo de doenças apresentam problemas, como a toxicidade, alto custo e/ou resistência parasitária.

Outro fator também relevante na escolha do tema baseia-se no fato do orientador deste trabalho atuar na pesquisa em leishmanioses desde o ano de 2000. Desenvolvendo projetos de pesquisa nas linhas da prevenção, sob a forma de vacinas; no diagnóstico laboratorial e desenvolvimento de novos produtos terapêuticos para as leishmanioses tegumentar e visceral.

Este estudo faz parte de uma das linhas de pesquisa que venho desenvolvendo, atualmente, no laboratório de pesquisa sob a coordenação do professor Dr. Eduardo Antonio Ferraz Coelho na UFMG, na qual objetivamos a busca por formas alternativas para o tratamento das leishmanioses, possibilitando a ampliação das opções terapêuticas uma vez que os fármacos atuais aplicados contra esse complexo de doenças apresentam problemas, como a toxicidade, alto custo e / ou resistência parasitária.

O trabalho possibilitou a produção de dois artigos científicos publicados em revistas de relevante impacto internacional, que serão apresentados neste documento, e no depósito de uma patente nacional.

A apresentação deste documento foi realizada de acordo com a Resolução nº 02/2013, de 18 de setembro de 2013; que regulamenta o formato dos trabalhos finais e de qualificação, estabelecendo condições para a marcação das defesas de teses e dissertações do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical da Faculdade de Medicina da UFMG.

## ***INTRODUÇÃO***

## 1. INTRODUÇÃO

As leishmanioses são doenças infecto-parasitárias de evolução aguda ou crônica, causadas por parasitos protozoários do gênero *Leishmania*, sendo endêmicas em 98 países no mundo. Devido à magnitude e complexidade clínica, biológica e epidemiológica, elas são consideradas um importante problema de saúde pública. Diferentes quadros clínicos podem ser observados nas leishmanioses, que dependem principalmente, da espécie infectante do parasito e do estado imunológico do hospedeiro infectado (GREVELINK e LERNER, 1996; NAKAMURA *et al.*, 2006).

Na apresentação clínica da leishmaniose tegumentar (LT), a doença pode-se apresentar sob três formas principais: leishmaniose cutânea (LC), leishmaniose mucosa (LM) e leishmaniose cutâneo-difusa (LCD) (WHO, 2014). A leishmaniose visceral (LV) é a forma clínica mais grave da doença, devido às frequentes complicações e potencial de evoluir para a morte, se não tratada (OMS, 2018).

As medidas profiláticas e de controle contra as leishmanioses visam, principalmente, à interrupção do ciclo biológico do parasito. Entretanto, o número de espécies de *Leishmania* sp., o caráter zoonótico da doença e a manutenção do ciclo silvestre, dificultam a adoção de medidas efetivas de controle. O diagnóstico da doença é baseado na avaliação das manifestações clínicas e na sua confirmação por meio de exames laboratoriais (TESH, 1995). O método parasitológico é conclusivo e tem alta especificidade, mas é um método invasivo e sua sensibilidade é variável, devido à qualidade da amostra colhida e da capacitação do técnico que a analisa (SUNDAR e RAI, 2002; TAVARES *et al.*, 2003). O diagnóstico sorológico da infecção por *Leishmania* na sua forma assintomática é complicado devido aos baixos títulos dos anticorpos de determinados antígenos e às reações-cruzadas com outras doenças (DAVIES *et al.*, 2003).

O tratamento de primeira escolha das leishmanioses se baseia na utilização de antimoniais pentavalentes, dentre os quais o antimoniato de N-metil glucamina (Glucantime®, Rhône Poulenc Rorer, França) e o estibogluconato de sódio (Pentostan®, Welcome Foundation, Inglaterra) são utilizados. No Brasil, o medicamento preconizado é o Glucantime®, no entanto, este fármaco causa efeitos colaterais como arritmias, toxicidade renal e hepática, dentre outros. A via de administração da medicação é parental, e apresenta longo tempo de tratamento, além de ser capaz de interagir com proteínas sulfidrilas de proteínas celulares causando perda de função e/ou formando complexos com ribonucleosídeos, o que induz a inespecificidade de ação do produto frente às células infectadas e não infectadas.

A anfotericina B livre e lipossomal, a pentamidina, a paramomicina e a miltefosina têm sido recomendadas nos casos de intolerância e/ou resistência ao tratamento convencional, sendo a anfotericina lipossomal o fármaco de segunda escolha no Brasil; entretanto, tais produtos também apresentam limitações devido aos efeitos colaterais, a necessidade de administração parenteral e endovenosa e/ou o custo elevado (SUNDAR *et al.*, 2009; GOTO e LINDOSO, 2010).

Frente às dificuldades enfrentadas pelos pacientes nos tratamentos preconizados, o abandono ou a interrupção do mesmo é comum, o que leva ao aumento da resistência dos parasitos aos fármacos utilizados (CROFT e COOMBS, 2003; VÉLEZ *et al.*, 2009). Também, há o fato do aumento do número de casos de recidiva à doença, que vem sendo observado em diversas regiões do mundo (VÉLEZ *et al.*, 2009).

Desta forma, a Organização Mundial da Saúde (OMS) tem preconizado a busca por novos produtos e o desenvolvimento de estratégias terapêuticas alternativas e de baixo custo para se tratar as leishmanioses. Estudos recentes realizados pelo nosso grupo de pesquisa demonstraram que a 8-hydroxi-quinolina apresentou atividade antileishmanial *in vitro* contra promastigotas e amastigotas de importantes espécies de *Leishmania* (DUARTE, *et al.*, 2016a). Além disso, quando foi incorporada a um sistema de *delivery* baseado em Poloxâmero 407, este produto foi eficaz no tratamento de camundongos BALB/c infectados com *L. amazonensis* ou *L. infantum* (DUARTE, *et al.*, 2016b; LAGE *et al.*, 2016).

No presente estudo, testamos um derivado de quinolina, o clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol), frente a duas espécies de *Leishmania* capazes de causar LV e LT no mundo. O clioquinol foi escolhido devido à sua propriedade quelante de metal apresentando atividades antibacterianas, antifúngicas e antiparasitárias já conhecidas (GHOLZ e ARONS, 1964; RITCHIE *et al.*, 2003; HONGMANEE, *et al.*, 2007; KIM *et al.*, 2012).

***REVISÃO DA LITERATURA***

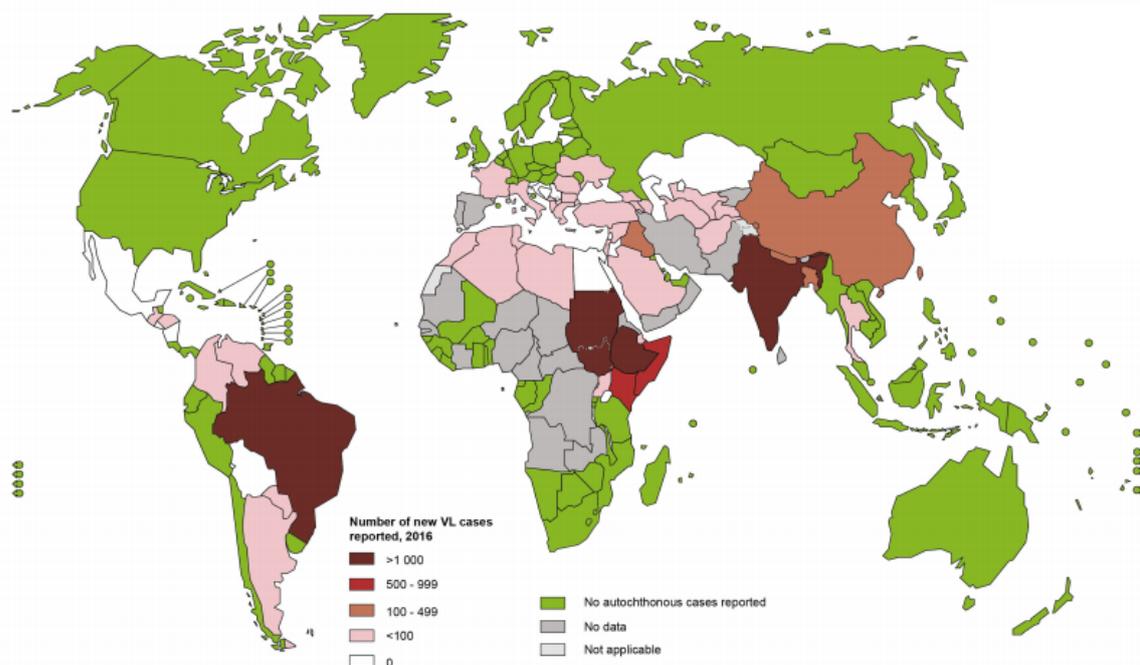
## 2. REVISÃO DA LITERATURA

### 2.1. EPIDEMIOLOGIA DAS LEISHMANIOSES

As leishmanioses são doenças causadas por protozoários do gênero *Leishmania*, que ocorrem em cinco continentes e são endêmicas em 97 países, possuindo elevada taxa de morbidade e mortalidade (ALVAR *et al.*, 2012). Este complexo de doenças é prevalente em áreas tropicais, subtropicais e Sul da Europa, com mais de 20 espécies de parasitos que causam a doença no homem (WHO, 2017).

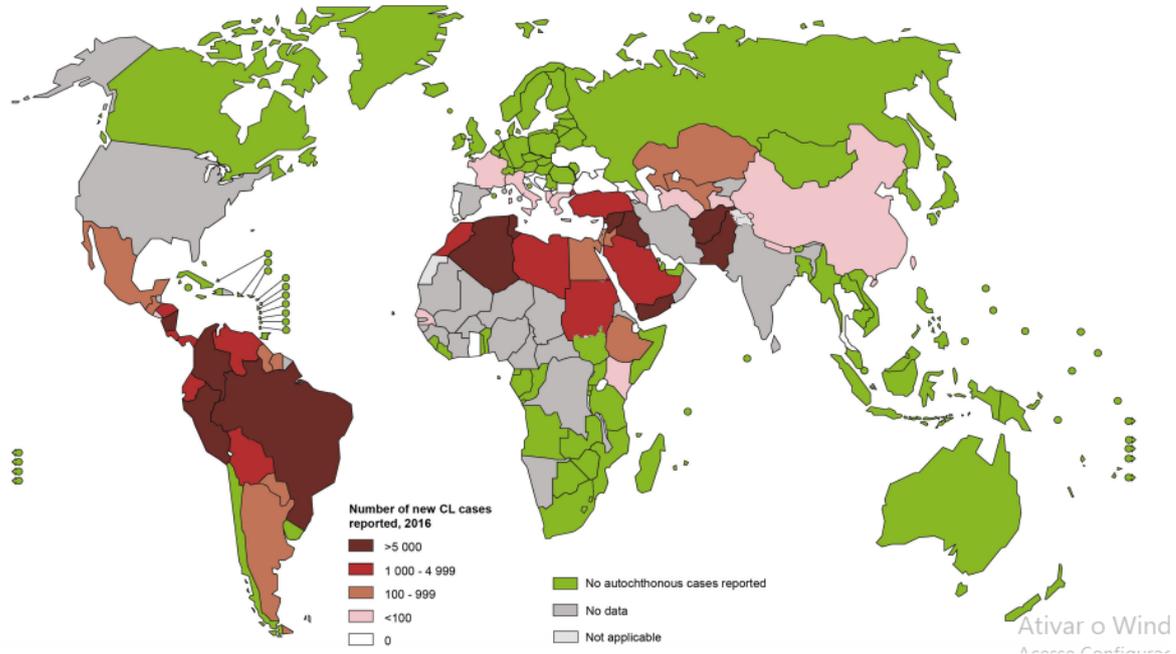
Elas acometem, principalmente, pessoas de classe econômica baixa, estando associadas à desnutrição, ao deslocamento da população, condições precárias de habitação, ao sistema imunológico debilitado e à falta de recursos. A diferença no avanço da doença está relacionada com a espécie do parasito infectante, bem como a suscetibilidade do hospedeiro, podendo variar entre distintas manifestações cutâneas até a forma visceral da doença (ASHFORD, 2000).

Estima-se que haja uma incidência anual de aproximadamente 0,2 a 0,4 milhões de casos de LV, sendo que 90% dos novos casos foram reportados em 7 países, Brasil, Etiópia, Índia, Quênia, Somália, Sudão do Sul e Sudão, registrando cerca de 60.000 mortes por ano (WHO, 2017). Segundo a WHO, 2018, foram reportados 55.530 novos casos de LV no ano de 2017 na América Latina e 4.297 no Brasil.



**Figura 1.** Distribuição mundial da Leishmaniose Visceral em 2016. Fonte: WHO, 2018

A LT também apresenta distribuição global, sendo endêmica na América Latina, sudoeste asiático e Índia. A distribuição da doença em 2016 está demonstrada na Figura 2.



**Figura 2.** Distribuição mundial da Leishmaniose Tegumentar em 2016. Fonte: WHO, 2018

Em 2018 foram reportados cerca de 0,7 a 1,2 milhões de LT no mundo (WHO, 2018), sendo que 10 países foram responsáveis por 84% dessas notificações (Afeganistão, Argélia, Brasil, Colômbia, Iraque, Paquistão Peru e Síria). Nas Américas, as formas cutânea e mucosa acometem pessoas em 20 países, sendo endêmica em 18 deles. Já os casos humanos de LV estão presentes em 12 países. No período entre 2001 e 2015, foram reportados 843.931 novos casos de LT, sendo 70% no Brasil, Colômbia e Peru (OPAS/OMS, 2017). Em 2017, foram reportados 17.809 novos casos de LT no Brasil (WHO, 2018).

## 2.2. A ETIOLOGIA E CICLO BIOLÓGICO DO PARASITO *Leishmania spp.*

As leishmanioses são doenças causadas por parasitos protozoários do gênero *Leishmania*, pertencente ao Sub-Reino *Protozoa*, Filo *Sarcomastigophora*, Ordem *Kinetoplastida* e Família *Trypanosomatidae*. Mais de 20 espécies de *Leishmania* podem causar a doença no homem (WHO, 2013) sendo que, nas Américas, atualmente 11 espécies dermatrópicas são reconhecidas por causar a patologia no homem e 8 espécies como capazes de causar a doença em animais (GRIMALDI e TESH, 1993; REITHINGER *et al.*, 2007; SHAW, 2007).

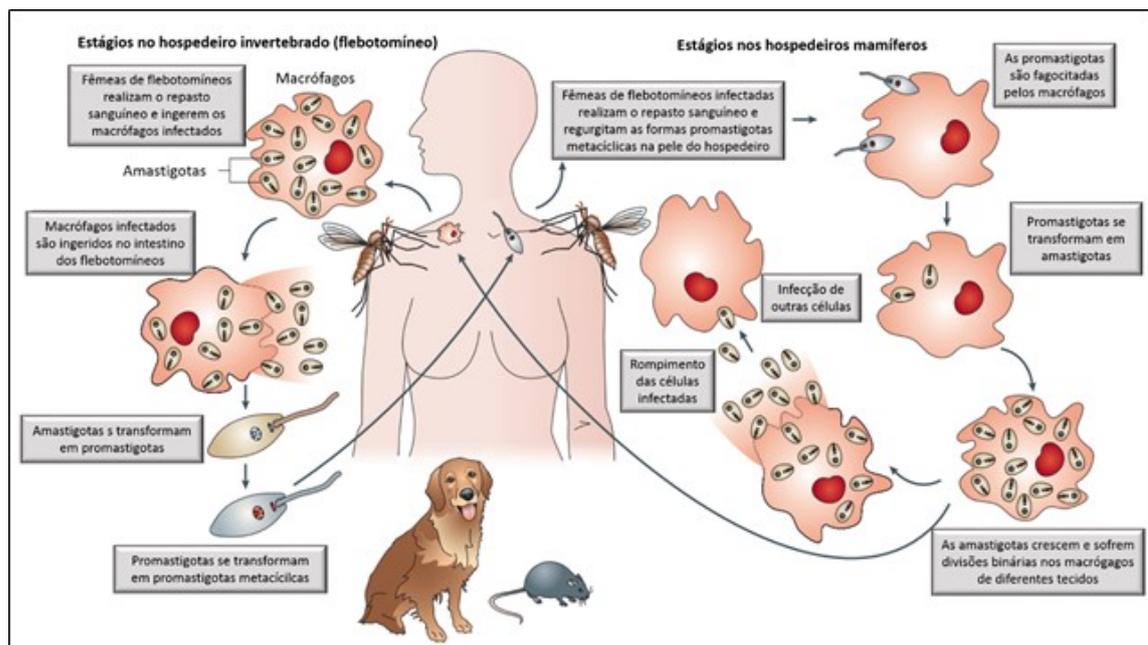
O vetor transmissor do parasito é um inseto da Ordem *Diptera*, Família *Psychodidae*, Sub-Família *Phlebotominae*, pertencente aos gêneros *Phlebotomus*, em países do Velho Mundo e *Lutzomyia*, nas Américas, sendo que apenas a fêmea possui hábitos hematófagos e é capaz de transmitir o parasito ao hospedeiro mamífero (GRIMALDI e TESH, 1993; SACKS e KAMHAWI, 2001; MOTA *et al.*, 2011; STRAZZULLA *et al.*, 2013; WHO, 2015). Estes insetos possuem cerca de 2 a 3 milímetros de comprimento, e cerca de 500 espécies de flebotomos são conhecidas, embora apenas cerca de 30 sejam capazes de transmitir os parasitos aos hospedeiros mamíferos (WHO, 2015).

Dentre os hospedeiros primários incluem humanos, cães e gatos domésticos, gambás, raposas e ratos (QUINNELL e COURTENAY, 2009). Os cães infectados são considerados como o principal reservatório urbano, responsáveis pela transmissão do parasito aos homens o que torna a leishmaniose visceral canina (LVC) não apenas um problema veterinário, mas também de saúde pública (BANETH *et al.*, 2008; TEIXEIRA-NETO *et al.*, 2010).

Os protozoários do gênero *Leishmania* caracteriza-se por apresentar duas formas evolutivas em seu ciclo biológico (ciclo de vida heteroxênico) nos organismos hospedeiros: amastigota, que é a forma intracelular obrigatória encontrada nos vertebrados, e a forma promastigota, que é encontrada no tubo digestivo dos vetores invertebrados (MOTA *et al.*, 2011). As formas amastigotas são formas arredondadas, com flagelo rudimentar, altamente infectivas e responsáveis pelo desenvolvimento da doença no hospedeiro mamífero. São parasitos intracelulares obrigatórios, sendo encontradas no interior de células fagocíticas, tais como macrófagos, neutrófilos, células dendríticas e células de Langherans. As formas promastigotas são formas alongadas, móveis, afiladas, possuem um longo flagelo, com núcleo único e têm o cinetoplasto localizado entre a porção anterior e o núcleo (GRIMALDI e TESH, 1993; ASHFORD, 2000; BASANO e CAMARGO, 2004).

A transmissão do parasito ao inseto ocorre quando o mesmo realiza uma hematofagia em um hospedeiro vertebrado infectado. O vetor ingere juntamente com o sangue as formas amastigotas, que em seu intestino tornam-se promastigotas procíclicas e migram para o trato digestório médio e anterior, onde se multiplicam e diferenciam-se em promastigotas metacíclicas, colonizando as glândulas salivares do vetor. Quando o vetor flebotomíneo fêmea infectado realiza um repasto sanguíneo, as formas promastigotas metacíclicas são regurgitadas na pele do homem. Prontamente, as células do sistema fagocítico-mononuclear, neutrófilos, células dendríticas e especialmente os macrófagos migram para o local e fagocitam os parasitos (KAYE e SCOTT, 2011; RIBEIRO-GOMES e SACKS, 2012;

HANDLER *et al.*, 2015). No interior dos macrófagos ocorre a diferenciação das formas promastigotas em amastigotas e também sua replicação por divisão binária, podendo resultar no rompimento das células infectadas e consequentemente a liberação dos parasitos, iniciando uma infecção no mamífero. A proliferação também pode ocorrer em outras células de diferentes tecidos, como nos linfonodos e no fígado (BARSKY *et al.*, 1978; REITHINGER *et al.*, 2007). A Figura 2 resume o ciclo biológico do agente infeccioso das leishmanioses.



**Figura 3.** Ciclo biológico do parasito *Leishmania*. O ciclo biológico do parasito é heteroxeno apresentando o inseto vetor como o hospedeiro invertebrado e o homem e o cão, como os hospedeiros vertebrados.

### 2.3. MANIFESTAÇÕES CLÍNICAS DAS LEISHMANIOSES

A patogenia das leishmanioses é determinada por fatores relacionados ao hospedeiro, como características genéticas, resposta imunológica, idade e estado nutricional; e por fatores relacionados aos parasitos, como a virulência da espécie infectante e por fatores relacionados ao vetor (LOCKSLEY *et al.*, 1999; TRIPATHI e SINGH e NAIK, 2007). O resultado desta complexa interação pode levar desde ao desenvolvimento de uma forma assintomática até a manifestação clínica aguda da doença (KANE *et al.*, 2001). Dessa forma, as leishmanioses podem ser classificadas em duas grandes categorias clínicas: LT e LV.

A LT pode compreender as formas clínicas: leishmaniose cutânea (LC), leishmanioses cutâneo-difusa (LCD) e leishmanioses mucosa (LM) (ASHFORD, 2000; KANE e MOSSER, 2000; DESJEUX, 2004; GOTO e LINDOSO, 2012). Tais patologias

podem causar desde uma lesão cutânea única, no local da picada do vetor e que pode apresentar cura espontânea, até lesões debilitantes, disseminadas pela epiderme e mucosa do paciente e que não apresentam cura espontânea, necessitando de tratamento quimioterápico (GRIMALDI e TESH, 1993).

A LC é a mais encontrada, ocorrendo em cerca de 90% dos pacientes. Sendo caracterizada pela presença de uma lesão cutânea primária que se desenvolve no local da picada do inseto vetor. A doença se manifesta em partes mais expostas do corpo, como face, braços e pernas. Inicialmente apresenta-se como um nódulo no local da picada que evolui formando uma úlcera granulosa contornada por uma borda elevada. Em alguns casos, nódulos satélites podem ocorrer nas proximidades da lesão inicial. Quando curados, deixam cicatrizes permanentes, as quais podem levar a uma condição de morbidade (STEBUT, 2014). A LC pode apresentar-se sob as formas clínicas:

- Cutâneo-localizada (LCL), responsável por 85% dos casos e que tem tendência à cicatrização espontânea, com boa resposta à terapêutica;
- Cutânea disseminada, mais rara, caracterizada pelo aparecimento de lesões papulares múltiplas que acometem diversas partes do corpo;
- Recidiva cútis (LRC), caracterizada por lesões nodulares em torno ou no interior da cicatriz de uma lesão prévia causada pelos parasitos.

A LCD constitui manifestação rara e grave, não apresentando cura espontânea. As lesões apresentam-se como nódulos, de forma anérgica e que raramente ulceram. As lesões espalham-se por todo o corpo e este quadro pode estar relacionado à ineficiência ou ausência de resposta celular por parte do sistema imune do hospedeiro. A LCD no Brasil é causada pela espécie *L. amazonensis* e constitui-se em uma forma clínica grave da doença, pois os pacientes apresentam lesões desfigurantes e incapacitantes, excluindo-os do seu meio de vida social, além de apresentar forte resistência ao tratamento (GONTIJO e CARVALHO, 2003; DESJEUX, 2004; MENDONÇA *et al.*, 2004).

A LM pode ocorrer simultaneamente com uma manifestação cutânea, no entanto, a doença geralmente ocorre meses ou anos após a ocorrência da LC; a espécie *L. braziliensis* é o principal agente etiológico da LM na América do Sul (Marsden, 1986). Os sintomas podem incluir comichão no nariz, obstrução, epistaxe, rinorreia, que evolui para formação de crostas, e sangramento. Inicialmente, a inflamação nasal e congestão são observadas em um exame da narina; no entanto, a ulceração e perfuração do septo podem também ocorrer. Partes do rosto, palato mole, faringe e laringe podem ser afetadas e, excepcionalmente, traqueia e

árvore respiratória superior; sendo que raramente, as conjuntivas oculares e mucosas de órgãos genitais e ânus podem também ser atingidas (WEIGLE e SARAVIA, 1996). Segundo a classificação proposta por MARZOCHI (1994) e considerando como parâmetros o tempo decorrido entre as lesões cutâneas e mucosas, o local de inoculação e a localização das lesões, a forma mucosa pode ser classificada em:

- Forma primária – situação em que a lesão de mucosa é causada pela picada do vetor diretamente na mucosa, restrita às mucosas labial e genital.
- Forma indeterminada – acometimento mucoso, sem identificação da porta de entrada, supondo-se que as lesões mucosas sejam originadas de infecção, sem manifestação cutânea clínica prévia.
- Forma tardia – caracterizada pelo aparecimento da lesão mucosa após alguns anos do surgimento da lesão cutânea, fato que costuma ocorrer nos dois primeiros anos. Mas, há relatos de sua ocorrência décadas após a erupção da lesão na pele.

A LV é a forma clínica mais grave da doença, devido às frequentes complicações e potencial de evoluir para a morte, se não tratada (OMS, 2018). Ela pode se apresentar de modo assintomático, agudo ou crônico. Os indivíduos assintomáticos desenvolvem sintomatologias pouco específicas, como febre baixa, tosse seca, diarreia, sudorese com a cura de forma espontânea ou a manutenção do parasito sem evolução clínica durante a vida. Na forma aguda, pode-se observar febre alta, palidez de mucosas e hepatoesplenomegalia discretas. E por último temos a forma crônica ou Kalazar clássica de evolução prolongada causando febre irregular, emagrecimento progressivo, hepatoesplenomegalia, edema generalizado, dispneia, cefaleia, dores musculares, dentre outros sintomas (ASHFORD, 2000).

As manifestações clínicas de acordo com cada espécie de parasito responsável em causar a infecção, e também os locais de ocorrência das doenças podem ser visualizados na tabela 1.

**Tabela 1.** Espécies de *Leishmania*. Adaptada de WHO EXPERT COMMITTEE ON THE CONTROL OF THE LEISHMANIASES; WHO, 2010.

<b>Manifestação Clínica</b>	<b>Espécie</b>
<b>Velho Mundo</b>	
	<b>Subgênero <i>Leishmania</i></b>
Leishmaniose Visceral	<i>Leishmania donovani</i> e <i>Leishmania infantum</i>
Leishmaniose Cutânea	<i>Leishmania major</i> , <i>Leishmania tropica</i> e <i>Leishmania aethiopica</i>
Leishmaniose Cutâneo-Difusa	<i>Leishmania aethiopica</i>
<b>Novo Mundo</b>	
	<b>Subgênero <i>Leishmania</i></b>
Leishmaniose Visceral	<i>Leishmania infantum</i>
Leishmaniose Cutânea	<i>Leishmania infantum</i> , <i>Leishmania mexicana</i> , <i>Leishmania pifanol</i> e <i>Leishmania amazonensis</i>
Leishmaniose Cutâneo-Difusa	<i>Leishmania mexicana</i> e <i>L. amazonensis</i>
<b>Novo Mundo</b>	
	<b>Subgênero <i>Viannia</i></b>
Leishmaniose Cutânea	<i>Leishmania brasiliensis</i> , <i>Leishmania guyanensis</i> , <i>Leishmania panamensis</i> e <i>Leishmania peruviana</i>
Leishmaniose Mucosa	<i>Leishmania brasiliensis</i> e <i>Leishmania panamensis</i>

## 2.4. IMUNOLOGIA DAS LEISHMANIOSES

A resistência à infecção contra *Leishmania* é relacionada à imunidade celular, representada pela produção de IFN- $\gamma$  nos hospedeiros mamíferos infectados (SCOTT, 2003). A importância do desenvolvimento de linfócitos T CD4<sup>+</sup> helper do tipo Th1 e linfócitos T CD8<sup>+</sup> no balanço entre as linhagens celulares Th1/Th2 foi demonstrada em modelos de camundongos infectados com *L. major* (SACKS e NOBEN-TRAUTH, 2002). Os estudos demonstraram a associação entre a resistência à infecção por *L. major* e o desenvolvimento de uma resposta Th1, com produção de citocinas como IFN- $\gamma$  e IL-12. Já a susceptibilidade à infecção parece ser devido ao desenvolvimento de uma resposta Th2, mediada pela produção de IL-4, IL-10 e TGF- $\beta$  (SCOTT, 2003).

A definição de um modelo animal relevante deve ser com base na finalidade do teste. No caso de modelos animais para o desenvolvimento de medicamentos, é de se esperar que eles devam imitar a infecção natural e exibem patologia, resposta imunológica e evolução comparável com a doença em humanos. Idealmente, a farmacocinética e farmacodinâmica do medicamento no modelo também devem ser comparáveis com os humanos. Os animais também devem ser fáceis de manter e manipular e de preferência não muito caros. Modelos animais de LC localizada e de LV estão bem estabelecidos, no entanto, ainda não encontraram bons

modelos para leishmaniose mucosa, difusa ou disseminada.

Sendo assim um grande avanço no estudo da imunologia das leishmanioses foi a elucidação do modelo de suscetibilidade, uma vez que camundongos BALB/c apresentam resposta do tipo T helper 1 (Th1) e T helper 2 (Th2) bem definidos quando da infecção com a espécie *L. major*, abrindo caminhos para a descrição dos papéis dos componentes celulares e humorais da resposta imunológica ao parasito *Leishmania* (MOSMANN *et al.*, 1986; LOCKSLEY *et al.*, 1987). Também, tal resposta auxilia na elucidação da relação parasito-hospedeiro e quanto às distintas espécies de *Leishmania*, favorecendo o entendimento das funções das células que compõem esse sistema complexo (NATALE e MELLO e MANZONI-DE-ALMEIDA; 2016).

Em camundongos resistentes à espécie *L. major*, células dendríticas produtoras de IL-12 migram rapidamente para o local da infecção. As células têm a capacidade de interagir com o parasito nos linfonodos drenantes e com LT Th1 que expressam o receptor funcional de IL-12 (IL-12R) (SCOTT, 2003). Resultados descritos por Heinzl *et al.* (1993) demonstram que a administração da proteína IL-12 na forma recombinante (rIL-12) altera a diferenciação de LT CD4<sup>+</sup> quando administrada na 1ª semana de infecção, promovendo uma resposta celular protetora em camundongos inicialmente susceptíveis à *L. major*. Observou-se uma redução significativa no edema e na carga parasitária de camundongos BALB/c tratados com rIL-12, caracterizada pela geração de uma resposta Th1, com níveis elevados de IFN- $\gamma$  e uma supressão da resposta Th2, pela reduzida produção de IL-4 (CHAKIR *et al.*, 2003).

Algumas linhagens de camundongos, como C57BL/6, C57BL/10, C3H e CBA são resistentes à infecção com *L. major* mesmo quando um grande número de parasitos (até 10<sup>7</sup>) é inoculado pela via subcutânea nos animais; enquanto outras linhagens, como BALB/c e SWR/J, apresentam um perfil de susceptibilidade.

A dicotomia da resposta imune Th1xTh2, evidente no modelo murino de BALB/c e infecção com *L. major*, não é observada quando a infecção ocorre com *L. donovani* e *L. infantum* (MIRALLES *et al.*, 1994; AHMED *et al.*, 2003). A infecção experimental em camundongos BALB/c com *L. infantum* evolui, inicialmente, no fígado, depois no baço e então na medula óssea (MARQUES-DA-SILVA *et al.*, 2005). Sinais clínicos, tais como hepatomegalia e esplenomegalia, são observados em camundongos infectados e um pico de carga parasitária ocorre no fígado entre 28 e 42 dias após a infecção enquanto que, no baço, a evolução da infecção ocorre mais lentamente atingindo o pico por volta de 8 a 10 semanas após o desafio (MELBY *et al.*, 1998). A via utilizada na infecção (intradérmica, endovenosa,

subcutânea) não altera o perfil de infecção, o que indica que o tropismo pelos órgãos internos corresponde às características intrínsecas do parasito (AHMED *et al.*, 2003; WILSON *et al.*, 2005). A imunidade protetora relacionada à LV relaciona-se ao desenvolvimento de uma resposta Th1 e ocorre na presença de citocinas ativadoras de macrófagos, tais como IFN- $\gamma$  e IL-12 (MURRAY *et al.*, 2002). Estudos indicam que o fenótipo de susceptibilidade se relaciona mais à incapacidade do desenvolvimento de uma resposta Th1 efetiva do que à elaboração de uma resposta Th2 exacerbada (MIRALLES *et al.*, 1994).

Dessa forma, embora citocinas tais como IL-4 e IL-10 tenham reconhecida importância por suas propriedades de desativar macrófagos e, provocar a progressão da infecção por *L. major*; no modelo de LV apenas a IL-10 tem sido apontada como promotora da doença (MURRAY *et al.*, 2002). Embora a IL-4 tenha a capacidade de inibir a ativação de macrófagos, sua importância na LV não tem sido comprovada. MIRALLES *et al.* (1994) sugeriram que a IL-4 não parece ser suficiente para influenciar a resposta Th1, nem o curso da infecção por *L. donovani* em camundongos BALB/c, estando ou não em níveis aumentados.

O controle da forma visceral da doença está associado ao padrão de resposta do tipo Th1, com produção das citocinas IFN- $\gamma$  ou TNF- $\alpha$  que culmina na ativação dos macrófagos e matam o parasito das espécies *L. infantum* e *L. donovani* (PEARSON e STEIGBIGEL, 1981; MURRAY e RUBIN e ROTHERMEL, 1983). Mesmo quando as manifestações clínicas não são apresentadas pelo hospedeiro humano, é produzida uma intensa resposta DTH (hipersensibilidade do tipo tardio) e alta produção de IL-2, IL-12 e IFN- $\gamma$  por células mononucleares de sangue periférico (CARVALHO *et al.*, 1985; SACKS e PERKINS, 1985). Em pacientes sintomáticos, há ausência de produção de IL-2 e de IFN- $\gamma$  e elevados níveis séricos de IL-10 (DE CAMARGO *et al.*, 2014; GOMES *et al.*, 2014).

A susceptibilidade e o desenvolvimento de LV em humanos são caracterizados pelo padrão de resposta Th2, com produção de citocinas IL-4 e IL-13, dentre outras, responsáveis por inibir células efetoras do sistema imune que levam à ativação clássica de macrófagos (BABALOO e KAYE e ESLAMI, 2001; THAKUR e MITRA e NARAYAN, 2003). A função da IL-17 ainda não é muito bem esclarecida na LV, entretanto, estudos correlacionam a presença de linfócitos Th17 secretando tal citocina em sinergismo com IFN- $\gamma$  (NASCIMENTO *et al.*, 2015) e IL-22 (PITTA *et al.*, 2009), exercendo papel protetor. Por outro lado, de acordo com o trabalho realizado por (ANDERSON *et al.*, 2007), há maior gravidade da doença pela presença das células T reguladoras, que exerceriam efeito supressor sobre linfócitos Th1 por meio de mecanismos reguladores como a secreção de IL-10 e TGF-

$\beta$ .

Diferenças no desenvolvimento da doença causada por *L. major* ou *L. amazonensis* sugerem que diferentes aspectos da resposta imune podem estar envolvidos nas infecções por tais espécies, de forma que o papel protetor ou indutor de suscetibilidade não se aplica a todos os hospedeiros e a todas as espécies de parasitos (SOONG *et al.*, 1997; CAMPBELL *et al.*, 2003).

A suscetibilidade à infecção por *L. amazonensis* em modelos murinos não está diretamente ligada a uma polarização de resposta do tipo Th2, mas a uma baixa produção de citocinas como IFN- $\gamma$ , IL-2, IL-12, dentre outras, o que mantém a sobrevivência do parasito e uma elevada carga parasitária (JI e SUN e SOONG, 2003). Estudos relataram que a infecção por essa espécie de parasito é capaz de dificultar a migração de células dendríticas do sítio de infecção para os linfonodos drenantes (HERMIDA *et al.*, 2014). Além de níveis baixos de IL-12, existe um comprometimento da expressão da cadeia  $\beta$ 2 de seu receptor (HEINZEL *et al.*, 1993; JONES *et al.*, 2000)

Em camundongos BALB/c, a infecção por *L. amazonensis* ocorre em maior nível quando há depleção de neutrófilos, em um mecanismo dependente das citocinas IL-17 e IL-10 (SOUSA *et al.*, 2014). No homem, neutrófilos infectados por formas promastigotas do parasito as produzem NETs (neutrophil extracellular traps) que eliminam os parasitos (GUIMARAES-COSTA *et al.*, 2009; GUIMARAES-COSTA *et al.*, 2014). Como são as primeiras células a chegarem ao sítio de infecção, os neutrófilos apresentam papel importante no controle do parasitismo no início da infecção (CARLSEN *et al.*, 2013; GUIMARAES-COSTA *et al.*, 2014).

Uma característica marcante da infecção por *L. amazonensis* é o papel que o IFN- $\gamma$  desempenha, uma vez que baixas concentrações dessa citocina induzem à proliferação de formas amastigotas em macrófagos infectados *in vitro*, assim como na infecção por *L. major*, onde o IFN- $\gamma$  é crucial na ativação de macrófagos, na morte do parasito e na resolução da infecção (QI *et al.*, 2004). Outra diferença na infecção por *L. amazonensis* é que as células T reguladoras parecem ter um papel de proteção, porém sua ação protetora não foi associada à produção de TGF- $\beta$  e IL-10 (JI *et al.*, 2005).

## 2.5. MEDIDAS DE CONTROLE DA DOENÇA

As medidas profiláticas e de controle contra as leishmanioses visam principalmente à

interrupção do ciclo biológico do parasito. Entretanto, o número de espécies de *Leishmania sp.*, o carácter zoonótico da doença e a manutenção do ciclo silvestre, dificultam a adoção de medidas efetivas de controle. O controle do vetor pode ser realizado pela aplicação de inseticidas no ambiente doméstico e peridoméstico. Entretanto, sua utilização apresenta relativa eficácia, principalmente, nas regiões florestais (TESH, 1995). No que se refere à LV, o cão, como importante hospedeiro reservatório doméstico do parasito, representa um importante elo na manutenção da transmissão da infecção entre o vetor e o homem (BARBIÉRI, 2006). Até o presente momento, a eliminação de cães infectados soropositivos constitui-se como a medida de controle adotada pelos órgãos competentes (TESH, 1995). A eliminação de reservatórios do parasito, como marsupiais e roedores silvestres, não é uma medida executável e ecologicamente correta. Existe também a possibilidade de adaptação do parasito a outros reservatórios existentes no ambiente (GRIMALDI e TESH, 1993; GRAMICCIA e GRADONI, 2005).

## 2.6. DIAGNÓSTICO DAS LEISHMANIOSES

O diagnóstico clínico das leishmanioses é um desafio, uma vez que a doença abrange um grande espectro de características clínicas (CHAPPUIS *et al.*, 2007). Os sinais são frequentemente confundidos com os de outras doenças, tais como lepra e câncer para LC e malária, esquistossomose, tripanossomíase, tuberculose e desnutrição para a LV (SINGH, 2006). Desse modo, o diagnóstico deve ser realizado com base na associação entre parâmetros clínicos e exames laboratoriais confirmatórios (TESH, 1995).

Ele é realizado com base na avaliação das manifestações clínicas e na confirmação por meio de exames laboratoriais (TESH, 1995). O primeiro teste de diagnóstico utilizado na detecção da doença foi o teste de intradermorreação de Montenegro (IDRM), que se baseia na resposta de células T de memória que tiveram contato prévio com o parasito, as suas desvantagens são as baixas especificidade e sensibilidade (WEIGLE *et al.*, 1991; DE PAIVA-CAVALCANTI *et al.*, 2015). O método parasitológico é conclusivo e tem alta especificidade, mas é um método invasivo e sua sensibilidade é variável, devido à qualidade da amostra colhida e da capacitação do técnico que a analisa (SUNDAR e RAI, 2002; TAVARES *et al.*, 2003).

Existem técnicas moleculares mais sensíveis para procurar o DNA do parasito; no entanto, eles são caros e exigem equipamentos sofisticados, limitando assim seu uso (SINGH e SUNDAR, 2015). Testes sorológicos tornam-se uma ferramenta diagnóstica ideal para a

detecção de anticorpos antileishmanial em soros de pacientes infectados, devido à sua simplicidade e baixo custo, e são considerados menos invasivos (ELMAHALLAWY. *et al*, 2014). Como consequência, métodos como imunofluorescência indireta (IFAT), teste de aglutinação direta (DAT), ensaio imunoenzimático (ELISA), teste de aglutinação em látex e testes imunocromatográficos têm sido empregados (FARAHMAND e NAHREVANIAN; 2016; SCHWARZ, N.G. *et al*, 2017). Entretanto, seu desempenho sorodiagnóstico é dificultado pela sensibilidade e / ou especificidade variável, levando à ocorrência de resultados falso-negativos ou falso-positivos (MATLASHEWSKI, G. *et al*. 2013).

## **2.7. TRATAMENTO DAS LEISHMANIOSES**

O tratamento das leishmanioses está disponível desde o início do século 20 (WHO, 2010). A apresentação clínica, a espécie de *Leishmania*, a ocorrência de falhas terapêuticas anteriores, a viabilidade do medicamento e a localização das lesões influenciam na escolha terapêutica. O objetivo do tratamento é a cura clínica das lesões e eliminação do parasito, ou o aprimoramento da capacidade do hospedeiro para a auto cura da lesão (GOTO e LINDOSO, 2010).

Embora cerca de 90% das lesões na LT possam se curar espontaneamente (entre 3 e 18 meses), o tratamento é importante, principalmente, nas lesões recentes, múltiplas e disseminadas; em portadores de imunossupressão e nos casos de ocorrência de lesões localizadas nas articulações (DAVIES *et al.*, 2003; MURRAY *et al.*, 2005). Acredita-se que a LM geralmente resulta da evolução da LC não-tratada ou com tratamento inadequado (BARRAL-NETO *et al.*, 1995). O tratamento da LT pode ser local ou sistêmico. O critério a favor da abordagem sistêmica é se as lesões têm envolvimento da mucosa ou dos linfonodos e/ ou se são refratárias ao tratamento local (PISCOPO e AZZOPARDI, 2006). Modalidades físicas de tratamento, incluindo crioterapia e aquecimento local por infravermelho vem sendo utilizadas, com índice de sucesso variável. A aplicação da pomada de paramomicina associada ao cloreto de metilbenzetônio apresenta taxa de cura de 74 a 85%, sendo mais efetiva do que o mesmo fármaco associado à ureia, entretanto com maior incidência de reações inflamatórias locais severas. Infiltração intralesional na derme ou na base da lesão com antimoniais pentavalentes pode ser feita. Esse procedimento é relativamente doloroso e necessita de ser feito a cada uma ou duas semanas, de três a oito vezes. O índice de cura com esse procedimento é 75%. Se não efetivo, deve-se considerar a abordagem sistêmica (PISCOPO e AZZOPARDI,

2006).

A abordagem de tratamento sistêmico compreende a terapia da LV e nos casos de LT não-responsivos ao tratamento tópico. Nesse contexto, a terapia convencional é a administração parenteral dos antimoniais pentavalentes: Pentostam<sup>®</sup> e Glucantime<sup>®</sup> (GOTO e LINDOSO, 2010; MURRAY *et al.*, 2005). O Glucantime<sup>®</sup> é o produto mais usado no Brasil e em países da América Latina (SANTOS *et al.*, 2008). Este medicamento atua sobre diversas vias do metabolismo dos parasitos, sendo sugerido que algumas particularidades químicas em sua composição podem contribuir para o efeito farmacológico. Ele parece atuar no mecanismo bioenergético das formas amastigotas dos parasitos, por meio de glicólise e  $\beta$ -oxidação que ocorrem nas organelas denominadas glicossomas. Outro mecanismo sugerido é a ligação com sítios sulfidrílicos incluindo os tripanotiona (que é uma forma não usual de glutathione contendo duas moléculas de glutathione conectadas por um espermina) levando à morte dos parasitos (ROBERTS *et al.*, 1995). Na LV, a dose é de 20 mg/kg/dia de antimonial pentavalente ( $Sb^{+5}$ ) durante 20 a 30 dias. (MURRAY *et al.*, 2005). A dose recomendada é de 10 a 20 mg/kg/dia de  $Sb^{+5}$  durante 20 dias na LC, 20 mg/kg/dia de  $Sb^{+5}$  durante 20 dias na LCD e 20 mg/kg/dia de  $Sb^{+5}$  durante 30 dias na LM (GOTO e LINDOSO, 2010).

A eficácia dos antimoniais é variável, dependendo da região geográfica, espécie de *Leishmania* e apresentação clínica. Infelizmente, o aumento de casos de falha terapêutica tem sido registrado em várias regiões do mundo, sendo que no Brasil observa-se a mesma em 16% dos pacientes em tratamento de LT (GOTO e LINDOSO, 2010). O regime de tratamento é ainda acompanhado por toxicidade relativamente frequente, manifestada por dores, artralgia, mialgia, náusea, edema e eritema. As queixas podem ser discretas ou moderadas e, raramente, exigem a suspensão do tratamento. Entretanto, efeitos adversos graves como cardiotoxicidade e falência renal podem ocorrer, principalmente, em pacientes idosos (MURRAY *et al.*, 2005). Assim, o fármaco atualmente considerado como primeira escolha está longe de ser satisfatório. Ressalta-se ainda que os antimoniais pentavalentes não podem ser administrados nas gestantes (GOTO e LINDOSO, 2010). O aparecimento de resistência de espécies de *Leishmania* aos antimoniais é relatado em alguns países, como na Índia (AMEEN, 2010; MANANDHAR *et al.*, 2008; PISCOPO e AZZOPARDI, 2006). Além disso, os antimoniais não são efetivos no tratamento da LM, especialmente, na manifestação severa da doença (PISCOPO e AZZOPARDI, 2006).

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

como AmpB, pentamidina e paramomicina (SANTOS *et al.*, 2008). Apesar de existirem alternativas de tratamento tanto para LV, quanto para LT, nenhum desses medicamentos foi desenvolvido com a finalidade exclusiva de eliminar o parasito do gênero *Leishmania*. Além disso, produzem inúmeros efeitos colaterais e suas formulações possuem preços elevados. A miltefosina é dos medicamentos de segunda escolha, sendo o primeiro medicamento oral para leishmaniose.

Este medicamento é uma alquilfosfocolina que tem atividade contra células cancerígenas e várias espécies de parasitos, assim como bactérias e fungos. Foi originalmente desenvolvida como um fármaco anticancerígeno, tendo sido aprovado na Índia como o primeiro tratamento oral para as leishmanioses (PEYRON *et al.*, 2005; NAGLE *et al.*, 2014). Desde 2002, a miltefosina é o único agente oral que é usada para o tratamento de todos os tipos de leishmaniose em diversos países no mundo (DORLO *et al.*, 2012). Este fármaco interfere na membrana celular sem interagir com o DNA e modula a permeabilidade da célula, a fluidez e composição de lipídios da membrana, o metabolismo dos fosfolipídios e a transdução do sinal de proliferação (JENDROSSEK e HANDRICK, 2003). Entretanto, apresenta eventos adversos que incluem problemas gastrointestinais, hepato e nefrotoxicidade. Outra limitação é a teratogenicidade da miltefosina, desta forma, mulheres em idade fértil precisam tomar contraceptivos durante o tratamento e um adicional de três meses depois, devido à meia-vida longa do medicamento (PANDEY *et al.*, 2009; DORLO *et al.*, 2012; NAGLE *et al.*, 2014). Outro problema enfrentado no tratamento com a miltefosina é o aparecimento de resistência de algumas espécies de *Leishmania* à sua ação farmacológica (DORLO *et al.*, 2012).

A AmpB é um antibiótico poliênico heptaeno de amplo espectro obtido de culturas de *Streptomyces nodosus* (DISMUKES, 2000). A AmpB tem uma estrutura química bastante complexa, apresenta características hidrofóbicas em decorrência da parte apolar de sua molécula, caracterizada pela presença de sete duplas conjugadas carbono-carbono; e características hidrofílicas, decorrentes da presença de inúmeros grupos hidroxila. Além disso, ela possui os grupos carboxílico e amino, que lhe conferem propriedades anfífilicas (DAMASCENO, 2010), sendo solúvel em água em pH abaixo de 2 ou acima de 11, porém nessas condições extremas, o fármaco não é estável (TORRADO *et al.*, 2008). Já em meio neutro, a AmpB se apresenta como um “zwitterion” (GOLENSER e DOMB, 2006), uma vez que os valores de pKa para o grupo amino protonado e para o grupo carboxílico são 10 e 5,7, respectivamente. A solubilidade da AmpB em água em pH fisiológico (6 a 7) é muito baixa,

sendo inferior a 1 mg/L (TORRADO *et al.*, 2008). Ressalta-se ainda que a AmpB é insolúvel em grande parte de solventes orgânicos (EGITO *et al.*, 2002). O fármaco é solúvel em dimetilsulfóxido (DMSO), levemente solúvel em dimetilformamida, muito levemente solúvel em metanol e praticamente insolúvel em álcool. Essa insolubilidade da AmpB em água leva à formação de agregados em concentrações definidas. A proporção relativa das diferentes espécies depende da concentração e é possível determinar o estado de agregação do fármaco pelo seu espectro de absorção no ultravioleta-visível (UV-Vis). Em concentrações abaixo de  $10^{-6}$  M, concentração de agregação crítica (CAC), o espectro de absorção é característico da forma monomérica solúvel, responsável por uma intensa banda a 409nm. Em contraste, em altas concentrações, as moléculas auto-associadas de AmpB formam oligômeros e, então, agregados de oligômeros e observa-se um novo espectro (EGITO *et al.*, 2002; TORRADO *et al.*, 2008).

A AmpB é recomendada como fármaco de segunda linha no tratamento da LT e LV, especialmente, quando há falha terapêutica com os fármacos antimoniais e no caso de pacientes soropositivos para o vírus da imunodeficiência humana (YARDLEY e CROFT, 1997). Além disso, é o fármaco de escolha para o tratamento de infecções fúngicas progressivas potencialmente graves (SHADKHAN *et al.*, 1997). O fármaco interage com o ergosterol, constituinte da membrana celular de fungos ou espécies de *Leishmania*, ou com o colesterol da membrana celular dos mamíferos, ainda que o antibiótico possua maior afinidade pelo ergosterol. A ligação ao ergosterol altera a permeabilidade celular seletiva, uma vez que há formação de poros e conseqüente extravasamento de íons (principalmente de íons potássio) e substâncias vitais. Distúrbios na atividade enzimática das membranas também ocorrem, provocando a morte celular (GOLENSER e DOMB, 2006).

Embora a AmpB apresente maior afinidade pelo ergosterol, muitos dos efeitos tóxicos que lhe são atribuídos decorrem da sua capacidade em se ligar ao colesterol e outros constituintes da membrana celular de mamíferos. As reações adversas agudas da AmpB, tais como febre, calafrios, tremores, náusea, vômitos e dor de cabeça, ocorrem frequentemente e estão relacionadas, principalmente, à infusão. Alterações cardiovasculares como hipotensão, hipertensão e arritmias cardíacas são observadas, ainda que com menor frequência. Hipocalemia, hipernatremia, diurese aumentada, hipomagnesemia, disfunção renal e efeitos tóxicos sobre a medula óssea (anemia, leucopenia e trombocitopenia) são associados com administrações repetidas do produto, necessárias no tratamento. A toxicidade está mais relacionada com a forma agregada da AmpB, que é mais tóxica que a monomérica. A forma

AmpB auto-associada solúvel em água propicia mudanças de permeabilidade nas membranas de células vermelhas e induz eventos citotóxicos (LEGRAND *et al.*, 1992).

A AmpB é comercializada como um pó liofilizado que é disperso no momento do uso. Para o tratamento da LT, recomenda-se a infusão de 20 doses em dias alternados de 1 mg/kg do fármaco reconstituída em 500 mL de solução de glicose durante 4 horas, sendo a dose total do tratamento 1 a 1,5 gramas de AmpB (GOTO e LINDOSO, 2010; HEPBURN, 2000). Para o tratamento da LV recomenda-se infusão lenta de 15 doses em dias alternados de 1 mg/kg ou 20 doses de 1 mg/kg diariamente (MURRAY *et al.*, 2005). Infelizmente, o excipiente desoxicolato de sódio é hemolítico e a formulação convencional é altamente nefrotóxica.

A redução da nefrotoxicidade da AmpB é o principal objetivo das formulações lipídicas que surgiram na década de 1990 (TORRADO *et al.*, 2008; VYAS e GUPTA, 2006). Além da formulação convencional, três preparações lipídicas de AmpB estão disponíveis comercialmente: a AmpB lipossomal (AmpBisome<sup>®</sup>), o complexo lipídico de AmpB (Abelcet<sup>®</sup>, ABLC) e a dispersão coloidal de AmpB (Amphocil<sup>®</sup>, ABCD) (VYAS e GUPTA, 2006). Todas essas formulações apresentam eficácia semelhante (GOTO e LINDOSO, 2010) e diferem quanto à estrutura, forma, tamanho, composição e conteúdo do fármaco.

As formulações lipídicas comercialmente disponíveis têm indicação regulamentada apenas para LV (VYAS e GUPTA, 2006). A AmpB lipossomal não tem sido usada na LT, mas relatos isolados de seu uso em casos de resistência aos tratamentos convencionais têm mostrado efetividade. A AmpB e sua formulação lipossomal vem sendo utilizadas com sucesso em casos complicados de LM (PISCOPO e AZZOPARDI, 2006).

A diminuição de toxicidade do tratamento com o uso das formulações lipídicas de AmpB, comparado ao tratamento convencional é bem estabelecida na literatura. 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 marcante redução da nefrotoxicidade, além da diminuição dos efeitos adversos relacionados à infusão (DISMUKES, 2000; VYAS e GUPTA, 2006).

Apesar da melhoria do índice terapêutico para as formulações lipídicas, a sua utilização permanece limitada, principalmente, pelo alto custo dos produtos (EGGER *et al.*, 2009). Portanto, o desenvolvimento de novos sistemas de *delivery* para o tratamento de leishmaniose, com a incorporação de drogas eficazes, no propósito de reduzir os seus efeitos colaterais, bem como apresentar um custo acessível para a população, pode ser considerado

como prioridade (ITALIA *et al.*, 2011).

Como já foi exposto, embora as formulações lipídicas comercialmente disponíveis tenham demonstrado redução de toxicidade em comparação à formulação convencional, tais produtos apresentam, geralmente, atividade similar. O aumento da eficácia foi obtido quando doses maiores foram usadas. Além disso, o uso mais abrangente das formulações lipídicas comerciais é limitado pelo seu alto custo. Sendo assim, diversos estudos têm sido conduzidos com intuito de desenvolver formulações alternativas. O foco destes trabalhos será o desenvolvimento de sistemas nano-estruturados que propiciem maior eficácia e menor toxicidade, em comparação à formulação convencional.

## 2.8 PRODUTOS NATURAIS

A utilização de extratos vegetais, óleos essenciais e produtos naturais, representam uma fonte rica de novas entidades químicas para a pesquisa e o desenvolvimento de novos medicamentos para doenças negligenciadas (TEMPONE *et al.*, 2005). O uso empírico de plantas medicinais tem demonstrado que partes como o caule, as raízes, folhas, sementes e os frutos têm eficiência no tratamento de diversas doenças, suscitando o interesse em seu estudo.

Desta forma, nos últimos anos, o recurso vegetal tornou-se uma importante fonte de produtos biologicamente ativos, sendo que 25% dos medicamentos atualmente disponíveis foram originados a partir de pesquisas com produtos naturais, alguns dos quais têm sido usados como matéria-prima de fármacos semissintéticos. Estudos farmacológicos identificaram possíveis moléculas bioativas, como quinolina, alcalóides, cumarinas, taninos, saponinas, ciclosporina, dentre outros, como capazes de serem utilizadas em um futuro tratamento de doenças, dentre as quais, as leishmanioses (VALADARES *et al.*, 2011; LAGE *et al.*, 2013; LAGE *et al.*, 2015; DUARTE *et al.*, 2016c).

Os compostos heterocíclicos vêm apresentando importância na construção de novos fármacos, dentre eles destacam-se a quinolina e seus derivados. As quinolinas são compostos formadas por azoto aromático heterocíclico e caracterizadas por apresentarem uma estrutura de anel duplo que contém um anel de benzeno fundido com piridina em dois átomos de carbono adjacentes. Por terem atividades biológicas diversificadas (SOLOMON e LEE, 2011; AFZAL *et al.*, 2015), dentre elas, antimalárica, antimicrobiana, antipsicóticos, anticancerígena, além de uma estrutura relativamente simples com versatilidade sintética que permite a geração de um grande número de derivados estruturalmente diversos; tais moléculas têm atraído o olhar da

comunidade científica, especialmente, de pesquisadores envolvidos na química dos produtos naturais e químicos orgânicos interessados na obtenção de drogas promissoras.

Dentre as moléculas derivadas da quinolina, a 8-HQN, um composto ligante de ferro (JI e ZHANG, 2005), está presente em uma variedade de plantas biologicamente ativas, sendo conhecido por apresentar efeitos biológicos distintos, como inibição de protease, no tratamento de câncer, pacientes infectados pelo HIV, e doenças neurodegenerativas, como doença de Alzheimer e Parkinson, bem como atividades antiparasitária e antibacteriana (LENTEZ *et al.*, 1999; COLLERY *et al.*, 2000; PRACHAYASITTIKUL *et al.*, 2013).

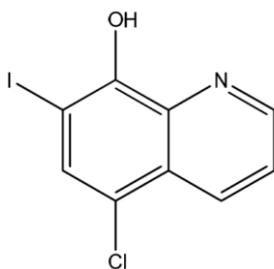
Estudos recentes realizados pelo nosso grupo de pesquisa demonstraram que a 8-HQN apresenta atividade antileishmanial *in vitro* contra promastigotas e amastigotas de importantes espécies de *Leishmania* no mundo (DUARTE, *et al.*, 2016a). Além disso, quando foi incorporada a um sistema de *delivery* baseado em poloxâmero 407, este produto foi eficaz no tratamento de camundongos BALB/c infectados com *L. amazonensis* ou *L. infantum* (DUARTE, *et al.*, 2016b; LAGE *et al.*, 2016). No presente estudo, testamos um derivado da 8-HQN, o clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol), frente a duas espécies de *Leishmania* capazes de causar LV e LT.

## 2.9 CLIOQUINOL

O clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol) é um medicamento que foi desenvolvido em 1899 como um antisséptico tópico. Este medicamento foi comercializado como antimicrobiano oral entre os anos de 1950 a 1970 para o tratamento de várias doenças intestinais e infecções de pele (MAO e SCHIMMER, 2008, BAREGGI e CORNELLI, 2010). Posteriormente, foi reaproveitado para tratar certas infecções fúngicas e protozoárias. O clioquinol vem sendo investigado para o tratamento de doenças como Alzheimer, Parkinson, Huntington e neoplasias (OLIVERI e VECCHIO, 2016). Um dos mecanismos de ação identificados do clioquinol refere-se à sua capacidade de interagir com os metais (propriedades quelantes de metais).

Atualmente o clioquinol é utilizado sob a forma de um creme (e em combinação com betametasona ou fluocinolona) no tratamento de doenças inflamatórias da pele tais como eczema, pé de atleta, coceira e micose. Sendo um componente dos medicamentos de prescrição Vioform®, Hidrocorte®, dentre muitos outros, que são antifúngicos tópicos.

No que se diz respeito à farmacocinética e metabolismo do clioquinol em ratos, sua absorção é rápida e sofre metabolismo de primeira passagem, transformando-se nos conjugados glucoronato e sulfato, que apresentam concentrações plasmáticas maiores que as do clioquinol livre (CHEN *et al.*, 1976; BAREGGI e CORNELLI, 2010). Em hamsters, as concentrações plasmáticas são muito baixas após a administração pela via oral, apresentando uma biodisponibilidade relativa de 12%, observada após administração de uma suspensão oral de clioquinol em carboximetilcelulose (BONDILOTTI *et al.*, 2007). Os estudos de farmacocinética em humanos são escassos. Um estudo que administrou doses de 250 a 1500 mg de clioquinol pela via oral em 6 indivíduos saudáveis, apresentou um tempo de meia-vida ( $t_{1/2}$ ) de 11 a 14 horas e um pico de concentração em torno de 4 horas após a administração (JACK e RIESS, 1973). Nos humanos o clioquinol apresenta uma menor metabolização na formação dos conjugados (CHEN *et al.*, 1976). O medicamento Hidrocorte® traz em sua bula que o clioquinol (um derivado hidroxiquinolônico halogenado) é o componente antimicrobiano, exerce uma ação bacteriostática ao invés de bactericida, é ativo contra um amplo espectro de micro-organismos patogênicos, incluindo fungos, por exemplo: *Candida*, *Microsporum*, *Trichofiton* e bactérias Gram-positivas, por exemplo: *Estafilococos*. Tem efeito inibitório apenas moderado sobre as bactérias Gram-negativas. A bula nos informa que após a aplicação tópica do creme dermatológico clioquinol + hidrocortisona, o clioquinol foi absorvido na proporção de cerca de 2% a 3%, conforme determinado por excreção urinária. Sendo o clioquinol excretado na urina, principalmente sob a forma de glicuronídeo, e em menor extensão, na forma de sulfato. Apenas traços de clioquinol não metabolizado são recuperados na urina.



**Figura 4.** Estrutura química do clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol).

## 2.10 REDIRECIONAMENTO DE FARMACO

O processo de descoberta e desenvolvimento de um novo medicamento é longo, caro e altamente regulamentado; uma vez que cada produto deve não só ser seguro e eficaz, mas a sua eficácia também deve ser comprovada em todos os grupos raciais e étnicos, bem como através

de diferentes faixas etárias (EICHBORN *et al.*, 2011).

O complexo caminho ligado às inovações científicas e tecnológicas para um fármaco inédito, leva de 2 a 3 anos para a descoberta e validação da finalidade, 0,5 a 1 ano para selecionar os protótipos com atividade biológica, mais 1 a 3 anos para otimizá-los, 1 a 2 anos para determinar propriedades ADMET (absorção, distribuição, metabolização, excreção e toxicidade) usando modelos animais. Para avaliar a segurança e eficácia em ensaios clínicos leva em média 5 anos e se tudo der certo, de 1 a 2 anos para obter aprovação e chegar no mercado (LI e JONES, 2012).

Em um estudo publicado em novembro de 2014, realizado pelo Centro para o estudo e desenvolvimento de fármaco (Tufts Center for the Study of Drug Development), estimou que para trazer um único fármaco novo para o mercado, o custo médio é de US \$ 2,6 bilhões em um período de tempo de aproximadamente 15 anos (DiMASI, 2014). Considerando que cerca de 90% dos medicamentos falham durante o desenvolvimento na fase I dos ensaios clínicos, este investimento é sempre de alto risco (LI e JONES, 2012). Estas questões exigem abordagens inovadoras que refletem as percepções de que nenhum alvo está sozinho, mas é incorporado em uma rede altamente complexa e heterogênea, e a reatividade cruzada de diferentes dosagens com outros alvos devem ser consideradas (EICHBORN *et al.* 2011).

Reposicionamento de fármaco (RF) ou reaproveitamento de fármaco é geralmente aplicado para a descoberta de novas atividades para uma medicação já usada clinicamente, ou seja, novos usos e aplicações terapêuticas (LIPINSKI, 2011). Trata-se do processo de encontrar novas utilizações fora do âmbito da indicação médica original de um medicamento aprovado ou composto que por alguma razão teve sua pesquisa descontinuada (MARUSINA *et al.*,2011).

## **2.11 NANOTECNOLOGIA**

O termo nanotecnologia remete à aplicação de materiais nanoparticulados e no desenvolvimento de sistemas em nanoescala, isto é, 1 a 100 nanômetros. Trata-se de uma área de ampla aplicabilidade que vêm contribuindo para o progresso na medicina, física, química, engenharia e nos mais diversos setores (MEHRAVAR, 2016). A nanotecnologia farmacêutica relaciona-se ao desenvolvimento, caracterização e aplicação de sistemas terapêuticos em sistema com escala nanométrica. Pesquisas de tais sistemas têm sido realizados ativamente no mundo com o propósito de direcionar e controlar a liberação de fármacos. A aplicação da nanotecnologia para fins terapêuticos e diagnósticos foi denominada “Nanomedicina” pelo

*National Institute of Health* nos Estados Unidos (WONG *et al.*, 2012).

Esta tecnologia data aos anos 1960, sendo que a primeira técnica a ser desenvolvida foi da microencapsulação, na qual polímeros e outras substâncias são transformados em partículas de tamanho micrométrico e após encapsulados. A microencapsulação é muito útil nas indústrias farmacêutica e cosmética, já que permite a proteção de substâncias sensíveis (lábeis ou voláteis), e o controle da liberação do fármaco, o que favorece na biodisponibilidade e conseqüentemente na redução da dose terapêutica e toxicidade. A microencapsulação contribuiu para utilização de técnicas mais eficientes, agora em escala nanométrica, legitimando o desenvolvimento de nanopartículas (KREUTER, 2014).

Nanopartículas poliméricas são sistemas utilizados para encapsulamento de fármacos, permitindo a liberação controlada e potencialização na biodisponibilidade e redução da toxicidade. Estas estruturas desempenham uma ferramenta tecnológica de características fundamentais como a biocompatibilidade, não imunogênica, atóxica e biodegradável. Nestes sistemas o agente terapêutico pode estar aprisionado na matriz polimérica ou adsorvido na superfície micelar (MENDES, *et al.*, 2017).

As micelas poliméricas podem ser classificadas em dois grandes grupos, sendo: as micelas surfactantes e as micelas poliméricas. Esta última se apresenta como sistemas transportadores coloidais compostos de polímeros sintéticos ou naturais. As micelas poliméricas são uma classe ampla de nanocarreadores e são constituídas por um invólucro polimérico de tamanho variável, disposto ao redor de um núcleo. (BERTRAND e LEROUX, 2012)

Os medicamentos nanotecnológicos oferecem segurança e maior eficácia terapêutica para os pacientes. E por um outro lado, estes sistemas são capazes de estender a rentabilidade econômica dos medicamentos patenteados e criar uma nova fonte de receita para as indústrias farmacêuticas, fato que incentiva novas pesquisas na área e avanços terapêuticos. A utilização de produtos nanotecnológicos geram diversas vantagens e entre elas destacam-se: a proteção do fármaco no sistema terapêutico contra possíveis instabilidades no organismo, promovendo manutenção de níveis plasmáticos em concentração constante; o aumento da eficácia terapêutica; a liberação progressiva e controlada do fármaco pelo condicionamento a estímulos do meio em que se encontram (sensíveis à variação de PH ou de temperatura); a diminuição expressiva da toxicidade pela redução de picos plasmáticos de concentração máxima; a diminuição da instabilidade e decomposição de fármacos sensíveis; a possibilidade de direcionamento a alvos específicos (sítio especificidade); a possibilidade de incorporação tanto

de substâncias hidrofílicas quanto lipofílicas nos dispositivos; a diminuição da dose terapêutica e do número de administrações e aumento da aceitação da terapia pelo paciente (PEIXOTO *et al.*, 2016). Embora sejam grandes as vantagens da nanotecnologia, existem inconvenientes que não podem ser ignorados, tais como a possibilidade de toxicidade dos insumos das nanopartículas, a ausência de biocompatibilidade dos materiais utilizados e, principalmente, o custo elevado de obtenção dos nanossistemas (LIN *et al.*, 2014).

***OBJETIVOS***

### 3. OBJETIVOS

#### 3.1. OBJETIVO GERAL

Avaliar a atividade antileishmanial *in vitro* e *in vivo* do clioquinol (ICHQ, 5-cloro-7-iodo-8-quinolinol, 5-cloro-8-hidroxi-7-iodoquinolina) contra diferentes espécies de *Leishmania*, no caso, *L. amazonensis*, e *L. infantum*; para o desenvolvimento de um tratamento a ser empregado contra as leishmanioses.

#### 3.2. OBJETIVOS ESPECÍFICOS

- 3.2.1. Avaliar a atividade antileishmanial do ICHQ contra formas promastigotas em fase estacionária de crescimento e amastigotas de *L. amazonensis* e *L. infantum* e a citotoxicidade em macrófagos peritoneais de camundongos e hemácias humanas.
- 3.2.2. Verificar o uso potencial do ICHQ na inibição da infecção de macrófagos por formas promastigotas em fase estacionária de crescimento de *Leishmania* spp.
- 3.2.3. Avaliar o mecanismo de ação do ICHQ sobre *Leishmania*.
- 3.2.4. Desenvolver formulações micelares incorporadas com ICHQ, infectar camundongos BALB/c com as espécies *L. amazonensis* ou *L. infantum* e realizar o tratamento utilizando as formulações descritas.
- 3.2.5. Avaliar a eficácia do tratamento dos animais por meio de leituras dos diâmetros das lesões (*L. amazonensis*) e da carga parasitária em fragmentos da lesão (*L. amazonensis*), baço, fígado, medula e linfonodos drenantes dos animais.
- 3.2.6. Avaliar a resposta celular por meio da produção de IFN- $\gamma$ , IL-2, IL-4, IL-10, IL-12, TNF- $\alpha$  e GM-CSF, além de óxido nítrico, por ELISA de captura e citometria de fluxo; e a resposta humoral pela avaliação dos níveis de IgG total, IgG1 e IgG2a específicos aos parasitos;
- 3.2.7. Avaliar a toxicidade do tratamento por meio da função hepática e renal nos animais infectados e tratados; bem como a melhor condição terapêutica utilizada.

## ***METODOLOGIA E RESULTADOS***

#### 4. METODOLOGIA E RESULTADOS

As seções de Metodologia e Resultados serão apresentadas sob a forma de artigos científicos, que foram diretamente derivados do projeto e que foram publicados em revistas internacionais de elevado impacto; conforme a Resolução nº 02/2013, de 18 de setembro de 2013 do Programa de Pós-Graduação em Ciências da Saúde: Infectologia e Medicina Tropical.

##### **Artigos científicos:**

**Artigo 1** – Antileishmanial activity, cytotoxicity and mechanism of action of clioquinol against *Leishmania infantum* and *Leishmania amazonensis* species. Basic Clin Pharmacol Toxicol. 2018 Sep;123(3):236-246. doi: 10.1111/bcpt.12990. Epub 2018 Apr 6.

**Artigo 2** – A Pluronic® F127-based polymeric micelle system containing an antileishmanial molecule is immunotherapeutic and effective in the treatment against *Leishmania amazonensis* infection. Parasitol Int. 2019 Feb;68(1):63-72. doi: 10.1016/j.parint.2018.10.005. Epub 2018 Oct 16.

#### 4.1. ARTIGO 1 – BREVE INTRODUÇÃO

O primeiro artigo a ser apresentado foi intitulado como “Antileishmanial Activity, Cytotoxicity and Mechanism of Action of Clioquinol Against *Leishmania infantum* and *Leishmania amazonensis* Species” e publicado na revista “*Basic & Clinical Pharmacology & Toxicology*” (doi: <https://doi.org/10.1111/bcpt.12990>). O estudo avaliou a atividade antileishmanial *in vitro* do clioquinol contra duas importantes espécies de *Leishmania* e a eficácia no tratamento de macrófagos infectados, bem como a inibição da infecção utilizando parasitos pré-tratados. Também, avaliou o mecanismo de ação da molécula em *L. amazonensis*.

## Antileishmanial Activity, Cytotoxicity and Mechanism of Action of Clioquinol Against *Leishmania infantum* and *Leishmania amazonensis* Species

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(Received 14 November 2017; Accepted 12 February 2018)

**Abstract:** In this study, a quinoline derivate, clioquinol (5-chloro-7-iodoquinolin-8-ol), was evaluated against *Leishmania amazonensis* and *Leishmania infantum* promastigotes and amastigotes. The cytotoxicity in murine macrophages and human red blood cells, as well as the efficacy in treating infected macrophages and the inhibition of infection using pre-treated parasites were also evaluated. Results showed that clioquinol inhibited *L. amazonensis* and *L. infantum* promastigotes with effective concentration 50% (EC<sub>50</sub>) values of  $2.55 \pm 0.25$  and  $1.44 \pm 0.35$  µg/mL, respectively, and of  $1.88 \pm 0.13$  and  $0.98 \pm 0.17$  µg/mL against axenic amastigotes, respectively. The cytotoxic EC<sub>50</sub> concentrations of clioquinol in murine macrophages and human red blood cells were, respectively,  $255 \pm 23$  and  $489 \pm 20$  µg/mL. With these results, the selectivity index was calculated, showing values of 99.9 and 177.1 against promastigotes, respectively, and of 135.6 and 260.1 against axenic amastigotes, respectively. Significant reductions in the percentage of infected macrophages after treatment using clioquinol were also observed, as well as when parasites were pre-treated with clioquinol and used to infect murine macrophages. The mechanism of action of clioquinol was investigated in *L. amazonensis*, and results revealed morphological and biochemical alterations in the clioquinol-treated parasites, including reduction in cell volume, loss of mitochondrial membrane potential, increase in the ROS production and rupture of the plasma membrane. The externalization of phosphatidylserine (PS) at the cell surface was evaluated in treated parasites that had been doubly labelled with annexin and propidium iodide (PI). The results showed no significant difference for PS exposure when compared to the untreated control, although a significant increase in the PI/annexin V-labelled cell population was found in the treated parasites. Results suggest that clioquinol induces a discontinuity of the parasite membrane, possibly related to a characteristic event of cell death caused by necrosis. This study demonstrates, for the first time, the antileishmanial activity of clioquinol against two relevant *Leishmania* species and suggests that the mitochondria of the parasites may be a possible biological target leading to parasite necrosis. Our findings suggest that clioquinol may have a potential application in treatment of leishmaniasis and further studies should be performed in infected mammalian hosts.

Leishmaniasis are diseases able to cause high mortality and morbidity worldwide, with 380 million people at risk and 70,000 deaths being registered per year caused by the disease [1]. This disease complex is caused by the parasites of the genus *Leishmania*, with visceral leishmaniasis (VL) caused by the *Leishmania donovani* complex species, including *L. donovani* and *L. infantum*, and tegumentary leishmaniasis (TL) caused by *L. braziliensis*, *L. guyanensis* and *L. amazonensis* species in the Americas [2,3].

Drugs are an important tool to control the disease, and the treatment has been based on the administration of the pentavalent antimonials. However, these regimens are accompanied

by several side effects, such as arrhythmias, renal and hepatic toxicity, among others [4]. In addition, parasite resistance to current drugs has increased [5]. Other compounds, such as amphotericin B (AmpB) and its liposomal formulations (pentamidine, paromomycin, miltefosine, among others), have also been employed for this purpose; however, side effects and/or high cost have been commonly reported [6,7].

Plants constitute an important source of new pharmaceuticals, and studies have been developed in an attempt to identify the bioactive molecules that could be employed for this purpose, such as quinoline, alkaloids, coumarins, tannins, saponins, cyclosporine, among others [8–11]. As a consequence, an increasing awareness of the potential of these compounds might lead to the development of novel drugs for the treatment of neglected diseases, as the impact of natural products on drug discovery is considerable, not only for cancer, but also for parasitic infections [12,13].

Naphthoquinones are found in distinct plant families and are used in the traditional medicine for the treatment of diseases,

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particularly as anticancer agents [14,15]. In addition, their derivatives present biological properties, such as antimicrobial and anticancer activities, which have stimulated the study of these bioactive compounds in the field of applied chemistry [16]. In this context, 8-hydroxyquinoline (8-HQN) has been characterized as a naphthoquinone-derived molecule presenting a variety of biological actions, such as protease inhibition, effect on cancer, Alzheimer's disease and Parkinson's disease, as well as antiparasitic and antibacterial effects [17–19].

This molecule presented an antileishmanial activity against different *Leishmania* species [20], and when it was incorporated to a Poloxamer 407-based delivery system, the compound proved to be effective in the treatment of *L. amazonensis*- or *L. infantum*-infected BALB/c mice [21,22]. In this study, an 8-HQN derivate, clioquinol (5-chloro-7-iodoquinolin-8-ol), which presented antibacterial, antifungal and antiamebic activities [23,24], was tested against two *Leishmania* species able to cause VL and TL around the world. The antileishmanial activity against *in vitro* stationary promastigotes and intramacrophages amastigotes of *L. amazonensis* and *L. infantum*, seeking to obtain the effective concentration 50% (EC<sub>50</sub>), as well as the 50% cytotoxic concentration (CC<sub>50</sub>), in murine macrophages and in human red blood cells (RBC<sub>50</sub>), was investigated. This study evaluated the efficacy of clioquinol in the treatment of infected macrophages, as well as the inhibition of infection using pre-treated parasites, and evaluated the mechanism of action of this molecule in *L. amazonensis*.

## Materials and Methods

**Ethics and mice.** This study was approved by the Committee for the Ethical Handling of Research Animals (CEUA) of the Federal University of Minas Gerais (UFMG), logged under protocol number 085/2017 and was approved by the Human Research Ethics Committee (COEP/UFMG), logged under protocol number CAAE-32343114.9.0000.5149. BALB/c mice (female, 8 weeks old, weighing 20–25 g) were purchased from the UFMG Institute of Biological Sciences and were maintained within specific pathogen-free conditions. Peritoneal macrophages were obtained through peritoneal lavage using 5 mL of cold PBS 1x (phosphate-buffered saline: 137 mM NaCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, at pH 7.4). The peritoneal exudate cells were centrifuged at 1000 × g for 10 min., and the pellet was resuspended in RPMI 1640 medium. Cells were then counted and plated into 96-well plates for the biological assays.

**Parasites.** *Leishmania amazonensis* (IFLA/BR/1967/PH-8) and *L. infantum* (MHOM/BR/1970/BH46) were used in this study. The parasites were grown in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which consisted of Schneider's medium plus 20% heat-inactivated foetal bovine serum (FBS, Sigma-Aldrich) and 20 mM L-glutamine pH 7.4 at 24°C. Stationary-phase promastigotes were cultured as described elsewhere [25]. To obtain the axenic amastigotes, a technical protocol was developed as described elsewhere, with few modifications [26]. Briefly, 1 × 10<sup>9</sup> stationary-phase promastigotes were washed three times in sterile phosphate buffer saline (PBS) 1x and incubated for 48 hr or 72 hr for *L. amazonensis* or *L. infantum*, respectively, in 5 mL FBS at 37°C. The parasites were then washed three times in PBS 1x, and their morphology was evaluated by staining performed with the Giemsa method.

**Antileishmanial activity.** The *in vitro* inhibition of *Leishmania* growth was evaluated by cultivating *L. infantum* or *L. amazonensis* stationary promastigotes and axenic amastigotes (1 × 10<sup>6</sup> cells) in the presence of clioquinol (0–20.0 µg/mL) (catalog number 130-26-7, Sigma-Aldrich) in 96-well culture plates (Nunc, Nunclon, Roskilde, Denmark) for 48 hr at 24°C. AmpB (0–1.0 µg/mL, Sigma-Aldrich) was used as a drug control. Cell viability was assessed using the colorimetric assay with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma-Aldrich) method. For this, MTT solutions were prepared at 5 mg/mL in PBS 1x, filtered and sterilized at the time of use, and 15 µL was added to each well. The optical density (O.D.) values were read in an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, San Jose, CA, USA), at 570 nm. The effective concentration 50% (EC<sub>50</sub>) of product was determined by applying a sigmoidal regression of a dose–response curve. The technical protocol was developed as described in Ref. [20].

**Cytotoxicity.** Cytotoxicity was evaluated in murine macrophages and human red blood cells. The inhibition of 50% of the macrophage's viability (CC<sub>50</sub>) was calculated by cultivating these cells (5 × 10<sup>5</sup> cells) with clioquinol (0–20.0 µg/mL) or AmpB (0–1.0 µg/mL) in 96-well plates (Nunc) for 48 hr at 37°C. Cell viability was evaluated by the MTT method, using the same concentrations of the reagent described above. The technical protocol was developed as described in Ref. [20]. The inhibitory concentration to 50% of human red blood cells (RBC<sub>50</sub>) was evaluated as an additional cytotoxicity parameter. For this, an incubation with clioquinol (0–20.0 µg/mL) or AmpB (0–1.0 µg/mL) and a 5% red blood cell (human O type) suspension was performed for 1 hr at 37°C. Then, the suspension was centrifuged by 1000 × g for 10 min., and the cell lyses percentage was determined spectrophotometrically at 570 nm. The absence (blank) or 100% of haemolysis were determined by replacing clioquinol for an equal volume of PBS 1x or distilled water, respectively, and the results were calculated by the haemolysis percentage as compared to the negative (PBS 1x) and positive (distilled water) controls, respectively.

**Treatment of infected macrophages.** Murine macrophages (5 × 10<sup>5</sup> cells) 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 for 24 hr at 37°C in 5% CO<sub>2</sub>. Stationary promastigotes (5 × 10<sup>6</sup> cells, in a ratio of 10 parasites per one macrophage) were then added to the wells, and cultures were incubated for 48 hr at 37°C in 5% CO<sub>2</sub>. Free parasites were removed by extensive washing with RPMI 1640 medium, while infected macrophages were treated with clioquinol (5.0, 10.0, and 15.0 µg/mL) or AmpB (0.1, 0.5, and 1.0 µg/mL) for 48 hr at 24°C in 5% CO<sub>2</sub>. After fixation with 4% paraformaldehyde, treated and infected macrophages were stained by Giemsa, and the percentage of infected cells, as well as the number of intramacrophage amastigotes of 200 cells, were determined in an optical microscope.

**Inhibition of infection using pre-treated parasites.** To evaluate the inhibition of infection using pre-treated parasites, stationary promastigotes (5 × 10<sup>6</sup> cells) were first incubated with clioquinol (5.0, 10.0 and 15.0 µg/mL) or AmpB (0.1, 0.5 and 1.0 µg/mL) for 1 hr at 24°C. After, treated parasites were washed three times in RPMI 1640 medium, quantified and incubated with murine macrophages (at a ratio of 10 parasites per one macrophage) for 24 hr at 37°C in 5% CO<sub>2</sub>. These were then washed and stained by Giemsa to evaluate both the percentage of infected macrophages as well as the number of intramacrophage amastigotes of 200 cells in an optical microscope.

**Morphology and cell volume in clioquinol-treated *L. amazonensis*.** To evaluate the cell volume and morphology, promastigotes (1 × 10<sup>7</sup>

cells) were untreated (control) or treated with 2.55 and 5.10 µg/mL of clioquinol (corresponding to 1 and 2 times the EC<sub>50</sub> value, respectively) for 6 hr at 25°C. Thereafter, parasites were harvested and fixed in 4% paraformaldehyde for 20 min., washed with PBS 1x, placed on glass slides and stained with Giemsa. Slides were analysed by optical microscopy (Olympus BX53, Shinjuku, Tokyo, Japan) and photographed (Olympus DP73), using a 100× objective lens. To assess the cell volume, treated or untreated parasites were analysed on a FACsCANTO II flow cytometer equipped with DIVA software (Joseph Trotter, Scripps Research Institute, La Jolla, CA, USA). A total of 10,000 events were acquired, analysing the FSC (forward scatter) parameter, which was correlated with the cell volume. Three independent experiments were carried out in duplicate.

**Mitochondrial membrane potential in clioquinol-treated parasites.** Studies for the evaluation of  $\Delta\Psi_m$  were performed using the Mitotracker® Red CM-H2XRos (MTk) and rhodamine 123 (Rh123) probes. For this, promastigotes ( $1 \times 10^7$  cells) were also treated with clioquinol (2.55 and 5.10 µg/mL) for 6 hr at 25°C. The cells were then harvested, washed and incubated with MTk (500 nM) or Rh123 (0.5 µg/mL) for 40 or 15 min., respectively. Cells stained with MTk were washed, resuspended in PBS 1× and transferred to 96-well clear bottom black microplates, at which time the fluorescence intensity was evaluated by a spectrofluorometer (FLx800), with an excitation wavelength of 540 nm and an emission of 600 nm. A potent mitochondrial oxidative phosphorylation uncoupler (FCCP; 5.08 µg/mL) was used as a positive control. Rh123-labelled parasites were analysed by a FACsCanto II flow cytometer equipped with DIVA software, and a total of 10,000 events were acquired in each sample using the FITC channel. The variation index (VI) of the fluorescence intensity of Rh123 was determined by the equation (MT-MC)/MC, in which MT is the mean of the treated parasites and MC is the mean of untreated parasites. Negative VI values indicated depolarization of parasite mitochondrial membrane potential. Miltefosine (18.0 µg/mL) was used as a control.

**Reactive oxygen species in clioquinol-treated *L. amazonensis*.** The ROS production in *L. amazonensis* promastigotes was evaluated in the treated parasites, as described in [27]. Briefly, promastigotes ( $1 \times 10^7$  cells) were incubated alone or with clioquinol (2.55 and 5.10 µg/mL) for 6 hr at 25°C. Parasites were incubated with a 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) fluorescent probe, for 30 min. in the dark at room temperature. The ROS production was evaluated fluorometrically with an excitation wavelength of 485 nm and an emission of 528 nm. Miltefosine (18.0 µg/mL) was used as a control.

**Plasma membrane integrity and phosphatidylserine (PS) exposure in clioquinol-treated parasites.** To evaluate the apoptosis/necrosis-related mechanisms in clioquinol-treated *L. amazonensis*, promastigotes ( $1 \times 10^7$  cells) were untreated or treated with clioquinol (2.55 and 5.10 µg/mL) for 6 hr at 25°C. After, they were washed in PBS 1× and incubated with 1.0 mg/mL of propidium iodide (PI), during 15 min. at room temperature. The plasma membrane integrity was evaluated by fluorescence intensity as described in [28]. Promastigotes warmed at 65°C were used as a positive control. To evaluate PS exposure, treated parasites were incubated with annexin V-FITC (Invitrogen, USA) for 15 min. at room temperature, at which time they were incubated with PI (1.0 µg/mL) for 15 min., and reactions were evaluated by flow cytometry. A total of 10,000 events were acquired using the FITC and PE channels, and miltefosine (18.0 µg/mL) was used as a control.

**Analysis of cell cycle in clioquinol-treated parasites.** The analysis of cell cycle was performed as described in [29]. Briefly, *L. amazonensis*

promastigotes ( $1 \times 10^7$  cells) were untreated or treated with clioquinol (2.55 and 5.10 µg/mL) for 6 hr at 25°C. After having been washed, parasites were permeabilized, incubated with ribonuclease (200.0 µg/mL) for 1 hr at 37°C and labelled with PI (7.0 µg/mL) for 20 min. Data acquisition and analysis were performed using a CytOFLEX flow cytometer (Beckman Coulter, Indianapolis, IN, USA) equipped with CytExpert 2.0 software (Beckman Coulter). A total of 5000 events were acquired using a PI610ND-A channel. Experiments were performed in three replicates, each one in duplicate.

**In vivo toxic effects.** BALB/c mice (n = 8 per group) were subcutaneously inoculated, once a day, in their left hind footpad with PBS 1x, AmpB (1.0 mg/kg of body-weight) or clioquinol (50.0 mg/kg of body-weight) for 10 days. Variations in body-weight and clinical signs were monitored daily. One day after the end of treatment, blood samples were collected for biochemical analysis, when the cardiac function was analysed by the dosage of creatine kinase-MB, while the hepatic function was analysed by quantifying aspartate aminotransferase (AST) and alanine aminotransferase (ALT). Nephrotoxicity was evaluated by examining the levels of blood urea nitrogen (BUN) and serum creatinine (CRTN). Experiments were performed using commercial kits (Labtest Diagnostica®, Belo Horizonte, Minas Gerais, Brazil), each of which was repeated twice.

**Statistical analysis.** The results were evaluated using Microsoft Excel (version 10.0) and analysed using GraphPad Prism™ (version 6.0 for Windows). The EC<sub>50</sub> and CC<sub>50</sub> values were calculated from the mean percentage reduction of the stationary promastigotes (EC<sub>50</sub>) or murine macrophages (CC<sub>50</sub>), respectively, when compared to that in the untreated controls. The curves were determined by applying sigmoidal regression to the logarithm concentration/response data. The one-way analysis of variance (ANOVA), followed by Dunnett's test or the Bonferroni post-test, was used to compare the groups. Differences were considered significant when  $p < 0.05$  (\*),  $p < 0.01$  (\*\*),  $p < 0.001$  (\*\*\*) and  $p < 0.0001$  (\*\*\*\*).

## Results

**Antileishmanial activity and cytotoxicity induced by clioquinol.** The *in vitro* antileishmanial activity of clioquinol was investigated against two *Leishmania* species, and AmpB was used as a control. Results showed EC<sub>50</sub> values of  $2.55 \pm 0.25$  and  $1.44 \pm 0.35$  µg/mL for clioquinol against *L. amazonensis* and *L. infantum* promastigotes, respectively, while these values were of  $0.10 \pm 0.02$  and  $0.07 \pm 0.03$  µg/mL, respectively, when using AmpB. The EC<sub>50</sub> values against *L. amazonensis* and *L. infantum* axenic amastigotes were of  $1.88 \pm 0.13$  and  $0.98 \pm 0.17$  µg/mL for clioquinol, respectively. Using AmpB, the values were of  $0.14 \pm 0.03$  and  $0.05 \pm 0.02$  µg/mL, respectively (table 1). Regarding cytotoxicity, the CC<sub>50</sub> values were of  $254.90 \pm 22.60$  and  $1.00 \pm 0.13$  µg/mL for clioquinol and AmpB, respectively. With these data, the selectivity index (SI) was calculated by the ratio between CC<sub>50</sub> and EC<sub>50</sub> values, and results were of 99.9 and 177.1 for clioquinol against *L. amazonensis* and *L. infantum* promastigotes, respectively, and of 135.6 and 260.1 against axenic amastigotes, respectively (table 2). For AmpB, these values were of 10.0 and 14.3 against *L. amazonensis* and *L. infantum* promastigotes, respectively, and of 7.1 and 20.0 against axenic amastigotes, respectively. Like an additional parameter of cytotoxicity, the haemolytic activity was also evaluated, and

Table 1.

Antileishmanial activity and cytotoxicity in murine macrophages.

Compounds	EC <sub>50</sub> <sup>1</sup> (µg/mL)				CC <sub>50</sub> <sup>2</sup> (µg/mL)
	Promastigotes		Axenic amastigotes		
	<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>	
Clioquinol	2.55 ± 0.25	1.44 ± 0.35	1.88 ± 0.13	0.98 ± 0.17	254.90 ± 22.60
Amphotericin B	0.10 ± 0.02	0.07 ± 0.03	0.14 ± 0.03	0.05 ± 0.02	1.00 ± 0.13

The promastigotes and axenic amastigotes of the parasites ( $1 \times 10^6$  cells) were incubated with clioquinol (0–20.0 µg/mL) for 48 hr at 24°C, at which time the cell viability was analysed by MTT assay. Amphotericin B (0–1.0 µg/mL) was used as a control. In addition, murine macrophages ( $5 \times 10^5$ ) were also incubated with the compounds, and the cytotoxicity was evaluated by the MTT method. The results were expressed as mean ± standard deviation of the groups.

<sup>1</sup>Inhibition of 50% of the *Leishmania* viability (EC<sub>50</sub>).

<sup>2</sup>Inhibitory concentration to 50% of murine macrophages (CC<sub>50</sub>). The data shown represent the mean ± standard deviation of three independent experiments.

Table 2.

Selectivity index and cytotoxicity in human red blood cells. The selectivity index of clioquinol was calculated by the ratio between the CC<sub>50</sub> and EC<sub>50</sub> values, which were obtained in experiments against the *Leishmania amazonensis* and *Leishmania infantum* promastigotes and axenic amastigotes.

Compounds	Selectivity index				RBC <sub>50</sub> <sup>1</sup> (µg/mL)
	Promastigotes		Axenic amastigotes		
	<i>L. amazonensis</i>	<i>L. infantum</i>	<i>L. amazonensis</i>	<i>L. infantum</i>	
Clioquinol	99.9	177.1	135.6	260.1	488.90 ± 19.50
Amphotericin B	10.0	14.3	7.1	20.0	13.50 ± 2.20

The inhibitory concentration to 50% of human red blood cells (RBC<sub>50</sub>)<sup>1</sup> was evaluated as cytotoxicity parameter. For this, an incubation with clioquinol (0–20.0 µg/mL) or AmpB (0–1.0 µg/mL) and a 5% red blood cell (human O type) suspension was performed for 1 hr at 37°C. Then, the suspension was centrifuged by 1000 × g for 10 min., and the cell lyses percentage was determined spectrophotometrically at 570 nm. The data shown represent the mean ± standard deviation of three independent experiments.

results were of 488.90 ± 19.5 and 13.50 ± 2.20 µg/mL for clioquinol and AmpB, respectively (table 2).

#### Treatment of infected macrophages.

The effect of the clioquinol in treating infected macrophages was evaluated. In the results using *L. infantum*, the infection percentage in treated and infected cells and the number of recovered amastigotes were of 10.5% ± 1.7% and 0.2 ± 0, respectively, when 15.0 µg/mL of clioquinol was used in the experiments. Using *L. amazonensis*, these values were of 6.6% ± 0.8% and 0.1 ± 0, respectively (table 3). The infectiveness reduction was calculated, and results in *L. infantum* showed a drop in infection in the order of 85.5% when infected macrophages were treated with 15.0 µg/mL of clioquinol, and of 92.7% when *L. amazonensis*-infected macrophages were treated with this molecule in the same concentration. Using AmpB, infection percentage in treated and infected cells and the number of recovered amastigotes were of 10.3% ± 1.8% and 0.2 ± 0, respectively, when 1.0 µg/mL of this molecule was employed. Using *L. amazonensis*, these values were of 11.2% ± 2.2% and 0.4 ± 0.1, respectively. The infectiveness reduction was also calculated and showed a drop in infection in the order of 85.8% in treated and *L. infantum*-infected cells, and of 87.5% when *L. amazonensis*-infected macrophages were

treated with AmpB. When macrophages were infected and untreated, infection percentage was of 72.3% ± 4.1% and 89.9% ± 4.5%, respectively, using *L. infantum* and *L. amazonensis*, while the number of recovered amastigotes was of 3.9 ± 0.2 and 7.7 ± 0.5, respectively.

#### Inhibition of infection using pre-treated parasites.

An analysis to evaluate the inhibitory potential of clioquinol in *Leishmania* was performed by incubating this molecule with parasite promastigotes, which were later used to infect murine macrophages. In the results, when *L. infantum* was incubated with clioquinol (15.0 µg/mL), the infection percentage and the number of recovered amastigotes were of 16.5% ± 3.5% and 0.3 ± 0.1, respectively, whereas against *L. amazonensis*, these values were of 19.8% ± 1.9% and 0.3 ± 0.1, respectively (table 4). Using AmpB (1.0 µg/mL), the infection percentage was of 18.7% ± 2.4% and 22.3% ± 3.2% for *L. infantum* and *L. amazonensis*, respectively, and the number of recovered amastigotes was of 0.5 ± 0.2 and 0.6 ± 0.1, respectively. When parasites were untreated and used to infect macrophages, the infection percentage was of 79.8% ± 3.3% and 93.4% ± 5.5% for *L. infantum* and *L. amazonensis*, respectively, and the number of recovered amastigotes was of 4.4 ± 0.5 and 8.6 ± 0.8, respectively.

Table 3.

Treatment of infected macrophages.

Products	Concentration (µg/mL)	Percentage of infected macrophages after treatment	Number of amastigotes per macrophage	Infectiveness reduction (%)
<i>Leishmania infantum</i>				
Clioquinol	15.0	10.5 ± 1.7	0.2 ± 0	85.5
	10.0	19.8 ± 3.2	0.7 ± 0.2	72.6
	5.0	31.3 ± 2.6	1.2 ± 0.3	56.7
	0	72.3 ± 4.1	3.9 ± 0.2	–
Amphotericin B	1.0	10.3 ± 1.8	0.2 ± 0	85.8
	0.5	26.7 ± 3.4	0.9 ± 0.3	63.1
	0.1	36.6 ± 2.8	1.8 ± 0.4	49.4
	0	72.3 ± 4.1	3.9 ± 0.2	–
<i>Leishmania amazonensis</i>				
Clioquinol	15.0	6.6 ± 0.8	0.1 ± 0	92.7
	10.0	8.4 ± 1.6	0.2 ± 0	90.6
	5.0	16.5 ± 2.2	0.4 ± 0.1	81.6
	0	89.9 ± 4.5	7.7 ± 0.5	–
Amphotericin B	1.0	11.2 ± 2.2	0.4 ± 0.1	87.5
	0.5	21.3 ± 3.2	0.9 ± 0.4	76.3
	0.1	33.4 ± 4.7	3.1 ± 0.5	62.8
	0	89.9 ± 4.5	7.7 ± 0.5	–

To evaluate the potential of clioquinol in treating infected macrophages, these cells ( $5 \times 10^5$ ) were previously infected with *L. amazonensis* or *L. infantum* stationary promastigotes (in a ratio of 10 parasites per one macrophage), and later treated with clioquinol (5.0, 10.0 and 15.0 µg/mL) for 48 hr at 24°C in 5% CO<sub>2</sub>. Amphotericin B (0.1, 0.5, and 1.0 µg/mL) was used as a drug control. The percentage of infected macrophages, the number of recovered amastigotes and the infection reduction in treated macrophages were calculated by applying a sigmoidal regression of concentration–inhibition curves determined by counting 200 cells per coverslip, in triplicate. The data shown represent the mean ± standard deviation of three independent experiments.

#### Mechanism of action in *L. amazonensis*.

This study used an optical microscopy and flow cytometry assay, aimed at evaluating whether or not treatment with clioquinol causes morphological alterations in *L. amazonensis* promastigotes. In the results, the treatment using clioquinol induced changes in the cell motility and morphological appearance. Photomicrography, when compared to untreated parasites, showed rounded promastigotes, enveloped flagellum and a subtle decrease in cell volume (fig. 1). In addition, flow cytometry assays confirmed a significant reduction in cell volume after the treatment with clioquinol (fig. 2). Alterations in the mitochondrial membrane potential ( $\Delta\Psi_m$ ) of the parasites were then evaluated. The results showed that treatment, when compared to negative control, induced a significant reduction in the MTK fluorescence intensity in the order of 14.99% and 16.09%, respectively (fig. 3). In addition, clioquinol-treated promastigotes and those labelled with the Rh123 fluorescent probe, showed a significant reduction in their fluorescence intensity in relation to untreated parasites. These results suggested that treatment using clioquinol induces  $\Delta\Psi_m$  alterations in promastigote forms. The fluorescent probe H2DCFDA was used to evaluate the ROS production in clioquinol-treated parasites, and the subsequent results revealed significant increases in the fluorescence intensity in the order of 3.99 and 4.06 times, respectively, when compared to the untreated controls (fig. 4), suggesting that the treatment with clioquinol induced oxidative stress in *L. amazonensis*.

Evaluating the plasma membrane integrity, clioquinol-treated promastigotes were labelled with PI and evaluated. Results, when compared to untreated controls, showed a significant

increase in their fluorescence intensity (fig. 5). These data indicated that an internalization of the fluorescent marker had occurred, suggesting that the treatment with clioquinol caused the rupture of the parasite membrane. As the loss of plasma membrane is one indicator of death by apoptosis, we analysed if the treatment could induce the externalization of PS at the cell surface. For this, promastigotes were treated and doubly labelled with annexin and PI and were then evaluated by flow cytometry. Results showed no significant difference for PS exposure (lower right quadrant) when compared to the untreated control. However, a significant population of PI-labelled cells (upper left quadrant) was directly proportional to the concentration of clioquinol used in the treatment, when compared to the untreated control (fig. 6). In addition, the treatment induced a significant increase in the PI/annexin V-labelled cell population (right upper quadrant). These results suggested that clioquinol induces a discontinuity of the parasite membrane, possibly related to a characteristic event of cell death caused by necrosis. We have investigated if the treatment with clioquinol could induce changes in the cell cycle. The results showed that clioquinol did not interfere in the cell cycle, and an increase in cells in the sub-G<sub>0</sub>/G<sub>1</sub> peak (DNA fragmentation), characteristic of cells in apoptosis, was not observed (fig. 7). By contrast, miltefosine presented a significant increase in the sub-G<sub>0</sub>/G<sub>1</sub> population (52.77%), when compared to untreated controls.

#### Toxicity in a mammalian model.

As the core purpose of this study is to develop a product to be used against leishmaniasis in infected mammalian hosts,

Table 4.

Immunotherapeutic action of cloiquinol.

Products	Concentration (µg/mL)	Percentage of infection using pre-treated parasites	Number of amastigotes per macrophage	Infectiveness reduction (%)
<i>Leishmania infantum</i>				
Clioquinol	15.0	16.5 ± 3.5	0.3 ± 0.1	79.3
	10.0	29.8 ± 2.8	0.9 ± 0.3	62.7
	5.0	38.7 ± 4.5	1.5 ± 0.2	51.5
	0	79.8 ± 3.3	4.4 ± 0.5	–
	Amphotericin B	1.0	18.7 ± 2.4	0.5 ± 0.2
Amphotericin B	0.5	33.2 ± 4.1	1.4 ± 0.4	58.4
	0.1	46.5 ± 3.7	2.3 ± 0.2	41.7
	0	79.8 ± 3.3	4.4 ± 0.5	–
	<i>Leishmania amazonensis</i>			
Clioquinol	15.0	19.8 ± 1.9	0.3 ± 0.1	78.8
	10.0	26.5 ± 3.3	0.8 ± 0.2	71.6
	5.0	40.4 ± 3.8	2.1 ± 0.5	56.7
	0	93.4 ± 5.5	8.6 ± 0.8	–
Amphotericin B	1.0	22.3 ± 3.2	0.6 ± 0.1	76.1
	0.5	32.3 ± 4.3	2.1 ± 0.5	65.4
	0.1	55.4 ± 3.7	4.9 ± 1.1	40.7
	0	93.4 ± 5.5	8.6 ± 0.8	–

The inhibitory effect of cloiquinol on the *Leishmania* infection in murine macrophages was evaluated using pre-treated *L. amazonensis* or *L. infantum* promastigotes to infect these cells. For this, parasites ( $5 \times 10^6$ ) were first incubated with cloiquinol (5.0, 10.0 and 15.0 µg/mL) or AmpB (0, 0.1, 0.5 and 1.0 µg/mL) for 1 hr at 24°C. Cells were then washed three times in an RPMI 1640 medium, quantified and incubated with macrophages ( $5 \times 10^5$  cells) for 24 hr at 37°C in 5% CO<sub>2</sub>. After, cells were washed and stained by Giemsa method to evaluate the percentage of infected macrophages, the number of intramacrophage amastigotes and the reduction of macrophage infection, which were determined by counting 200 cells in triplicate. The data shown represent the mean ± standard deviation of three independent experiments.

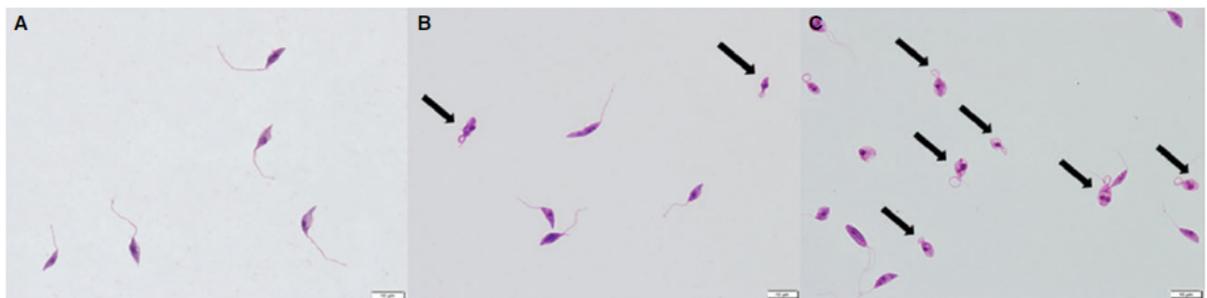


Fig. 1. Clioquinol causes changes in the morphology of *Leishmania* promastigotes. To verify whether the compound cloiquinol causes changes in the *Leishmania amazonensis* morphology, promastigotes ( $1 \times 10^7$  cells) were untreated (A) or treated with 2.55 µg/mL (B) or 5.10 µg/mL (C) of the compound, then fixed in 4% paraformaldehyde, placed on glass slides and stained with Giemsa. Slides were analysed by optical microscopy and photographed at 100 times magnification. The arrows show promastigotes with rolled flagella and altered cell morphology. Images are representative of three independent experiments performed in triplicate.

the *in vivo* side effects induced by cloiquinol in BALB/c mice have been analysed through clinical and biochemical evaluations in the animals that received the treatment for 10 days. As a control, mice received saline or AmpB. The levels of the cardiac, hepatic and renal markers were measured, which demonstrated that cloiquinol-treated mice presented unchanged amounts of these enzymes, while AmpB-treated animals presented higher levels of AST, ALT, BUN and CRTN, reflecting hepatic and renal toxicity. In addition, loss of weight and weakness were signals observed in AmpB-treated mice, which also showed a negative variation in the order of 6% in their body-weight. By contrast, cloiquinol-treated animals showed

no clinical alteration, as well as a positive body-weight variation of 5% was observed at the end of the experiment.

### Discussion

The antileishmanial drugs currently available to treat against leishmaniases present limitations due to the high cost of the liposomal AmpB formulations; the systemic toxicity of the old therapeutics, as well as the difficult to administer them; and the increase in parasite resistance. In this context, there is an urgent need to discover new, safe and more effective products to be applied against this disease [30]. In this light, plants are

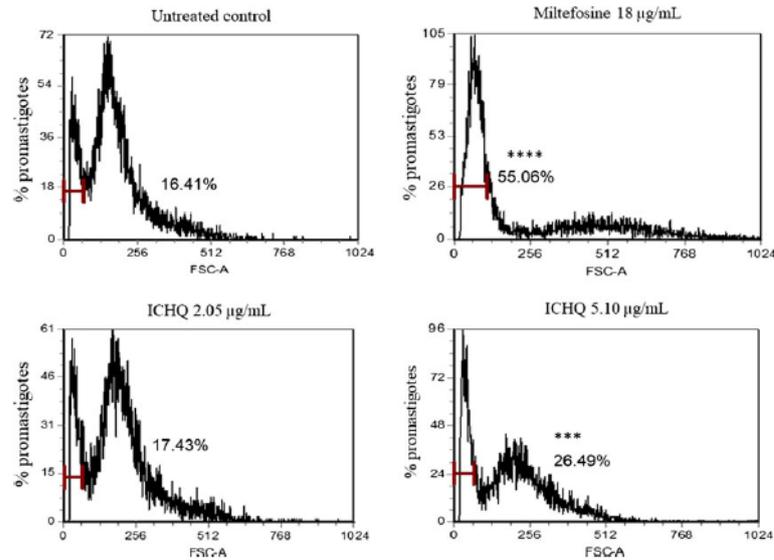


Fig. 2. Clioquinol alters the cell volume of *Leishmania amazonensis* promastigotes. To verify whether the clioquinol alters the cell volume, promastigotes ( $1 \times 10^7$  cells) were treated with clioquinol (2.55 or 5.10  $\mu\text{g/mL}$ ). After, they were washed with PBS 1 $\times$  and evaluated by flow cytometry aiming to observe the FSC parameter (Forward Scatter). The evaluation was interpreted using the *FCS Express V3* software, and the statistical differences were indicated by Dunnett's test: (\*\*\*)  $p < 0.001$  and (\*\*\*\*)  $p < 0.0001$ . ICHQ, clioquinol.

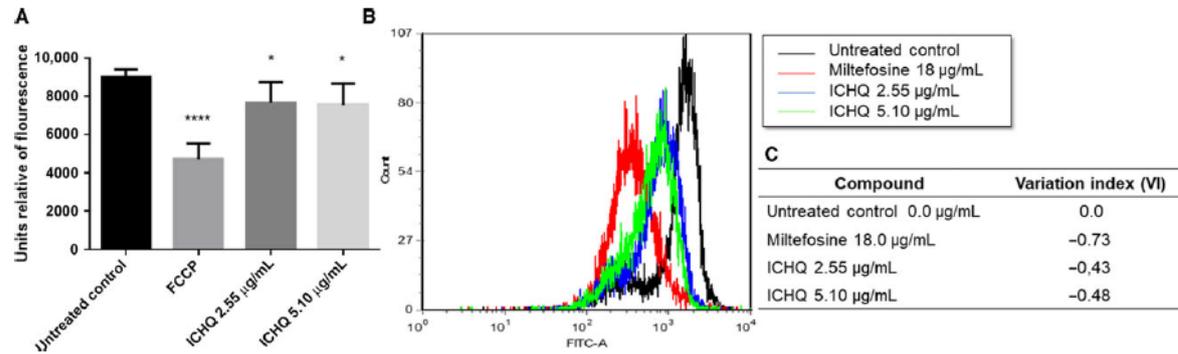


Fig. 3. Clioquinol induces the generation of ROS in *Leishmania amazonensis*. To verify whether clioquinol induces generation of ROS in *L. amazonensis*, promastigotes ( $1 \times 10^7$  cells) were treated with clioquinol (2.55 and 5.10  $\mu\text{g/mL}$ ) for 6 hr and incubated with the fluorescent probe H2DCFDA. The fluorescence intensity was determined by a spectrofluorometer. Miltefosine (18.0  $\mu\text{g/mL}$ ) was used as a positive control. Bars indicate the mean plus standard deviation of the groups, which were calculated with results obtained in three independent experiments. The statistical differences were indicated by Dunnett's test: (\*)  $p < 0.05$  and (\*\*\*\*)  $p < 0.0001$ . ICHQ, clioquinol.

considered to be an authentic source to identify new and effective biological compounds, which should be exploited for the development of new antileishmanial compounds [31]. It has been estimated that there are about 250,000 medicinal plant species in the world. Nevertheless, the biological activity of only 6% of them has been studied, and about 1% of medicinal compounds have been developed and are being tested in clinical trials [7,32].

The pharmacological properties of the quinoline ring illustrate the number of commercial products containing this heterocyclic system [33]. Quinoline-based compounds present a variety of biological actions, such as antimalarial and antileishmanial agents [28,34,35]. As a consequence, this study

illustrates the leishmanicidal activity of 8-HQN, a quinoline derivative, against distinct *Leishmania* species [20]. Thus, as part of a wider survey on this class of molecules, the present study investigated the antileishmanial activity of clioquinol, a quinoline derivative, against stationary promastigotes and intracellular amastigotes of *L. amazonensis* and *L. infantum*, as well as evaluated the mechanism of action and the *in vivo* effects of this molecule when administered in naive BALB/c mice.

In this study, clioquinol exhibited a strong and selective antileishmanial activity against *in vitro* and *in vivo* *L. infantum* and *L. amazonensis* promastigotes and amastigotes. This effect was accompanied by a low toxicity against murine macrophages

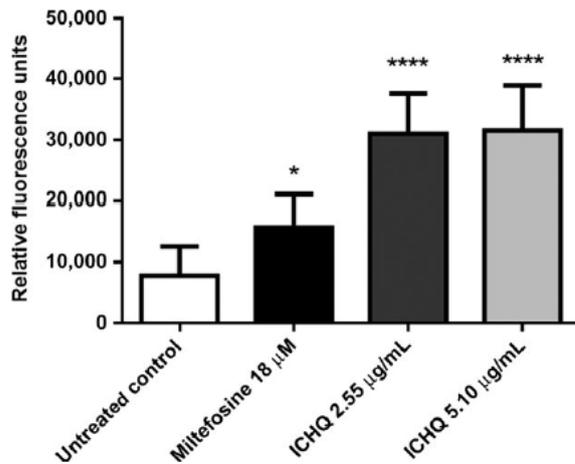


Fig. 4. Cloiquinol induces change in  $\Delta\Psi_m$  of *Leishmania amazonensis*. To assess whether or not cloiquinol induces the depolarization in  $\Delta\Psi_m$  of the parasites, promastigotes ( $1 \times 10^7$  cells) were treated with cloiquinol (2.55 and 5.10  $\mu\text{g/mL}$ ), FCCP (5.08  $\mu\text{g/mL}$ ) or miltefosine (18.0  $\mu\text{g/mL}$ ). The cells were then washed and incubated separately with MTK (500 nM) and Rh123 (0.5  $\mu\text{g/mL}$ ), and the parasites were analysed by a spectrofluorometer and a flow cytometry, respectively. Also, the variation index (VI) of the fluorescence intensity of Rh123 was determined by the  $(\text{MT}-\text{MC})/\text{MC}$  equation, given that MT is the mean of the treated parasites and MC is the mean of untreated parasites. Negative VI values indicate depolarization of the mitochondrial membrane potential. (A) Changes in  $\Delta\Psi_m$  assessed by MTK marking. (B) Representation of the fluorescence intensity of the groups for Rh123. (C) VI values for each treatment group. Bars indicate the mean plus standard deviation of the groups, which were calculated with results obtained in three independent experiments. The statistical differences were indicated by Dunnett's test: (\*)  $p < 0.05$  and (\*\*\*\*)  $p < 0.0001$ . ICHQ, cloiquinol.

and human red blood cells, with no toxic effects observed in any of the tested concentrations of this molecule. In addition, the selectivity index of cloiquinol was much higher than AmpB, a known drug used to treat leishmaniasis; consequently, additional assays were performed to elucidate the mechanism of action of cloiquinol related to parasite death.

Several studies have shown that mitochondria are important targets in the mechanism of death in Trypanosomatids. As this organelle is unique in these protozoa, the collapse of the mitochondrial membrane potential is known as one characteristic metabolic process of cell death in these parasites [28,36–38]. There are multiple sources of ROS to the cells [9,39], and the mitochondrial collapse associated with cell membrane depolarization has been related to apoptosis and/or necrosis events [40]. Here, treatment with cloiquinol induced a significant increase in the production of ROS in all of the tested concentrations. In fact, the  $\Delta\Psi_m$  of the parasites was measured, and the results showed that treatment with cloiquinol reduced the parasite's  $\Delta\Psi_m$ , when compared to untreated controls. Others have also associated the action of quinoline derivatives with the *Leishmania* mitochondria dysfunction [41,42].

In this context, the mechanism of cell death triggered by cloiquinol was also evaluated as regards their effect on the plasma membrane of the parasites. The results showed that the

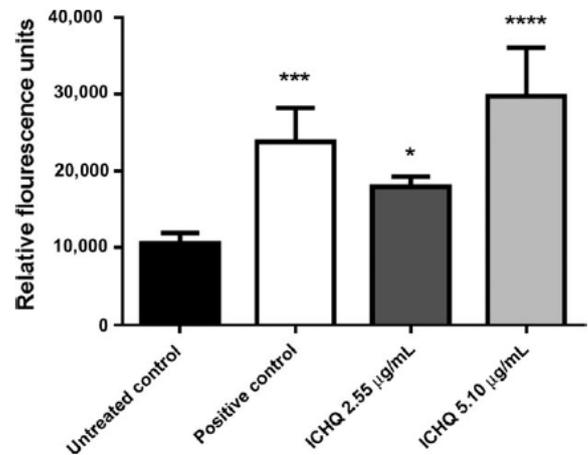


Fig. 5. Treatment with cloiquinol causes alteration in the *Leishmania amazonensis* plasma membrane. To verify whether cloiquinol causes rupture in the parasite's plasma membrane, promastigotes ( $1 \times 10^7$  cells) were untreated or treated with cloiquinol (2.55 and 5.10  $\mu\text{g/mL}$ ) and incubated for 6 hr. Subsequently, cells were incubated with propidium iodide, and the fluorescence intensity was determined by spectrofluorometer. Heated (65°C) promastigotes were used as a positive control. Bars indicate the mean plus standard deviation of the groups, which were calculated with results obtained in three independent experiments. The statistical differences were indicated by Dunnett's test: (\*)  $p < 0.05$ , (\*\*\*)  $p < 0.001$ , and (\*\*\*\*)  $p < 0.0001$ . ICHQ, cloiquinol.

treatment with this compound altered the integrity of the plasma membrane in *Leishmania*. Our data showed that cloiquinol induced a significant permeabilization of the parasite membrane, when compared to the untreated cells, thus demonstrating that the parasites most likely undergo cell death by necrosis. Although cloiquinol-treated promastigotes induced the depolarization of  $\Delta\Psi_m$  and cell shrinkage (both feature characteristics that are suggestive of apoptosis), neither PS exposure on the external leaflet of the plasma membrane nor  $G_0/G_1$  phase cell cycle arrest was found. In this context, it can be concluded that the mechanism of death induced by cloiquinol was compatible with necrosis.

As the purpose of this study was to identify new therapeutics to be used in the *in vivo* treatment against leishmaniasis, a toxicity study was performed in BALB/c mice, which received high daily doses of cloiquinol for 10 days. AmpB was used as a control. One day after the end of treatment, high levels of renal and hepatic enzymes were found in the AmpB-treated mice, as compared to those that had received cloiquinol, indicating an organic toxicity in the animals caused by AmpB. Others have also found hepato and nephrotoxicity caused by AmpB in murine models treated against leishmaniasis [43–46]. As a consequence, we can infer that the administration of cloiquinol in these mammalian hosts can be considered safe, as no toxicity was observed in the animals, thus suggesting a wide safety margin in therapeutic doses of this compound, as well as opening the possibility to use this molecule in future studies for the treatment of *Leishmania*-infected hosts.

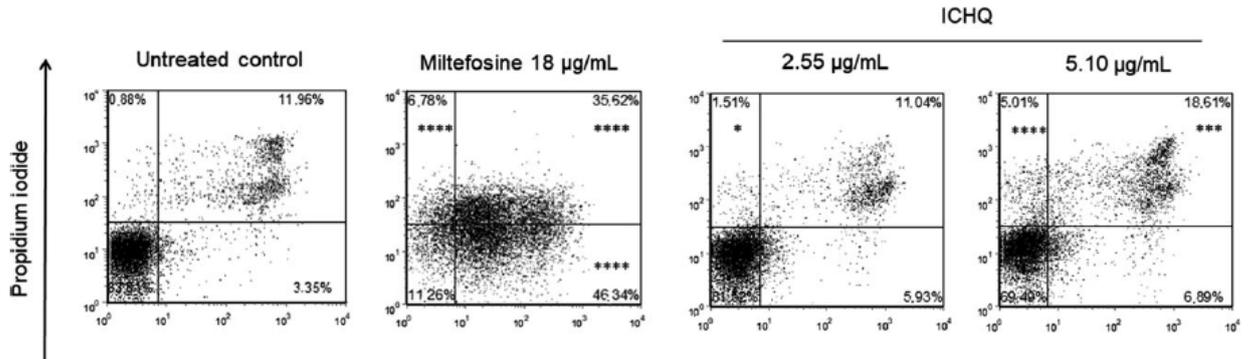


Fig. 6. Clioquinol not induces phosphatidylserine exposure in *Leishmania amazonensis*. Promastigotes ( $1 \times 10^7$  cells) were incubated with clioquinol (2.55 and 5.10  $\mu\text{g}/\text{mL}$ ) for 6 hr and doubly labelled with annexin V-FITC and PI. Analyses were performed by flow cytometry. A total of 10,000 events were acquired using the FITC and PE channels. Miltefosine (18.0  $\mu\text{g}/\text{mL}$ ) was used as a positive control. The upper left quadrants show a percentage of PI-labelled cells, while the lower shows unmarked cells. The lower right quadrants show annexin V-labelled cells, while the upper right quadrants show double-labelled cells with PI and annexin V. The dot plots presented are representative of three independent experiments. Statistical differences were performed on GraphPad Prism 6.0 software by analysis of variance (One-Way ANOVA), and differences were analysed by Dunnett's test: (\*)  $p < 0.05$ , (\*\*\*)  $p < 0.001$ , and (\*\*\*\*)  $p < 0.0001$ . ICHQ, clioquinol.

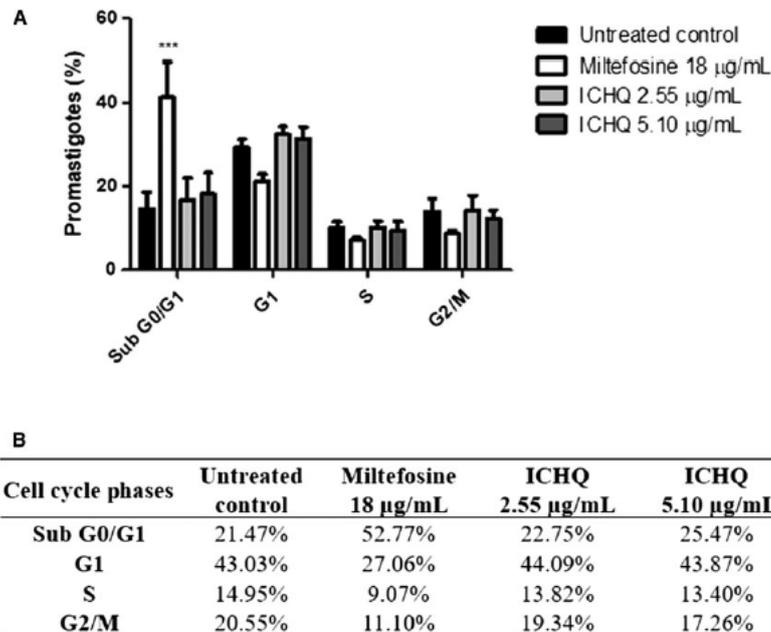


Fig. 7. Clioquinol does not interfere in the *Leishmania amazonensis* cell cycle. Promastigotes ( $1 \times 10^7$  cells) were untreated (negative control) or treated with clioquinol (2.55 and 5.10  $\mu\text{g}/\text{mL}$ ) for 6 hr, permeabilized with 70% ethanol, incubated with ribonuclease and labelled with PI. DNA content was analysed by flow cytometry, and a total of 5000 events were acquired using a PI610ND-A channel. Graph form (A) and percentage of cells (B) present in each phase of the cell cycle are shown. Bars indicate the mean plus standard deviation of the groups, which were calculated with results obtained in three independent experiments. The statistical differences were indicated by the Bonferroni post-test: (\*\*\*\*)  $p < 0.0001$ . ICHQ, clioquinol.

In summary, the data from the present study showed that clioquinol presents a selective antileishmanial activity against two important *Leishmania* species in the world, as well as a rapid *in vitro* effect, manifesting changes in membrane permeability, mitochondrial functionality and parasite morphology. In addition, the ability of clioquinol to reduce

*Leishmania* infection in murine macrophages, as well as its effectiveness in inhibiting the infection of these cells using pre-treated parasites, demonstrates that clioquinol should be tested in future studies to treat mammalian models against *Leishmania* spp. infection, as well as to inhibit parasite infection.

### Acknowledgements

This work was supported by grants from Pró-Reitoria de Pesquisa da Universidade Federal de Minas Gerais (Edital 02/2017), FAPEMIG (CBB-APQ-00819-12, CBB-APQ-01778-2014, CBB-APQ-01712/15) and CNPq (APQ-482976/2012-8, APQ-488237/2013-0, and APQ-467640/2014-9). MACF, FL and JDG are grant recipients of FAPEMIG/CAPES. EAFC, RJA and ESC are grant recipients of CNPq.

### Conflict of interest

The authors confirm that they have no conflict of interests in relation to this work.

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#### 4.1. ARTIGO 1 – CONCLUSÃO

Os resultados apresentados mostraram que o clioquinol apresentou atividade antileishmanial seletiva contra duas importantes espécies de *Leishmania* no mundo *in vitro*, causando alterações na permeabilidade da membrana, na funcionalidade mitocondrial e morfologia parasitária na espécie *L. amazonensis*. Além disso, a capacidade do clioquinol para reduzir a infecção por *Leishmania* em macrófagos de camundongos, bem como eficácia na inibição da infecção dessas células usando parasitos pré-tratados, demonstrou que o clioquinol pode ser testado em estudos futuros para tratar modelos de mamíferos contra a infecção por *Leishmania spp.*, bem como para inibir a infecção pelo parasito.

## 4.2. ARTIGO 2 – BREVE INTRODUÇÃO

O segundo artigo a ser apresentado foi intitulado como “A Pluronic® F127-based polymeric micelle system containing an antileishmanial molecule is immunotherapeutic and effective in the treatment against *Leishmania amazonensis* infection” e publicado na revista internacional “*Parasitology International*” (doi: <https://doi.org/10.1016/j.parint.2018.10.005>). No artigo, ICHQ foi incorporado em um sistema de micelas poliméricas baseadas em Poloxâmero 407 (ICHQ/M), e sua atividade antileishmanial foi avaliada *in vivo* em camundongos BALB/c infectados com *L. amazonensis*. A anfotericina B (AmpB) e sua formulação lipossomal (Ambisome®) foram utilizadas como controle. Avaliações parasitológicas e imunológicas foram realizadas 30 dias após o tratamento.



## A Pluronic® F127-based polymeric micelle system containing an antileishmanial molecule is immunotherapeutic and effective in the treatment against *Leishmania amazonensis* infection

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### ARTICLE INFO

#### Keywords:

Visceral leishmaniasis  
Treatment  
Toxicity  
Delivery systems  
5-chloro-7-iodoquinolin-8-ol

### ABSTRACT

Clioquinol (5-chloro-7-iodoquinolin-8-ol or ICHQ) was recently showed to presents an *in vitro* effective antileishmanial action, causing changes in membrane permeability, mitochondrial functionality, and parasite morphology. In the present study, ICHQ was incorporated into a Poloxamer 407-based polymeric micelles system (ICHQ/M), and its antileishmanial activity was *in vivo* evaluated in *L. amazonensis*-infected BALB/c mice. Amphotericin B (AmpB) and its liposomal formulation (Ambisome®) were used as controls. Parasitological and immunological evaluations were performed 30 days after the treatment. Results indicated more significant reductions in the average lesion diameter and parasite burden in ICHQ or ICHQ/M-treated mice, which were associated with the development of a polarized Th1 immune response, based on production of high levels of IFN- $\gamma$ , IL-12, TNF- $\alpha$ , GM-CSF, and antileishmanial IgG2a antibody. Control groups' mice produced high levels of IL-4, IL-10, and IgG1 isotype antibody. No organic toxicity was found by using ICHQ or ICHQ/M to treat the animals, although those receiving AmpB and Ambisome® have presented higher levels of renal and hepatic damage markers. In conclusion, results suggested that the ICHQ/M composition can be considered as an antileishmanial candidate to be tested against human leishmaniasis.

### 1. Introduction

Leishmaniasis is a disease complex considered endemic in 98 countries, with 350 million people at risk of contracting the infection by the *Leishmania* parasite [1]. It is a neglected disease that presents high morbidity and mortality in tropical and subtropical regions in the world, occurring mainly in Africa, Asia, Southern Europe, and South and Central Americas. Tegumentary leishmaniasis (TL), the more

common clinical form of disease, can be caused by *Leishmania major*, *L. tropica*, *L. aethiops*, *L. panamensis*, *L. mexicana*, *L. braziliensis*, and *L. amazonensis* species; whereas visceral leishmaniasis (VL) is caused by *L. donovani* and *L. infantum* species [2]. TL causes self-healing cutaneous lesions, although the uncontrolled parasite replication leading to mutilation and morbidity is observed in the patients. On the other hand, VL, which can be fatal if acute and untreated, results from an infection in phagocytic cells within the reticulum-endothelial system due to

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<https://doi.org/10.1016/j.parint.2018.10.005>

Received 11 September 2018; Received in revised form 12 October 2018; Accepted 15 October 2018

Available online 16 October 2018

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metastasis of parasites and infected cells from the initial site of infection [3].

*Leishmania amazonensis* is one of the major etiologic agents of disease in the Americas, and it is associated with a broad spectrum of human leishmaniasis, ranging from cutaneous to visceral form [4,5]. This parasite species presents a wide geographical distribution, which overlaps with transmission areas of other species [6,7]. Murine models susceptible to the *L. amazonensis* infection develop an antileishmanial Th2 immune response, which is characterized by production of high levels of IL-4, IL-10, IL-13, among other anti-inflammatory cytokines; while the resistance has been associated with the development of Th1 response, which is characterized by production of IFN- $\gamma$ , IL-2, IL-12, among other pro-inflammatory molecules [8,9].

The parasitological diagnosis of leishmaniasis is considered the gold standard to identify the infection; however, it requires an invasive procedure of collect of the samples, such as aspirates in organs as spleen and bone marrow, or lesion or mucosal fragments; being performed only in specialized health centers and by trained professionals [10]. In addition, the sensitivity of the tests is variable, depending on the presence of the parasite in the collected sample [11]. There are more sensitive molecular techniques to search for the parasite DNA; however, they are expensive and require sophisticated equipment, thus limiting their use [12]. Serological tests become an ideal diagnostic tool for detection of antileishmanial antibodies in sera of infected patients, due to its simplicity and low cost, and are considered less invasive [13]. As consequence, methods such as indirect immunofluorescence, direct agglutination test, enzyme-linked immunosorbent assay (ELISA), latex agglutination test, and immunochromatographic tests have been employed [14,15]. However, their serodiagnostic performance is hampered by variable sensitivity and/or specificity, thus leading to the occurrence of false-negative or false-positive results [16].

Among the more successfully tested antigens, the rK39 is a prominent. This 39-amino acid repetitive immunodominant B-cell epitope kinesin-related protein is conserved among viscerotropic *Leishmania* species [17]. It has been employed for the serodiagnosis of disease, as incorporated in rapid tests, such as rK39 dipstick and immunochromatographic tests. However, this antigen appears to be more suited for the detection of symptomatic cases with severe disease than those asymptomatic or in healthy people living in endemic areas of leishmaniasis [18]. Faced with these difficulties, an effective treatment against disease should be performed, aiming to allow the cure of the patients, as well as to inhibit the occurrence of spreading of the infection caused by dissemination of the parasites between sandflies and mammalian hosts.

The treatment against leishmaniasis is based on the use of pentavalent antimonials, although other drugs, such as amphotericin B (AmpB), pentamidine, miltefosine, and paramomycin, are used [19]. However, problems related with the toxicity and teratogenicity, treatment duration, high cost, administration route, and/or development of resistant strains have limited the efficacy of the therapeutics [20–22]. AmpB is able to binds to the membrane sterols of parasites, hampering their cell permeability and leading to the loss of cations, such as K<sup>+</sup>, causing cell death. The toxicity of this drug is derived from its interaction with sterols in bilayer membranes, such as cell walls, causing pore formation in the membranes, in turn leading either to their rupture. To reduce the toxicity of this drug, AmpB-containing lipid formulations have been developed. A liposomal formulation (AmBisome®), an AmpB colloidal dispersion (Amphocil®), and an AmpB-lipid complex (Abelcet®) have been used for the treatment of leishmaniasis. The World Health Organization has recommended the use of these products, based on their higher levels of efficacy and safety, besides low toxicity when compared to the use of free drug. However, the main factor limiting the widespread use of these formulations has been their high cost [23,24]. In this context, and due to few current alternatives available on the market, the identification of new compounds to be used in the treatment against leishmaniasis should be considered,

aiming to find novel effective, safe and economically feasible antileishmanial agents.

The growing interest in applying herbal extracts, essential oils, and natural products to treat against human disease has increased in the last decades, aiming to identify new antiparasitic targets derived from vegetable sources, as based on secondary metabolites, of which naphthoquinones and quinolines are examples [25,26]. This alternative is required, since the drug discovery is a long and expensive process, requiring an average of 10 to 20 years before a new product is identified and developed for human clinical applications. In the case of leishmaniasis, there are few new antileishmanial drugs in the pipeline, and drug resistance is increasing in distinct regions in the world [26].

Quinones are natural aromatic metabolites found in plant families, as well as in algae, fungi, and bacteria. They include anthraquinones, benzoquinones, and naphthoquinones [27,28], which present biological properties, such as microbicidal, antileishmanial and anticancer action [29–31]. Recently, a quinoline-derivate molecule, namely clioquinol, 5-chloro-7-iodoquinolin-8-ol or ICHQ, presented an *in vitro* effective antileishmanial activity against *L. amazonensis* and *L. infantum* species. The inhibition 50% of the *Leishmania* viability (IC<sub>50</sub>) against *L. amazonensis* and *L. infantum* promastigotes was of 2.55 ± 0.25 and 1.44 ± 0.35 µg/mL, respectively, and of 1.88 ± 0.13 and 0.98 ± 0.17 µg/mL against the amastigote forms, respectively. On the other hand, the inhibition of 50% of the macrophage viability (CC<sub>50</sub>) was of 255 ± 23 µg/mL, with selectivity index of 99.9 and 177.1 against promastigotes, respectively, and of 135.6 and 260.1 against amastigotes, respectively [32].

In this context, in the present study, the *in vivo* antileishmanial activity of ICHQ was evaluated in chronically *L. amazonensis*-infected BALB/c mice. This molecule was incorporated to a Poloxamer 407 (Pluronic® F127)-based micelle system, and the therapeutic efficacy of

the product was compared with the use of free AmpB and AmBisome®. The compounds were administered in the infected mice, and the formulation efficacy was investigated 30 days after the treatment, by means of the evaluation of the immune response and parasite load in the treated and infected animals.

## 2. Materials and methods

### 2.1. Pharmaceuticals and formulation of the ICHQ-containing micelle system

Poloxamer 407 (Pluronic® F127) and ICHQ were purchased from Sigma-Aldrich (St. Louis, MO, USA), with catalog numbers 16,758 and 130–26-7, respectively. AmpB (Cristália, São Paulo, São Paulo, Brazil) was resuspended (1 mg) in methanol/DMSO (9:1 v/v) solution, and was maintained at –80° until use. AmBisome® (Gilead Sciences, Inc. San Dimas, USA) was obtained as a lyophilized powder (50 mg), and resuspended in water to prepare a stock solution (4 mg/mL). ICHQ-containing micelles (ICHQ/M) were prepared as described [26]. Briefly, Poloxamer 407 (18% w/w) was diluted in phosphate buffer (PBS) pH 7.4 under magnetic agitation for 18 h, at 4 °C. Eight milligrams of ICHQ were added to 500 µL of dichloromethane PA, and solubilized by using vortex. The mixture was added to the previously prepared solution under vigorous magnetic agitation and in an ice bath, until a viscous emulsion has been obtained. The dichloromethane was evaporated using rotary evaporate (Buchi, Flawil, Switzerland), and the formulation was obtained as a transparent yellow gel at room temperature. Empty micelles (18% w/w) were prepared using the same technical protocol.

### 2.2. Mice and parasites

BALB/c mice (female, 8 weeks age) were obtained from the breeding facilities of the Department of Biochemistry and Immunology, Institute of Biological Sciences, Federal University of Minas Gerais (UFMG), Belo Horizonte, Minas Gerais, Brazil. Animals were

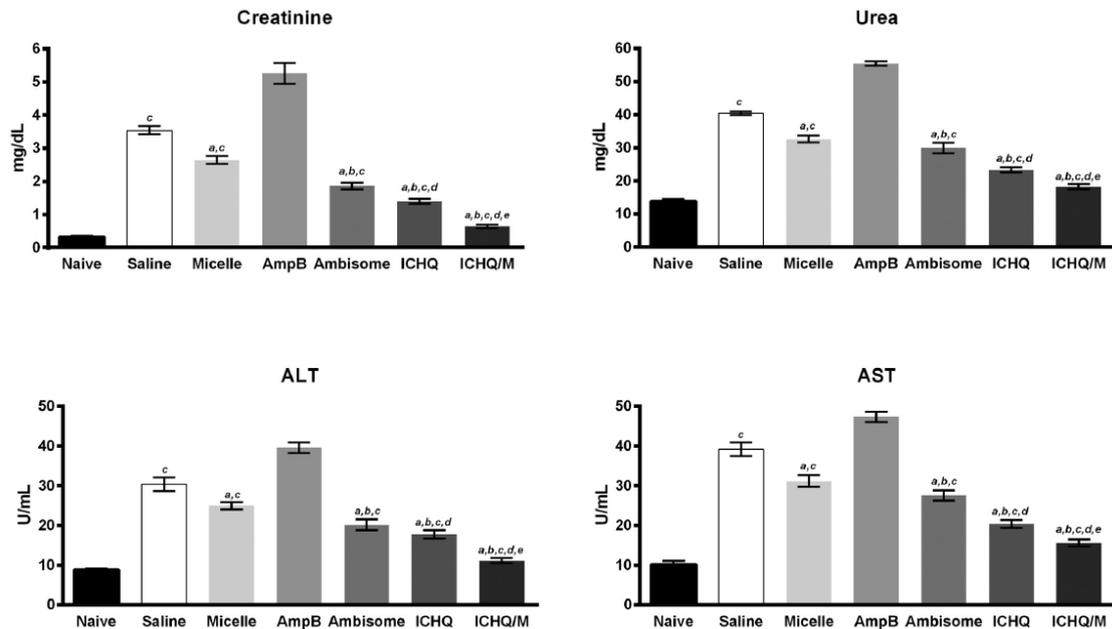


Fig. 1. Toxicity induced in the treated and infected animals. The levels of creatinine, urea, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated in sera samples of infected and treated mice ( $n = 10$  per group), 30 days after the treatment. Samples of naive (non-infected and non-treated) mice were used as control. Bars represent the mean  $\pm$  standard deviation of the groups. The letters a, b, c, d, and e indicate statistically significant differences in relation to the saline, micelle, AmpB, Ambisome®, and ICHQ groups, respectively ( $P < 0.0001$ ).

maintained under pathogen-free conditions. The study was approved by Committee for the Ethical Handling of Research Animals (CEUA) of UFMG (protocol number 085/2017). *L. amazonensis* (IFLA/BR/1967/PH-8) stationary promastigotes were grown in complete Schneider's medium (Sigma-Aldrich, St. Louis, MO, USA), which was composed by Schneider's medium added with 20% heat-inactivated fetal bovine serum (FBS, Sigma-Aldrich, USA), 20 mM L-glutamine, 200 U/mL penicillin, 100  $\mu$ g/mL streptomycin, and 50  $\mu$ g/mL gentamicin pH 7.4 at 24 °C. The soluble *Leishmania* antigenic (SLA) extract was prepared as described [33].

### 2.3. Infection and treatment schedules

BALB/c mice ( $n = 10$  per group) were infected with  $10^7$  *L. amazonensis* stationary promastigotes through subcutaneous injection in the base of the tail, after trichotomy. After the development of ulcerated lesions (at approximately 50 days post-infection), animals were divided into groups according to lesion size (2 to 3 mm), aiming to ensure similar average lesion diameter between the groups. Then, mice were treated with one of the following therapeutic schedules: (a) Control group (saline): mice received 50  $\mu$ L of PBS pH 7.4 by subcutaneous route, once a day during 15 days; (b) Empty micelle (micelle) group: mice received 50  $\mu$ L of non-incorporated micelle (10 mg/kg body weight) by subcutaneous route, every two days and during 15 days; (c) Amphotericin B (AmpB) group: mice received 50  $\mu$ L of a solution containing 1 mg/kg body weight by intraperitoneal route, every two days and during 15 days; (d) Ambisome® group: mice received 50  $\mu$ L of a solution containing 2 mg/kg body weight by intravenous route, every five days and during 15 days; (e) ICHQ group: mice received 50  $\mu$ L of a solution containing 10 mg/kg body weight by subcutaneous route, every two days and during 15 days; and (f) ICHQ/micelle (ICHQ/M) group: mice received 50  $\mu$ L of micelle incorporated with ICHQ (5 mg/kg

body weight) by subcutaneous route, every two days and during 15 days. Further observations including the occurrence of nodules, as well as metastasis and other clinical signals in the treated and infected animals were performed. After the treatment, the lesion average diameter was measured weekly using an electronic caliper (799-6/150 model, Starrett®, Brazil). Thirty days after the treatment, animals were euthanized and parasitological and immunological parameters were evaluated to investigate the treatment efficacy.

### 2.4. Assays for toxicity

To evaluate the toxicity of the treatment, the hepatic function was analyzed by dosage of the alanine transaminase (ALT) and aspartate transaminase (AST), whereas the nephrotoxicity was evaluated by the levels of blood urea nitrogen and serum creatinine in serum samples of the treated and infected animals using commercial kits (Labtest Diagnostica®, Belo Horizonte). Serum samples from naive (non-treated and non-infected) mice ( $n = 6$ ) were used as control.

### 2.5. Determination of the parasite burden

#### 2.5.1. Limiting dilution technique

The parasitism was evaluated in the infected tissue, draining lymph node, spleen, and liver of the animals, by limiting dilution technique [34] and RT-PCR assay [35]. For this, tissue and organs were weighed and homogenized using a glass tissue grinder in sterile PBS  $1 \times$ . Tissue debris were removed by centrifugation at  $150 \times g$  and cells were concentrated by centrifugation at  $2000 \times g$ . The pellet was resuspended in 1 mL of complete Schneider's medium and 220  $\mu$ L 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

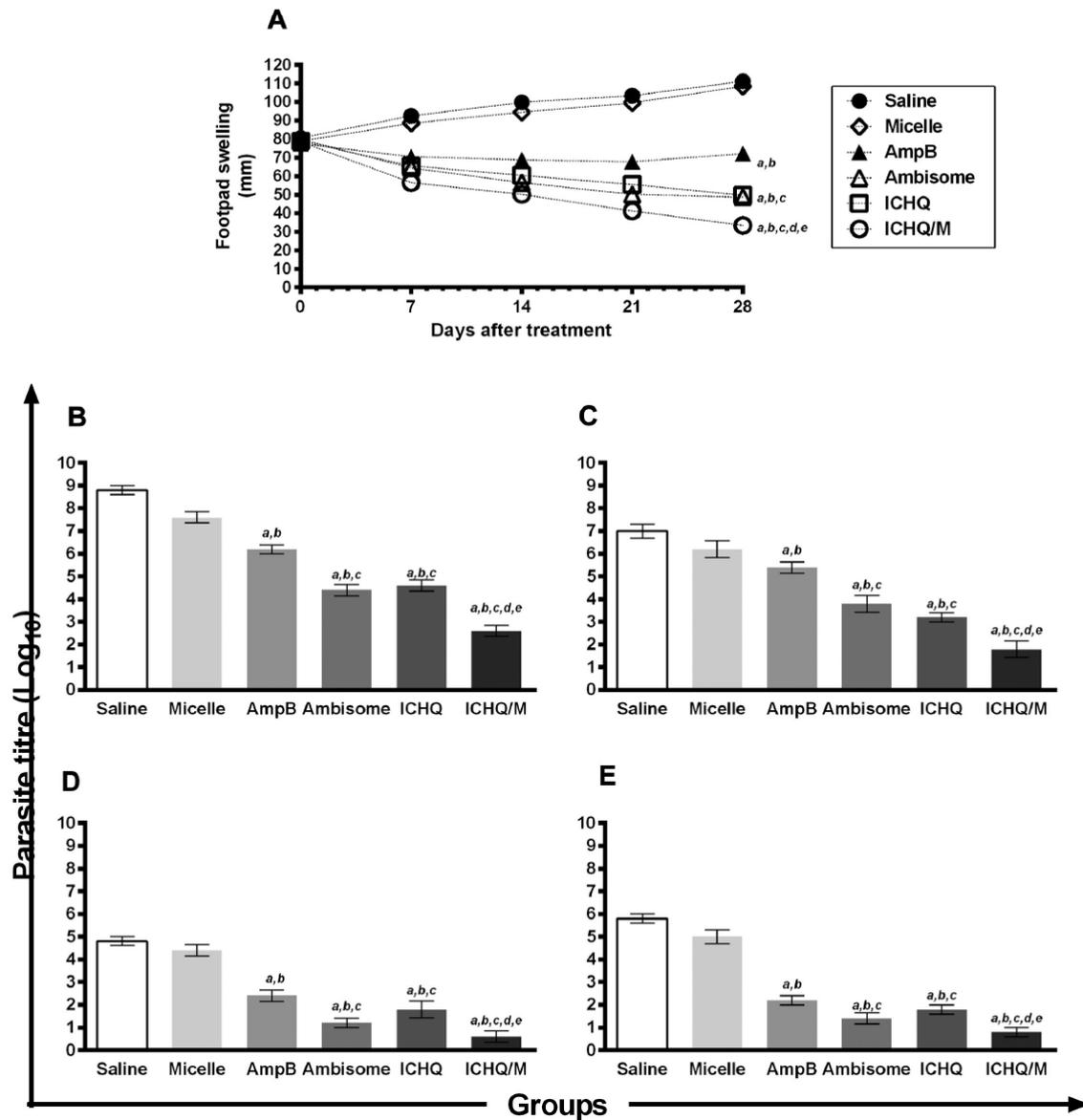


Fig. 2. Evaluation of infection and parasite burden in the treated animals. BALB/c mice were subcutaneously infected with  $10^7$  *L. amazonensis* stationary promastigotes. At approximately 50 days post-infection, when animals presented lesion size between 2 and 3 mm, they were divided into groups ( $n = 10$  per group), and received saline or were treated with micelle, AmpB, Ambisome®, ICHQ or ICHQ/M. The lesion development was monitored weekly and during 4 weeks. The lines represent the lesion average diameter (area) expressed as the mean  $\pm$  standard deviation of the groups (A). The parasite load was evaluated 30 days after the treatments in the infected tissue (B), draining lymph nodes (C), spleen (D), and liver (E), by a limiting dilution technique. Bars represent the mean  $\pm$  standard deviation of the groups. The letters *a*, *b*, *c*, *d*, and *e* indicate statistically significant differences in relation to the saline, micelle, AmpB, Ambisome®, and ICHQ groups, respectively ( $P < 0.001$ ).

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.

#### 2.5.2. RT-PCR

The parasite load was also evaluated by RT-PCR technique. For this,

lesion fragment DNA was extracted using a phenol-chloroform method. Five hundred microliters of lysis buffer solution [50 mM Tris, 50 mM NaCl, and 10 mM EDTA (pH 8.0)], 1% Triton X-100 and 20  $\mu$ L of proteinase K solution (20 mg/mL, Promega) were added to each fragment (20 mg). The mixture was incubated for 16 h at 37 °C, and 500  $\mu$ L of phenol (Sigma-Aldrich) were added, homogenized by inversion for 10 min and centrifuged at  $10,000 \times g$  for 5 min. The supernatant was

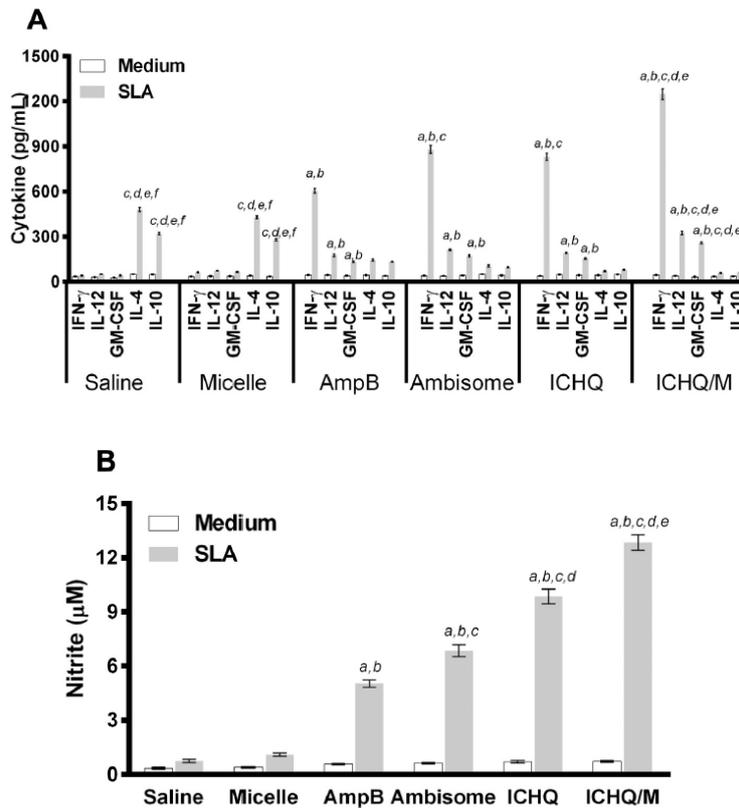


Fig. 3. Cellular response developed after the treatment. Single cells suspensions were obtained from the spleen of treated and infected mice ( $n = 10$  per group), 30 days after the treatment. Then, cells ( $5 \times 10^6$ ) were unstimulated (medium, background control) or stimulated with *L. amazonensis* SLA ( $25 \mu\text{g}/\text{mL}$ ), for 48 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . IFN- $\gamma$ , IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the supernatants by a capture ELISA (A). The nitrite production was evaluated in the cell supernatant by Griess reaction (B). Bars represent the mean  $\pm$  standard deviation of the groups. The letters a, b, c, d, and e indicate statistically significant differences in relation to the saline, micelle, AmpB, Ambisome<sup>®</sup>, and ICHQ groups, respectively ( $P < 0.001$ ).

transferred to a new tube and this step was repeated. Fifty-hundred microliters of chloroform-isoamyl alcohol (24:1; Sigma-Aldrich) were added and the solution was homogenized by inversion and centrifuged at  $10,000 \times g$  for 5 min. The sample was transferred for a new tube, and 500  $\mu\text{L}$  of ethanol 95% (v/v) were added. The material was incubated for 10 min in ice bath for DNA precipitation, and thus centrifuged at  $14,000 \times g$  for 10 min. Then, 500  $\mu\text{L}$  of ethanol 70% (v/v) were added to pellet, which was resuspended and centrifuged in the same conditions. The supernatant was removed and the sample was dried at room temperature. The resulting DNA was resuspended in 100  $\mu\text{L}$  of milli-Q water, and was amplified using the *Forward* (CCTATTTACACCAACC CCCAGT) and *Reverse* (GGGTAGGGCGTCTGCGAAA) primers. The mouse  $\beta$ -actin gene (*Forward*: CAGAGCAAGAGAGGTATCC and *Reverse*: TCATTGTAGAAGGTGTGGTGC) was used as endogenous control and to verify sample's integrity. Standard curves were obtained from DNA extracted from  $10^8$  parasites for kDNA and from  $10^8$  peritoneal macrophages for  $\beta$ -actin, under the same conditions used to extract the other samples. PCR was performed on StepOne<sup>™</sup> Instrument (48 wells-plate; Life Technologies) using  $2 \times$  SYBR<sup>®</sup> Green PCR Master Mix (5  $\mu\text{L}$ ,

Applied Biosystems), with 2 mM of each primer (1  $\mu\text{L}$ ) and 4  $\mu\text{L}$  of DNA (5 ng/ $\mu\text{L}$ ). Samples were incubated at  $95^\circ\text{C}$  for 10 min and submitted to 40 cycles of  $95^\circ\text{C}$  for 15 s and  $60^\circ\text{C}$  for 1 min. Results were converted into number of parasites (in log) per nucleated cell (multiplied by one thousand to facilitate visualization).

## 2.6. Cellular response

### 2.6.1. Capture ELISA

Splenocytes were collected to evaluate the cellular response 30 days after the treatment. For this, cells ( $5 \times 10^6$ ) were plated in 24-well plates (Nunc) in duplicate and incubated in complete DMEM (medium), which was composed by the medium plus 20% FBS and 20 mM  $\text{l}$ -glutamine pH 7.4. Then, cells were unstimulated (medium, background control) or stimulated with *L. amazonensis* SLA ( $25 \mu\text{g}/\text{mL}$ ), for 48 h at  $37^\circ\text{C}$  in 5%  $\text{CO}_2$ . IFN- $\gamma$ , IL-4, IL-10, IL-12p70 and GM-CSF levels were measured in the culture supernatants by capture ELISA, by using commercial kits (BD Pharmingen<sup>®</sup>, San Diego, CA, USA), according to the manufacturer's instructions. The nitrite production was evaluated in the cell supernatant by Griess reaction, according described [35]. Aiming to block the IL-12,  $\text{CD4}^+$  and  $\text{CD8}^+$  mediated T cell cytokine release, splenocytes of treated mice were *in vitro* stimulated with *L. amazonensis* SLA ( $25 \mu\text{g}/\text{mL}$ ) and incubated with 5  $\mu\text{g}/\text{mL}$  of monoclonal antibodies (mAb) against mouse IL-12 (C17.8), CD4 (GK 1.5) or CD8 (53–6.7). Appropriate isotype-matched controls – rat IgG2a

(R35–95) and rat IgG2b (95–1) – were employed in the assays. Antibodies (no azide/low endotoxin<sup>™</sup>) were purchased from BD (Pharmingen<sup>®</sup>).

### 2.6.2. Flow cytometry

A flow cytometry assay was also performed to evaluate the IFN- $\gamma$ , TNF- $\alpha$  and IL-10-producing  $\text{CD4}^+$  and  $\text{CD8}^+$  T cell frequency. For this, splenocytes ( $5 \times 10^6$ ) were incubated in complete RPMI 1640 medium

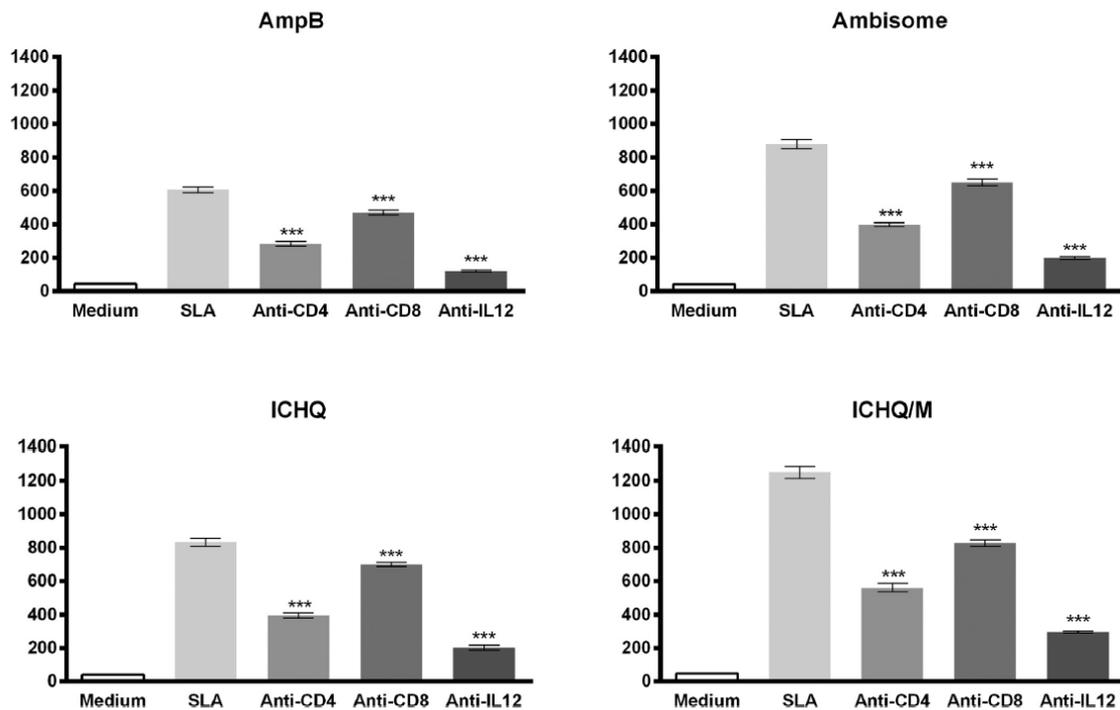


Fig. 4. Evaluation of the IFN- $\gamma$  production by CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the treated and infected animals. Spleen cells ( $5 \times 10^6$  cells) of AmpB, Ambisome®, ICHQ or ICHQ/M groups' mice ( $n = 10$  per group) were stimulated with SLA (25  $\mu$ g/mL), for 48 h at 37 °C in 5% CO<sub>2</sub>, in the absence (control) or presence of monoclonal antibodies (mAb) against mouse IL-12, CD4<sup>+</sup> or CD8<sup>+</sup>. The supernatants were collected, and the IFN- $\gamma$  production was analyzed by capture ELISA. Bars represent the mean  $\pm$  standard deviation of the groups. (\*\*\*) indicates the existence of statistically significant difference in relation to the unstimulated or SLA-stimulated cultures (P < 0.001).

in polypropylene tubes (Pharmingen®), and were unstimulated (medium) or stimulated with *L. amazonensis* SLA (25  $\mu$ g/mL), for 48 h at 37 °C in 5% CO<sub>2</sub>. Then, cells were incubated with phorbol myristate acetate (PMA, 25 ng/mL) and ionomycin (1  $\mu$ g/mL), which were diluted in complete RPMI 1640 medium. The IFN- $\gamma$ , TNF- $\alpha$  and IL-10-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequency was evaluated following an analysis based on their relative flow cytometry size (forward laser scatter – FSC) and granularity (side laser scatter – SSC) graphs. After the selection of the interest region R1 containing FSCLow and SSCLow phenotype cells, graphs of density plot distribution of CD4/FL1 or CD8/FL1 versus IFN- $\gamma$ /FL2<sup>+</sup>, TNF- $\alpha$ /FL2<sup>+</sup>, and IL-10/FL2<sup>+</sup> cells were performed to determine the IFN- $\gamma$ <sup>+</sup>, TNF- $\alpha$ <sup>+</sup>, and IL-10<sup>+</sup> T cell frequency in the cultures. Results were expressed as indexes, which were calculated by the ratio between the cytokine-producing T cell percentages versus the values found in the unstimulated cultures (SLA/CC ratio).

## 2.7. Humoral response

The antileishmanial IgG1 and IgG2a isotypes production was evaluated in serum samples from the treated and infected animals. For this, SLA was used as an antigen (1.0  $\mu$ g per well), and serum samples were diluted at 1:100 in PBS-T (PBS 1  $\times$  plus 0.05% Tween 20), with an incubation occurring for 1 h at 37 °C. After washing plates seven times, anti-mouse IgG total, IgG1 and IgG2a horseradish-peroxidase conjugated antibodies (Sigma-Aldrich) were added (in 1:10,000, 1:5000 and 1:10,000 dilutions, respectively, in PBS-T), and reaction was developed by incubation with 2  $\mu$ L H<sub>2</sub>O<sub>2</sub>, 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  $\mu$ L H<sub>2</sub>SO<sub>4</sub> 2 N. The optical density was determined by an ELISA microplate spectrophotometer (Molecular Devices, Spectra Max Plus, Canada), at 492 nm.

## 2.8. 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 with P < 0.05. The experiments were repeated, and the results were similar between them. Data shown in this study are representative of the first experiment.

## 3. Results

### 3.1. Toxicological study in the treated and infected mice

A toxicological study was performed in the treated and infected animals 30 days after the treatment. Results showed significant increase in serum enzymes, which were associated with renal and hepatic damage, since significantly higher levels of ALT, AST, urea, and creatinine were found in the AmpB-treated animals, when compared to the values found in the ICHQ and ICHQ/M groups. These groups presented also lower values of these enzymes in comparison to results obtained in the Ambisome® group (Fig. 1). Clinical symptoms such as ataxia and weakness were observed in the AmpB group, with 10% reduction in the

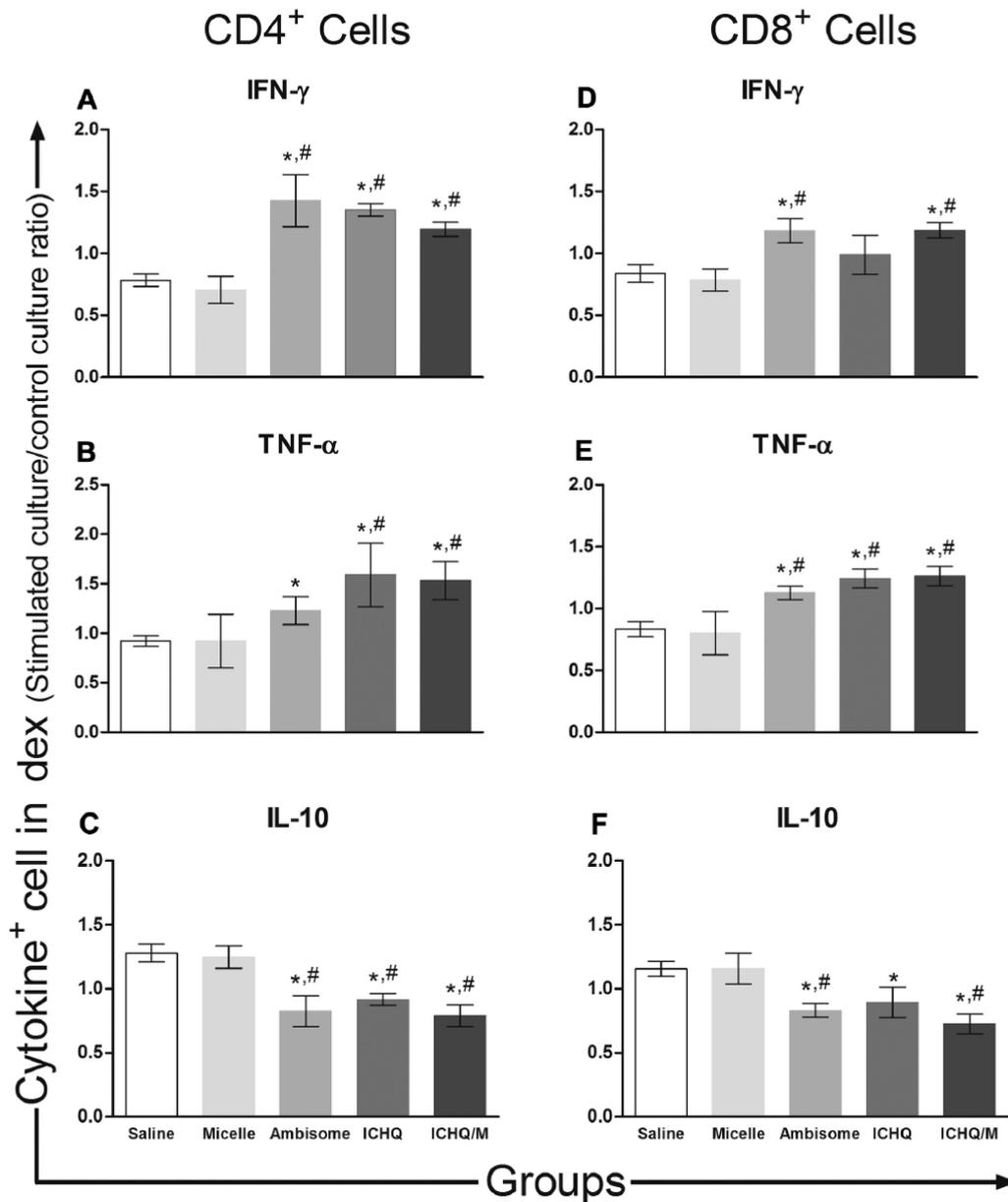


Fig. 5. Profile of intracytoplasmic cytokine-producing T cells. BALB/c mice were subcutaneously infected with  $10^7$  *L. amazonensis* stationary promastigotes, and after 50 days they received saline or were treated with micelle, Ambisome®, ICHQ or ICHQ/M. The cytokine (IFN- $\gamma$ , TNF- $\alpha$ , and IL-10)-producing CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequency in their spleen cells (n = 10 per group) was evaluated 30 days after the treatment. Results were reported as cytokine indexes, which were calculated by the ratio of cytokine<sup>+</sup>-cells present in the SLA-stimulated cultures versus those found in the unstimulated (control) cultures (SLA/CC ratio). Bars represent the mean  $\pm$  standard deviation of the groups for CD4<sup>+</sup> (panels A, B and C) and CD8<sup>+</sup> (panels 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).

animals' body weight, possibly reflecting the *in vivo* toxicity of the drug. On the other hand, ICHQ and ICHQ/M-treated mice showed a positive variation in their body weight, in the order of 4% and 8%, respectively (data not shown).

### 3.2. Parasitological evaluation in the treated and infected animals

The efficacy of the therapeutics was evaluated 30 days after the treatment of *L. amazonensis*-infected BALB/c mice, by measuring the average lesion diameter and parasite burden in the infected tissue, liver, spleen and draining lymph nodes of the animals. Results showed that

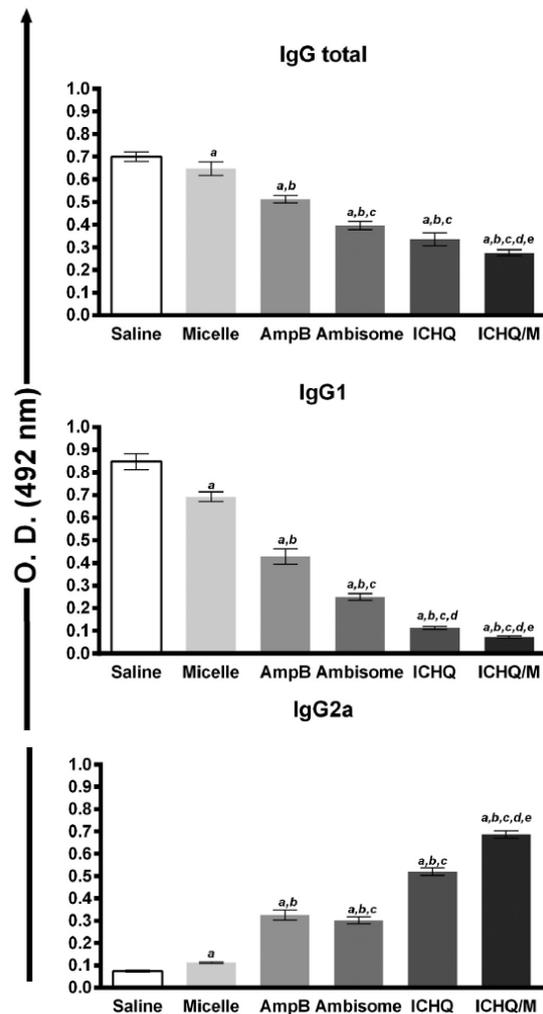


Fig. 6. Humoral response developed after the treatment. Serum samples were obtained of infected and treated mice ( $n = 10$  per group) 30 days after the treatments, and the antileishmanial IgG total, IgG1 and IgG2a production was investigated. Bars represent the mean  $\pm$  standard deviation of the groups. The letters a, b, c, d, and e indicate statistically significant differences in relation to the saline, micelle, AmpB, Ambisome<sup>®</sup>, and ICHQ groups, respectively ( $P < 0.0001$ ).

ICHQ and ICHQ/M-treated mice displayed significant reductions in their average lesion diameter, when compared to values found in the saline, micelle and AmpB groups (Fig. 2A). Data obtained in the ICHQ and Ambisome<sup>®</sup> groups were similar; however, animals that were treated with ICHQ/M presented lower results in the average lesion diameter, when compared to the others. The parasite burden showed also that ICHQ/M group' mice presented the best protective result, while the ICHQ and Ambisome<sup>®</sup> groups showed similar parasitism found in the tissue and organs (Fig. 2B). The RT-PCR assay showed also significant reductions in the parasite load in the Ambisome<sup>®</sup>, ICHQ, and ICHQ/M groups, in the order of 55.0%, 64.0%, and 88.0%, respectively, when compared with the saline group (data not shown).

### 3.3. Cellular and humoral response generated in the treated and infected mice

The immune response developed in the treated and infected animals was evaluated 30 days after the treatment. Results showed a antileishmanial Th1 profile generated in all treated animals, based on significantly higher levels of IFN- $\gamma$ , IL-12 and GM-CSF cytokines, when compared to the saline and micelle groups. Between the treated animals, those receiving ICHQ or ICHQ/M presented a more polarized Th1 response, when compared to the results found in the AmpB and Ambisome<sup>®</sup> groups (Fig. 3A). In addition, saline and micelle groups' mice produced higher levels of antileishmanial IL-4 and IL-10. The nitrite production was also measured in the culture supernatant, and results showed that ICHQ or ICHQ/M-treated animals presented higher levels of this molecule; in comparison to the values obtained in AmpB and Ambisome<sup>®</sup> groups' mice (Fig. 3B). The involvement of CD4<sup>+</sup> and CD8<sup>+</sup> T cells in the IFN- $\gamma$  production was evaluated. Results showed this cytokine was significantly inhibited when anti-CD4 antibody was added to the *in vitro* cultures. Otherwise, the addition of anti-CD8 antibody decreased also the IFN- $\gamma$  production in the cultures; although the levels of this molecule have been higher, when compared to the use of anti-CD4 antibody (Fig. 4).

The flow cytometry assay showed also that ICHQ or ICHQ/M-treated mice presented the higher IFN- $\gamma$  and TNF- $\alpha$ -producing CD4<sup>+</sup> and CD8<sup>+</sup> T cell frequency, when compared to the other groups (Fig. 5). On the other hand, saline and micelle groups' mice showed higher IL-10<sup>+</sup> T cell frequency. The humoral response was also evaluated, and results showed that ICHQ or ICHQ/M-treated mice produced higher levels of antileishmanial IgG2a isotype, when compared to the IgG1 levels, thus indicating also a polarized Th1 response in these animals (Fig. 6).

## 4. Discussion

This work represents a continuation of a previous study developed to identify new antileishmanial molecules, where ICHQ showed a selective *in vitro* antileishmanial action against *L. infantum* and *L. amazonensis* species, leading to alterations in the membrane permeability, mitochondrial function, and parasite morphology [32]. ICHQ was also effective in reduce the infection in treated and infected macrophages, as well as to inhibit the infection of these cells by using pretreated parasites. In the present study, the *in vivo* antileishmanial activity of ICHQ, which was incorporated to a Poloxamer 407-based micelle system, was evaluated against *L. amazonensis* infection in BALB/c mice. Immunological and parasitological analyses showed that this composition was highly effective in treat chronically infected mice, based on significant reductions in the parasite burden analyzed in different organs of the animals.

The inherent disadvantages related with the toxicity of traditional drugs have spurred the pharmaceutical industry to identify new delivery systems, since the design and development of new products is a long and expensive task [36,37]. In this context, AmpB has been applied in the treatment of leishmaniasis [38,39], since it interacts with the ergosterol in the parasite membrane causing leishmanicidal action, although it also forms pores in cholesterol-containing membranes present in mammalian hosts, thus explaining the cytotoxic activity of this drug [40]. AmpB-containing lipid formulations are less toxic to the patients; however, their high cost limits their application in underdeveloped countries [21,41,42]. As a consequence, new studies developed to identify new delivery systems to old or new candidates will to reduce their toxicity, as well as allow to the production of formulations with a lower cost [43,44].

Copolymers based on combinations between hydrophilic and hydrophobic products have been used to incorporate antileishmanial molecules [26,45–47]. In particular, a Poloxamer 407-based copolymer was recently used in association with a quinoline derivate, namely 8-

hydroxyquinoline (8-HQN), and the formed compost was well-successfully used to treat *L. amazonensis*-infected BALB/c mice [46]. In the present work, ICHQ was incorporated to this delivery system and applied in a lower dose in infected animals. Our results pointed out to most significant reductions in the lesion average diameter and parasite load in the treated animals, when compared to values found in the controls. Although Ambisome® has been also effective in treat infected animals; the parasite burden was higher in comparison to the values found in the ICHQ/M group. This fact can be considered relevant, since ICHQ-containing polymeric micelles are cheaper to produce, with an estimated value of ten times less in comparison for the production of AmpB-containing liposomal formulations.

Parasitological and immunological analyses were developed 30 days after the treatment, since our purpose was to wait the end of the administration of the therapeutics to investigate their long-term efficacy [48]. Higher periods of time were not possible to be investigated, due to the severity of the infection in the controls, as well as by the Ethical aspects in our University. Interestingly, despite the heavy infection in the saline group, ICHQ or ICHQ/M-treated mice showed significantly lower lesion diameter, which was correlated with significant reductions in the parasite burden in infection site (infected tissue and popliteal lymph nodes) and organs (liver and spleen) of the animals, when compared to the results found in the other groups. Although free AmpB or Ambisome®-treated mice have also presented reduction in the parasitism, it was less significant in comparison to the values found in the ICHQ and ICHQ/M groups. Similar results were found when other natural or synthetic molecules were associated to delivery systems and

administered in *Leishmania*-infected mice, in comparison to the use of old and/or conventional drugs [49–51].

An important problem related to the use of AmpB is the organic toxicity found in the treated hosts. Alterations in renal and hepatic functions are important dose-limiting side effects, and the dosage of organic markers, such as AST, ALT, urea and creatinine, could help to evaluate internal damages caused by this product [52–54]. In our study, higher levels of AST, ALT, urea and creatinine were found in AmpB-treated animals. These mice showed also reductions in their body weight, possibly reflecting the toxicity of the drug. On the other hand, upon the treatment with ICHQ or ICHQ/M, the enzymatic markers were found in low levels, characterizing the absence or low toxicity induced by these products in the treated mice. Although Ambisome® group mice have showed also low levels of these enzymes, the values were higher in comparison to those found in the ICHQ and ICHQ/M groups.

The immune response evaluated after the treatment showed that ICHQ or ICHQ/M groups' mice produced higher levels of Th1 cytokines (IFN- $\gamma$ , IL-12, TNF- $\alpha$ , and GM-CSF), besides high levels of parasite-specific IgG2a isotype and antileishmanial nitrite, whereas control groups' animals produced higher levels of Th2 cytokines (IL-4 and IL-10). In this context, our results pointed out to the profile of the development of an immune stimulation induced by ICHQ and ICHQ/M through Th1 cytokine-driven and macrophage-mediated mechanisms, which were NO-dependent, to treat *L. amazonensis*-infected animals. Similar immunological correlates using other antileishmanial molecules were also found by others [9,55,56].

Works evaluating antileishmanial molecules have showed that mitochondria are important targets in *Leishmania* parasites, which are responsible by their mechanism of death. Since this organelle is unique in these protozoa, the collapse of the mitochondrial membrane potential can causes the *Leishmania* death by means of events related with apoptosis and/or necrosis [55–57]. In fact, quinolines, such as ICHQ, have showed to alter the *Leishmania* membrane potential, thus leading to an increase in the production of reactive oxygen species and parasite's death. Previous results using ICHQ showed that it induced significant permeabilization of the *Leishmania* membrane, when compared

to the untreated cells, corroborating the findings related with other quinoline derivatives [58].

As a limiting factor of the present study, distinct therapeutic

regimens, such as the reduction of the number of doses, parasitological investigation in other periods of time after the treatment, and the inclusion of other antileishmanial molecules as additional controls were not performed. Nevertheless, we believe that the present work can be considered adequate, since the ICHQ-containing micellar composition showed an *in vivo* effective action against *Leishmania*, as well as did not cause toxicity in the treated animals. As a consequence, and due to its stability, low cost, and easily of production, the ICHQ/M formulation could be considered as an antileishmanial candidate to be tested against human disease.

#### Conflict of interest

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

#### Acknowledgments

The authors thank the Program for Technological Development in Tools for Health-PDTIS-FIOCRUZ for use of its facilities. This work was supported by grants from CAPES, FAPEMIG (CBB-APQ-00819-12 and CBB-APQ-01778-2014) and CNPq (APQ-482976/2012-8, APQ-488237/2013-0, and APQ-467640/2014-9). MACF is a grant recipient of FAPEMIG/CAPES. EAFB, BMR and DMS are grants recipient of CNPq.

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## 4.2. ARTIGO 2 – CONCLUSÃO

A análise imunológica e parasitológica dos camundongos infectados com *L. amazonensis* e tratados mostrou que a composição micelar contendo ICHQ foi altamente eficaz na redução da carga parasitária nos animais. A análise imunológica demonstrou que a proteção foi correlacionada com o desenvolvimento de uma resposta imune Th1, caracterizada por níveis elevados de IFN- $\gamma$ , IL-12 e GM-CSF, além de baixos níveis de IL-4 e IL-10. Dessa forma, o sistema micelar composto com ICHQ pode ser considerado como uma alternativa para o tratamento futuro contra a leishmaniose tegumentar.

***CONCLUSÃO GERAL***

## 5. CONCLUSÃO GERAL

Os resultados sugerem que o clioquinol (ICHQ) foi eficaz em estudos in vitro contra distintas espécies de *Leishmania* e no tratamento de um modelo de mamífero infectado experimentalmente com *L. amazonensis* e, conseqüentemente, pode ser considerado como um candidato a estudos futuros visando à melhoria das condições de tratamento contra as leishmanioses tegumentar e visceral.

***PERSPECTIVAS***

## 6. PERSPECTIVAS

- Desenvolver uma formulação tópica contendo ICHQ.
- Realizar estudo *in vivo* avaliando a ação do ICHQ micelar contra a leishmaniose visceral e em outros modelos de mamíferos, tais como o hamster.

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## 8. ANEXO

### 8.1. PATENTE



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#### 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 023737 0

#### Dados do Depositante (71)

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Depositante 1 de 1

Nome ou Razão Social: UNIVERSIDADE FEDERAL DE MINAS GERAIS

Tipo de Pessoa: Pessoa Jurídica

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**PETICIONAMENTO  
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Petição Eletrônica em 03/11/2017 às 15:15, Petição 870170084818

**Dados do Pedido**

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Natureza Patente: 10 - Patente de Invenção (PI)

Título da Invenção ou Modelo de "COMPOSIÇÕES FARMACÊUTICAS LEISHMANICIDAS

Utilidade (54): CONTENDÓ CLIOQUINOL E USO"

Resumo: A presente tecnologia trata de composições farmacêuticas contendo um derivado de quinolinas, o clioquinol, que possui atividade leishmanicida seletiva contra formas promastigotas e amastigotas de *L. amazonensis* e *L. infantum*. As composições descritas na presente invenção podem ser usadas no tratamento das leishmanioses tegumentar e visceral em espécies de mamíferos, incluindo no homem e cão.

Figura a publicar: 1

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**PETICIONAMENTO  
ELETRÔNICO**

Esta solicitação foi enviada pelo sistema Peticionamento Eletrônico em 03/11/2017 às 15:15, Petição 870170084818