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NOVA PROPOSTA PARA O TRATAMENTO DA LEISHMANIOSE TEGUMENTAR USANDO ANTIMONIATO DE MEGLUMINA E OXIRANOS EM ASSOCIAÇÃO

LUIZ FILIPE GONÇALVES DE OLIVEIRA

RIO DE JANEIRO
Maio de 2018

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Orientador: Dr. Carlos Roberto Alves

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Rio de Janeiro, 30 de maio de 2018

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Ata da defesa de tese de doutorado em Biologia Parasitária de **Luiz Filipe Gonçalves de Oliveira**, sob orientação do Dr. Carlos Roberto Alves. Ao trigésimo dia do mês de maio de dois mil e dezoito, realizou-se às nove horas, no Auditório Maria Deane/FIOCRUZ, o exame da tese de doutorado intitulada: **"NOVA PROPOSTA PARA O TRATAMENTO DA LEISHMANIOSE TEGUMENTAR USANDO ANTIMONIATO DE MEGLUMINA E OXIRANOS EM ASSOCIAÇÃO."** No programa de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz, como parte dos requisitos para obtenção do título de Doutor em Ciências - área de concentração: Genética e Bioquímica, na linha de pesquisa: Estudos Bioquímicos e Moleculares de Agentes Infecciosos e Parasitários e Vetores. A banca examinadora foi constituída pelos Professores: Dr. Armando de Oliveira Schubach - INI/FIOCRUZ (Presidente), Drª. Maria de Nazaré Correia Soeiro - IOC/FIOCRUZ, Dr. Fernando de Carvalho Silva - UFF/RJ e como suplentes: Dr. Alcides José Monteiro da Silva – UFRJ/RJ e Dr. Helvécio Vinícius Antunes Rocha – FARMANGUINHOS/FIOCRUZ. Após arguir o candidato e considerando que o mesmo demonstrou capacidade no trato do tema escolhido e sistematização da apresentação dos dados, a banca examinadora pronunciou-se pela APROVAÇÃO da defesa da tese de doutorado. De acordo com o regulamento do Curso de Pós-Graduação em Biologia Parasitária do Instituto Oswaldo Cruz, a outorga do título de Doutor em Ciências está condicionada à emissão de documento comprobatório de conclusão do curso. Uma vez encerrado o exame, o Coordenador do Programa, Dr. Rafael Maciel de Freitas, assinou a presente ata tomando ciência da decisão dos membros da banca examinadora. Rio de Janeiro, 30 de maio de 2018.

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“Se, a princípio, a ideia não é absurda, então não há esperança para ela.”
(Albert Einstein)

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Luiz Filipe Gonçalves de Oliveira

Esta tese porpõe uma nova abordagem para o tratamento da leishmaniose tegumentar com o antimoniato de N-metilglucamina (antimoniato de meglumina - AM) associado a dois derivados oxiranos. O estudo foi conduzido em modelo murino de infecção *in vitro* e *in vivo* por *Leishmania (Leishmania) amazonensis*. Na primeira etapa do estudo foram descritas alterações histológicas causadas por epoxi-α-lapachona, epoximetil-lausona e AM em camundongos BALB/c não infectados, bem como a predição de algumas de suas propriedades farmacocinéticas. Os resultados indicaram que tanto os oxiranos quanto o antimoniato de meglumina induzem alterações histopatológicas nos órgãos analisados. O epoximetil-lausona foi o mais tóxico para o tecido pulmonar, enquanto os danos mais graves no coração foram causados pelo epoxi-α-lapachona. O AM causou alterações leves a moderadas nos tecidos cardíacos e pulmonares, mas sem qualquer efeito detectado nos tecidos cerebrais. Na segunda etapa foi necessário avaliar a eficácia do epoximetil-lausona sobre a infecção de macrófagos e camundongos BALB/c infectados por *L.(L.) amazonensis*. Em amastigotas intracelulares, o IC_{50} do epoximetil-lausona foi ligeiramente superior ao do AM (7,41 ± 0,2 e 4,43 ± 0,25 µM, respectivamente), sendo o efeito mais evidente após 48 horas de exposição (18 vezes e 7,4 vezes inferiores, respectivamente). Os promastigotas também foram afetados pelo composto, porém o IC_{50} foi seis vezes maior (45,45 ± 5,0µM), indicando sua especificidade sobre os amastigotas intracelulares. A análise de citotoxicidade revelou que o epoximetil-lausona tem um efeito menor (1,7 ×) comparado ao AM (40,05 ± 3,0 e 24,14 ± 2,6 µM). O tratamento com três doses do epoximetil-lausona reduziu a lesão da pata dos animais infectados em 27 %, enquanto a redução obtida com o AM chegou a 31% nas doses baixa e intermediária, e 64% com a dose mais alta, comparado ao grupo controle. Alterações ultraestruturais detectadas nos amastigotas da lesão constataram comprometimento da integridade dos parasitos. Na etapa final deste estudo foi demonstrado o efeito do tratamento com o AM associado aos oxiranos epoxi-α-lapachona e epoximetil-lausona sobre a infecção experimental *in vitro* e *in vivo*. Os compostos foram testados individualmente e em combinações, seguindo as razões: 3:1; 1:1 e 1:3 (p/v) sobre macrófagos infectados. Todos os compostos, assim como suas combinações mostraram índices endocíticos muito inferiores ao grupo controle, sendo as maiores reduções obtidas nas razões de 3:1. O tratamento de camundongos BALB/c com os compostos individualmente e combinados nas mesmas razões levou a reduções significativas das lesões. O melhor efeito das combinações foi observado nas razões de 3:1. Os resultados indicaram que a associação do AM com os oxiranos produz um incremento no efeito leishmanicida, e pode ser considerada uma nova abordagem para o tratamento da leishmaniose cutânea.

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NEW PROPOSAL FOR TEGUMENTARY LEISHMANIASIS TREATMENT USING MEGLUMINE ANTIMONIATE AND OXIRANE IN ASSOCIATION

ABSTRACT

PHD THESIS IN PARASITE BIOLOGY

Luiz Filipe Gonçalves de Oliveira

This thesis proposes a new approach for the treatment of tegumentary leishmaniasis with meglumine antimoniate (MA) associated to two oxirane derivatives. The study was conducted in murine model of *in vitro* and *in vivo* infection caused by *Leishmania (Leishmania) amazonensis*. In the first part of the study, histological changes induced by epoxy-α-lapachone, epoxymethyl-lawsone and MA were described in non infected BALB/c mice, as well as the prediction of their pharmacokinetic properties. The results indicated that both oxiranes and MA induce histopathological changes in the examinated organs. Epoxymethyl-lawsone was the most toxic to lung tissue, while the most severe damage to the heart was caused by epoxy-α-lapachone. MA caused mild to moderate changes in cardiac and pulmonary tissues, but with no detected effect in brain tissues. The second step was to assess the efficacy of epoxymethyl-lawsone on the macrophage infected and on BALB/c mice infected by *L. (L.) amazonensis*. In intracellular amastigotes, the IC_{50} value of epoxymethyl-lawsone was lightly superior to MA (7.41 ± 0.2 and 4.43 ± 0.25 μM, respectively) and the effect became more evident after 48 hours of exposure (18-fold and 7.4-fold lower, respectively). Promastigotes were also affected by the compound, but the IC_{50} was six-fold higher (45.45 ± 5.0 μM), indicating their specificity by intracellular amastigotes. Cytotoxicity assays revealed that epoxymethyl-lawsone has lower effect (1.7-fold) compared to MA (40.05 ± 3.0 e 24.14 ± 2.6 μM). Treatment with three doses of epoxymethyl-lawsone reduced the paw lesion of the infected mice by 27%, while the reduction obtained with MA reached 31% at the low and intermediate doses and 64% with the high dose compared to the control group. Ultrastructural changes detected in amastigotes from lesions showed the integrity of parasites clearly affected. In the final stage of this study, the effect of the treatment with MA associated to oxiranes epoxi-α-lapachone and epoxymethyl-lawsone on the experimental infection *in vitro* and *in vivo* was demonstrated. The compounds were tested alone and in combinations following the ratios: 3:1; 1:1 and 1:3 (w/v) on infected macrophages. All compounds as well as its combinations showed endocytic indexes much lower than the control group, with the highest reductions obtained in the 3: 1 ratio. Treatment of BALB / c mice with drugs alone and combined in the same ratios led to significant reductions in the lesions. The best effect of the combination was observed in the 3:1 ratio. The results indicated that the association of MA with oxiranes produces enhance in the leishmanicidal effect and may be considered a new approach for the treatment of cutaneous leishmaniasis.

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LISTA DE SIGLAS E ABREVIATURAS

AM	Antimoniato de meglumina
ATP	Trifosfato de adenosina
CR1	Receptor de complemento tipo 1
CR3	Receptor de complemento tipo 3
DNA	Ácido desoxirribonucleico
EGS	Estibogluconato de sódio
ELISA	Ensaio de imunoabsorção enzimática
Fc	Fração cristalizável
FDA	*Food and Drug Administration*
gp63	Glicoproteína 63
GSH	Glutationa reduzida
IgG	Imunoglobulina G
kDa	Quilo Dalton
LC	Leishmaniose cutânea
LCD	Leishmaniose cutâneo difusa
LM	Leishmaniose mucocutânea
LPG	Lipofosfoglicano
LPS	Lipopolissacarídeo
LTA	Leishmaniose tegumentar americana
LV	Leishmaniose visceral
mRNA	Ácido ribonucleico mensageiro
OMS	Organização Mundial da Saúde
OPAS	Organização Panamericana de Saúde
PCR	Reação em cadeia de polimerase
PPG	Proteofosfoglicano
TNF-α	Fator de necrose tumoral
TR	Tripanotiona redutase
VP	Vacúolo parasitóforo

1. INTRODUÇÃO

1.1. Considerações gerais

1.1.1 Breve histórico

Leishmanioses são doenças infecciosas, não contagiosas, causadas por protozoários do gênero *Leishmania*. No Velho Mundo ocorrem desde o Senegal, na África até a Índia e Mongólia, sul da França e Namíbia (Blum et al., 2004). Trata-se de uma doença humana muito antiga, existindo há milênios. Há descrições de lesões reminiscentes de ferida oriental em tábuas na biblioteca do rei assírio Assurbanipal do século VII a.C., mas acredita-se que podem ser derivadas de textos datados de 1500–2500 a.C., (*apud* Steverding, 2017). Os antigos centros de distribuição de especiarias no passado originaram nomes como botão-do-oriente, botão-de-Delhi, botão-de-Bagdá, botão-de-Aleppo, entre outros (*apud* Oumeish, 1999).

O Papiro Ebers, um dos tratados médicos mais antigos de que se tem registro, foi escrito no Antigo Egito por volta de 1550 a.C. e descreve uma doença com características muito similares às da leishmaniose cutânea, denominada botão do Nilo. No Velho Testamento, há referência a uma das "pragas do Egito", descrita como tumores e úlceras que poderia ter sido baseada em uma epidemia de leishmaniose cutânea (Bíblia - **6ª praga**, *Ex*. 9:9). O livro de Deuteronômio é constituído por uma série de discursos promulgados por Moisés para preparar uma aliança entre Deus (*Javé*) e o seu povo (Israel). Trata-se na verdade de um conjunto de regras sócio-político-religiosas que serviu de base para a reforma realizada pelo rei Ezequias que, entre 716 e 701 a.C, preparou Israel para a guerra com os assírios. No seu capítulo 28, o texto fala das bênçãos concedidas aos que obedecerem às leis, e das maldições que recairão no caso de desobediência: "*Javé ferirá você com úlceras do Egito, com tumores, crostas e sarnas, que você não conseguirá curar*" (Bíblia - **Maldições**, *Dt 28:27*). Outra evidência para a presença da leishmaniose durante a antiguidade surgiu de um estudo paleoparasitológico realizado em 42 múmias egípcias de um túmulo do Império do Oriente em West Thebes (2050–1650 a.C). Neste trabalho foi detectado DNA mitocondrial de *Leishmania donovani* em quatro espécimes, sugerindo que a forma visceral da doença já estava presente no antigo Egito (Zink et al., 2006)

A primeira descrição exata da ferida oriental foi feita na Idade Média, século X pelo filósofo e médico persa conhecido com Avicena (Abū ʿAlī al-Ḥusayn ibn ʿAbd

Allāh ibn Sīnā). O polímata árabe descreveu criteriosamente uma doença batizada de úlcera de Balkh, uma cidade ao norte do Afeganistão (Manson-Bahr, 1996).

No Novo Mundo, estão descritas em cerâmica pré-colombiana desde o século V lesões faciais desfigurantes reminiscentes sugestivas de leishmaniose mucocutânea. Quatro crânios femininos datados do século XI encontrados no cemitério arqueológico de Coyo Oriente, no deserto de San Pedro de Atacama no norte do Chile, levantam evidências morfológicas e moleculares da leishmaniose na América do Sul (Costa et al., 2009).

Em 1756, o médico e naturalista escocês Alexander Russell publicou um relato clínico detalhado das formas secas e úmidas da ferida oriental enquanto clinicava em Aleppo. Ele descreveu detalhadamente o desenvolvimento das lesões, narrando que as doenças curam dentro de oito meses a um ano. Russell relata neste documento que "*... pelo que observei, é infinitamente melhor não aplicar nada, do que qualquer um dos inúmeros medicamentos que eles fazem uso...*", mas também escreveu que descobrira que um emplastro mercurial era mais eficaz (Russel, 1756).

A busca pelos agentes causadores das diferentes formas de leishmaniose iniciou-se ao longo do século XIX, mas a descrição inicial dos parasitos foi atribuída a James Homer Wright em 1903. O patologista americano descreveu parasitos isolados de uma criança com suspeita de ferida oriental e os classificou como *Helcosoma tropicum* (Wright, 1903). Em 1906, o médico e zoólogo alemão Max Lühe mudou o nome para *Leishmania tropica* (Lühe, 1906). No entanto, há vários indícios de que os parasitos já haviam sido observados por David Douglas Cunningham em 1885, porém o médico escocês não percebeu o que eram de fato (Cunningham, 1885). Em 1898, o médico do exército russo Piotr Fokich Borovsky reconheceu que os microorganismos presentes nas feridas orientais eram protozoários, porém suas observações foram publicadas em um periódico russo obscuro e por isso passaram despercebidas (Hoare, 1938).

Entretanto, a doença foi descrita como síndrome clínica em 1903, pelo médico do exército escocês William Leishman, a partir de observações feitas em um grupo de soldados britânicos recém-chegados da Índia com perda excessiva de peso e esplenomegalia. O patologista ao analisar células do baço de um dos soldados mortos pela "febre Dum-Dum", notou a presença de pequenas células arredondadas (Leishman, 1903). Ainda em 1903, o professor de fisiologia da universidade de Madras, Charles Donovan analisou, de forma independente, aspirados esplênicos de

um menino hindu com febre irregular e encontrou corpúsculos arredondados muito semelhantes àqueles que Leishman havia descrito (Donovan, 1903). Nesse mesmo ano, Charles Louis Alphonse Laveran e Félix Étienne Pierre Mesnil classificaram esse organismo como *Piroplasma donovani* (Laveran, 1903), e Ronald Ross propôs sua reclassificação para *Leishmania donovani*, criando o gênero *Leishmania* (Ross, 1903).

Havia suspeitas de que moscas de areia seriam os vetores destes parasitos (Wenyon, 1911), mas só em 1921 dois irmãos franceses, o químico Edmond Sergent e o biólogo Étienne Sergent demonstraram que a escarificação de uma suspensão de moscas na pele de voluntários resultou no desenvolvimento de lesões orientais típicas (Sergent, 1921). No entanto, o resultado deste experimento não fora aceito como prova conclusiva de que tais insetos seriam os vetores da ferida oriental. Somente em 1941 o mecanismo de transmissão foi finalmente demonstrado quando o parasitologista britânico-israelense Saul Adler conseguiu infectar cinco voluntários através da picada de flebotomíneos infectados experimentalmente com *Leishmania tropica* (provavelmente *Leishmania major*) em laboratório (Adler, 1941). No ano seguinte, Swaminath e colaboradores demonstraram que os flebotomíneos eram também os vetores do kala-azar (Swaminath et al., 1942).

No Brasil, Adolpho Lutz e Arthur Neiva iniciaram, ainda em 1912, a catalogação das espécies incluídas no gênero *Phlebotomus*, como contribuição à entomologia sistemática brasileira, e relataram suas primeiras impressões sobre os insetos: "*O genero **Phlebotomus** pertence ás **Psychodidas** e contem mosquitinhos cujos habitos correspondem aos de muitas **Culicidas** e **Cerapogoninas**, sendo que as femeas se alimentam repetidas vezes de sangue e também atacam o homem. Assim parecem habilitadas para a transmissão de moléstias*..." (Lutz & Neiva, 1912). Em 1922 o médico brasileiro Henrique de Beaurepaire Rohan Aragão relacionou os flebotomíneos como os responsáveis pela transmissão de leishmaniose na América do Sul (Aragão, 1922). Posteriormente descobriram que esses flebotomíneos do Novo Mundo pertenciam ao gênero *Lutzomyia* (Steverding, 2017).

A leishmaniose no Brasil é conhecida desde 1855, quando Alexandre Cerqueira encontrou lesões de pele muito similares às do botão-do-oriente (*in* Teixeira, 1995). Em 1908 em São Paulo, Artur Neiva participou da descrição de numerosos casos de leishmaniose durante a construção da Estrada de Ferro Noroeste do Brasil, principalmente na cidade de Bauru, tornando-a conhecida como "úlcera de Bauru"

(Azevedo, 1994). A confirmação aconteceu quando Adolpho Carlos Lindenberg encontrou, em 1909, o parasito em úlceras cutâneas e nasobucofaríngeas de indivíduos que trabalhavam em áreas de desmatamentos na construção de rodovias no interior de São Paulo (Lindenberg, 1909). Na década de 50, houve uma diminuição geral da ocorrência de casos de leishmaniose tegumentar, mas, nos 30 anos seguintes a doença apresentou franca expansão (Marzochi, 1992).

Em 1926, João Montenegro desenvolveu um teste cutâneo utilizando uma suspensão de microrganismos mortos obtidos a partir de cultura, conhecido como intradermorreação de Montenegro (Montenegro, 1926). O teste se baseia na reação de hipersensibilidade retardada medida pela presença de inflamação, eritema ou erupção cutânea (Guedes et al., 1990). Sua sensibilidade varia entre 86 e 100% e especificidade de aproximadamente 100%, e representa o principal exame complementar para o diagnóstico da leishmaniose tegumentar americana em áreas endêmicas, sendo ainda hoje de grande importância para países como o Brasil (Furtado, 1980).

A partir de 1969, os testes de imunofluorescência indireta (Nery-Guimarães et al., 1969; Walton et al., 1972; Matossian et al., 1975) e ensaios imunoenzimáticos (*Enzyme Linked Immunosorbent Assay* - ELISA) (Baldelli et al., 1978; Edrissian & Darabian, 1979) foram aplicados no diagnóstico da doença. Somente na década de 1990, a introdução dos testes para detecção do ácido desoxirribonucleico (*deoxyribonucleic acid* - DNA) do cinetoplasto pela reação em cadeia de polimerase (*Polymerase Chain Reaction* - PCR) veio a contribuir com o aumento da sensibilidade do diagnóstico, permitindo a diferenciação das espécies em amostras de tecido e sangue (Rodgers et al., 1990) .

O tratamento atual da leishmaniose foi baseado na proposta de Gaspar Vianna que, em 1912, foi capaz de tratar efetivamente pacientes com leishmaniose cutânea pela injeção de tártaro emético (Vianna, 1912) (Figura 1A). Essa abordagem foi inspirada na então promessa dos efeitos microbicidas que os antimoniais apresentaram contra outras espécies de tripanossomatídeos (Rey, 1962).

Em 1915, esse tratamento também se mostrou eficaz contra a leishmaniose visceral, conforme relatado Giovanni Di Cristina e Giuseppe Caronia na Itália (Di Cristina & Caronia, 1915) e por Leonard Rogers na Índia (Cook, 2006). No entanto, o tratamento tinha desvantagens, pois o medicamento é altamente tóxico para os

pacientes, e considerado muito instável no clima tropical. Havia ainda relatos de que o antimônio não teria efeito benéfico em outros estudos (Shortt, 1945; Berman, 1988).

Essa controvérsia levou a mais estudos e, em 1920, um novo composto antimonial foi desenvolvido pelo médico e cientista indiano Upendranath Brahmachari para tratar o calazar, ureia estibamina (Brahmachari, 1928) (Figura 1B). Os próximos avanços no tratamento da leishmaniose foram alcançados apenas décadas depois com o advento dos antimoniais pentavalentes: antimonil (V) gluconato de sódio em 1937 (Kikuth & Schmidt, 1937), antimoniato de N-metil-meglumina (Gailliot, 1941) (Figura 1C) e estibogluconato de sódio em 1945 (Goodwin, 1995) (Figura 1D). Esses fármacos são associados a menos efeitos tóxicos para os pacientes.

A primeira obtenção do antimoniato de meglumina está descrita na patente britânica da Rhone Poulenc (Gailliot, 1941). As matérias-primas empregadas foram a N-metil-D-glucamina (NMG) e o pentacloreto de antimônio ($SbCl_5$) (Método 1) ou tricloreto de antimônio ($SbCl_3$) (Método 2). No primeiro método, uma solução aquosa de NMG em dietilamina é adicionada ao $SbCl_5$ dissolvido em clorofórmio (Figura 2 A). No segundo método, o $SbCl_3$ é oxidado ao Sb^{5+} utilizando H_2O_2, e o produto resultante é então misturado à NMG (Figura 2 B) (Carvalho et al., 2015).

1.1.2. Importância

Atualmente, a leishmaniose é considerada pela Organização Mundial da Saúde (OMS) uma das doenças mais negligenciadas. É encontrada em 98 países no mundo, sendo endêmica em 82 destes. É bem estabelecido que as leishmanioses possam ser causadas por mais de 21 espécies de *Leishmania* e transmitidas por mais de 90 espécies de flebotomíneos (família Psychodidae, Subfamília Phlebotominae). A OMS estima que cerca de 350 milhões de pessoas estejam sob o risco de contrair a doença, e que a incidência anual seja de cerca de 2 milhões de casos. É reconhecidamente um grande problema de saúde pública devido às altas taxas de incidência e prevalência, ampla distribuição nos trópicos e capacidade de produzir deformidades, causando significativo impacto social e econômico (WHO, 2010).

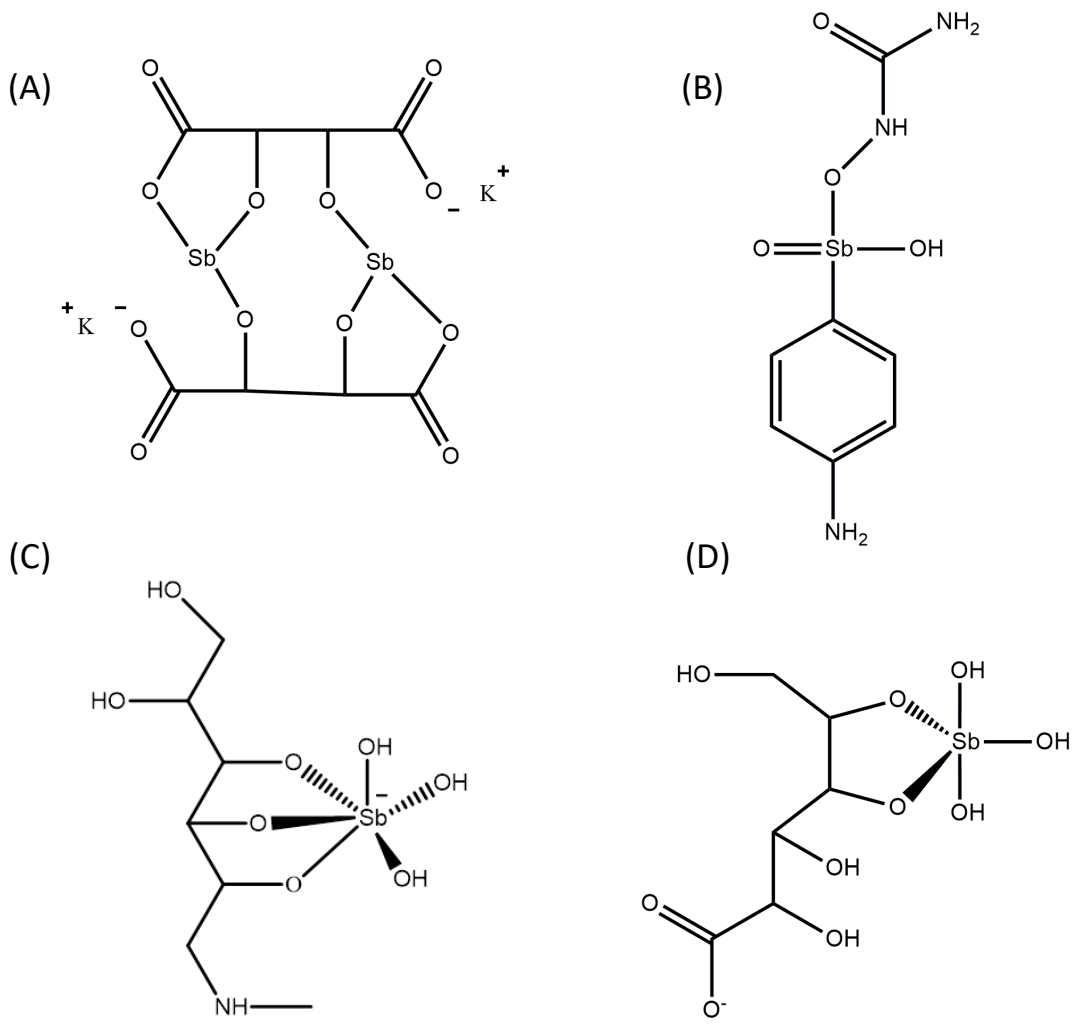

Figura 1: Estrutura química dos compostos orgânicos derivados do antimônio usados para tratar a leishmaniose. (A) Tartarato de potássio e antimônio (Tártaro emético), (B) Ureia estibamina, (C) Antimoniato de N-metil-glucamina, (D) Estibogluconato de sódio.

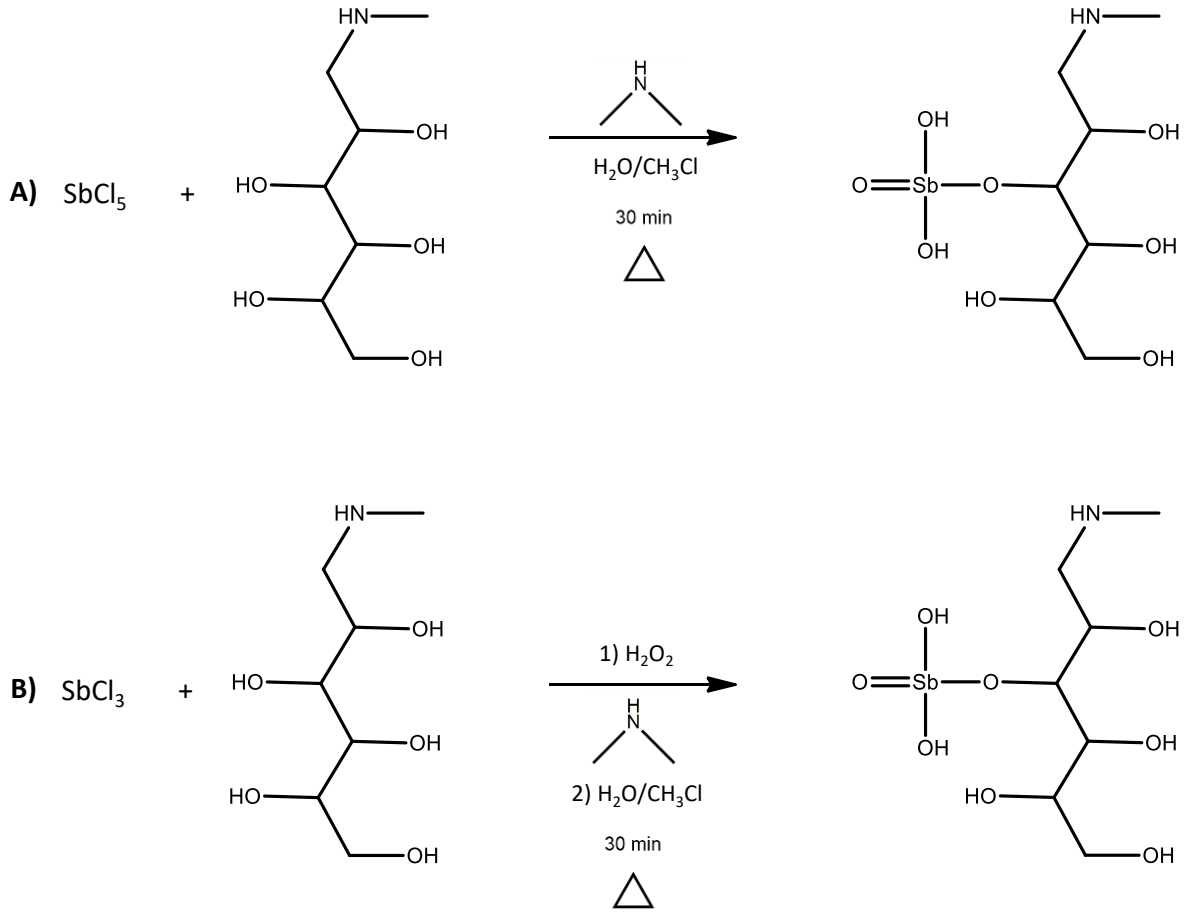

Figura 2: Processo de obtenção do antimoniato de N-metil-glucamina descrito na patente britânica da Rhone Poulenc (1941). Adaptado de Carvalho et al., 2015.

A doença é tradicionalmente classificada em quatro apresentações clínicas principais: leishmaniose visceral (LV), leishmaniose cutânea (LC), leishmaniose mucocutânea (LM) e leishmaniose dérmica pós-calazar. Na LV, também conhecida como calazar, há comprometimento de órgãos internos, sendo fatal em mais de 95 % dos casos não tratados. A forma tegumentar da doença se divide em LC, forma mais comum caracterizada por lesões cutâneas em partes expostas do corpo, deixando cicatrizes ao longo da vida, e a LM que pode levar à destruição parcial ou total das membranas mucosas do nariz, boca e garganta, levando à incapacidade moderada a grave (WHO, 2010).

A dinâmica da infecção é afetada por vários fatores que dificultam o controle da doença, incluindo: (*i*) a pobreza e condições sanitárias domésticas precárias, como a falta de manejo adequado de resíduos e/ou esgoto a céu aberto, podem aumentar os locais de criadouros de flebotomíneos; (*ii*) a desnutrição, definida como baixa ingestão de proteínas, ferro, vitamina A e zinco, poderia aumentar o risco de infecção por espécies de *Leishmania* viscetrópicas; e (*iii*) mudanças ambientais também podem afetar a incidência de leishmaniose, incluindo a urbanização, a domesticação do ciclo de transmissão e assentamentos em áreas florestais (WHO, 2017).

1.1.3. Agente etiológico e seu ciclo biológico

Os protozoários do gênero *Leishmania* pertencem à ordem Kinetoplastida, da família Trypanosomatidae. O gênero é dividido em três subgêneros: *Leishmania*, *Viannia* e *Sauroleishmania*. Os subgêneros *Leishmania* e *Viannia* foram inicialmente separados de acordo com a região para onde os promastigotas metacíclicos migram e se aderem durante seu desenvolvimento no inseto vetor. Promastigotas do subgênero *Leishmania* seguem para a válvula estomodeal, localizada na interseção entre os intestinos médio e anterior do inseto, enquanto os promastigotas de *Viannia* se dirigem para a região pilórica do intestino posterior (Sacks & Kamhawi, 2001). Análises filogenéticas baseadas em sequências de DNA comprovaram a existência desta separação. O subgênero *Sauroleishmania* afeta somente répteis e, portanto, não apresentam importância médica (Bates, 2007).

O parasito é dimórfico apresentando duas formas principais, *(i)* promastigotas extracelulares com flagelo livre, que se multiplicam e se desenvolvem no trato digestivo de insetos flebotomíneos, e *(ii)* amastigotas intracelulares sem flagelo livre

que se desenvolvem em macrófagos de mamíferos (Teixeira et al., 2013). Os amastigotas irão infectar principalmente os macrófagos da pele e mucosas, e de órgãos do sistema reticuloendotelial como fígado, baço, medula óssea e gânglios linfáticos (Kamhawi, 2006; Bates, 2007).

Embora os macrófagos sejam considerados a célula hospedeira mais importante para a manutenção da infecção, vários outros tipos celulares demonstraram a capacidade de endocitar *Leishmania in vitro* ou *in vivo*. Estes incluem neutrófilos (Chang, 1981; Pearson & Steigbigel, 1981; Pearson et al., 1983; van Zandbergen et al., 2004), eosinófilos (Grimaldi et al., 1984; Pearson et al., 1987; Vasconcellos et al., 1992; Oliveira et al., 1998), células dendríticas (Williams, 1988; Moll et al., 1999), células epiteliais (Belle, 1958) e fibroblastos (Bogdan et al., 2000; Macedo-Silva et al., 2014). No interior destas células, os amastigotas se multiplicam em vacúolos parasitóforos (VP), cuja biogênese envolve a aquisição de marcadores de membrana de endossomas tardios das células hospedeiras (Desjardins et al., 1994; Lang et al., 1994; Russell & Chakraborty, 1992; Courret et al., 2002). A aquisição desses marcadores é um evento coordenado que resulta em um VP maduro, presumivelmente necessário para a sobrevivência e multiplicação dos parasitos.

O parasito alcança o inseto vetor quando sugado durante o repasto sanguíneo de flebotomíneos fêmeas, com sangue infectado por amastigotas livres no interior de macrófagos. No interior do trato digestivo do flebotomíneo ocorrem muitas alterações fisiológicas como a secreção de proteases hidrolíticas pelo epitélio digestivo e a formação de uma membrana ao redor do sangue infectado, denominada matriz peritrófica. Esta matriz é secretada pelo epitélio digestivo do inseto, sendo composta por proteínas e proteoglicanos e suas funções parecem estar relacionadas com a proteção do próprio epitélio contra danos às vilosidades e infecções por microorganismos patogênicos (Pimenta et al., 1997; Sacks & Kamhawi, 2001).

Os parasitos transformam-se em promastigotas entre 12 e 18 horas após o repasto sanguíneo, adaptando-se às novas condições existentes. São inicialmente pró-cíclicos e permanecem pequenos ovoides apresentando pouca motilidade. Posteriormente, ocorre rápida multiplicação destas formas que começam a transformar-se em promastigotas longos e delgados com grande motilidade, promastigotas nectomonados. Todo este processo ocorre ainda dentro da matriz peritrófica que parece criar uma barreira contra a rápida difusão das enzimas

proteolíticas, limitando assim sua exposição aos parasitos. Se durante esta fase inicial a matriz parece favorecer a sobrevivência do parasito, mais tarde se torna uma barreira física ao seu desenvolvimento (Sacks & Kamhawi, 2001).

Estudos envolvendo *Phlebotomus mongolensis* infectados por *L. (L.) donovani* e *P. papatasi* infectados por *L. (V.) panamensis* nos quais a matriz peritrófica não foi desfeita durante a digestão do sangue resultaram em aprisionamento e posterior excreção dos parasitos do trato digestivo do inseto (Feng, 1951; Walters et al., 1992). Enzimas quitinolíticas derivadas dos parasitos têm sido apontadas como as responsáveis pela lise da parte anterior da membrana (Schlein et al., 1991). Foi observado também que a persistência da infecção depende da ligação do protozoário às células epiteliais do trato intestinal do inseto, mediada por uma superfície de lipofosfoglicano (*lipophosphoglycan* - LPG) (Sacks et al., 2000; Soares et al., 2002). Evidência importante sobre o papel do LPG nas interações entre o parasito e o epitélio do vetor foi demonstrada através do uso de mutantes defeituosas em LPG que foram incapazes de se ligar e sustentar a infecção no intestino do inseto (Sacks et al., 2000). Uma proteína de 65 kDa foi identificada em *Phlebotomus papasi* como o receptor para LPG de *L.(L.) major* (Dillon & Lane, 1999). Variações na estrutura dos LPG têm sido implicadas na especificidade de várias espécies de *Leishmania* às diferentes espécies de flebotomíneos (Sacks et al., 1995; Kamhawi et al., 2000; Sacks & Kamhawi, 2001).

Posteriormente, os promastigotas nectomonados transformam-se em promastigotas haptomonadas, formas mais curtas que se multiplicam rapidamente e, em promastigotas metacíclicos, formas curtas e delgadas com alta motilidade, com um flagelo no mínimo duas vezes maior que seu corpo. Estas formas não são comumente vistas em divisão. Nesta fase, ocorre uma migração destas formas para a região torácica do inseto, levando a um acúmulo de parasitos atrás da válvula cárdica. Esta migração tem sido geralmente atribuída a um gradiente de concentração de açúcares seguido pelos parasitos. A capacidade dos promastigotas migrarem quimiotaxicamente na presença de açúcares tem sido demonstrada *in vitro*, entretanto também tem sido vista na ausência de uma “refeição” rica em açúcares (Warburg & Schlein, 1986). Formas haptomonadas ligam-se via hemidesmossomos ao epitélio intestinal do vetor, enquanto as formas metacíclicas permanecem nadando livremente no lúmen, migrando inclusive para regiões como esôfago, faringe e probóscida do inseto. A incapacidade das formas metacíclicas de

se ligarem às células do epitélio intestinal do vetor pode ser explicada, pelo menos em parte, pela perda do potencial intrínseco de ligação, caracterizado por modificações estruturais ocorridas na superfície do LPG, que fazem com que os açúcares terminais responsáveis pela ligação ao epitélio não estejam mais expostos (Sacks, 1992; Mahoney et al., 1999).

O acúmulo de grandes quantidades de promastigotas metacíclicos nas regiões anteriores do intestino, incluindo sua presença na probóscida, sugere que estas formas são inoculadas durante o repasto sanguíneo. Há um consenso de que uma transmissão eficiente envolva a formação de um “tampão” biológico que prejudica a ingestão de sangue (Killick-Kendrick et al., 1977; Killick-Kendrick & Molyneux, 1981; Beach et al., 1985). Isso deve promover a regurgitação das formas promastigotas infectivas do intestino anterior ou atrás da válvula estomodeal à medida que o inseto tenta deslocar o tampão para conseguir se alimentar (Sacks & Kamhawi, 2001).

Durante o repasto sanguíneo, o inseto vetor transfere as formas promastigotas metacíclicas para a pele do hospedeiro mamífero juntamente com uma pequena quantidade de saliva (Sacks & Kamhawi, 2001). A saliva suprime a atividade leishmanicida do macrófago por inibir a produção de óxido nítrico (NO), acelerando o desenvolvimento da lesão (Fig. 5). Isto tem sido atribuído a um peptídeo salivar, o maxadilan, um agonista seletivo do polipeptídeo ativador da adenilato-ciclase pituitária tipo 1, que inibe a produção do Fator de Necrose Tumoral α (TNF-α) por macrófagos estimulados por LPS (Bozza et al., 1998; Soares et al., 1998) e diminui sua capacidade de produzir NO e eliminar parasitos de *Leishmania* em *vitro* (David et al., 1997).

As glicoproteínas de superfície do parasito estão envolvidas no processo de ligação com os receptores da célula hospedeira, sendo a glicoproteína 63 (gp63) e o LPG os principais glicoconjugados envolvidos neste processo (Alexander et al., 1999). Embora não possua um mecanismo ativo de entrada na célula, os parasitos de *Leishmania* se utilizam desses ligantes de superfície, sendo fagocitados por meio de endocitose mediada por receptores (Alexander et al., 1999). O LPG e gp63 também desempenham papéis cruciais na proteção dos parasitos da atividade microbicida dos macrófagos. Os promastigotas acessam preferencialmente os macrófagos via receptores CR3 e CR1, não conseguindo desencadear o estresse oxidativo nos macrófagos (revisado por Brittingham & Mosser, 1996). O LPG também inibe de forma transitória a fusão fagossomo-endossômica (Desjardins &

Descoteaux, 1997), elimina os radicais de oxigênio gerados durante a “explosão respiratória” (Chan et al., 1989), inibe atividade da proteína quinase C (Giorgione et al., 1996), e suprime a expressão de NOS2 de macrófagos e a produção de NO (Proudfoot et al., 1996). O gp63 também foi associado à supressão do estresse oxidativo (Sorensen et al., 1994), e fortes evidências sugerem que a atividade de proteases protege o parasita da citólise lisossomal e degradação (Seay et al., 1996).

1.1.4. Leishmaniose Tegumentar Americana

A leishmaniose tegumentar americana (LTA) é uma zoonose endêmica de difícil controle que acomete a pele e mucosas, caracterizada por lesões cutâneas nos sítios primários de inoculação dos parasitos (Sampaio, 1999). A forma mais comum é representada por uma úlcera única, mas a doença apresenta uma variedade de manifestações clínicas, como lesões múltiplas, lesões nodulares e lesões cutâneo-mucosas que afetam regiões como as mucosas nasal e oral, faringe e laringe. Esta forma pode ser desfigurante e, em alguns casos, evoluir para o óbito por acometimento respiratório secundário (Marsden, 1986).

A LTA distribui-se amplamente no continente americano, estendendo-se desde o sul dos Estados Unidos até o norte da Argentina (Marzochi, 1992). No Brasil, tem sido notificada em todos os estados, constituindo, portanto, uma das afecções dermatológicas que merece maior atenção, pela sua magnitude, pelo risco de causar deformidades no ser humano e pelo envolvimento psicológico do doente com impacto no campo social e econômico (Gontijo & Carvalho, 2003; Ludwig, 2009).

De 2001 a 2006 no Brasil, foram registrados mais de 173 mil casos de leishmaniose tegumentar. Nos últimos 10 anos, foram notificados cerca de 234 mil novos casos de LTA, evidenciando que as ações de vigilância e controle no País não conseguem conter a progressão da doença. No período de 2001 a 2006, verificamos uma média anual de 28.979 casos novos registrados, enquanto no período de 2007 a 2017, a média anual de 21.272 casos novos (Figura 3). O coeficiente mais elevado em todo este período foi observado em 2003, quando a doença atingiu 17,42 casos por 100 mil habitantes (Tabela 1).

Na região Norte, há registros de casos em 82% dos municípios, e no Nordeste ocorre em praticamente todos os municípios. Em Mato Grosso, 100% dos municípios têm casos registrados. As regiões Sudeste e Sul também apresentam surtos epidêmicos, principalmente nos estados do Espírito Santo, Minas Gerais e Paraná (Vale & Furtado, 2005; Brasil, 2017).

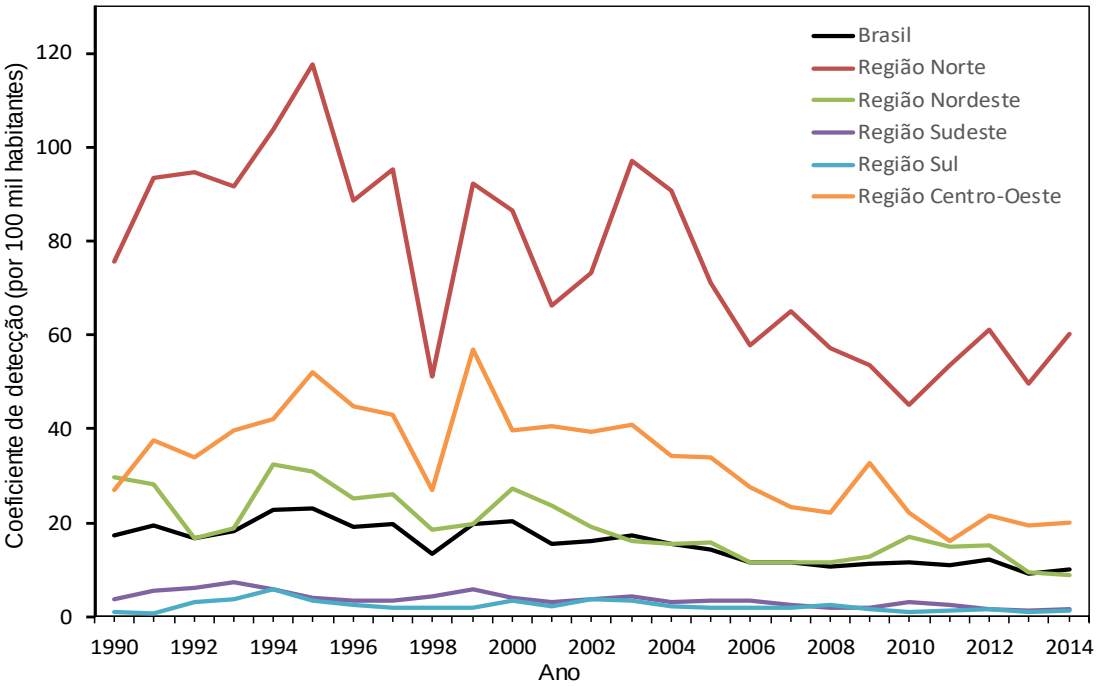

Figura 3: Taxa de incidência da leishmaniose tegumentar por região no Brasil no período de 1990 a 2014. Os dados são apresentaddos como coeficiente de detecção dos casos de leishmaniose tegumentar por 100 mil habitantes, cerca de 29 anos após a regulamentação da notificação compulsória da doença (Decreto nº 49.974-A de 21 de janeiro de 1961), (fonte: Rede Interagencial de Informações para a Saúde).

Tabela 1: Número de casos notificados e confirmados de leishmaniose tegumentar por forma clínica registados no Sistema de Informação de Agravos de Notificação -

	PERÍODO					
	2001 a 2006			**2007 a 2017**		
REGIÃO	**CUTÂNEA**	**MUCOSA**	**TOTAL**	**CUTÂNEA**	**MUCOSA**	**TOTAL**
Norte	63.385	4.813	68.198	94.723	5.591	100.314
Nordeste	52.266	1.969	54.235	69.820	2.381	72.201
Sudeste	16.049	1.852	17.901	18.912	2.499	21.411
Sul	3.919	573	4.492	4.115	695	4.810
Centro-oeste	26.819	2.232	29.051	32.268	2998	35.266
TOTAL	**162.438**	**11.439**	**173.877**	**219.838**	**14.164**	**234.002**

SINAN*

(*) http://portalsinan.saude.gov.br/ (acessado em 22/03/2018)

A LTA é endêmica em várias regiões das Américas Central e do Sul. Existem 12 espécies responsáveis pela doença nas Américas, entretanto, três merecem maior atenção: *Leishmania* (*Viannia*) *brasiliensis*, *Leishmania (Viannia) guyanensis e Leishmania* (*Leishmania*) amazonensis (Gontijo & Carvalho, 2003; Silveira et al., 2004).

L. (V.) braziliensis - mais amplamente distribuída, ocorrendo no Amazonas, Pará, Ceará, Amapá, Paraíba, Bahia, Espírito Santo, Rio de Janeiro, São Paulo, Paraná, Minas Gerais, Goiás, Mato Grosso e Brasília (Rangel & Lainson, 2003). Em estados da Região Norte, como Amazonas e Pará, a dispersão da infecção vem sendo atribuída a *Lutzomyia wellcomei* durante a estação das chuvas (Chagas et al., 2016). O vetor não apresenta hábitos domiciliares, portanto, o ser humano é infectado quando entra na mata. Os hospedeiros naturais mais prováveis são roedores silvestres como *Bolomys lasiurus* e *Nectomys squamipes* (Brasil, 2017).

Nos outros estados, o ser humano tem adquirido a doença após a devastação das florestas, deslocando os vetores para regiões peridomiciliares. No Ceará, a principal forma de transmissão é a periurbana, onde *Psychodopygus wellcomei* é o principal vetor e está relacionado com áreas de florestas (Ready et al., 1983). No estado de Minas Gerais e em áreas do interior da Bahia (Três Braços), o flebotomíneo incriminado é *Lutzomyia whitmani*, encontrado em áreas peridomiciliares, em plantações de bananeiras e florestas secundárias (Cuba et al., 1984; Carvalho et al., 2010). As lesões são, geralmente, únicas e em áreas expostas durante o trabalho rural, como braços, pernas e faces (Brasil, 2017).

Lutzomyia intermedia é o vetor encontrado com maior frequência nas áreas domiciliares, ocorrendo raramente em ambiente florestal (Silva & Gomes, 2001). Nestas áreas, a endemia perde a relação com atividades ocupacionais, atingindo todas as faixas etárias e ambos os sexos, tendendo a casos familiares, principalmente em residências próximas às encostas de morros. Apesar do encontro frequente de algumas espécies de animais domésticos como cão, equinos, mulas e roedores domésticos infectados, não foi possível demonstrar que animais silvestres atuam como reservatórios de *L. (V.) braziliensis*, o que levanta a hipótese de que possa haver outros ciclos onde o ser humano e animais domésticos participem (Cunha et al., 2006).

L. (V.) guyanensis - ocorre em florestas de terra firme, principalmente ao Norte da Bacia Amazônica (Amapá, Amazônia, Roraima e Pará) e algumas regiões das Guianas. O ciclo epidemiológico desta espécie envolve vários mamíferos arborícolas como hospedeiros naturais, tais como a preguiça de dois dedos (*Choloepus didactylus*) e o tamanduá (*Tamandua tetradactyla*), (Laison & Shaw, 1987). *Lutzomyia umbratilis* é o principal vetor, porém *Lu. anduzei* tem sido encontrado também parasitado por *L.* (*V.*) *guyanensis*. De modo geral, estes insetos não são inclinados a picar o homem, apenas quando são perturbados por atividades associadas ao desmatamento, penetração em áreas de florestas virgens e atividades militares, acometendo principalmente jovens e adultos do sexo masculino. Em algumas áreas periurbanas como as de Manaus no Estado do Amazonas, que sofreram desflorestamentos, estabeleceram-se conjuntos habitacionais muito próximos à mata, resultando em aproximação de alguns animais silvestres, como o gambá *Didelphis marsupialis*, atraídos pelo lixo urbano trazendo para estas áreas o risco de infecção (Arias et al., 1981).

Leishmania (L.) amazonensis - ocorre em florestas da Amazônia (Amazonas, Pará, Rondônia, Tocantins e sudoeste do Maranhão) e também em alguns locais da Bahia, Minas Gerais, São Paulo e Goiás (Lainson, 1997; Basano & Camargo 2004). Seus hospedeiros principais são os marsupiais e roedores como *Proechymis* e *Oryzomys capito*. Devido à atividade noturna de seus vetores (*Lu*. *flaviscutelana* e *Lu. olmeca nociva*) e a pouca atratividade destes flebotomíneos pelo ser humano, a doença em humanos é relativamente rara, restrita a caçadores e pescadores que penetram na floresta à noite (Basano & Camargo 2004). As lesões são geralmente ulcerosas e únicas, porém, alguns indivíduos podem desenvolver leishmaniose cutânea difusa (LCD), apresentando infiltrações, pápulas e tubérculos envolvendo várias áreas do corpo (Gontijo & Carvalho, 2003).

Existem outras espécies de *Leishmania* que foram descritas recentemente: *L*. (*V*.) *lainsoni, L. (V.) naiffi*, *L. (V.) lindenberg* e *L. (V.) shawi*. A primeira é responsável por alguns casos na região de Tucuruí (Pará), onde foram isolados parasitos em alguns animais da espécie *Cunicuens paca* e o vetor suspeito era *Lu. ubiquitalis*. *Leishmania (Viannia) naiffi* também tem sido isolada de edentados (*Dasypus novemcictus*) no Pará. Os prováveis vetores são *Lu. Ayrosai* e *Lu. paraensis*. *Leishmania (Viannia) shawi* tem casos descritos no Acre e Pará e seu vetor é *Lu.*

whitmani. Esta espécie tem sido encontrada em macacos, preguiças e alguns procionídeos (Silveira et al., 1987; Silveira et al., 1991).

1.1.5. Modelo murino de infecção experimental em leishmaniose

1.1.5.1. Macrófagos murinos como modelo celular de infecção por Leishmania sp.

Macrófagos são células do sistema reticuloendotelial ou sistema mononuclear-fagocitário, originadas a partir de monócitos do sangue que migram para o tecido conjuntivo e se diferenciam (Appelberg, 2012). Apresentam forma variável, geralmente oval, com contornos irregulares, núcleo descentrado e citoplasma abundante. Podem apresentar pseudópodos resultantes da emissão de sistemas de microtúbulos, o que lhes confere um movimento ameboide e podem direcionar o sua locomoção (Jessen & Moe, 1972). Além da fagocitose de células apoptóticas e patógenos, desempenham várias outras funções, dentre as quais a apresentação de antígeno aos linfócitos T sendo, por isso também designadas como células apresentadoras de antígeno (Schindl et al., 2006). Como células fagocíticas, os macrófagos contêm quantidades de lisossomos em seu citoplasma, onde se acumulam numerosas enzimas hidrolíticas tais como lipases, nucleases e glicosilases que apresentam atividade máxima de degradação de partículas em pH ácido (Appelberg, 2012).

Os macrófagos são as principais células infectadas por *Leishmania*, sendo indispensáveis para sua sobrevivência e persistência da infecção. A interação dos parasitos com as células hospedeiras ocorre através de proteinas de superfície como os receptores de complemento (CR1 e CR3 - *Complement receptors types* 1, 3), receptor de fibronectina e receptor de manose (Alexander et al., 1999). Os glicoconjugados de superfície do parasito como lipofosfoglicano (*lipophosphoglycan* - LPG), glicoproteína de 63kDa (gp63) e proteofosfoglicano (*proteophosphoglycan* - PPG) são cruciais para endocitose inicial de promastigotas e posterior sobrevivência dentro da célula hospedeira. No caso de amastigotas, sua cobertura com Imunoglobulina G (IgG) do hospedeiro favorece a ligação a receptores da região Fc, da IgG, presentes em macrófagos, facilitando sua entrada (Liu & Uzonna, 2012).

A infecção de macrófagos murinos residentes por *Leishmania* sp tem sido amplamente utilizada como modelo para ensaios com novos fármacos. No entanto, a persistência da infecção *in vitro* depende de fatores como infectividade e a

virulência da cepa do parasito, caracterizada como a capacidade do parasito em subverter os mecanismos microbicidas do macrófago, incluindo a geração de radicais livres de oxigênio e produção de óxido nítrico (Chan et al., 1989; Proudfoot et al., 1995; Proudfoot et al., 1996; Dejardins & Descoteaux, 1997). Promastigotas de *L. (L.) infantum* exibiram infectividade limitada em macrófagos murinos *in vitro* em relação aos promastigotas de *L. (L.) donovani* que mostraram infecções notáveis. Além disso, as taxas de multiplicação de amastigotas nas infecções por *L. (L.) infantum* não foram significativas quando comparadas às de amastigotas de *L. (L.) donovani* (Mèndez et al., 1996).

De forma geral, os modelos *in vitro* devem garantir não apenas a internalização de promastigotas, mas também a transformação, sobrevivência e multiplicação dos parasitos na forma amastigota (Berman et al., 1979; Pearson et al., 1981). Estudos recentes mostraram que clones de *L.(V.) braziliensis* (MCAN/BR/1998/R619) apresentam padrões distintos de infecção em macrófagos murinos que podem variar desde alta carga parasitária a baixas taxas de multiplicação em 24 horas até a resolução completa da infecção em 72 horas de infecção (Cysne-Finkelstein et al., 2018). Outras cepas do subgênero *Viannia* também demonstraram capacidade de infectar macrófagos, mas sem conseguir se replicar no interior da célula hospedeira (Matta et al., 2010).

O modelo de infecção deste estudo utilizou *L. (L.) amazonensis* (MHOM/BR/73/LTB0016), uma cepa considerada muito virulenta com altas taxas de internalização e multiplicação dos parasitos (média de cerca de 30 parasitos por macrófago) já em 24 horas de infecção (Figura 4D). Outra característica de espécies do subgênero *Leishmania*, tais como *L. (L.) major* e *L. (L.) mexicana*, é que a infecção produz vacúolos parasitóforos grandes contendo vários amastigotas, ao contrário daqueles geralmente observados em infecções por espécies do subgênero *Viannia* (Chang et al., 2003; Castro et al., 2006; Menezes et al., 2013).

1.1.5.2. Camundongos como modelo de infecção experimental por Leishmania sp.

Modelos de infecção experimental utilizando animais de laboratório são essenciais para a compreensão de aspectos imunológicos e perfis inflamatórios da infecção por *Leishmania* sp, além de permitir avaliar os efeitos de novos fármacos, já que análises envolvendo culturas celulares ou amostras de tecido podem levar a resultados equivocados ou controversos. A escolha de um modelo animal deve

considerar pelo menos os três aspectos: (i) fisiologia razoavelmente compatível com a humana, (ii) possibilidade da análise de diversos aspectos da infecção e (iii) manutenção relativamente simples (Pereira & Alves, 2008).

O modelo de infecção por *Leishmania* em murinos tem sido amplamente utilizado em estudos para melhor compreender o ciclo de vida, o processo infeccioso, a interação parasito-hospedeiro, e testar compostos que tenham apresentado atividade leishmanicida significativa em modelos de infecção usando cultura de macrófagos.

Apesar do estado geral de saúde e condição fisiológica do hospedeiro influenciar a progressão da doença, a predisposição genética exerce maior papel no quadro patológico geral. De fato, diversos estudos com camundongos demonstraram que dentre linhagens existentes, duas destas (BALB/c e C57BL/6) representam indivíduos susceptíveis e não susceptíveis (Titus et al., 1985). Linhagens BALB/c desenvolvem infecção progressiva com lesões que não cicatrizam, metástases, necrose do membro e morte, em algumas semanas. Contrariamente, linhagens C57BL/6 apresentam lesões que podem curar espontaneamente (Mendes Wanderley et al., 2012).

O modelo de infecção experimental adotado nesta tese utilizou a linhagem BALB/c infectada por *L. (L.) amazonensis* no coxim plantar (Figura 4A). Os camundongos BALB/c são altamente susceptíveis ao parasito e desenvolvem lesões crônicas nas patas cujos volumes podem ser facilmente mensurados (Figura 4B). Além das características genéticas do animal, a evolução da infecção pode ser influenciada por fatores como o tamanho do inóculo, o local de inoculação e a cepa do parasito.

A importância do inóculo inicial pode ser entendida num modelo proposto para simular o parasitimo por um longo período de duração. Neste tipo de modelo, os promastigotas metacíclicos foram inoculados na derme da orelha de camundongos BALB/c e a análise dos resultados demonstrou que o estabelecimento das lesões cutâneas foi diretamente proporcional ao inóculo inicial aplicado. Nos camundongos infectados com apenas 10 parasitos, as lesões foram raras, enquanto a maioria dos que foram infectados com 100 parasitos apresentou lesões. Todos os camundongos infectados com 1.000 parasitos desenvolveram lesões progressivas (Courret, et al., 2003).

De maneira geral, a linhagem BALB/c quando infectada por *L. (L.) amazonensis* pode desenvolver metástases nas patas, orelha, cauda, nariz e mucosa oral,

podendo ser considerada bom modelo também para a leishmaniose mucocutânea. As lesões metastáticas nestes animais causam destruição da região nasal com muitos macrófagos intensamente parasitados por amastigotas sob a superfície epitelial, com destruição óssea e extensa reação inflamatória (Cupolilo et al., 2003).

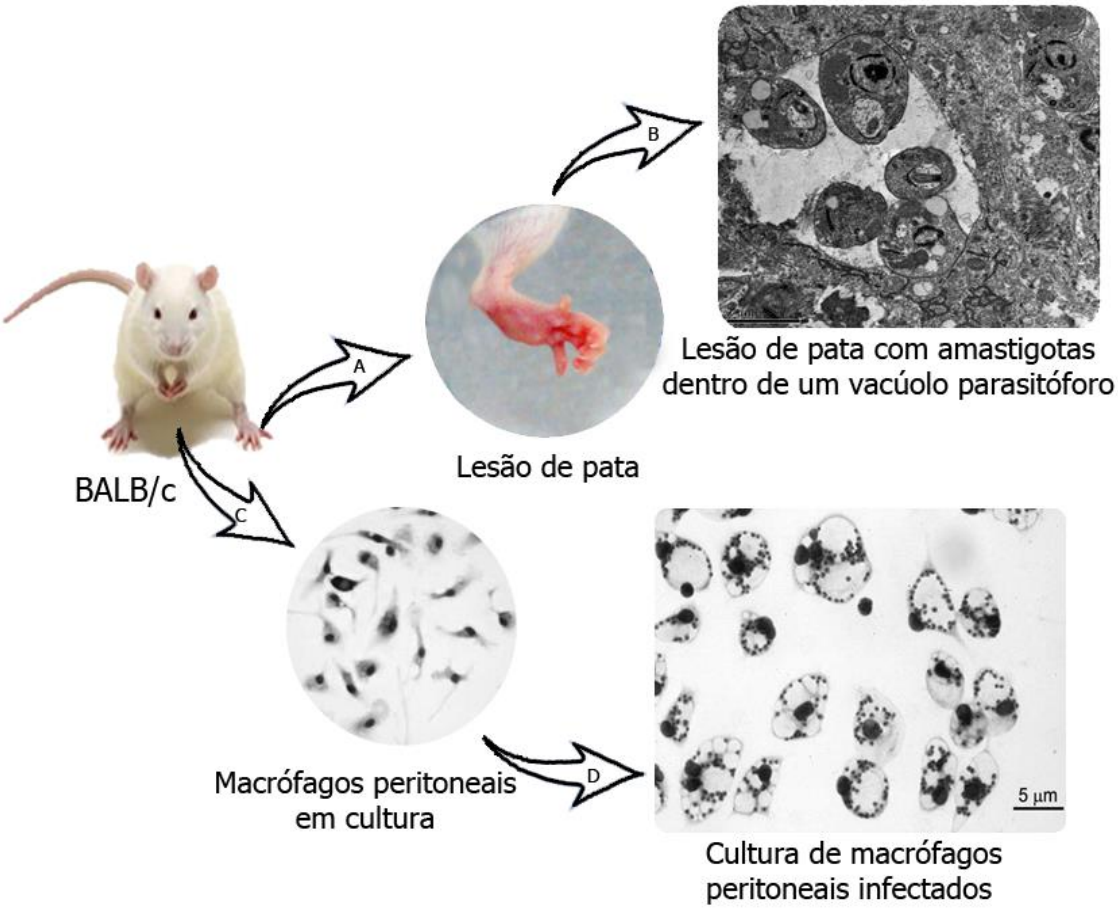

Figura 4: Representação do modelo experimental murino. Camundongos BALB/c são linhagens isogênicas susceptíveis à infecção por *Leishmania (L.) amazonensis*. Os parasitos quando inoculados no coxim plantar dos animais causam lesões progressivas que se tornam visíveis pelo aumento do volume das patas (A). Análise por microscopia eletrônica de transmissão mostrando vacúolos parasitóforos (VP) contendo múltiplos amastigotas (A), característica da infecção por esta espécie (B). Ensaios de infecções *in vitro* podem se feitos com macrófagos peritoneais aderidos a lamínulas (C), nos quais também se detecta multiplos amastigotas por vacúolos parasitóforos (D).

1.1.6. Quimioterapia das leishmanioses: Características farmacológicas e efeitos adversos

Apesar do longo período desde que os parasitos de *Leishmania* foram identificados e descritos, a leishmaniose continua afetando milhões de pessoas em regiões tropicais e subtropicais no mundo. Esta situação deve-se à inexistência de opções eficazes e seguras de tratamento, práticas de controle inadequado e pouco interesse das principais indústrias farmacêuticas para pesquisar novas alternativas de tratamento. Esse desinteresse caracteriza a leishmaniose como uma doença tropical negligenciada (WHO, 2017).

1.1.6.1 Medicamentos de primeira linha - Antimoniais pentavalentes

Os compostos antimoniais pentavalentes (Sb^{5+}) têm sido usados há mais de 60 anos e continuam sendo considerados pela Organização Mundial da Saúde os medicamentos de primeira linha para quimioterapia de todas as formas clínicas da leishmaniose. A longevidade deste regime de tratamento pode ser explicada pelo risco de toxicidade associado aos fármacos ditos de segunda linha anfotericina B e pentamidina que apresentam um índice terapêutico supostamente menor (Berman, 1988). Estes fármacos estão recomendados nos casos em que há contraindicação, intolerabilidade ou resistência aos antimoniais pentavalentes (Haldar et al., 2011). Na Tabela 2 estão apresentadas informações relevantes sobre regime terapêutico, efeitos adversos e mecanismo de ação (ANEXO 2).

Atualmente estão disponíveis duas formulações destes agentes: estibogluconato de sódio (Pentostam®- EGS) e antimoniato de meglumina (Glucantime® - AM). Enquanto o primeiro é usado principalmente em países de língua inglesa, o segundo é predominantemente comercializado em países de língua francesa, espanhola e portuguesa (Berman 1988). Tanto a eficácia quanto a toxicidade destes compostos estão relacionadas com o seu teor de Sb^{5+} e, apesar de suas diferenças estruturais, são considerados terapeuticamente similares, mesmo sabendo que a formulação do Pentostam contém quase 20% mais Sb^{5+} que o Glucantime (Berman, 1988; WHO, 2010).

Até o momento, o mecanismo de ação preciso dos antimoniais pentavalentes contra o parasito permanece desconhecido. No entanto, parece ser multifatorial, como observado para outros compostos à base de metais pesados, em vez de ser devido a uma rota específica. No nível molecular, acredita-se que o antimônio se

ligue aos grupos sulfidrila de certas proteínas, causando alterações em suas estruturas e, eventualmente, prejudicando a sua função (Frézard, 2009) (Tabela 2).

Há evidências sugerindo que a forma biologicamente ativa do metal pesado no composto é, de fato, o antimônio trivalente (Sb^{3+}). Assim, o antimonial pentavalente atuaria como pró-fármaco (Goodwin & Page, 1943). Outros autores têm apoiado essa hipótese, com base na detecção da biorredução de Sb^{5+} em Sb^{3+} após a administração intramuscular de antimoniato de meglumina em pacientes com leishmaniose (Miekeley et al., 2002). O agente redutor implicado na biotransformação foi a glutationa reduzida (GSH), um tiol presente em grandes quantidades no ambiente intracelular (Frèzard et al., 2001; Ferreira et al., 2003) (Tabela 2).

Na busca pelo entendimento de como os antimoniais exercem efeito leishmanicida, foi observado que estes compostos podem inibir certas fases do ciclo do metabolismo energético de amastigotas. Observações experimentais indicaram que exposição *in vitro* de *L. (L.) mexicana* ao EGS resultou em uma diminuição dose-dependente na viabilidade celular e da produção de CO_2 oriunda de glicose e palmitato nas culturas de parasitos. A partir desses dados, pode ser inferido que as enzimas glicolíticas e os componentes da via de oxidação de ácidos graxos são inibidos por EGS, mas não a via da hexose monofosfato e o ciclo do ácido cítrico, levando a um esgotamento dos níveis intracelulares de ATP (Berman et al., 1985) (Tabela 2).

Há evidências que o Sb^{3+} inibe a atividade da tripanotiona redutase (TR) em *L. (L.) donovani*, uma enzima essencial para a sobrevivência do parasito dentro de macrófagos (Wyllie et al., 2004). Além disso, foi constatado que o Sb^{3+} se liga, com alta afinidade, ao sítio ativo da TR de *L. (L.) infantum*, inibindo sua atividade enzimática. A inibição dessa enzima pelos antimoniais tem sido descrita como um passo crucial para a atividade leishmanicida desses compostos (Baioco et al., 2009) (Tabela 2).

Os mecanismos microbicidas adicionais dos antimoniais podem incluir: *(i)* indução de apoptose em amastigotas por Sb^{3+}, como observado pela fragmentação de DNA e exposição de fosfatidilserinas na superfície externa da membrana plasmática em parasitos expostos aos antimoniais (Sudhandiran & Shaha, 2003); *(ii)* inibição de topoisomerases (Lucumi et al., 1998); *(iii)* formação de complexos com

ribonucleosídeos (Demicheli et al., 2002); e *(iv)* interferência na translocação de purinas pré-formadas (Carter et al., 2000).

O uso de antimônio pentavalente é caracterizado por um espectro de efeitos adversos leves a graves. Tais efeitos incluem dores musculoesqueléticas, distúrbios gastrointestinais, cefaleia e anorexia, além de toxicidade cardíaca, hepática e pancreática, que pode em alguns casos levar à morte (Tabela 2).

1.1.6.2 Medicamentos de segunda linha

Anfotericina B - O desoxicolato de anfotericina B é um antibiótico poliênico (Figura 5A) obtido de *Streptomyces nodosus*, com atividade antifúngica bem conhecida e relatada como eficaz contra promastigotas e amastigotas de *Leishmania* tanto *in vitro* quanto *in vivo* (Yardley & Croft, 2000). Este medicamento foi aplicado para o tratamento de LV na Índia e no Brasil por muitos anos e provou ser um tratamento eficaz, porém de difícil administração. A anfotericina B também mostrou ser eficaz contra a forma mucosa da doença, na qual as recaídas são comuns (Sampaio & Mardsen, 1997; Herwaldt, 1999; Sundar, 2001; Wolday et al., 2001). Tentativas de reduzir os efeitos colaterais da anfotericina B levaram ao desenvolvimento de formulações lipídicas desse fármaco que a encapsulam em micelas. As partículas lipídicas são rapidamente removidas da circulação do paciente por fagócitos mononucleares que, em seguida, liberam grandes quantidades do composto dentro das células infectadas, aumentando assim seus efeitos antiparasitários.

Atualmente, três formulações lipídicas a base de anfotericina B estão disponíveis: anfotericina B lipossomal (AmBisome; Nexstar, EUA); anfotericina B complexo lipídico (Abelcet, ABLC; Liposome Co., EUA), e anfotericina B dispersão coloidal (Amphocil, Amphotec; Sequus, EUA) (Sievers et al., 1996; Sundar, 2001; Wolday et al., 2001). Estas formulações são similares ao desoxicolato de anfotericina B em sua eficácia, mas são significativamente menos tóxicas. A formulação lipossomal da anfotericina B é utilizada preferencialmente para o tratamento da LV, mas devido ao seu elevado custo, sua utilização nos países em desenvolvimento é restrita (Brynceton, 2001).

O mecanismo de ação da anfotericina B deve-se à sua reação com esterois que contêm uma substituição metílica no carbono 24 (episterol e ergosterol) na membrana celular do parasito, formando poros que alteram o equilíbrio iônico, a permeabilidade celular e, eventualmente, levam à morte celular (Bolard, 1986). No

entanto, este fármaco também pode se ligar a moléculas de colesterol presentes na membrana celular das células do hospedeiro, causando efeitos colaterais nos pacientes (Bolard, 1986; Brajtburg & Bolard, 1996). Este fármaco é altamente eficaz contra *Leishmania*; em ensaios com hamsters ou macacos infectados com *L. (L.) donovani*, foi demostrado que a anfotericina B foi mais potente do que os antimoniais pentavalentes em ensaios *in vitro* contra o parasito (Bolard, 1986; Murrey et al., 1993; Sundar, 2001).

Apesar de sua alta efetividade, a anfotericina B desoxicolato é usada apenas como medicamento de segunda escolha devido a seus efeitos adversos que podem ser graves e muito inconvenientes, incluindo a necessidade de administração parenteral, terapia longa e monitoramento clínico constante. Assim, é usualmente utilizado apenas nos casos em que o tratamento com antimonial pentavalente não produziu resposta adequada. Porém é considerado o fármaco de primeira escolha para o tratamento de gestantes e de casos mais graves (Sundar, 2001; Wolday et al., 2001).

Pentamidina - A pentamidina é uma diamina aromática (Figura 5B) utilizada no tratamento de pacientes que não respondem à terapia com antimoniais. Este medicamento também é aplicado no tratamento de casos incipientes de tripanossomíase rodesiense ou gambiense (Doua et al., 1996). Foi introduzida pela primeira vez como agente leishmanicida em 1952 e tem sido usado no tratamento de várias formas clínicas de leishmaniose. No entanto, sua alta toxicidade aliada a uma suposta baixa efetividade no Velho Mundo em comparação às outras opções de tratamento, levou a suspensão da sua utilização em vários países (Jha et al., 1991; Sundar, 2001; Ouellette et al., 2004).

O mecanismo de ação da pentamidina parece estar relacionado à sua capacidade de ligar DNA cinetoplasto nos parasitos e afetar assim sua sobrevivência. No entanto, esta hipótese exige investigação adicional e outros efeitos potenciais da pentamidina sobre os parasitos devem ser abordados (Brynceton, 2001; Ouellette et al., 2004).

O isotionato de pentamidina é preferencialmente administrado por via intravenosa. infusão ou, alternativamente, intramuscular, como é prontamente absorvido e sai da circulação rapidamente. A dose recomendada é de 7 mg / kg (correspondendo a 4 mg de pentamidina base) em intervalos de 48 horas. Alternativamente, uma dose de

2 mg / kg de pentamidina base pode ser administrada num total de sete injeções. A dose total de pentamidina base no tratamento não deve exceder 2 g (Monzote, 2009; WHO, 2010; Brasil, 2017).

Os efeitos adversos mais comuns relacionados ao uso de isotionato de pentamidina são dor e abscessos estéreis no local de injeção, náuseas, vômitos, tonturas, mal-estar, mialgia, artralgia, cefaleia, hipotensão, síncope, citólise de células beta do pâncreas, hipoglicemia e hiperglicemia (Revisado por Oliveira et al., 2011). Em casos extremos, pode ocorrer cardiotoxicidade, levando a arritmia fatal. Outro efeito tóxico importante da pentamidina é o desenvolvimento de diabetes insulino-dependente em pacientes tratados; este efeito tem uma taxa de incidência de 12,5% nos casos onde a dose total do tratamento se aproxima de 2g (Sundar, 2001; Brasil, 2017).

Miltefosine - O miltefosine, um hexadecilfosfocolina (Figura 5C), foi originalmente desenvolvido como agente antineoplásico oral (para o tratamento de câncer de pele). Após uma série de estudos clínicos entre 1997 e 2000, foi aprovado sob o nome comercial Impavido®, tornando-se, em alguns países, o primeiro tratamento oral para leishmaniose (Croft & Engel, 2006). Seu mecanismo de ação contra os parasitos de *Leishmania* parece ser através da modulação de receptores de superfície celular que afetam muitos processos celulares relevantes, incluindo a homeostase do íon cálcio, mecanismos de remodelação éter-lipídica, síntese de fosfatidilcolina, transdução de sinal, metabolismo do inositol, ativação de fosfolipase e proteína quinase C, bem como outras vias mitogênicas e apoptóticas (Verma & Dey, 2004). Miltefosine também aumenta a citotoxicidade em macrófagos causando estresse oxidativo e estimulando o consumo de glicose celular com a produção de espécies reativas de oxigênio, tais como H_2O_2 e superóxido O_2, levando eventualmente à morte dos parasitos dentro dessas células (Croft et al., 2003).

O miltefosine tem sido utilizado em doses de 2-2,5 mg/kg/dia ou 50 mg duas vezes por dia durante 28 dias. Vale ressaltar que a eficácia deste medicamento no tratamento de LC no Novo Mundo é considerada suficiente, mas limitada para LV (Minodier & Parola, 2007; Monzote, 2009; WHO, 2010). (Tabela 2).

Os efeitos adversos mais comuns observados com o miltefosine estão relacionados ao trato gastrointestinal e incluem diarreia e vômito. Estes efeitos ocorrem em mais de 30% dos pacientes tratados e seu uso é contraindicado durante

gravidez devido aos seus efeitos teratogênicos. Graves sintomas podem ocorrer quando doses tão altas quanto 200 mg/dia são usados (Fisher et al., 2001; WHO, 2010).

Paromomicina - A paromomicina, também conhecida como aminosidina, é o único aminoglicosídeo (Figura 5D) com atividade leishmanicida clinicamente importante; ambas as formas visceral e cutânea podem ser tratadas com este antibiótico. Devido à sua má absorção oral, uma formulação parenteral para o tratamento da LV e uma formulação tópica para o tratamento da LC foram desenvolvidas (Ben Salah et al., 1995; Croft et al., 2002; Sundar et al., 2007). A paromomicina foi testada contra LV na dose de 15-20 mg/kg de sulfato de paromomicina por 21 dias (WHO, 2010). Para tratar a LC, três formulações tópicas foram utilizadas: 15% de paromomicina com 12% de cloreto de metilbenzetônio; 15% de paromomicina com 10% de ureia; e 15% de paromomicina com gentamicina a 0,5%. Todas essas formulações são administradas duas vezes ao dia por até 20 dias. Estas formulações mostraram resultados variados dependendo da espécie de *Leishmania* envolvida. Uma vantagem adicional da paromomicina foi observada quando da sua combinação com antimoniais; neste caso, a paromomicina ajudou a reduzir a duração da terapia de 30 dias para 17 a 21 dias (Monzote, 2009).

O mecanismo de ação exato da paromomicina requer mais elucidação, mas foi reportado que ela inibe a síntese protéica em protozoários, ligando-se à subunidade ribossômica 30S, causando um acúmulo de complexos anormais de iniciação (Sundar & Chakravarty, 2008). Semelhante a outros aminoglicosídeos, a paromomicina tem vários efeitos adversos, incluindo ototoxicidade, nefrotoxicidade, lesão do oitavo nervo craniano e anormalidades da função hepática (Sundar et al., 2007) (Tabela 2).

Tabela 2*: Medicamentos atualmente utilizados no tratamento da leishmaniose, seus efeitos adversos mais frequentes e mecanismo de ação.

FÁRMACOS	POSOLOGIA	EFEITOS ADVERSOS		MECANISMO DE AÇÃO
		QUEIXAS CLÍNICAS	ALTERAÇÕES LABORATORIAS/TOXICIDADE	
PRIMEIRA LINHA				
Antimoniais pentavalentes	LC: 10–20 mg Sb^{5+}/kg/dia, por no mínimo 20 dias or até a cicatrização das lesões. LV ou LM: 20 mg Sb^{5+}/ kg/dia por 20–30 dias (Berman, 1988; Brasil, 2017; WHO 2010).	Mialgia, artralgia, cefaleia, náuseas, vômitos, dor abdominal, e edema no local de aplicação (Herwaldt and Berman, 1992; Oliveira et al., 2011).	Distúrbios da repolarização ventricular (tais como alterações da onda T e do segmento ST); arritmia; aumento transitório leve a moderado das transaminases e/ou amilases (Herwaldt and Berman 1992; Oliveira et al., 2011).	Possível inibição da glicose e/ou oxidação de ácidos graxos; efluxo ativo de tióis como glutationa e tripanotiona e/ou inibição da tripanotiona redutase, causando estresse oxidativo no parasito (Berman et al., 1985; Cunningham and Fairlamb, 1995; Ameen, 2007).
SEGUNDA LINHA				
Anfotericina B (deoxicolato)	LC: 0,5–1 mg/kg/dia (dose total de 1–1,5 g); LV ou LM: 1 mg/kg/dia (dose total de 2.5–3 g) por 20 dias em dias subsequentes ou alternados (Monzote, 2009).	Hiperpirexia, flebite, cefaleia, calafrios, astenia, mialgia e artralgia, vômitos e hipotensão (Sundar, 2001).	Nefrotoxicidade (diminuição da filtração glomerular), hipocalemia, distúrbios hepáticos e depressão da medula óssea (Sundar, 2001; Brasil, 2017).	Formação de estruturas semelhantes a canais (poros) abrangendo toda a bicamada lipídica pela interação com os esterois da membrana causando alteração na permeabilidade aos cátions, água e glicose, afetando também as enzimas da membrana (Bolard, 1986; Neumann et al., 2009).
Pentamidina (isotionato)	4 – 7 mg/kg de sal de isethionato em dias alternados (dose total de 2g) (Brasil, 2017; WHO, 2010).	Dores musculoesqueléticas, anorexia, dor abdominal, náuseas, cefaleia, dor leve a moderada no local de aplicação (Oliveira et al., 2011).	Rabdomiólise, hipotensão, hiperglicemia ou hipoglicemia e aritmia, diabetes insulino dependente** (Sundar, 2001; Delobel e Pradinaud, 2003).	Ligação ao cinetoplasto do parasito causando profundas alterações ultraestruturais (Soeiro et al., 2013).
Miltefosine	Crianças entre 2–11 anos: 2,5 mg/kg/dia; pacientes com 12 anos ou mais: 50 mg/dia (peso \ 25 kg); 100 mg/dia (peso / 25–50 kg); 150 mg/dia (peso maiores / 50 mg) (WHO, 2010).	Alterações gastrointestinais (principalmente diarréia e vômito) (Croft and Engel, 2006; Dorlo et al., 2012).	Anormalidades transitórias afetando as funções renal e hepática; teratogenicidade (Croft e Engel, 2006; Dorlo et al., 2012).	Inibição da via de sobrevivência PI3 K-Akt / PKB de maneira dose-dependente, levando à apoptose (Ruiter et al., 2003)
Paramomicina (aminosidina)	LV: 15 – 20 mg/kg de sulfato de paramomicina (11–15 mg da base) por 21 dias; LC: pomada duas vezes ao dia por 20 dias (Monzote, 2009; WHO, 2010).	Erupções cutâneas, prurido local e ardência*** (Sundar, 2001).	Oto- e/ou nefrotoxicidade (Sundar et al., 2007).	Inibição da síntese de proteínas pela ligação à subunidade 30 S ribossômica do complexo ribossômico 30 – 50 S no início do códon de mRNA, causando acumulação de um complexo de iniciação inativo (Sundar e Chakravarty, 2008).

*Tabela adapatada do anexo 2;
**Efeito tóxico relacionado ao tratamento prolongado e / ou uso de altas doses,
*** efeitos adversos relacionados a formulações tópicas.

1.1.6.3 Derivados azólicos

Os muitos compostos azólicos têm sido amplamente utilizados como agentes antifúngicos orais, pois são bem tolerados pelos pacientes e eficientes no tratamento dessas infecções (Lestner & Hope, 2013; Rani et al., 2013). Eles têm sido sugeridos para o tratamento clínico da leishmaniose, pois esses fármacos demonstraram atividade leishmanicida *in vitro* e *in vivo*, inibindo a biossíntese do ergosterol nos parasitos e afetando sua membrana celular.

O fluconazol demonstrou resultados promissores no tratamento da leishmaniose cutânea ou visceral causada por parasitos de ambos os subgêneros (Jha, 1998; Alrajhi et al., 2002; Sousa et al., 2011), embora sua eficiência, aplicabilidade ou dosagem necessária ainda estejam em debate (Zulunov et al., 2002; Morizot et al., 2007; Emad et al., 2011; Torres & Suaréz, 2012).

O itraconazol apresenta dados contraditórios na literatura: há relatos de casos clínicos em que pacientes foram tratados com sucesso para leishmaniose cutânea com este azol (Consigli et al., 2006; Baroni 2009) mas, em um estudo clínico maior, observou-se que as taxas de cura dos pacientes com leishmaniose cutânea foram semelhantes às do grupo placebo (Nassiri-Kashani et al., 2005).

O cetoconazol também foi usado em estudos com um pequeno número de pacientes infectados com espécies do Velho e Novo Mundo e apresentou uma taxa de cura aceitável (Weinrauch et al.,1983; Weinrauch et al., 1987; Saenz et al, 1990). No entanto, esses resultados ainda exigem confirmação por estudos maiores.

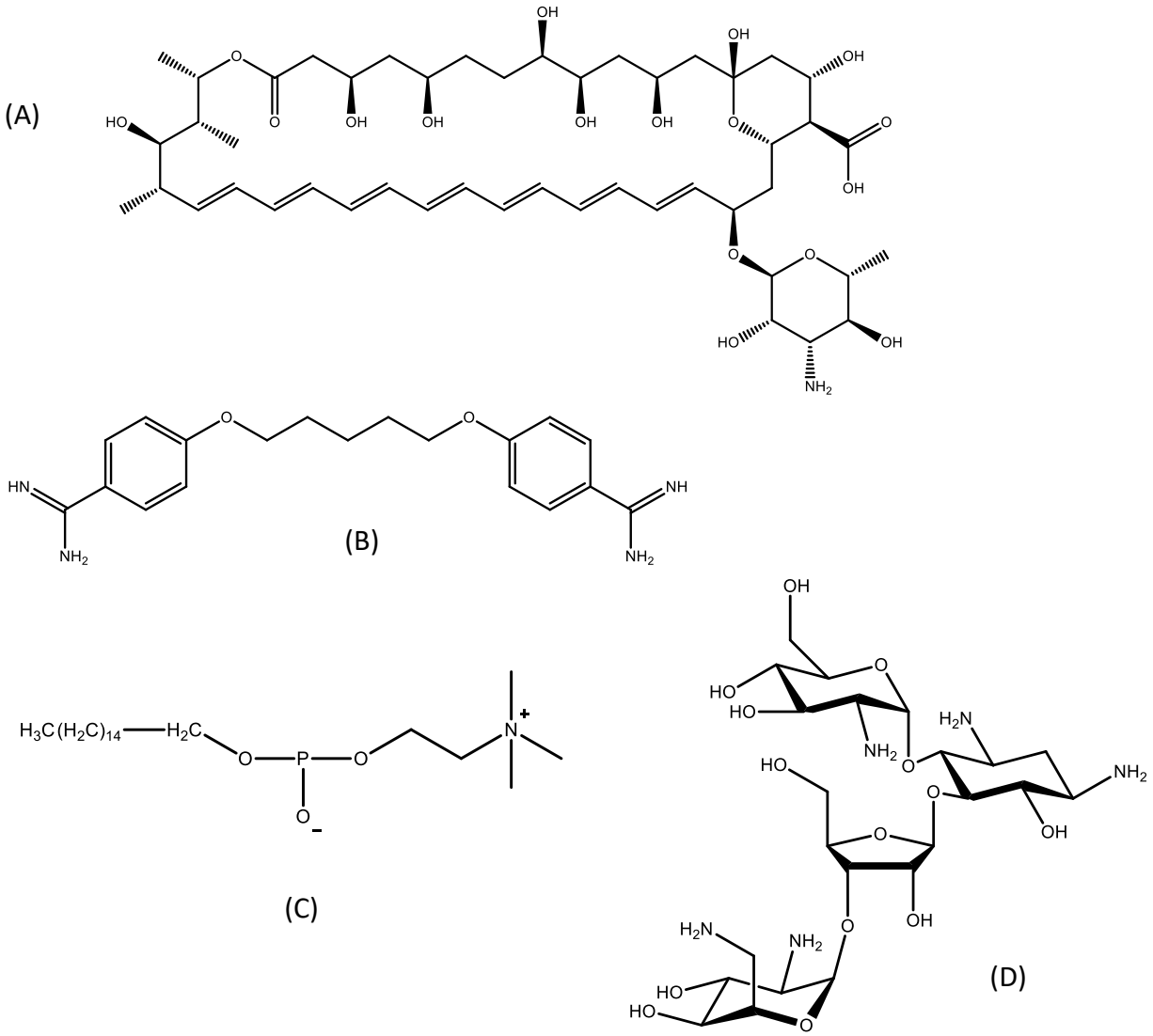

Figura 5: Estrutura química dos fármacos de segunda escolha usados para o tratamento da leishmaniose. (A) Anfotericina B, (B) isotionato de pentamidina, (C) Miltefosine, (D) Paramomicina.

1.1.7. Desenvolvimento de novos fármacos para leishmanioses

Nas últimas décadas, a pesquisa por fármacos leishmanicidas tem utilizado diferentes abordagens e novas ferramentas, incluindo: (a) conhecimentos em biologia, metabolismo e genoma do parasito, (b) aplicação de ferramentas de bioinformática, e (c) estabelecimento cada vez maior de redes de colaboração para apoiar o desenvolvimento de novos fármacos (Monzote, 2009).

A química medicinal é uma ciência diretamente aplicada ao desenvolvimento de novos fármacos que recebeu grandes avanços das áreas de biologia molecular e estrutural, e de química computacional. A geração de modificações estruturais numa molécula precursora sabidamente ativa para se obter novos derivados tem sido uma abordagem bem-sucedida para o desenho de novos fármacos (Liñares et al., 2006).

1.1.7.1. Produtos naturais e seus derivados como alternativas para o tratamento da leishmaniose

Muitos grupos de pesquisa têm procurado por novos fármacos antiparasitários de origem vegetal, principalmente metabólitos secundários. Produtos naturais continuam a desempenhar um papel importante na terapêutica clínica. De fato, entre 1981 e 2010, 1355 novos fármacos foram aprovados pelo FDA, dos quais 4,7% eram produtos naturais ou seus derivados e 22% continham grupos farmacofóricos derivados de produtos naturais (Newman & Cragg, 2012). Nesse contexto, uma variedade de produtos naturais obtidos a partir de extratos de plantas tem se mostrado ativa contra protozoários de *Leishmania*. Entre estas, as quinonas são moléculas consideradas interessantes em química medicinal devido ao seu espectro de atividade biológica e propriedades químicas (Monks et al., 1992).

Quinonas são substâncias orgânicas oriundas de metabólitos aromáticos naturais encontrados em várias famílias de plantas, bem como em fungos, algas e bactérias. Neste grupo de substâncias estão incluídas as benzoquinonas, antraquinonas e naftoquinonas (López López et al., 2014). Uma importante propriedade química das quinonas é a sua capacidade de agir como agente oxidante ou de desidrogenação. Esta propriedade redox é conduzida pela formação de um sistema totalmente aromático (Hillard et al., 2008; de Paiva et al., 2015). Recentemente, o interesse pelas quinonas e seus derivados aumentou não só devido ao seu papel nos processos bioquímicos, mas também pela sua atividade contra uma ampla variedade de agentes infecciosos (Babula et al., 2009). Na natureza, compostos

como as ubiquinonas (coenzima Q) e menaquinonas (vitamina K) estão envolvidos em importantes etapas do ciclo de vida, atuando principalmente como componentes da cadeia transportadora de elétrons, na coagulação sanguínea e na fotossíntese (Monks & Jones, 2002).

Dentre as quinonas, as 1,4-naftoquinonas são compostos cujo interesse em química medicinal é alto devido a sua capacidade de controlar a infecção e a multiplicação de tripanossomatídeos de interesse médico (Pinto & de Castro, 2009). Exemplos de 1,4-naftoquinonas que mostraram atividade contra espécies de *Leishmania* e *Trypanosoma cruzi* são o lapachol, isolado de árvores brasileiras pertencentes ao gênero *Tabebuia*, e seus derivados α-lapachona e β-lapachona.

O lapachol é conhecido desde 1858 e tem sido identificado desde então como constituinte de várias plantas das famílias Bignoniaceae, Verbenaceae e Proteaceae, sendo sua ocorrência maior na família Bignoniácea, particularmente no gênero *Tabebuia*, popularmente conhecida como ipê (Silva et al., 2003).

O principal interesse no lapachol está relacionado com sua capacidade de induzir estresse oxidativo através da formação intracelular de espécies reativas do oxigênio, como o peróxido de hidrogênio (H_2O_2), o ânion-radical superóxido ($O_2^{\bullet -}$) e o radical hidroxila ($HO^{\bullet}$). Estas espécies podem danificar alguns componentes celulares importantes, tanto de células normais, como de células neoplásicas. Isso altera o balanço natural de sinais que interferem na divisão celular em pontos específicos da evolução morfogênica natural, e pode induzir a apoptose, caso o estresse oxidativo não seja completamente eliminado (Amarantes-Mendes & Green, 1999).

Por essa razão, o lapachol chegou a ser avaliado clinicamente no tratamento do carcinoma de Walker-256 (Subramanian et al., 1998) e do sarcoma de Yoshida (Rao et al., 1968) e, apesar de ter promovido a regressão definitiva das neoplasias em aproximadamente 30% dos pacientes, os resultados dos ensaios clínicos desaprovaram seu uso devido à ocorrência de efeitos colaterais que agravaram o quadro clínico dos pacientes, tais como anemia, aumento do tempo de coagulação e problemas gastrintestinais.

Adicionalmente, o lapachol e seus derivados mostraram amplo espectro de atividade farmacológica, como atividade antimicrobiana e antifúngica (Gafner et al., 1996); atividade cercaricida (prevenção da penetração de cercárias *Schistosoma mansoni* na pele) (Austin, 1974); ação moluscicida (atividade contra caramujos Biomphalaria glabrata, hospedeiro intermediário do Schistosoma mansoni (dos

Santos et al., 2000); leishmanicida [ação intracelular nas formas amastigotas de *Leishmania (Viannia) braziliensis*] (Teixeira et al., 2001; Kayser et al., 2000); tripanossomicida (Goulart et a., 1997); antimalárico (Carvalho et al., 1988); uso contra enteroviroses (Pinto et al. 1987); anti-inflamatória (de Almeida et al., 1990), entre outras.

No entanto, a toxicidade associada ao lapachol e seus derivados limitou seu potencial para uso no tratamento da leishmaniose e de outras doenças (Lima et al., 2004). Na busca por derivados menos tóxicos para as células de mamíferos, a modificação química do centro quinonoide da α-lapachona e da 2-hidroxi-1,4-naftoquinona (lausona), seguida de uma epoxidação, gerou os oxiranos epoxi-α-lapachona e epoximetil-lausona, respectivamente (Carneiro et al., 2012). Foi demonstrado que o epoxi-α-lapachona é capaz de matar formas promastigotas de *L. (V.) braziliensis* e *L. (L.) amazonensis* e amastigotas intracelulares em macrófagos humanos (Silva et al., 2014).

Além disso, uma redução significativa no tamanho da lesão da pata de camundongos BALB/c infectados por *L. (L.) amazonensis* foi observada após quatro semanas de tratamento e afetou negativamente a atividade de serino-protease de promastigotas e amastigotas (ANEXO1).

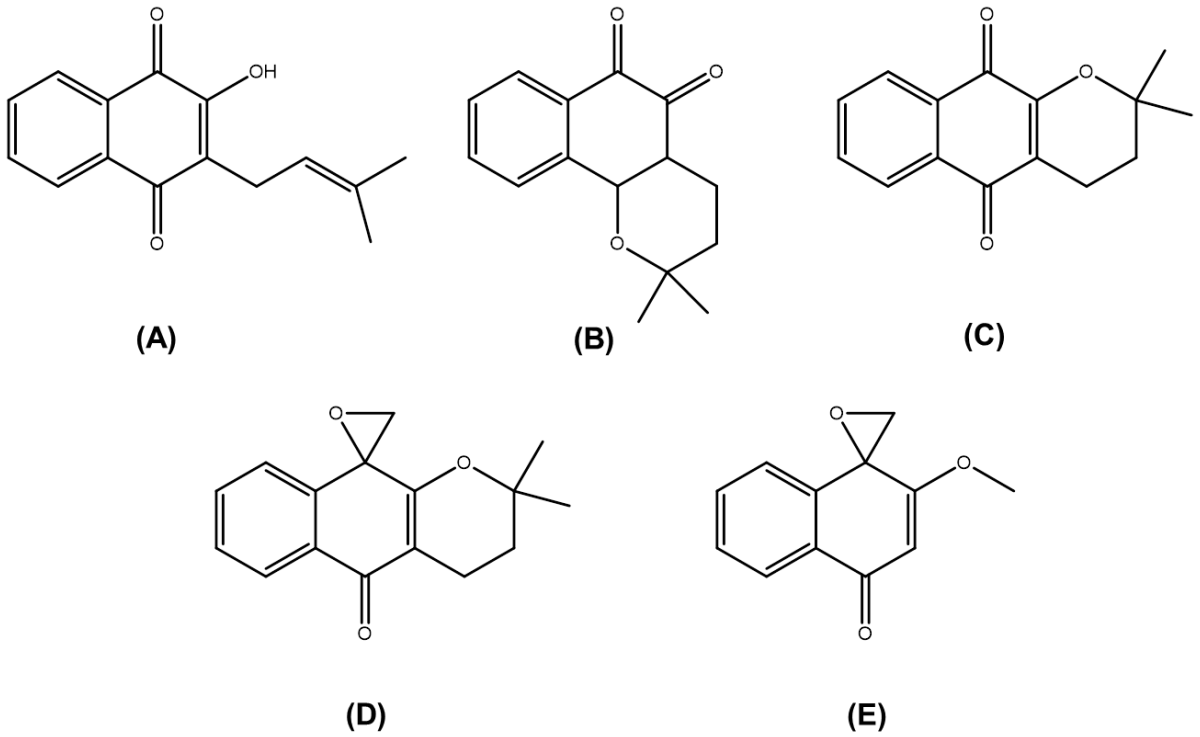

Figura 6: Estrutura química das principais naftoquinonas e oxiranos ativos. (A) Lapachol (CID 3884, $C_{15}H_{14}O_3$); (B) β-lapachona (CID 3885, $C_{15}H_{14}O_3$); (C) α-lapachona (CID 72732, $C_{15}H_{14}O_3$); (D) Epoxi-α-lapachona (CID 12000280, $C_{16}H_{16}O_3$); (E) Epoximetil-lausona ($C_{12}H_{10}O_3$) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top).

2. JUSTIFICATIVA

As leishmanioses estão em expansão em território nacional, apesar do número de casos tenha diminuído nos últimos anos. Isso pode ser atribuído, pelo menos em parte, a alguns fatores relacionados ao tratamento, tais como (i) falha terapêutica dos medicamentos de primeira linha, (ii) toxicidade elevada dos medicamentos de segunda linha, (iii) emergência de cepas resistentes, (iv) baixa adesão dos pacientes aos esquemas de tratamento e (v) via de administração parenteral com injeções diárias (de Oliveira et al., 1995; Romero et al., 2001). Além disso, as indústrias farmacêuticas de capital nacional e estrangeiro pouco têm investido em pesquisa para o desenvolvimento de alternativas ao tratamento da leishmaniose, o que explica o baixo número de fármacos e formulações em uso na clínica, com baixa toxidade e efetividade comprovada.

Desse modo, a OMS recomenda e apóia pesquisas para novos medicamentos contra as leishmanioses (WHO, 2010). De fato, temos presenciado nas últimas décadas um aumento no conhecimento básico da biologia do parasito, avanços significativos na área de química medicinal e síntese, e o desenvolvimento de ferramentas de bioinformática como estratégias pré-clínicas usadas para identificar potenciais candidatos a fármacos (Swinney & Anthony, 2011).

No entanto, abordagens baseadas na melhoria do tratamento com os medicamentos usados têm tido mais sucesso do que àquelas com base no desenho de novas entidades químicas. Os avanços incluem o desenvolvimento de formulações mais seguras para os fármacos já existentes, a avaliação de medicamentos usados para tratar doenças não relacionadas, combinações de fármacos e novos protocolos terapêuticos (Murray, 2004).

A hipótese apresentada nesta tese é que a associação entre o antimoniato de meglumina e os oxiranos possa produzir efeitos leishmanicidas aumentados e, possivelmente, associados à diminuição dos efeitos adversos, por redução da dose aplicada. Tal proposta pode significar um novo enfoque para a quimioterapia das leishmanioses.

3. OBJETIVOS

3.1 Objetivo Geral

Reunir evidências do potencial terapêutico dos derivados oxiranos em combinação com antimoniato de meglumina no tratamento da infecção tegumentar experimental por *L. (L.) amazonensis* em modelo murino.

3.2 Objetivos específicos

- Caracterizar as alterações histopatológicas no pulmão, coração, rim, fígado, cérebro e cerebelo de camundongos BALB /c sadios induzidas por epoxi-α-lapachona, epoximetil-lausona e antimoniato de meglumina.
- Estimar parâmetros farmacocinéticos e toxicológicos *in silico* dos oxiranos em comparação ao antimoniato de meglumina.
- Avaliar o efeito citotóxico causado pela exposição em cultura de macrófagos murinos ao epoxi-α-lapachona, epoximetil-lausona e antimoniato de meglumina.
- Determinar a atividade leishmanicida do epoximetil-lausona sobre amastigotas intracelulares e promastigotas, bem como seu efeito no controle da lesão de camundongos infectados.
- Avaliar os efeitos do tratamento com o antimoniato de meglumina e epoxi-α-lapachona e epoximetil-lausona, isolados e em combinação, sobre infecção de macrófagos e no controle de lesões de camundongos.

4. MÉTODOS E RESULTADOS

4.1. DOCUMENTO 1:

Oliveira LFG, Souza-Silva F, Cysne-Finkelstein L, Rabelo K, Amorim JF, Azevedo AS, Bourguignon SC, Ferreira VF, Paes MV, Alves CR. Evidence for Tissue Toxicity in BALB/c Exposed to a Long-Term Treatment with Oxiranes Compared to Meglumine Antimoniate. Biomed Res Int. 2017; 2017:9840210.

Nesta etapa do estudo foi oportuno avaliar as propriedades farmacocinéticas do epoxi-α-lapachona e do epoximetil-lausona e o perfil toxicológico em comparação ao antimoniato de meglumina, caracterizado como alterações histológicas, induzidas por estes fármacos em tecidos de camundongos BALB / c não infectados. Os efeitos destes compostos no fígado, rim, pulmão, coração e tecidos cerebrais de camundongos saudáveis foram examinados.

Os dados apresentados mostram que tanto os oxiranos quanto o antimoniato de meglumina neste esquema de tratamento induziram alterações em todos os tecidos de camundongos BALB / c, sendo o pulmão, coração e cérebro os mais afetados. O epoximetil-lausona foi o mais tóxico para o tecido pulmonar, enquanto os danos mais graves no coração foram causados pelo epoxi-α-lapachona. O antimoniato de meglumina causou alterações leves a moderadas nos tecidos cardíacos e pulmonares, mas não causou qualquer alteração nos tecidos cerebrais.

Com os dados desta publicação foi possível alcançar os dois primeiros objetivos deste trabalho de tese.

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Research Article

Evidence for Tissue Toxicity in BALB/c Exposed to a Long-Term Treatment with Oxiranes Compared to Meglumine Antimoniate

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Leishmaniasis remains a serious public health problem in developing countries without effective control, whether by vaccination or chemotherapy. Part of the failure of leishmaniasis control is due to the lack of new less toxic and more effective drugs able to eliminate both the lesions and the parasite. Oxiranes derived from naphthoquinones now being assayed are promising drugs for the treatment of this group of diseases. The predicted pharmacokinetic properties and toxicological profiles of epoxy-α-lapachone and epoxymethoxy-lawsone have now been compared to those of meglumine antimoniate, and histological changes induced by these drugs in noninfected BALB/c mice tissues are described. Effects of these compounds on liver, kidney, lung, heart, and cerebral tissues of healthy mice were examined. The data presented show that both these oxiranes and meglumine antimoniate induce changes in all BALB/c mice tissues, with the lung, heart, and brain being the most affected. Epoxymethoxy-lawsone was the most toxic to lung tissue, while most severe damage was caused in the heart by epoxy-α-lapachone. Meglumine antimoniate caused mild-to-moderate changes in heart and lung tissues.

1. Introduction

Leishmaniasis represents a group of parasitic diseases caused by more than 20 *Leismania* spp. parasites, which are transmitted to humans by the bite of infected female phlebotomine sandflies. The epidemiology of leishmaniasis depends on the characteristics of the parasite species, the local ecological characteristics of the transmission sites, current and past exposure of the human population to the parasite, and human behavior [1]. Different forms of leishmaniasis represent an important public health problem in the Americas, due to their widespread distribution and high prevalence. Besides that,

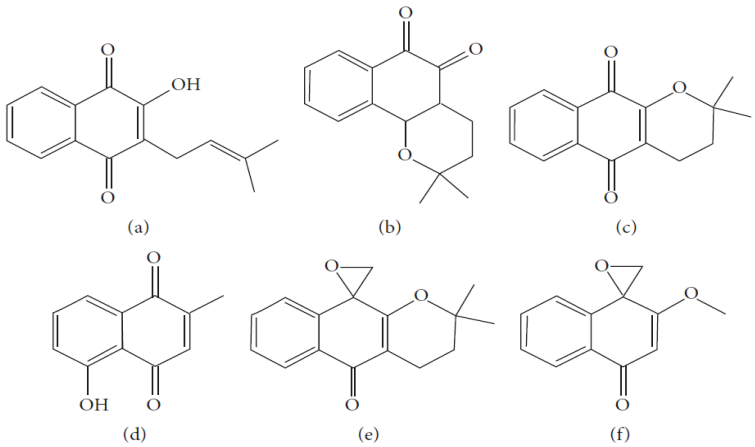

FIGURE 1: Chemical structure of main active naphthoquinones and oxiranes. (a) Lapachol (CID 3884; molecular formula, $C_{15}H_{14}O_3$; molecular weight, 242.274 g/mol); (b) β-lapachone (CID 3885; molecular formula, $C_{15}H_{14}O_3$; molecular weight, 242.274 g/mol); (c) α-lapachone (CID 72732; molecular formula, $C_{15}H_{14}O_3$; molecular weight, 242.274 g/mol); (d) plumbagin (CID 10205; molecular formula, $C_{11}H_8O_3$; molecular weight, 188.182 g/mol); (e) epoxy-α-lapachone (CID 12000280; molecular formula, $C_{16}H_{16}O_3$; molecular weight, 256.301 g/mol); (f) epoxymethoxy-lawsone (molecular formula, $C_{12}H_{10}O_3$; molecular weight, 202.210 g/mol) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top).

the risk factors of transmission are linked to socioeconomic and environmental patterns, which make it difficult to control the disease. Human infections result in a variety of clinical symptoms involving the skin, the mucosa of the upper respiratory tract, and the visceral organs, with difficult treatment for severe cases and with little assurance of parasitological cure [2].

Leishmaniasis treatment was initially described in 1912 by Vianna, when the curative action of a trivalent antimonial (Sb^{3+}) on the skin form of the disease was discovered [3]. However, due to their severe toxic side effects trivalent antimonials were replaced after 1940 by pentavalent antimonials (Sb^{5+}), which have remained even nowadays as the drug of choice for the treatment of all forms of leishmaniasis. In cases where Sb^{5+} treatment is unsuccessful, second-choice drugs, as amphotericin B and pentamidine, are used [4]. The wide variety of adverse effects attributed to these drugs is well described in the literature, ranging from clinical complaints such as musculoskeletal pain and nausea and vomiting to serious toxic effects on heart, liver, and kidney [5]. The most hazardous side effect associated with antimonials is undoubtedly cardiotoxicity, characterized by several changes in the cardiovascular system, particularly altered ventricular repolarization [reviewed in [6]]. Also increasing resistance to pentavalent antimonials has been reported requiring the use of higher doses and longer-term therapy, increasing the toxic risk [7, 8]. Drug resistance has been reported in India, with patients failing to respond or showing a relapse after therapy with antimony drugs [9]. Resistance to the current therapy with antimony drug has also been reported in many other countries including Afghanistan, Iran, Brazil, Peru, and Saudi Arabia [10].

In this context, the search for novel less toxic antileishmanial drugs has been the subject of several studies. Interest in applying herbal extracts, essential oils, and natural products against leishmaniasis has increased in recent years [11]. Many research groups have searched for new potential antiparasitic drugs from vegetable sources, mainly based on secondary metabolites, of which naphthoquinones are an example [12].

Quinones are a wide and varied family of natural aromatic metabolites found in several plant families, as well as in fungi, algae, and bacteria. These quinones include benzoquinones, anthraquinones, and naphthoquinones [13]. An important chemical property of quinones is their ability to act as oxidizing or dehydrogenating agents. This redox property is driven by the formation of a fully aromatic system [14, 15]. In recent years, interest in quinones and their derivatives has increased not only due to role in biochemical processes but also for their activity against a wide range of infectious agents [16]. In nature, these compounds, such as ubiquinones (coenzyme Q) and menaquinones (vitamin K), are involved in important stages of the life cycle, acting primarily as components of the electron transport chains and in photosynthesis [17].

1,4-Naphthoquinones are derived from naphthalene by the introduction of two carbonyl groups in the 1,4 positions or, less commonly, in the 1,2 positions (1,2-naphthoquinones) [13]. Their interest in medicinal chemistry derives from their ability to control infection by and multiplication of trypanosomatids of medical interest [18]. Examples of quinones which have been described as effective against *Leishmania* species and *Trypanosoma cruzi* include lapachol (Figure 1(a)), β-lapachone (Figure 1(b)), and α-lapachone (Figure 1(c)), first isolated from the heartwood of trees of the Bignoniaceae family (*Tabebuia* sp.), and plumbagin (Figure 1(d)), extracted

from *Plumbago scandens* L. roots and other *Plantago* spp. [19–21].

However, the toxicity of the lapachone derivatives limits their potential for use in the treatment in leishmaniasis and other diseases. Chemical modifications were performed on these compounds to address this issue and modification of the quinonoid center of α-lapachone followed by epoxidation generated the epoxy-α-lapachone (Figure 1(e)) and epoxymethoxy-lawsone (Figure 1(f)), derivatives potentially less toxic for mammalian cells [22, 23].

Epoxy-α-lapachone led to a dose- and time-dependent decrease in the numbers of promastigotes of *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*. This compound was able to kill amastigotes inside human macrophages [24]. In addition, this reduction of the lesion size in the paw of BALB/c mice infected by *L. (L.) amazonensis* was observed until six weeks after treatment and negatively affected the activity of serine proteinase of both promastigote and amastigote forms [25].

The potential use of these oxiranes derived from natural 1,4-naphtoquinones against *Leishmania* parasites to treat the infection in humans made it relevant to evaluate the prediction of pharmacokinetic properties and possible toxic effects of epoxy-α-lapachone and epoxymethoxy-lawsone comparing to meglumine antimoniate and describe histological changes induced by these drugs in noninfected BALB/c mice tissues. Additionally, the possible biochemical mechanisms associated with cell damage in these tissues are discussed. The data gathered here aggregate new evidences on histological changes in mammalian host tissues induced by oxiranes and meglumine antimoniate.

2. Materials and Methods

2.1. Chemicals and Culture Reagents. Dimethyl sulfoxide (DMSO), penicillin, streptomycin, and Schneider's Drosophila medium were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was acquired from Cultilab S/A (Brazil). Meglumine antimoniate (Glucantime) was purchased from Sanofi-Aventis Farmacêutica (Suzano, Brazil). Propylene glycol was obtained from Vetec Quimica. Epoxy-α-lapachone and epoxymethoxy-lawsone compounds were synthesized by the Department of Organic Chemistry of the Instituto de Química, Universidade Federal Fluminense.

2.2. In Silico Analysis. In silico assessing of the pharmacokinetics and toxicity parameters of oxiranes compounds and meglumine antimoniate was performed here. Pharmacokinetic parameters (absorption, distribution, metabolism, and excretion) and toxicological were tested using the web server platform "pkCSM: predicting small-molecule pharmacokinetic properties using graph-based signatures" (available in http://bleoberis.bioc.cam.ac.uk/pkcsm/prediction). Data on absorption, distribution, metabolism, excretion (ADME), and toxicity (T) of those compounds are presented in Table 1.

2.3. Parasite Cultures. *Leishmania (Leishmania) amazonensis* (MHOM/BR/73/LTB0016) was obtained from the Leishmania collection of the Instituto Oswaldo Cruz (Fiocruz). In vitro promastigote cultures were maintained at 28°C in Schneider's medium (pH 7.2) containing 1 mM L-glutamine, 10% FCS, 100 IU/mL penicillin, and 100 μg/mL streptomycin, with frequent subpassages to maintain the parasites in the logarithmic growth phase. The parasites were in the stationary growth phase after 5 days of culture in Schneider's medium.

2.4. Experimental Treatments. All experiments were conducted with 5-to-7-week-old BALB/c mice weighing approximately 22 g. The animals were obtained from the animal breeding center of Fiocruz, and all experimental procedures were performed as approved by the Committee for the Ethical Use of Animals of Instituto Oswaldo Cruz (L-052/2015). A group of BALB/c mice were inoculated subcutaneously, in the left footpad, with 1.0×10^4 promastigotes and the lesion sizes were measured weekly. Treatments of infected and noninfected mice were performed with either meglumine antimoniate (Glucantime) as a comparative control for treatment efficacy 22.7 mg/Kg/day (4.1 μM of Sb^{5+}) or epoxy-α-lapachone at 22.7 mg/Kg/day (1.9 μM/Kg/day) and epoxymethoxy-lawsone at 11.4 mg/Kg/day (1.2 μM/Kg/day) and diluted in a mix of DMSO : propylene glycol : saline (1 : 12 : 7 and 1 : 9 : 10, resp.). The drugs were administered subcutaneously in the dorsal region of each mouse in a dose of 100 μL per animal. Negative-control groups were included, in which sterile PBS or the dilution mix of DMSO : propylene glycol : saline with higher concentration of propylene glycol (1 : 12 : 7) was administered during treatment. All treatments were made daily from Monday to Friday, until 20 doses.

2.5. Histopathological Study. Organs (liver, lung, heart, kidney, and brain/cerebellum) were collected from six BALB/c mice of each group (drugs, vehicle, or no treatment). Fragments of the tissues were excised, cleaved, fixed in buffered formalin (10%), and blocked in paraffin. Tissue sections, 4 μm thick, were cut, deparaffinized in three baths of xylene, and rehydrated with increasing concentrations of ethanol (70% to 100%). Hematoxylin and eosin (HE) were used finally to stain the tissue sections for histological examination. Tissue damage of liver, lung, heart, brain, and cerebellum were semiquantitatively assessed based on intensity and focal or diffuse character. A total of 10 fragments of tissue for each animal were examined and results were plotted as the media of damage values. For each lesion a parameter was assigned with a numerical value between 0 and 4, according to the severity and extent of the damage: 0 = absent, 1 = mild, 2 = moderate, 3 = severe, and 4 = severe-diffuse. The tissue alterations quantified in the liver were circulatory changes (hemorrhage and edema) and inflammatory infiltrates in the central and portal vein and necrosis. For quantification of lung tissue alterations, circulatory alterations, the thickening of the alveolar septa, and bronchiolar inflammatory infiltrates were considered. In the case of kidney tissue, measurements took into account circulatory changes (hemorrhage, edema, and vascular congestion); cortical infiltrates; disorganization of the cortical area and regions of the collecting, proximal, and distal tubules; and destructed epithelial lining of distal convoluted tubules. Tissue injuries were measured over the

Table 1: In silico pharmacokinetic and toxicological parameters for oxirane compounds and meglumine antimoniate.

Predicted properties	Epoxy-α-lapachone	Epoxy-methoxy-lapachol	Meglumine antimoniate	Interpretation
Absorption				
Caco2 permeability	1.595	1.584	−0.443	>0,90
Intestinal absorption	100	100	27.93	% absorbed
Skin Permeability	−3.242	−3.249	−2.893	>−2.5 low skin permeability
P-Glycoprotein substrate	Yes	Yes	Yes	Yes/no
P-Glycoprotein inhibitor	No	No	No	Yes/no
Distribution				
Volume of distribution	0.201	−0.012	−0.35	Low: <−0.15; high: >0.45
Fraction unbound (human)	0.283	0.392	0.985	0.0 to 1.0
BBB permeability	0.325	0.260	−1.287	Low: <−1/high: >0.30
CNS permeability	−2.087	−2.014	−4.761	Positive: >−2; negative: <−3
Metabolism				
CYP3A4 substrate	Yes	No	No	Yes/no
CYP2D6 substrate	No	No	No	Yes/no
CYP3A4 inhibitor	No	No	No	Yes/no
CYP2D6 inhibitor	No	No	No	Yes/no
CYP1A2 inhibitor	Yes	Yes	No	Yes/no
Excretion				
Total clearance	0.07	0.197	−0.154	log mL/min/kg
Toxicity				
AMES toxicity	No	Yes	No	Yes/no
Max. tolerated dose (human)	0.536	0.925	0.947	log mg/kg/day
Oral Rat Acute Toxicity (LD50)	1.975	1.962	1.184	mol/kg
Oral Rat Chronic Toxicity (LOAEL)	1.728	2.290	1.218	log mg/kg_bw/day
Hepatotoxicity	No	No	No	Yes/no
Skin sensitisation	No	No	No	Yes/no

whole slide to be quantified by optical microscopy Olympus BX53 using Image Pro Plus software and all analyses were performed in a blind manner without prior knowledge of the group.

3. Results

3.1. In Silico Pharmacokinetic and Toxicity Parameters of Oxirane Compounds and Meglumine Antimoniate. A computational study was performed for the prediction of ADME profile of each of the oxiranes derived from 1,4-naphthoquinones as well as meglumine antimoniate and the main results are presented in Table 1. Both oxiranes are completely absorbed by the oral and intestinal mucosa, as demonstrated by Caco-2 monolayer test and the intestinal absorption predicted method. Meglumine antimoniate showed the lowest values for oral and intestinal absorption as was already known. However, all these compounds may theoretically act as P-glycoprotein substrates. Epoxy-α-lapachone showed a moderate distribution volume while epoxymethoxy-lawsone value was very low. On the other hand, the unbound fraction of epoxy-α-lapachone was lower than that of epoxymethoxy-lawsone (0.28 and 0.39, resp.). Meglumine antimoniate presented a very high unbound fraction (0.98), suggesting that this drug has the highest bioavailability. The ability of a drug to cross into the brain is an important parameter to consider in an attempt to reduce adverse effects or to improve the pharmacological activity of drugs that act within the brain. Two tests were conducted to predict this capability: blood-brain barrier (BBB) permeability and central nervous system (CNS) permeability. The latter is a more direct measure. The results indicated that both oxiranes might pass through into the brain.

Regarding the metabolism of these drugs, epoxy-α-lapachone is a substrate for CYP3A4. Surprisingly, epoxymethoxy-lawsone and meglumine antimoniate are not, at least theoretically, substrates for cytochrome P450 enzymes. Besides that, oxiranes may act as inhibitors of CYP1A2. Considering the toxicity, according to Table 1, epoxymethoxy-lawsone could have a mutagenic potential (AMES positive test) but it seems to be better tolerated than epoxy-α-lapachone (higher Maximum Tolerated Dose).

3.2. Histopathological Analysis of Healthy Mice Treated with Oxirane Derivatives and Meglumine Antimoniate. Histopathological analysis was performed in healthy BALB/c mice following a previous assay where we showed that these oxirane compounds and meglumine antimoniate were able

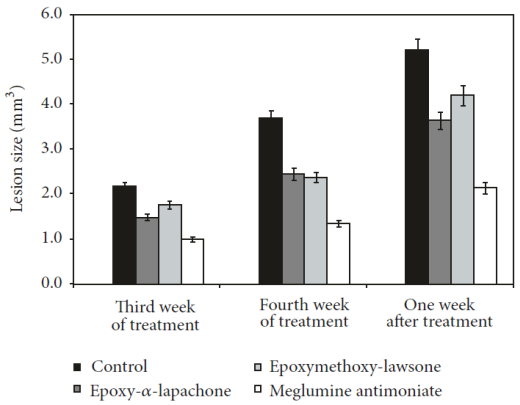

FIGURE 2: Treatment of infections in mice caused by *Leishmania* (*L.*) *amazonensis*. BALB/c mice were inoculated subcutaneously, in the left footpad, with 1.0×10^4 promastigotes. After 4 weeks of infection, the mice were treated weekly with 22.7 mg/Kg/day (1.9 μM/Kg/day) of epoxy-α-lapachone (dark grey square), 11.4 mg/Kg/day (1.2 μM/Kg/day) of epoxymethoxy-lawsone (light grey square), and 22.7 mg/Kg/day (4.1 μM of Sb^{5+}/Kg/day) of meglumine antimoniate (white square), administered subcutaneously with five animals per group. Negative controls were treated with a mix of DMSO : propylene glycol : saline with higher concentration of propylene glycol (1 : 12 : 7) (black square). Lesion sizes (mm^3) were measured weekly and the results of third and fourth weeks of treatment and one week after treatment are shown. The data are presented by the mean and standard deviation (SD), from five mice.

to control the size of the lesion in mice infected with *L. (L.) amazonensis*. The largest difference was observed one week after the treatment whose main results are summarized in Figure 2. The most effective doses were selected to assess the tissue-specific toxicity. Epoxymethoxy-lawsone was as effective as epoxy-α-lapachone even when tested at a lower concentration. The best results were obtained when the reference drug was used.

In general, the drugs were able to induce changes in all tissues analyzed. The most affected organs were lung, heart, and brain. Epoxymethoxy-lawsone was the most toxic to the lung tissue, while most severe damage to the heart and brain was provoked by epoxy-α-lapachone. As expected, meglumine antimoniate induced severe changes in heart tissue and curiously caused changes to the lung tissue (Table 2).

3.2.1. Histopathological Analysis of the Lung. As expected, in lung tissue of nontreated mice a regular structure of alveoli and alveolar septa was observed (Figure 3(A)). In BALB/c mice, treatment with epoxy-α-lapachone and epoxymethoxy-lawsone caused severe septum thickening and the appearance of mononuclear inflammatory infiltrates around the bronchiole and pulmonary veins, suggesting an interstitial pneumonia (Figures 3(B)–3(D)). Extensive areas of hemorrhage were found in the epoxymethoxy-lawsone treated group (Figures 3(E)–3(G)). The reference drug, meglumine antimoniate, induced similar changes such as moderate septum thickening (Figure 3(H)) and both severe peribronchiolar and perivascular infiltrates (Figure 3(I)).

3.2.2. Histopathological Analysis of the Heart. A normal cardiac structure with branching fibers, central nuclei, and intercalated discs was observed in nontreated animal tissues (Figure 4(A)). On the other hand, oxiranes induced parenchyma alterations, such as severe-diffuse interstitial necrosis, leading to a degeneration of cardiac fibers (Figures 4(B) and 4(C)). In addition, a difference in the pattern of necrosis was observed. In the case of epoxy-α-lapachone there is a more degenerative diffuse necrosis with focal areas of fiber regeneration, while with epoxymethoxy-lawsone areas of severe necrosis were noted, but there are regions where the fibers structures are still preserved. Treatment with meglumine antimoniate also showed severe necrosis (not shown). Mononuclear infiltrates were noted in all treatments ranging from severe in meglumine antimoniate and epoxymethoxy-lawsone to severe-diffuse in epoxy-α-lapachone (Figures 4(B)–4(D)).

3.2.3. Histopathological Analysis of the Kidney. As expected, in nontreated mice a normal structure with preserved distal and proximal convoluted tubules, intact renal glomerulus, and collector tubules was observed (Figures 5(A) and 5(B)), but both oxiranes caused several alterations in kidney tissue. Disorganization of the proximal and distal tubules was associated with all treatments; however, the most severe damage was provoked by epoxy-α-lapachone followed by meglumine antimoniate (moderate) and epoxymethoxy-lawsone (mild) (Figures 5(C) and 5(E)). We have detected severe destructed epithelial lining of distal convoluted tubules in the cortical area when mice were treated with oxiranes (Figures 5(C) and 5(E)). Mild-to-moderate cortical and medullar vascular congestion was found with the oxirane compounds (Figure 5(E)). Cortical and medullar infiltrates were related to all drugs ranging from moderate to severe (Figures 5(C)–5(F)). Additionally, epoxymethoxy-lawsone provoked severe vascular congestion in the medullar area (Figure 5(F)).

3.2.4. Histopathological Analysis of the Liver. Liver tissue obtained from nontreated mice presented hepatocytes, sinusoidal capillaries, portal space, and central vein with regular structure (Figure 6(A)). Parenchymal damage was observed in both oxiranes treatments. The most significant changes detected with oxirane derivatives were necrosis and increased cellularity (Figures 6(B)–6(D)). These changes were more notable when epoxy-α-lapachone was used (Figures 6(B) and 6(C)). A mild hemorrhage and edema were also found in this group. No alterations were detected in the antimonial treated group (Figure 6(E)).

3.2.5. Histopathological Analysis of the Brain/Cerebellum. Brain and cerebellum of nontreated mice showed regular architecture of the cerebral and cerebellar cortex (Figures

Table 2: Semiquantitative analysis of histological changes in tissues of mice treated with oxiranes derivate of natural 1,4-naphthoquinones and meglumine antimoniate.

Changes	Epoxy-α-lapachone	Epoxymethoxy-lawsone	Meglumine antimoniate
Lung			
Thickening of the alveolar septa	+++	+++	++
Peribronchiolar infiltrates	+++	+++	+++
Perivascular infiltrates	++	++++	+++
Hemorrhage	++	++++	+
Heart			
Necrosis	++++	+++	+++
Degeneration of cardiac fibers	++++	+++	0
Mononuclear infiltrates	++++	+++	+++
Kidney			
Disorganization of the proximal and distal tubules	+++	++	++
Destructed epithelial lining of distal convoluted tubules	+++	+++	0
Disorganization collecting tubules regions	+++	++	0
Cortical infiltrates	++	+++	++
Medullar infiltrates	+++	++	++
Cortical hemorrhage	++	0	0
Medullar hemorrhage	+	+	0
Vascular congestion in the cortical area	+	++	0
Vascular congestion in the medullar area	+	+++	0
Liver			
Necrosis	+++	++	0
Mononuclear infiltrates	+++	+++	0
Hemorrhage	+	0	0
Edema	+	0	0
Brain/Cerebellum			
Mononuclear infiltrates in Pia Mater	++++	++	0
Disorganization of the cortical region	++++	++	0
Reactive gliosis	++++	++	0
Mononuclear infiltrates in parenchyma	++++	++	0
Degeneration in Purkinje Neuron	++++	+++	0

Grades were assigned using a subjective scale of 0 to ++++ for the different changes. 0 = absent, + = mild, ++ = moderate, +++ = severe, and ++++ = severe-diffuse.

7(A) and 7(G)). In BALB/c mice treated with epoxy-α-lapachone were observed thickening of the pia mater associated with intense diffuse infiltrates of the cortex and severe-diffuse reactive gliosis, suggesting an increase in the number of glial cells, mainly astrocytes (Figures 7(B) and 7(C)). The group treated with epoxymethoxy-lawsone showed similar but less severe alterations. Infiltrates in the cortex area were discrete in mice of this group (Figures 7(D) and 7(E)). Cerebellum of mice treated with oxiranes showed degeneration in Purkinje neurons (Figures 7(H) and 7(I)). No changes were observed in brain or cerebellum of mice treated with meglumine antimoniate (Figure 7(F)). The changes detected in brain and cerebellum of animals treated with oxiranes indicated a blood-brain barrier disruption, showing that these molecules are able to pass through into the central nervous system (SNC).

4. Discussion

New approaches in the chemotherapy of *Leishmania* spp. infections are well studied in murine models. However, drug efficacy studies may overestimate the beneficial effect of the interventions. The approach applied here consists in an effort to identify and describe toxicity profile of a new class of synthetic compounds, the oxiranes, combining an in silico prediction of pharmacokinetic parameters with histopathological analysis. Data obtained here with an inbred strain of mice, non-*Leishmania*-infected, emphasize the importance of monitoring and improving the understanding of the toxic effects on human tissues due to resemblance in physiology.

The present study shows strong evidence that the treatment with oxiranes epoxy-α-lapachone and epoxymethoxy-lawsone at dose of 22.7 mg/Kg/day and 11.4 mg/Kg/day, respectively, administrated daily from Monday to Friday, until 20 doses in healthy mice caused substantial histological changes in lung, heart, kidney, liver, and brain of healthy mice. The same protocol which had been previously applied to treat mice infected by *L. (L.) amazonensis* controlled the size of the lesion. In this context, analyses were based on the higher dose considered as the most effective in controlling progression of the lesion in mice. Further studies are necessary for the follow-up of animals in the weeks after treatment, to provide data about persistence or reversibility of the changes found.

The toxic effects of oxiranes detected in mice tissues may be explained by the chemical nature of these compounds

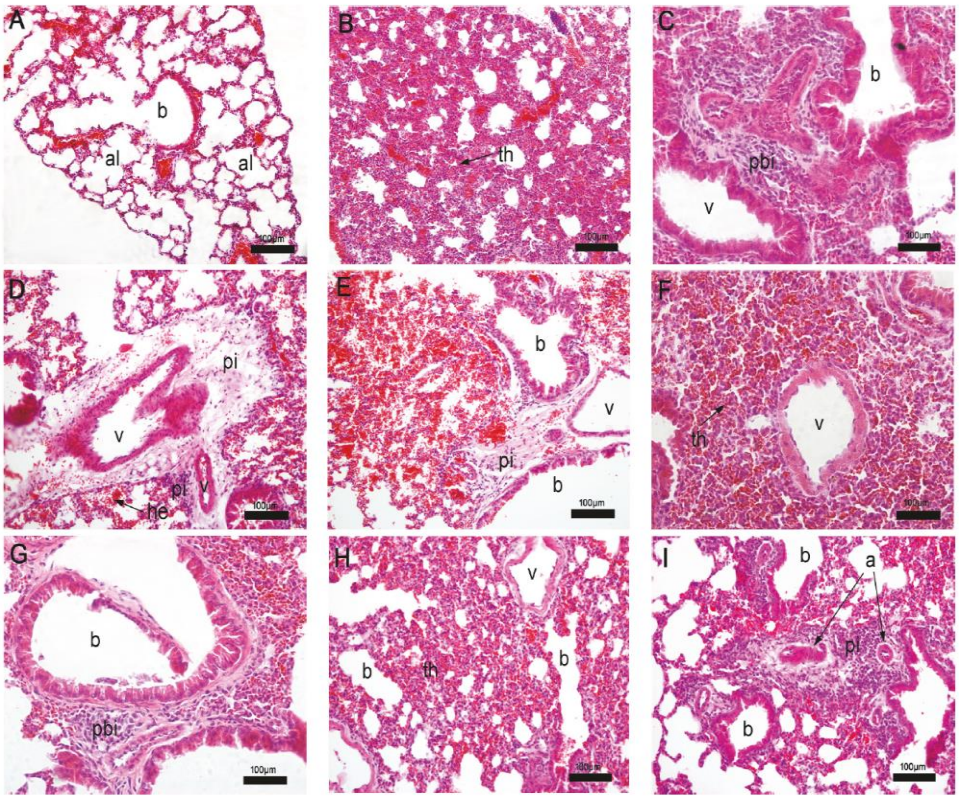

Figure 3: Histopathological analysis of mice lung tissue. Nontreated BALB/c mice (A) presenting normal aspect of alveoli and bronchiole and after treatment with epoxy-α-lapachone (B, C, D) and epoxymethoxy-lawsone (E, F, G) showing pulmonary alterations, including severe septal thickening, hemorrhage, presence of peribronchiole, and perivascular infiltration. After meglumine antimoniate treatment (H, I), moderate septal thickening and severe perivascular infiltration are observed. Lung tissues were stained with hematoxylin and eosin. Tissue images are representative of 10-fragment analysis by animals ($n = 6$), for each treatment group. Alveoli (al), bronchiole (b), hemorrhage (he), peribronchiole (pbi), perivascular infiltration (pi), pulmonary vein (v), and septal thickening (th).

and their putative pharmacokinetic effects as assessed. Oxiranes are synthetic compounds derived from natural naphthoquinones and bioactive against *Trypanosoma cruzi* [26] and *Leishmania* spp. [24]. Epoxy-α-lapachone and epoxymethoxy-lawsone were based on α-lapachone and 2-hydroxy-1,4-naphthoquinone (lawsone), respectively [27]. For this reason, such molecules might share the toxic properties already described for these quinones.

The injuries observed may be due to their high distribution in the tissues and the oxidation/reduction mechanism earlier described for quinones. Quinones are oxidants agents and electrophiles, and the relative contribution of these properties to both toxic and therapeutic activities can mainly be influenced by the effects of their substituents and the characteristics of the quinone core [17]. The redox cycling of quinones may be initiated by either a one- or two-electron reduction. The one electron reduction of quinones is catalyzed by NADPH-cytochrome P450 reductase and other flavoprotein enzymes, leading to unstable semiquinones. Semiquinones transfer electrons to molecular oxygen (O_2) and return to their original quinoidal structure, thus generating a superoxide anion radical ($O_2^{-\bullet}$). Superoxide can be converted into hydrogen peroxide (H_2O_2) via a superoxide dismutase- (SOD-) catalyzed reaction, followed by the formation of a hydroxyl radical ($HO^{\bullet}$) through the iron catalyzed reduction of peroxide via the Fenton reaction (Supplemental file, in Supplementary Material available online at https://doi.org/10.1155/2017/9840210). All of these reactive oxygen species are powerful oxidizing agents and are probably responsible for damage to macromolecules such as DNA, proteins, and lipids, leading to oxidative stress and apoptosis in the cells [13, 17, 18, 28]. Thus, it is possible that some of these pathways would be overactivated in the lung due to a high concentration of molecular oxygen and iron ion present in pulmonary alveoli, which could explain the extensive damage found in this organ.

Another mechanism for signs of toxicity found in tissues exposed to naphthoquinones is that these compounds act as electrophiles forming covalent bonds with nucleophilic functions in biological molecules in an arylation reaction. When the nucleophile is a thiol group, the reaction generates a thioether, which is usually stable [28]. Therefore, the molecular basis for the quinone cytotoxicity has been attributed to the alkylation of essential protein thiol and amine groups as

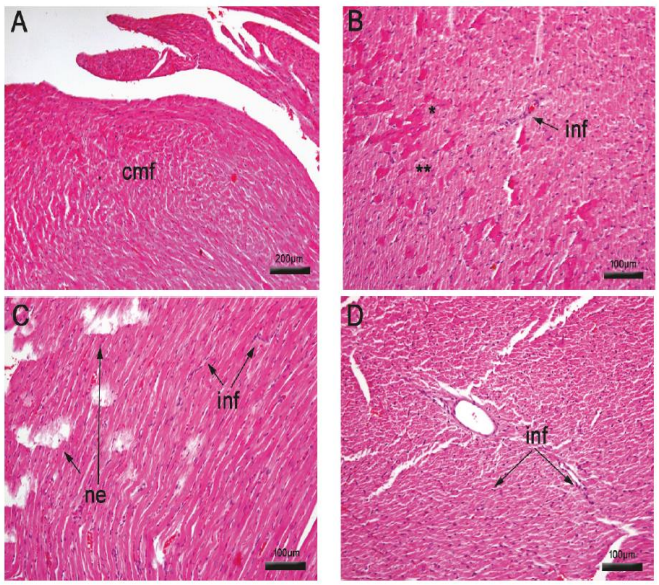

Figure 4: Histopathological analysis of mice heart tissue. Nontreated BALB/c mice presented normal structure (A) and after treatment with epoxy-α-lapachone (B) showed regenerated ($*$) and degenerated cardiac fibers ($**$) and epoxymethoxy-lawsone resulted in diffuse necrosis areas (C). A difference in the necrosis pattern was noted. In the case of epoxy-α-lapachone there is a more degenerative diffuse necrosis with focal areas of fiber regeneration while with epoxymethoxy-lawsone areas of severe necrosis are seen, but there are regions where the fiber structure is still preserved. Meglumine antimoniate caused moderate mononuclear infiltrates (D). Heart tissues were stained with hematoxylin and eosin. Tissue images are representative of 10-fragment analysis by animals ($n = 6$), for each treatment group. Cardiac muscular fibers (cmf), necrosis (ne), and infiltrates (inf).

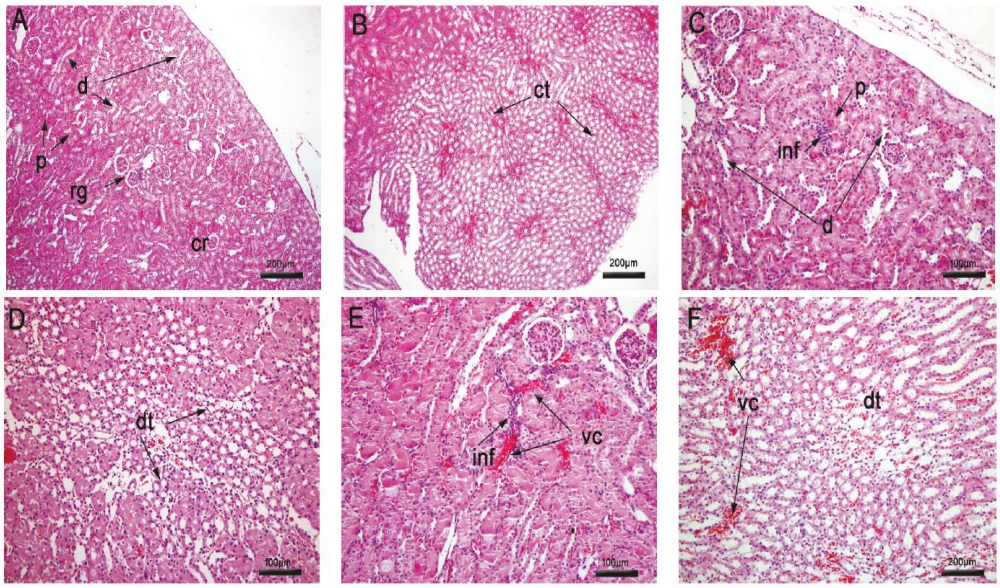

Figure 5: Histopathological analysis of mice kidney tissue. Nontreated BALB/c mice showed regular cortical and medullar regions (A, B) and after treatment with epoxy-α-lapachone showed mononuclear infiltrates near the proximal (p) tubules in focal areas of the cortical region and destructed epithelial lining of distal (d) convoluted tubules (C, D). Degenerate collector tubules were observed in diffuse areas of the medullar region. Epoxymethoxy-lawsone produced mononuclear infiltrates and vascular congestion in diffuse areas (E) and focal areas of vascular congestion and degenerate collector tubules (F). Kidney tissues were stained with hematoxylin and eosin. Tissue images are representative of 10-fragment analysis by animals ($n = 6$), for each treatment group. Collector tubules (tc), degenerate collector tubules (dt), epithelial lining of distal convoluted tubules (d), infiltrates (inf), molecular region (mr), proximal convoluted tubules (p), renal glomerulus (rg), and vascular congestion (vc).

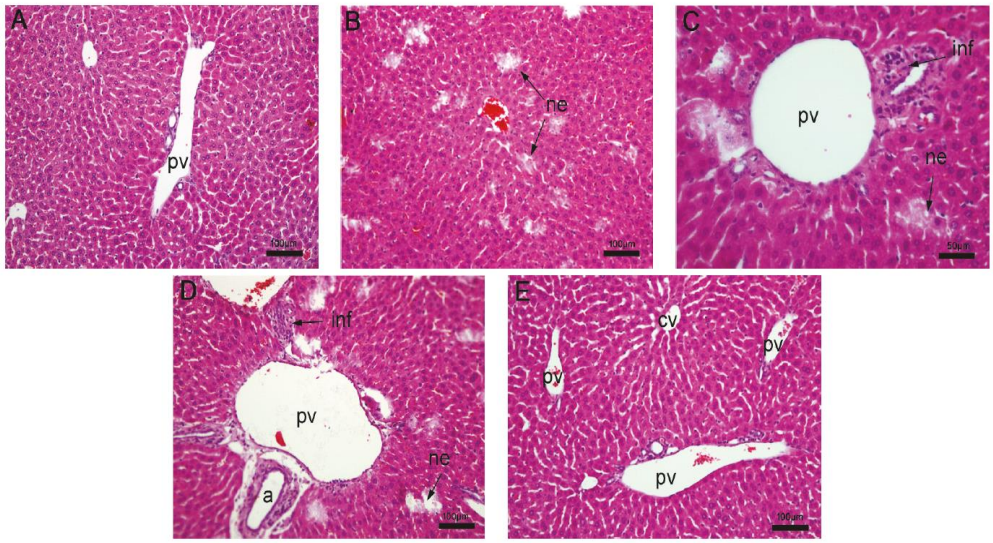

FIGURE 6: Histology analysis of the liver. (A) Nontreated BALB/c mice presenting normal aspect. Liver sections of mice treated with epoxy-α-lapachone showing diffuse areas of necrosis around the portal space and hepatic parenchyma and mononuclear infiltrates near portal vein and biliary ducts (B, C). In BALB/c mice treated with epoxymethoxy-lawsone triad portal space focal necrosis and infiltrates around biliary ducts and arterioles were noted (D, E). Mice treated with meglumine antimoniate retained the hepatic parenchyma with regular structure. Liver tissues were stained with hematoxylin and eosin. Tissue images are representative of 10-fragment analysis by animals ($n = 6$), for each treatment group. Arterioles (a), central vein (cv), infiltrates (inf), necrosis (ne), and portal vein (pv).

well as the oxidation of essential protein thiols by activated reactive oxygen species [17].

Our results detected vascular changes in lung as severe-diffuse hemorrhage and intense perivascular infiltrates, besides vascular congestion in both the cortical and medullar areas and a mild medullar hemorrhage in kidney of mice treated with epoxymethoxy-lawsone.

The fact that meglumine antimoniate has caused important changes in mice tissues, with relevant toxic effects in both heart and lung tissues, is a matter of concern. Pentavalent antimonials have been used for decades, and complaints related to lung function by the patients are very rare. In addition, the most toxic effects refer to heart function, particularly ventricular repolarization disorders and an increase of corrected QT interval (QTc) [5]. It was demonstrated that both trivalent potassium antimony (III) tartrate and sodium stibogluconate, regardless of their different oxidation states, increased cardiac calcium currents at therapeutic concentrations, whereas three major cardiac potassium currents (I_{Kr}, I_{Ks}, and I_{K1}) were not affected. Cardiac calcium currents were especially sensitive to the trivalent antimony compound. According to authors, as calcium currents regulate the plateau phase of the cardiac action potential and an increased amplitude provokes a delay in cardiac repolarization, this finding may explain the occurrence of development QTc prolongation which can lead to ventricular tachycardia, *torsades de pointes,* and other arrhythmias in patients treated with antimonial drugs [29]. Indeed, we have evidence that meglumine antimoniate induced severe necrosis in murine cardiac tissue with presence of very intense mononuclear infiltration. These data suggest that such foci of necrosis may be responsible, in part, for the remarkable electrocardiographic changes during treatment with antimonials; however, supplementary assays are needed to prove this hypothesis.

Data obtained by in silico analysis indicate a possible application of oxirane compounds by oral or topical route. This might allow exploration of alternative ways for the administration of these compounds aimed at reducing their toxic effects. Furthermore, a high distribution predicted for oxiranes in brain tissues suggested by this analysis was confirmed by the histological changes found in the brain and cerebellum of mice, indicating that these compounds pass into the SNC. Findings such as inflammation and thickening of the pia mater and reactive gliosis indicate encephalitis, which was most severe and intense with epoxy-α-lapachone treatment. This relevant information must be considered in any formulation as a guide to reduce side effects and toxicity.

On the other hand, the negative prediction of toxic effects on hepatocytes was not confirmed. We have detected several foci of severe-to-moderate necrosis and intense inflammatory infiltrate in hepatic tissue of animals treated with oxiranes. It is demonstrated that the application of computational technologies to predict kinetic properties and to identify the toxicity potential based on the physicochemical and structural characteristics of the chemical substances raises relevant theoretical issues but must be further assessed by biological assays.

5. Conclusions

In conclusion, data presented here add evidence for toxicity induced by oxiranes and meglumine antimoniate, characterized by histological changes in different vital organs of healthy

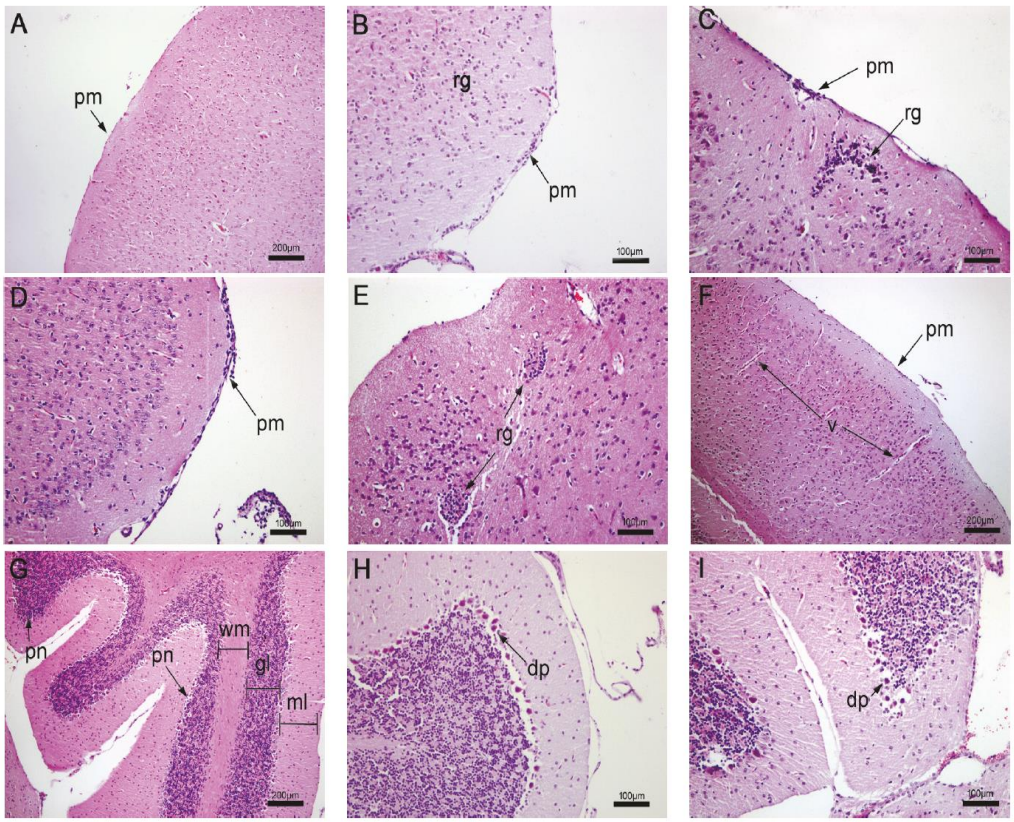

Figure 7: Histopathological analysis of mice brain and cerebellum. A regular architecture of the cerebral and cerebellar cortex of nontreated BALB/c mice is shown (A, G). Treated mice with epoxy-α-lapachone (B, C) and epoxymethoxy-lawsone (D, E) showed thickening of pia mater and reactive gliosis. No alterations were observed. Meglumine antimoniate in the cortex (F). Cerebellum of mice treated with oxiranes showed degeneration in Purkinje neurons (H, I). Alterations detected in brain and cerebellum of animals treated with oxiranes indicated a blood-brain barrier disruption. Brain tissues were stained with hematoxylin and eosin. Tissue images are representative of 10-fragment analysis by animals ($n = 6$), for each treatment group. Cortex region (cr), degenerate Purkinje neuron (dp), granular layer (gl), infiltrates (inf), molecular layer (mL), pia mater (pm), Purkinje neuron (pn), reactive gliose (rg), vein (v), and white matter (wm).

mice. Toxic and adverse effects of drugs are related to several factors such as dose, time, and frequency of exposure, route of administration, and pharmacokinetic parameters. Nonetheless, an active molecule that initially has been shown to be toxic should not be discarded before considering alternatives such as reduction of dose or duration of treatment, combined use with other drugs, and study of different formulations. The best example is amphotericin B, a very active molecule against different microorganisms but also very toxic to mammalian cells in which concepts of drug delivery systems involving liposomal formulations with lower toxicity have been applied [30].

Disclosure

Carlos R. Alves and Vitor F. Ferreira are fellow researchers at Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) institution. Luiz F. G. Oliveira is a doctoral fellow at Fundação Oswaldo Cruz (Fiocruz) institution. Franklin S. Silva is a postdoctoral fellow at Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) institution.

Conflicts of Interest

All authors declare no competing financial interest.

Authors' Contributions

All authors approved the final version of the manuscript. Luiz Filipe Gonçalves Oliveira and Franklin Souza-Silva equally contributed to this paper.

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4.2 DOCUMENTO 2:

Oliveira LFG, Souza-Silva F, de Castro Côrtes LM, Cysne-Finkelstein L, de Souza Pereira MC, de Oliveira Junior FO, Pinho RT, Corte Real S, Bourguignon SC, Ferreira VF, Alves CR. Antileishmanial Activity of 2-Methoxy-4H-spiro-[naphthalene-1,2'-oxiran]-4-one (Epoxymethoxy-lawsone): A Promising New Drug Candidate for Leishmaniasis Treatment. Molecules. 2018 Apr 10;23(4).

Na continuidade do nosso estudo, foi investigada a eficácia do epoximetil-lausona e antimoniato de meglumina em formas amastigotas intracelulares de *Leishmania (Leishmania) amazonensis* e em camundongos BALB/c infectados.

Num ensaio com amastigotas intracelulares, o valor da metade da concentração inibitória máxima (IC_{50}) para o epoximetoxi-lauson foi ligeiramente superior (7,41± 0,2 e 4,43 ± 0,25 µM) ao encontrado para o antimoniato de meglumina (7,41± 0,2 e 4,43 ± 0,25 µM). A eficácia de ambos os fármacos se tornou mais evidente após 48 horas de exposição, quando o composto oxirano e o fármaco de referência alcançaram valores de IC_{50} 18 vezes e 7,4 vezes inferiores, respectivamente. Os promastigotas também foram afetados por epoximetil-lausona após 24 horas de incubação (IC_{50} = 45,45 ± 5,0µM), mas com IC_{50} 6 vezes maior do que aquele encontrado para amastigotas intracelulares. A análise de citotoxicidade revelou que o epoximetil-lausona (CC_{50} = 40,05 ± 3,0 µM) tem um efeito 1,7 vezes menor do que o antimoniato de meglumina (CC_{50} = 24,14 ± 2,6 µM), indicando ser menos citotóxico. O tratamento com epoximetil-lausona em lesões da pata de camundongos BALB/c levou a uma redução significativa de 27% ($p < 0,05$) do tamanho da lesão para todas as doses administradas, em comparação com o grupo controle. A redução da lesão também foi detectada após o tratamento com antimoniato de meglumina, atingindo 31,0% (0,23 mg de Sb^{5+}/Kg /dia e 2,27 mg de Sb^{5+}/Kg /dia) e 64,0% (22,7 mg de Sb^{5+}/Kg/dia). Além disso, alterações ultraestruturais de formas amastigotas da lesão de camundongos foram evidenciadas.

O conjunto de dados reunidos aqui indica que o epoximetil-lausona tem efeitos pronunciados sobre parasitos e merece avançar para o estágio pré-clínico. Com esta publicação alcançamos os objetivos relativos ao estudo dos efeitos leishmanicidas do epoximetoxi-lauson e antimoniato de meglumina sobre macrófagos de camundongos BALC/c *in vitro* e os efeitos destes fármamcos sobre o controle da infecção causada por *L.* (*L.*) *amazonensis* neste modelo experimental.

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Antileishmanial Activity of 2-Methoxy-4H-spiro-[naphthalene-1,2′-oxiran]-4-one (Epoxymethoxy-lawsone): A Promising New Drug Candidate for Leishmaniasis Treatment

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Abstract: Epoxymethoxy-lawsone is a naphthoquinone derivative promising as drug candidate for the treatment of leishmaniases. In the present work the effectiveness of Epoxymethoxy-lawsone, and meglumine antimoniate on *Leishmania* (*Leishmania*) *amazonensis* parasites and on mice paw lesions of infected BALB/c mice was assessed. In an intracellular amastigotes assay, the half-maximal inhibitory concentration (IC_{50}) value for Epoxymethoxy-lawsone was slightly higher (1.7-fold) than that found for meglumine antimoniate. The efficacy of both drugs became more evident after 48 h of exposure when either the oxirane compound and reference drug reached 18-fold and 7.4-fold lower IC_{50} values (0.40 ± 0.001 μM and 0.60 ± 0.02 μM), respectively. Promastigotes were also affected by Epoxymethoxy-lawsone after 24 h of incubation (IC_{50} = 45.45 ± 5.0 μM), but with IC_{50} 6-fold higher than those found for intracellular amastigotes. Cytotoxicity analysis revealed that Epoxymethoxy-lawsone (CC_{50} = 40.05 ± μM) has 1.7-fold higher effects than meglumine antimoniate (CC_{50} = 24.14 ± 2.6 μM). Treatment of the paw lesion in infected BALB/c mice with epoxymethoxy-lawsone led to a significant 27% reduction ($p < 0.05$) of the lesion size, for all administrated doses, compared to the control group. Lesion reduction was also detected after mice treatment with meglumine antimoniate, reaching 31.0% (0.23 mg of Sb(V)/Kg/day and 2.27 mg of Sb(V)/Kg/day) and 64.0% (22.7 mg of Sb(V)/Kg/day). In addition, mice lesion ultrastructural changes were evidenced in amastigotes. The set of data gathered here indicate that Epoxymethoxy-lawsone has pronounced effects on parasites and merits furthering to the preclinical stage.

Keywords: antileishmanial activity; oxiranes; naphthoquinones; epoxymethoxy-lawsone; meglumine antimoniate

1. Introduction

Leishmaniasis is classified as one of the neglected tropical diseases by the World Health Organization which estimates that 350 million people are at risk of contracting this infection, while nearly two million new cases occur annually [1]. The infection is caused by more than 20 *Leishmania* species, which are transmitted by inoculation of promastigote forms in humans through the bite of infected female phlebotomine sandflies. In the mammalian host, these parasites differentiate into amastigote forms inside cells and affect skin, mucosa, and cartilage, causing cutaneous leishmaniasis (CL). However; some species can infect internal tissues and organs, such as the liver, spleen, and bone marrow, causing visceral leishmaniasis (VL). Mucosal leishmaniasis (ML) is a metastatic outcome of the cutaneous form in which the parasites become disseminated to the oropharyngeal mucosa [2]. The epidemiology of leishmaniasis depends on the characteristics of the parasite species, the ecological features of the transmission sites, and the degree of current or past exposure of the population to the parasite. Furthermore, the risk factors of transmission are linked to socioeconomic and environmental patterns, which can make disease control more difficult [3].

Despite some important recent advances in the diagnosis, treatment and cost reduction of key drugs, both mortality and morbidity show a worrying increasing trend worldwide. This can be attributed to several factors, including lack of a vaccine, ineffective vector control and limitations of current drugs used to treat the infection [4,5].

Pentavalent antimonials, such as meglumine antimoniate (Figure 1), have been used since the 1940s and remain the first-choice drugs to treat all clinical forms of leishmaniasis, due to the even higher risks of toxicity associated with the second line drugs amphotericin B and pentamidine. These second line drugs are only used when there is a contraindication, intolerability or resistance to the first line drugs [6]. Nevertheless, pentavalent antimonials are frequently associated with high frequencies of mild to severe adverse effects, including musculoskeletal pain, gastrointestinal disorders, headache and anorexia, as well as cardiac, hepatic and pancreatic toxicity, leading in some cases to death [7]. At the same time, the large pharmaceutical companies have made little investment in research to develop therapeutic alternatives for leishmaniasis which explains the paucity of compounds and formulations with low toxicity and proven effectiveness in clinical use.

Consequently, the search for plant products is gaining special attention because they are theoretically more accessible, usually cheap and can be made accessible to lower income population who are the most affected by the disease [8]. A variety of natural products obtained from plant extracts has proved to be active against *Leishmania* species. Among these, the 1,4-naphthoquinones are considered attractive structures in medicinal chemistry due to their biological activities and chemical properties [9]. Examples of 1,4-naphthoquinones that have shown activity against *Leishmania* species and *Trypanosoma cruzi* are lapachol, isolated from Brazilian trees belonging to the genus *Tabebuia* and its derivatives α-lapachone and β-lapachone [10]. Most of the lapachone derivatives however, exhibit significant toxicity that limits their potential as new drugs [11].

In a search for less toxic derivatives for mammalian cells, chemical modification of the quinonoid center of α-lapachone and 2-hydroxy-1,4-naphthoquinone (lawsone) followed by an epoxidation, generated the oxiranes epoxy-α-lapachone and Epoxymethoxy-lawsone (Figure 1), respectively [12]. We have demonstrated that epoxy-α-lapachone was capable to kill promastigote forms of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* and intracellular amastigotes in human macrophages [13]. Furthermore, reduction of the lesion size in the paw of BALB/c mice infected was observed after four weeks of treatment [14].

Previous data showed that Epoxymethoxy-lawsone has a significant effect on control of BALB/c mice paw lesion caused by *L.* (*L.*) *amazonensis* [16]. In the present study, unequivocal evidence is presented of the antileishmanial activity of this oxirane compound on intracellular amastigotes and promastigote forms as well as in the control of the paw lesion caused by *L.* (*L.*) *amazonensis*.

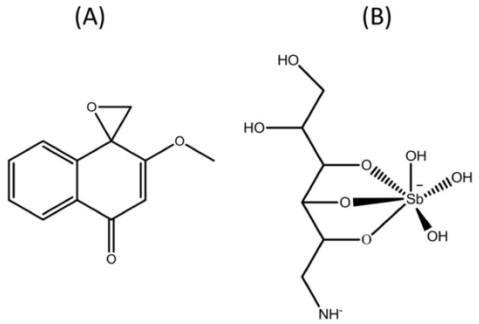

Figure 1. Chemical structure of drugs. (**A**) 2-methoxy-4H-spiro[naphthalene-1,2′-oxiran]-4-one, also known as Epoxymethoxy-lawsone ($C_{12}H_{10}O_3$, 202.21 g/mol) and (**B**) meglumine antimoniate known commercially as Glucantime® ($C_7H_{18}NO_8Sb$, 365.98 g/mol—structure proposed by Frézard et al. [15]).

2. Results

2.1. Drug Effects against Intracellular Amastigotes and Promastigotes of Leishmania (L.) amazonensis

BALB/c mice macrophages infected with *L.* (*L.*) *amazonensis* treated with Epoxymethoxy-lawsone showed a significant decrease in the number of viable parasites compared to control cultures. Reference drug meglumine antimoniate also exhibited significant effects. Both drugs were able to kill intracellular amastigotes in a dose-dependent manner at 24 and 48 h of exposure (Figure 2). All concentrations tested resulted in endocytic index (EI) lower than the control groups incubated with medium or 0.8% DMSO (Figure 2). The inhibitory effect on the multiplication rate at the highest concentration of epoxymethoxy-lawsone (25 µM) was 82.2% and 98.3% at 24 and 48 h, compared to the controls EI = 2980 and EI = 2816, respectively. Meglumine antimoniate (4.1 µM) also caused a pronounced inhibitory effect on the parasite multiplication (46% and 88.4% at 24 and 48 h, respectively). Half-maximal inhibitory concentration (IC_{50}) determined for epoxymethoxy-lawsone in 24 h was about 18-fold higher than 48 h, while for meglumine antimoniate the difference between two incubating times was 7.4-fold (Table 1). To evaluate possible macrophage activation by the drugs, we determined nitric oxide (NO) production in the cultures supernatants, however no significant change in the NO levels for both treatments were observed (0.9 ± 0.01 mM) compared to the control group (data not shown).

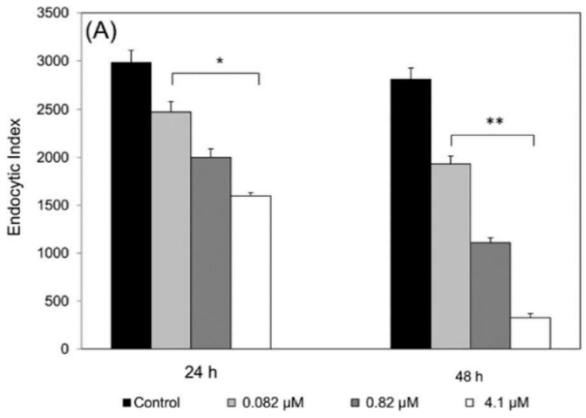

Figure 2. *Cont.*

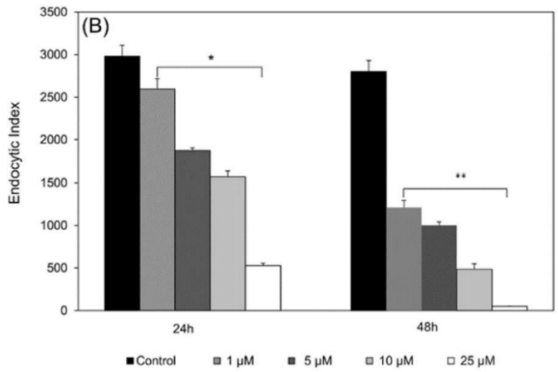

Figure 2. Effects of the drugs on the endocytic index of the *Leishmania* (*L.*) *amazonensis* amastigotes in mice macrophages. Meglumine antimoniate (**A**) and epoxymethoxy-lawsone (**B**) were co-incubated in cultures of BALB/c mice macrophages infected with *L.* (*L.*) *amazonensis* for 24 h and 48 h. Control cultures (black bars) were treated with RPMI 1640 medium only or with 1% of DMSO, respectively. The results are expressed as the mean and standard deviation of three assays. All concentration points analyzed showed statistical significance from their respective controls: (*) $p \leq 0.042$; (**) $p \leq 0.009$; (***) $p \leq 0.0003$).

Table 1. Effects of drugs on the murine macrophage cells and on the *Leishmania* (*L.*) *amazonensis* parasites.

Drug	CC_{50} (µM)	IC_{50} (µM)		Selectivity *	Specificity **
	Murine macrophage 72 h	Promastigote 24 h	Intracellular amastigote 24 h	Intracellular amastigote 48 h		
Epoxymethoxy-lawsone	40.05 ± 3.0	45.45 ± 5.0	7.41 ± 0.2	0.40 ± 0.001	5.40	6.13
Meglumine antimoniate	24.14 ± 2.6	ND	4.43 ± 0.25	0.60 ± 0.02	5.45	ND

The values are expressed as concentration of drugs (µM) causing 50% of cellular cytotoxicity (CC) and inhibitory concentration of parasite multiplication, (IC) effects and represent the average and standard deviation (±) of three independent experiments. Data of murine macrophages CC_{50} and promastigotes IC_{50} were obtained by ATP-bioluminescence, and intracellular amastigote IC_{50} by endocytic index assays. Selectivity (*) is defined as the ratio between parasite IC_{50} and murine macrophages CC_{50}. Specificity (**) is the ratio between promastigote IC_{50} and intracellular amastigote IC_{50}. Specificity values higher than two were chosen to define a compound as more active against the intracellular amastigote stage.

Epoxymethoxy-lawsone also inhibited the growth of free-living promastigote and the IC_{50} determined for 24 h was six-fold higher than that found for intracellular amastigotes, suggesting that the oxirane compound has a stage-specific effect. Table 1 presents the selectivity and specificity indexes for two drugs. Selectivity values for both drugs were very similar (>5.00), indicating that the toxic concentration for a mammalian cell (in this case murine macrophages) is five-fold higher than those toxic to the parasite. Additionally, we have assessed the effects of Epoxymethoxy-lawsone in human macrophages infected by *L.* (*L.*) *amazonensis* (data not shown). In these assays, the drug at concentrations of 1 µM and 10 µM caused a reduction of 65.4% and 87.9%, respectively, when compared with control (0.8% DMSO).

2.2. Effects In Vivo of Treatment on Experimental Cutaneous Lesions Caused by Leishmania (L.) amazonensis

BALB/c mice treated with three different concentrations of Epoxymethoxy-lawsone and meglumine antimoniate showed significantly reductions in the lesion size after 4 weeks of treatment compared to the control group (Figure 3). Maximum lesion sizes measure after 11 weeks post-infection was 5.8 ± 0.11 mm^3 in control group treated with vehicle. Despite the fact no dose-response association was observed in the Epoxymethoxy-lawsone treatment, the effects of the compound on lesion reductions (27.3%, 27.5% and 27.2% for 11.4 mg/Kg/day, 1.14 mg/Kg/day and 0.11 mg/Kg/day, respectively) were all statically significant of those detected in the vehicle groups ($p < 0.03$). Lesion reduction was also observed in mice groups which received the intermediate and lower doses of

meglumine antimoniate, reaching to 31.0% (0.23 mg of Sb(V)/Kg/day and 2.27 mg of Sb(V)/Kg/day) of untreated group ($p < 0.03$). The highest lesion reduction value was observed in the mice group treated with meglumine antimoniate at a dose of 22.7 mg of Sb(V)/Kg/day, reaching 64% of the control value ($p < 0.02$).

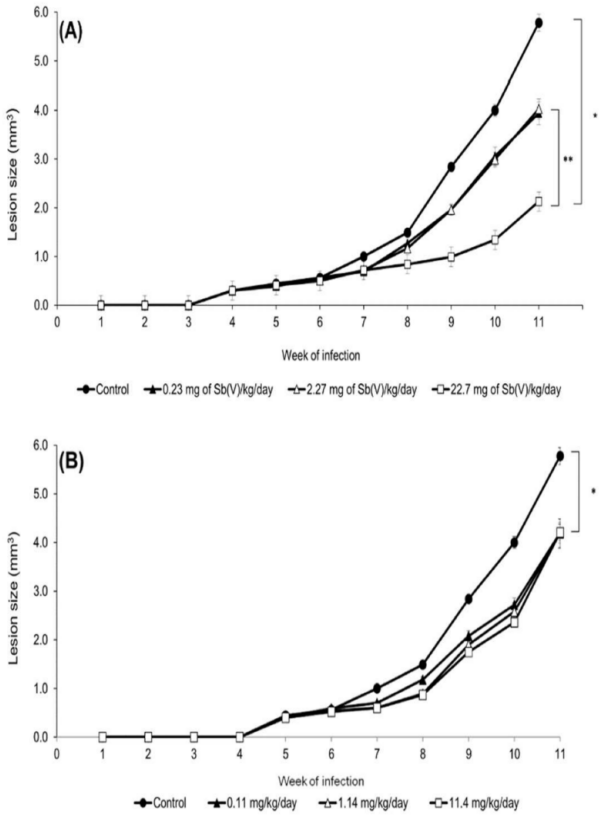

Figure 3. Treatment of experimental infection in BALB/c mice caused by *Leishmania* (*L.*) *amazonensis*. Mice were inoculated subcutaneously, in the left footpad, with 1.0×10^4 promastigotes at the logarithmic phase of growth. After 4 weeks of infection, mice were treated daily with meglumine antimoniate (**A**) or Epoxymethoxy-lawsone (**B**) at three different concentrations administered subcutaneously in groups with five animals. The control group was treated with a mix of DMSO/propylene glycol/saline (1:9:10). The lesion sizes were measured weekly and the results are represented as means with standard deviations from three independent experiments. Analyzed points exhibited significant differences from the control, (*) $p \leq 0.03$, and with the groups, (**) $p \leq 0.02$.

2.3. Effects of the Treatments in BALB/c Skin Lesions by Light Microscopy and Transmission Electron Microscopy

The skin lesions of untreated and treated mice groups were examined regarding the presence of both amastigotes and parasitophorous vacuoles (PV) which are indicators of the infection. Semiquantitative analysis of the skin lesion fragments showed a dose-response effect in the reduction of parasite load and number of vacuoles for Epoxymethoxy-lawsone treatment compared to untreated group, while mice treated with higher and intermediate doses of meglumine antimoniate exhibited no difference in the parasite load (Table 2 and Figure 4). In the lower doses, we have observed a similar quantity of vacuoles of those found in the untreated group; however, the parasite load was lower in the treated groups (Table 2).

Table 2. Semiquantitative analysis of BALB/c mice skin lesions caused by *Leishmania* (*L.*) *amazonensis*.

Mice Groups	Treatment Dose (mg/kg/Day)	Vacuoles	Amastigotes
Untreated	-	++++	++++
Epoxymethoxy-lawsone	11.4	+	+
	1.14	+	+
	0.11	++++	+++
Meglumine antimoniate	22.7	++	++
	2.27	+++	++
	0.23	++++	+++

(+) = mild, (++) = moderate, (+++) = severe, (++++) = severe-diffuse.

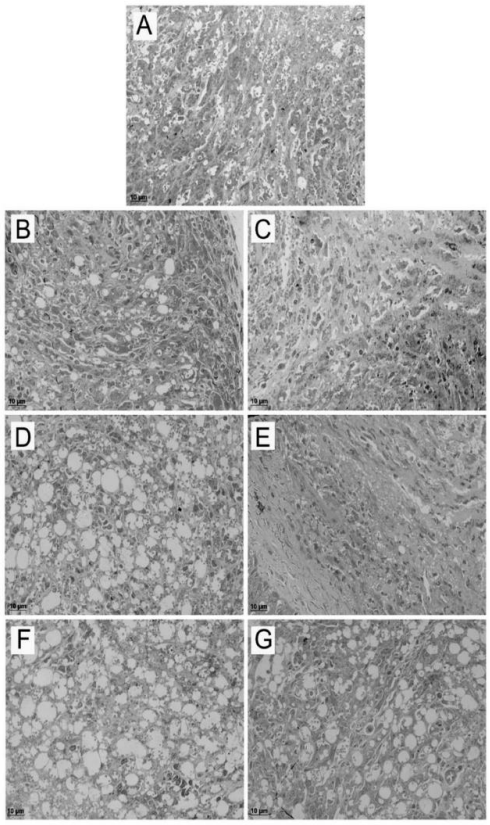

Figure 4. Analysis of mice lesions by light microscopy. Semithin sections of mice skin lesions of untreated (**A**) and treated groups with three doses of meglumine antimoniate (**B**: 22.7 mg of Sb(V)/kg/day; **D**: 2.27 mg of Sb(V)/kg/day; **F**: 0.23 mg of Sb(V)/kg/day) and Epoxymethoxy-lawsone (**C**: 11.4 mg/kg/day; **E**: 1.14 mg/kg/day; **G**: 0.11 mg/kg/day). Lesions were extracted one week after the end of a four weeks treatment course. The images are representatives of ten fragments of each group mice.

Ultrastructural changes in amastigotes from skin mice lesion were assessed by transmission electron microscopy. The ultrastructural analysis of untreated amastigotes within parasitophorous vacuoles (PV) demonstrating their normal characteristics such as rounded or oval shape and cytoplasmic components as bar-shaped kinetoplast (K), nucleus (N) and flagellar pocket (Fp) with emerging flagellum (Figure 5A). Amastigotes from mice groups exposed to both drugs exhibited different degrees of damage. Amastigotes (P) from meglumine antimoniate group showed dense nuclear chromatin with an altered profile and, in most images analyzed, the nucleoli were not

observed (Figure 5B). Mice treated with Epoxymethoxy-lawsone, showed amastigotes from their lesions presenting rarefied cytoplasm (asterisk), kinetoplast with an atypical condensation (arrow head) and either a very dense chromatin (thin arrows) and nucleolus (Nu) (Figure 5C, D). Several of these ultrastructural alterations were also detected in the meglumine antimoniate treatment mice group (data not shown).

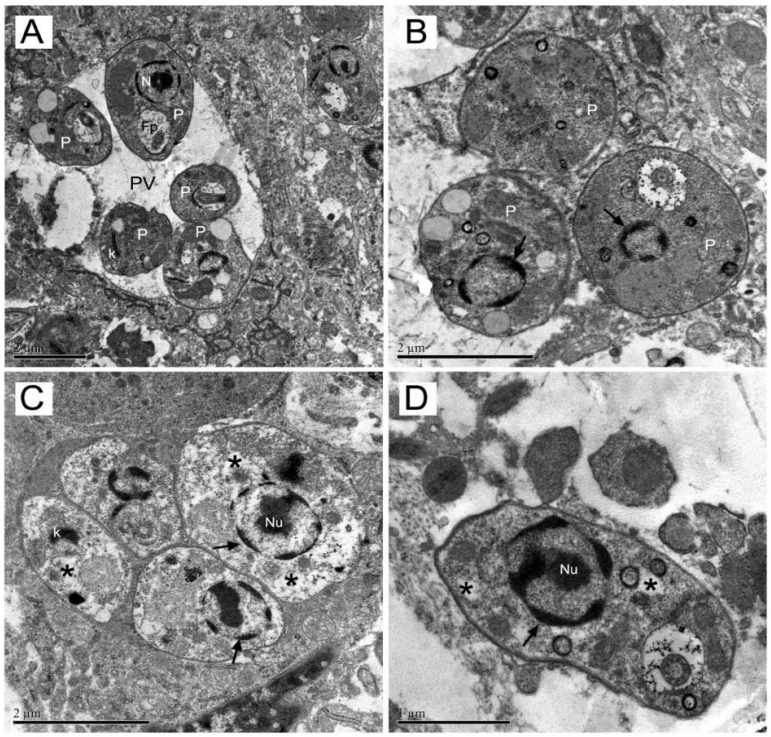

Figure 5. Transmission electron microscopy of mice skin lesion. The ultrastructural analysis of amastigotes from lesion were performed in untreated (**A**) or mice treated with meglumine antimoniate or Epoxymethoxy-lawsone at higher doses (22.7 mg of Sb(V)/kg/day and 11.4 mg/kg/day, respectively). In (**A**): amastigotes with no morphological changes within parasitophorous vacuoles (PV); bar-shaped kinetoplast (K); nuclei (N) and flagellar pocket (Fp). In (**B**): Amastigotes (P) show dense nuclear chromatin (thin arrow) with altered profile and absence of nucleoli (→). In (**C**): Amastigotes (P) with rarefied cytoplasm (*); kinetoplast with an atypical condensation (k) and dense nuclear chromatin (→) and nucleolus (Nu). In (**D**): Amastigote (P) shows dense nuclear chromatin with altered profile and absence of nucleoli. The images are representatives of ten selections of each group mice.

3. Discussion

Leishmaniasis remains as one of the most neglected tropical diseases, affecting mainly the poorer populations in developing countries. Current treatment, based on pentavalent antimony, is associated with severe side effects, including cardiotoxicity, hepatotoxicity and pancreatic toxicity [7]. Furthermore, high cost and technological dependence should be considered by endemic countries, increasing the need for new, more efficient and less toxic drugs. In this scenario, we highlight plant-derived compounds such as the naphthoquinones and its derivatives. Recently studies have demonstrated antileishmanial activity of epoxy-α-lapachone on macrophage infection and in the treatment of experimental murine infection with low cytotoxicity in mammalian cells [13]. Here, we tested Epoxymethoxy-lawsone, a new oxirane derivative that also exhibited lower toxicity on mammalian cell against both in vitro and in vivo infection.

The approach proposed here to evaluate leishmanicidal effects of Epoxymethoxy-lawsone was successful since we have proved its in vitro action on the multiplication rate of intracellular amastigotes and promastigotes growth, as well as in vivo ability to control the experimental mice paw lesion infection induced by *L.* (*L.*) *amazonensis*. In this study, we administrated meglumine antimoniate as reference control due to be a first-choice drug for all forms of human leishmaniasis treatment. Our results showed a similar selectivity index for both drugs, and their IC_{50} values were slightly different. Besides that, reference drug showed higher cytotoxicity on macrophages than oxirane compound (1.7-fold). Selectivity index is a key parameter to consider when developing antimicrobial compound and the higher reach this value the safer it will be, in theory, the therapeutic use of the drug, as exhibited for Epoxymethoxy-lawsone here.

Recently, a series of recommendations on criteria for selecting hit and lead compounds in drug discovery was published [17]. Selectivity values may vary considerably according to the microorganism and its environment in the host, but general recommendation is that this value should be greater than 10-fold using a mammalian cell line, as HepG2 or Vero cells [17]. Indeed, one of the most crucial elements for the success of drug screening is to apply assays that faithfully reproduce the microenvironment of a pathogen causing the disease. For this reason, we used murine macrophages due to this type of cell is the main target of *Leishmania spp* infection. Additionally, Epoxymethoxy-lawsone demonstrated other relevant parameters for the *Leishmania* life cycle that is the specificity by the intracellular stage. It should be noted that Epoxymethoxy-lawsone showed higher potency than epoxy-α-lapachone in infected macrophage cultures, represented by an IC_{50} value about five-fold lower for 24 h of exposure (7.41 μM compared to 37 μM), with similar cytotoxicity profile [13].

We have evidence that Epoxymethoxy-lawsone reduced of lesion size in BALB/c mice paws due decreasing the parasite load, but not by reduction of the inflammatory process only, since was not possible to distinguish the lesion reduction effects among the three doses tested. In addition, this idea is reinforced by the observation that both Epoxymethoxy-lawsone and meglumine antimoniate did not induce nitric oxide production in BALB/c mice macrophages infection. It is important to emphasize that drug efficacy measured solely by changes in lesion size can be misleading since a typical lesion is composed by a complex profile of inflammatory cells and amastigotes contained in vacuoles within macrophages of the skin [18].

In addition, the effects of drugs were perceived by the presence of vacuoles organelles with remaining parasite after elimination, mainly in mice groups treated with meglumine antimoniate as result of lesions treated by an effective drug. On the other hand, in the Epoxymethoxy-lawsone groups, lesion tissues showed a lower number of vacuoles. These depleted organelles were extensively detected, indicating a drastic reduction of parasite load.

To prove the drastic effect of treatments on the parasites from paw lesions, we decide to investigate ultrastructural alterations suffered by amastigotes to assess the integrity of parasites in skin tissue. Transmission electron microscopy analysis applied here revealed drastic changes in amastigotes ultrastructure which clearly affected parasite integrity. Epoxymethoxy-lawsone caused more pronounced changes than the reference drug.

Some of these alterations, such as those found in the amastigotes nuclei, are indicative of apoptosis events [19]. We are sure that those disorders found are in accordance with amastigotes inability to multiply in the macrophage mice lesions thus decreasing the paw lesion size in treated mice.

The data presented here suggest that Epoxymethoxy-lawsone is capable of crossing the plasma membrane of the macrophages and acts by directly killing amastigotes, but the mode of action of the drug on *L.* (*L.*) *amazonensis* is not well known yet. As this compound is also derived from the α-lapachone molecule by an epoxidation reaction, and shares most of chemical and structural features with epoxy-α-lapachone, is possible that the new oxirane also acts by inhibiting proteases from *Leishmania* sp. and other trypanosomatids, as previously reported [12,14,20,21], however further studies are needed to prove this hypothesis.

4. Materials and Methods

4.1. Chemicals and Culture Material

Dimethyl sulfoxide (DMSO), osmium tetroxide solution (OsO_4), Epoxy Embedding Medium kit (Epon), penicillin, streptomycin, Lab-Tek chamber slides, Greiner CELLSTAR® 96 well plates, RPMI 1640 medium and Schneider's Drosophila medium were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal calf serum (FCS) was acquired from Cultilab S/A (São Paulo, Brazil). CellTiter-Glo® luminescent cell viability assay was acquired from Promega Corporation (Madison, WI, USA). Meglumine antimoniate (Glucantime®) was kindly provided by Dr. Armando de Oliveira Schubach team (INI/Fiocruz). Propylene glycol was obtained from Vetec Quimica (Rio de Janeiro, Brasil). The Epoxymethoxy-lawsone compound was synthesized by the Department of Organic Chemistry of the Instituto de Química, Universidade Federal Fluminense and the powder was stored at 2 to 8 °C until its further use in assays.

4.2. Cell Culture

Peritoneal macrophages were harvested from BALB/c mice as previously described [22]. Cells were recovered after centrifugation (2 ×, 1800 × *g*, 10 min, 4 °C) in RPMI 1640 medium containing 10% FCS. Subsequently, cells were seeded at a density of 5×10^5 cells/well in Lab-Tek chamber slides and maintained at 37 °C in a 5% of CO_2 atmosphere for 24 h. Non-adherent cells were removed by washing the culture plates with RPMI 1640 medium. Monolayers of murine peritoneal macrophages were used in leshmanicidal assays.

4.3. Parasite Cultures

Leishmania (*Leishmania*) *amazonensis* (strain MHOM/BR/73/LTB0016) was obtained from the *Leishmania* collection (Coleção de Leishmania do Instituto Oswaldo Cruz—CLIOC) of the Instituto Oswaldo Cruz (IOC). In vitro promastigote cultures were maintained at 28 °C in Schneider's medium (pH 7.2) containing 1 mM L-glutamine, 10% FCS, 100 IU/mL penicillin, and 100 µg/mL streptomycin, with frequent subpassages to maintain the parasites in the logarithmic growth phase.

4.4. Activity against Promastigotes

Parasites were seeded on 96-well plates (1×10^5 per well) in Schneider's medium, were treated for 24 h at 28 °C with Epoxymethoxy-lawsone in a concentration ranging from 1.6 µM to 100 µM. Parasite viability was assessed by measuring ATP production using CellTiter-Glo® (50 µL/well) and the luminescent signal was measured using a FlexStation 3 reader (Molecular Devices, Sunnyvale, CA, USA) [23]. Drug efficacy determined by half maximal inhibitory concentration (IC_{50}) was calculated by linear regression.

4.5. Activity against Intracellular Amastigotes

To evaluate the activity of compounds against intracellular amastigotes, macrophages were infected by promastigotes in a proportion of 10:1 (parasite:cell) for 4 h of interaction, at 37 °C followed by washing with PBS and addition of RPMI medium containing 5% FCS. After 4 h of infection, the cultures were treated for 24 h or 48 h at 37 °C with Epoxymethoxy-lawsone (1 to 25 µM) and meglumine antimoniate (0.082 to 4.1 µM) and then, fixed with 100% methanol and Giemsa-stained. The level of infection and number of intracellular parasites was determined by random counting of at least 300 cells. The endocytic index was calculated by multiplying the percentage of infected cells by the mean number of parasites per infected cell. Drug inhibition concentrations (IC_{50}) were calculated by regression analysis of dose-response curves. Selectivity index (SI) was calculated by the following formula: SI = CC_{50} (macrophage cytotoxicity) divided by IC_{50} (antiparasitic activity) for both drugs. The experiments were carried out in triplicate.

4.6. Toxicity to Mammalian Cells

BALB/c mice macrophages grown on 96-well plates were treated with epoxymethoxy-lawsone (1.6 to 100 μM) and meglumine antimoniate (4.1–200.0 μM of Sb^{5+}) for 72 h at 37 °C. Then, macrophages viability was determined by incubation with CellTiter-Glo® (20 μL/well) for 3 minutes at room temperature under agitation. Luminescence was measured using a FlexStation 3 reader (Molecular Devices). The CC_{50}, concentration of compound that reduces 50% of mammalian cell viability was determined by linear regression. Control was incubated with dimethyl sulfoxide (DMSO) in concentrations ≤1%.

4.7. Experimental Murine Infection

Experimental infection was conducted with 5- to 7-week-old BALB/c mice weighing approximately 22 g. Mice were inoculated in the footpad of the left hind limb with 1.0×10^4 promastigotes of *L.* (*L.*) *amazonensis* in the stationary growth phase (after 5 days of culture in Schneider's medium) in a total volume of 50 μL of phosphate-buffered saline (PBS) at 10 mM.

4.8. Mice Treatment Schedules

The experimental treatments were performed with either antimoniate meglumine, as a comparative control for treatment efficacy and Epoxymethoxy-lawsone diluted in a mixture of DMSO/propylene glycol/saline (1:9:10, defined as vehicle) in the following doses: 22.7 mg of Sb(V)Kg/day, 2.27 mg of Sb(V)/Kg/day and 0.23 mg of Sb(V)/Kg/day (corresponding to 4.1 μM, 0.41 μM and 0.041 μM of Sb(V)) and 11.4 mg/Kg/day, 1.14 mg/Kg/day and 0.11 mg/Kg/day (corresponding to 1.2 μM, 0.12 μM, and 0.012 μM of oxirane compound). Drugs were administered subcutaneously in the dorsal region of each mouse in a dose of 100 μL per animal. Treatments were carried out for four weeks with daily injections (five consecutive days with a two-day pause until 20 doses), starting four weeks after challenge infection, when the paw lesions had already become noticeable. Negative-control groups were treated with vehicle used to dissolve the oxirane compound. The lesions were evaluated weekly by measuring the height and width of the paw and calculating lesion areas (obtained by multiplying these measures in mm^2) with a digital caliper.

4.9. Processing of Samples for Transmission Electron Microscopy

Lesions were excised with a surgical scissors after 20 days of treatment (50 days post infection), washed in PBS and fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer pH 7.2 with 3.5% sucrose for 1h/4 °C. Samples were post-fixed with 1% OsO_4 (1 h/4 °C) in cacodylate buffer and, after washing, the samples were dehydrated in serial acetone concentrations (30%, 50%, 70%, 90% and 100%). Finally, samples were embedded in PolyBed 812 resin and polymerized at 72 h/ 60 °C. After polymerization, semi-thin sections were made in an Ultracut S ultramicrotome (Leica, Vienna, Austria) stained with toluidine blue and eosin and analyzed under a light microscope - Axio imager 2 (Zeiss, Göttingen, Germany). After selecting the areas of interest, ultra-thin sections were obtained in Leica Ultracut S ultramicrotome, collected in 300 mesh copper grids, contrasted with 5% and lead citrate and analyzed in the transmission electron microscope - JEOL JEM-1011 (Boston, MA, USA).

4.10. Semiquantitative Analysis of Fragments

All fragments were semiquantitatively assessed based on the intensity and focal or diffuse character of the infection, considering presence of vacuoles and quantity of amastigotes and the results were plotted as the media of both amastigote number and vacuoles in macrophages. For each fragment a parameter was assigned with a numerical value between + and ++++, according to the intensity and extent of the infection: + = mild, ++ = moderate, +++ = severe, and ++++ = severe-diffuse. The samples were analyzed under light microscope (Zeiss Axio imager m2).

4.11. Ethical Aspects

Mice experimental procedures performed here were approved by the Committee for the Ethical Use of Animals of Instituto Oswaldo Cruz (L-052/2015). The animals were obtained from the animal breeding center of Fundação Oswaldo Cruz (Fiocruz). The additional assays with human cells from healthy donors were approved by the Committee of Ethics Fiocruz (C.E. Fiocruz protocol number 535/09).

4.12. Statistical Analysis

To compare results, Student's test was applied; data matrices were considered statistically different when the P value was less than 0.05. Statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA).

5. Conclusions

The set of results gathered here prove that chemical modification made on the 2-hydroxy-1,4-naphthoquinone generated another effective and with low toxicity derivative from same chemical synthesis series of other oxirane compounds **12**. We are sure that Epoxymethoxy-lawsone has reached the minimum requirements to advance to the preclinical stage, including in vitro and in vivo complementary tests.

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Author Contributions: C.R.A. and L.F.G.O. conceived and designed the experiments; L.F.G.O., F.S.S., L.M.C.C. and L.C.F. performed the experiments; C.R.A., L.F.G.O., M.C.S.P., F.O.O.J., R.T.P. and S.C.R analyzed the data; C.R.A, M.C.S.P., R.T.P., S.C.R, S.C.B and V.F.F. contributed reagents/materials/analysis tools; C.R.A. and L.F.G.O. wrote the paper.

Conflicts of Interest: The authors declare no conflict of interest.

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Sample Availability: Samples of the compounds are not available from the authors.

4.3 DOCUMENTO 3

Oliveira LFG, Souza-Silva F, de Castro Côrtes LM, Veloso LB, Cysne-Finkelstein L, de Pereira BAS, Lechuga GC, Bourguignon SC, Ferreira VF, Alves CR. Enhancing of leishmanicidal effect of the association of meglumine antimoniate and oxiranes (epoxy-α-lapachone and epoxymethoxy-lawsone) in an experimental challenge model in mice infected by *Leishmania (Leishmania) amazonensis* (artigo submetido a publicação).

Na última etapa do trabalho, foram estudados os efeitos da associação do antimoniato de meglumina (AM) com os oxiranos epoxi-α-lapachona (LAP) e epoximetoxi-lauson (LAU) sobre a infecção *in vitro* (macrófagos) e *in vivo* (camundongos BALB/c), causadas por *Leishmania (Leishmania) amazonensis*.

Os compostos foram testados individualmente e em combinações seguindo as razões fixas 3:1; 1:1 e 1:3 (p/v) sobre amastigotas intracelulares. Todos os fármacos, assim como suas combinações, apresentaram índices endocíticos muito inferiores ao grupo controle. Os maiores valores de redução foram obtidos quando AM/LAP e AM/LAU estavam na razão 3:1, chegando a 98,3% e 93,6% em relação ao controle, respectivamente. Camundongos BALB/c tratados com os compostos individualmente e nas mesmas razões mostraram reduções significativas no tamanho da lesão após quatro semanas de tratamento, em comparação com o grupo controle. Com relação aos fármacos isolados, LAP e LAU apresentaram melhores efeitos (40%), enquanto AM exibiu a menor redução observada (30%). O melhor efeito produzido na combinação também foi observado com AM/LAP e AM/LAW na razão 3:1 (38,3% e 45,5%, respectivamente), o que pode indicar que os oxiranos potencializam os efeitos leishmanicidas do AM.

Os resultados aqui mostrados indicam que o antimoniato de meglumina associado aos oxiranos apresenta um acréscimo na atividade leishmanicida sobre a infecção experimental *in vitro* e *in vivo* e pode ser considerada uma nova abordagem para o tratamento da leishmaniose cutânea.

Title:

Enhancing of leishmanicidal effect of the association of meglumine antimoniate and oxiranes (epoxy-α-lapachone and epoxymethyl-lawsone) in an experimental challenge model in mice infected by *Leishmania (Leishmania) amazonensis*

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Abstract

Cutaneous leishmaniasis is a neglected tropical disease which presents high incidence and morbidity rates, affecting mainly the poorest people in developing countries. Although many studies have been analyzing potential novel antileishmanial drugs, pentavalent antimonials remain the first-line drugs for leishmaniasis treatment. Their overall effects against Leishmania parasites are indubitable, but the therapy has shown severe adverse effects. An alternative to minimize this issue may be a treatment based on its combination with other drugs; an approach which could reduce the adverse effects while maintaining or even enhancing the effects against the parasites. In this study we analyzed the potential of the association of meglumine antimoniate (MA) with the oxiranes epoxy-α-lapachone (LAP) or epoxymethyl-lawsone (LAW), as a new approach to treat cutaneous leishmaniasis. We demonstrate that association between these drugs produces an increment in the leishmanicidal activity on *L. (L.) amazonensis* both in vitro and in vivo when compared to the activity of MA individually. The compounds were tested alone or in combinations (following fixed-ratios of 3:1; 1:1 and 1:3) against intracellular amastigotes in peritoneal macrophages being able to reduce intracellular parasites numbers, as measured by the endocytic index, in all conditions tested. The most effective conditions were the treatments with MA/LAP or MA/LAW in a 3:1 ratio where reductions of 98.3% and 93.6% in the endocytic index were observed, respectively. BALB/c mice challenged with *L. (L.) amazonensis* showed significant reductions in the lesions mean size after four weeks of treatment with the compounds alone or their associations in similar fixed-ratios. While MA, LAP or LAW alone were able to control lesions growth when compared to untreated animals (reduction of lesions mean size of 30%, 40% and 40%, respectively), the association of MA/LAW in 3:1 ratio presented the highest impact on lesion growth, leading to a reduction of lesion mean size of 45.5%. The results gathered herein indicate that the association of meglumine antimoniate to oxiranes leads to an increment in the antileishmanial activity and it is a promising approach for the treatment of cutaneous leishmaniasis.

Keywords: Combination treatment, meglumine antimoniate, oxiranes, epoxy-α-lapachone, epoxymethyl-lawsone, experimental leishmaniasis, *Leishmania (Leishmania) amazonesis*.

1. Introduction

Cutaneous leishmaniasis (CL) is an infectious disease, transmitted by female sandflies, which presents high incidence and morbidity rates and affects mainly the poorest people in developing countries, where access to health services is often precarious (1). The World Health Organization estimates that global prevalence is around 12 million cases per year, occurring in 98 countries, but with around 90% of the cutaneous and mucocutaneous cases concentrated in seven developing countries, including Brazil (2). Leishmaniasis is considered as an important neglected tropical disease due to its extensive distribution in tropical areas of the globe and risk to produce deformities (3).

Despite almost seven decades of researches assaying alternative therapies to replace pentavalent antimonials, these drugs remain the first-line treatment for most forms of leishmaniasis worldwide (4). Pentavalent antimonials therapy is associated to mild or severe adverse effects, being often accompanied by pain and swelling at site of the intramuscular application and several systemic symptons, which include nausea, vomiting, weakness and myalgia, abdominal colic, diarrhea, skin rashes and variable transient increase of transaminases and/or amylases (5, 6). The most serious adverse effect associated to pentavalent antimonials threrapy is undoubtedly cardiotoxicity, characterized by ventricular repolarization disorders (as alterations in T wave and ST segment) and arrhythmia, which can lead to sudden death (5, 6). The second-line drugs, applied when antimonials cannot be resorted, amphotericin B and pentamidine isethionate present even higher toxicity and thus their use are only recommended in cases of contraindication, intolerance, low therapeutic response or resistance to antimonials (7). These drugs are associated to a range of adverse effects, being the most serious nephrotoxicity, detected by a decreasing of glomerular filtration and insulin-dependent diabetes, respectively (8, 9, 10). Additionally, other antileishmanial medicines such as paramomycin, miltefosine, pentoxifylline and ketoconazole are also available in some countries; however, some are still under clinical investigation (2).

Drug combination therapy is a successful strategy in the treatment of several infectious diseases such as malaria, tuberculosis and AIDS, and has been applied for leishmaniasis treatment, mainly in endemic countries (2, 10). Treatments using combined drugs have potential advantages, such as (i) shortening the course of

treatment; (ii) minimize the risk of selection of drug-resistant parasites; and, (iii) reducing the total dose of medicines needed, thereby decreasing both incidence of adverse effects and costs (2, 11).

Approaches involving the pentavalent antimonials associated to other drugs have been conducted, but some results are still controversial (12, 13, 14, 15, 16). Even though there are known advantages of drug combination therapies for treating visceral leishmaniasis, as a markedly reduced risk of resistance development, the therapeutic decisions for applying these treatments must be based on the individual benefit/risk ratio of the drugs, the settings of the health service providing the treatment, the local availability of the antileishmanial medicines and epidemiological issues (2,17).

For CL, topical treatment can be used as a first treatment option whenever possible, but in many cases of New World CL, systemic treatment might be indicated due to the risk of mucosal spread. Besides, the risk of severe adverse effects is acceptable for patient suffers from numerous, face-disfiguring or complicated lesions, but not for those with a mild form of the disease (2, 11).

Naphtoquinones are natural compounds considered to be a promising approach for new drugs, as they were shown to have antibacterial (18, 19), antifungal (20-22), antiviral (23, 24), antitumor (25-30), antimalarial (31-33) and antileishmanial activities (34, 35). However, several of their derivatives exhibited significant toxicity, therefore presenting a difficulty for using these compounds in treatments. Recently, we have demonstrated that the oxiranes epoxy-α-lapachone (LAP) and epoxymethyl-lawsone (LAW) (Figure 1), synthetic compounds generated by epoxidation of one carbonyl in the quinonoid center of α-lapachone and 2-hydroxy-1,4-naphthoquinone (lawsone), were effective against *Leishmania* in both *in vitro* and *in vivo* experimental infections and showed low toxicity for mammalian cells (36, 37, 38).

LAP led to a dose- and time-dependent decrease in the growth rate of Leishmania (Viannia) braziliensis and *L. (L.) amazonensis* promastigote cultures, killed amastigotes inside human macrophages (36), and reduced the lesion mean size in BALB/c mice infected by *L. (L.) amazonensis* (37). LAW affected promastigotes viability after 24 h of exposure and demonstrated an activity on intracellular amastigotes similar to that of the reference drug meglumine antimoniate.

LAW was also able to hinder lesion growth in *L. (L.) amazonensis* infected BALB/c mice (38).

Studies focusing on identifying drug associations with promising leishmanicidal activities in experimental infection have been frequent, as these may lead to improving the efficacy of treatments and/or reducing the doses administered (39, 40). Thereby, following this research line, the objective of the present study is to determine whether association of meglumine antimoniate (MA) with epoxy-α-lapachone or epoxymethyl-lawsone may develop into a novel approach for the CL treatment. Herein, we demonstrate an enhanced leishmanicidal effect produced by such combination treatments on *L. (L.) amazonensis*, either in macrophage cultures infected by this parasite or in experimentally challenged BALB/c mice.

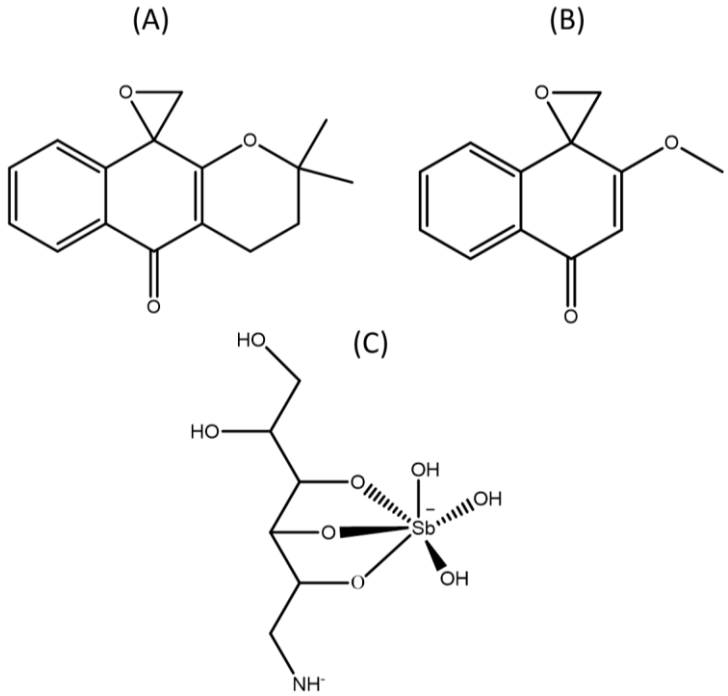

Figure 1: Chemical structure of used drugs. (A) 2,2-dimethyl-3,4-dihydrospiro[benzo[g]chromene-10,2'-oxiran]-5-one, also known as epoxy-α-lapachone ($C_{16}H_{16}O_3$, 256.296 g/mol; CID 12000280) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top), (B) 2-methoxy-4*H*-spiro[naphthalene-1,2'-oxiran]-4-one, also known as epoxymethyl-lawsone ($C_{12}H_{10}O_3$, 202.21 g/mol), and (C) meglumine antimoniate known commercially as Glucantime ($C_7H_{18}NO_8Sb$, 365.98 g/mol).

2. Results and Discussion

Leishmaniasis represents a major public health issue in the Americas due to their distribution in several countries and high prevalence (41). Treatment is a pivotal measure to control this disease; however effectiveness of current options have been questioned due to the increasing number of cases of resistance and toxicity, pointing toward the need for novel drug proposals (42). In recent years, efforts have been performed to improve antimonial chemotherapy. Several studies added further information on its chemical structure (43), mechanisms of action, new methods of preparation and potential incorporation into different formulations (as reviewed in 44). In this context, a promising strategy is the association of pentavalent antimonials with oxiranes, compounds that showed significant antileishmanial activity; such association could reduce the necessary drug doses, maintain or improve the baseline efficacy of pentavalent antimonials treatment and impact no increment in the treatment toxicity. In addition, a combination of two drugs with rather different mechanisms of action over the parasites may have the benefit of hindering the emergence of resistant strains. Therefore, we devised in the present study a new approach for the CL chemotherapy based on combination treatment of meglumine antimoniate with epoxy-α-lapachone or epoxymethyl-lawsone.

Initially, we investigated the combination effects of MA with the oxiranes LAP and LAW on peritoneal macrophages of BALB/c mice infected in vitro with L. (L.) amazonensis. The drugs were tested alone (at a concentration equivalent to half of IC_{50} values previously determined) or in combinations following fixed-ratios of 3:1; 1:1 and 1:3. All drugs as well as its combinations exhibited impact over the macrophage-infecting parasites, as determined by the endocytic index (EI): treated cultures had EI values lower than untreated cultures (Figure 1). The effects of MA or LAW alone on the parasites displayed a similar pattern: the EI was reduced in 82% and 80%, respectively. LAP alone showed the lowest impact over the parasites (EI reduced in 75%). Some of the drug combinations were sensibly more effective over the intracellular parasites: MA/LAP or MA/LAW at 3:1 ratio led to reduction of 98.3% and 93.6% reduction on the EI of the control, respectively. In relation to respective drugs alone, the ratio combinations showed reductions of 90.5% and 93.2% (MA/LAP) and 64.6 % and 67.7 % (MA/LAW). No significant difference in 1:3 and 1:1 ratios was detected. No significant difference in 1:3 and 1:1 ratios was detected. Cytotoxicity

assays performed using either the drugs alone or its combinations, in the ratios established above, indicated no significant change in the viability of treated macrophage cultures when compared to untreated controls, either culture medium alone or added with 0.8% DMSO, used as diluent of the compounds, (data not shown). This set of results established the feasibility of proceeding to in vivo experiments, aiming to analyze the effects of combination treatments on the progression of lesions in experimentally challenged mice.

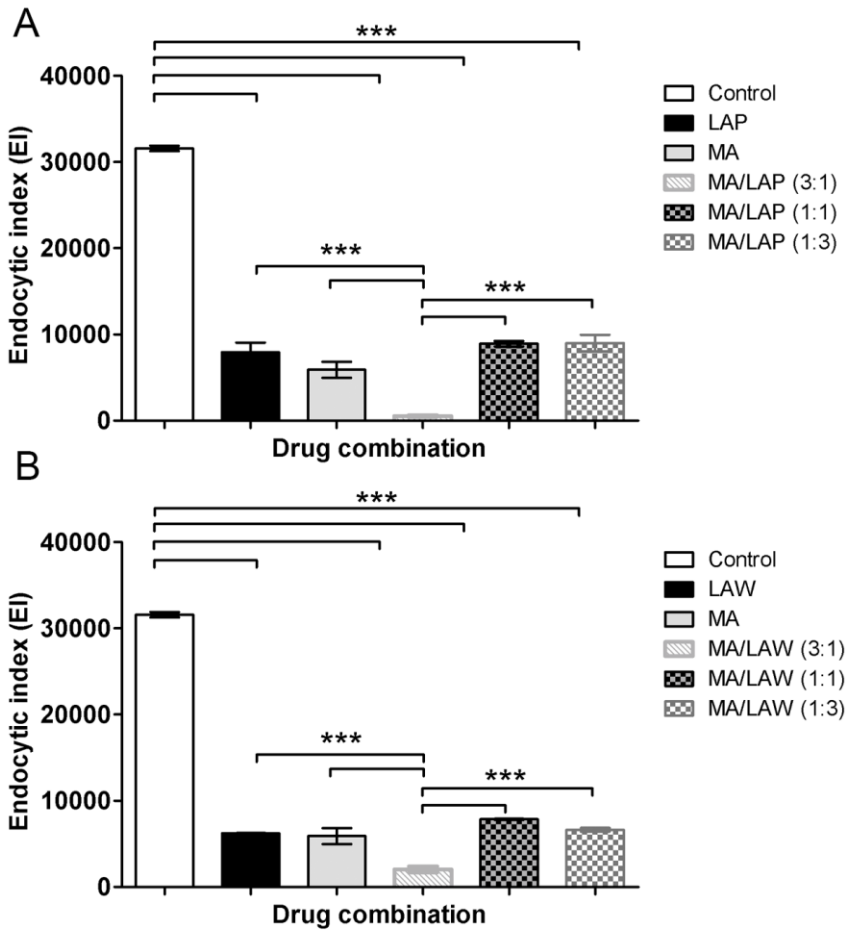

Figure 2: Effects of combination treatments with oxiranes and meglumine antimoniate on the endocytic index of in peritoneal murine macrophages infected *in vitro* with *Leishmania (L.) amazonensis*. Cultures of peritoneal macrophages isolated from BALB/c mice were infected with L. (L.) amazonensis and incubated (24 h, 37°C) with either meglumine antimoniate (MA), epoxy-α-lapachone (LAP) or epoxymethyl-lawsone (LAW) alone or combined in fixed-ratio of 3:1, 1:1 and 1:3. Control cultures (white bars) were treated with 0.8% DMSO. The endocytic index was determined by counting intracellular parasites in at least 300 random cells. The results are expressed as the mean and standard deviation of three independent assays. Difference between groups was analyzed with one-way ANOVA, Bonferroni post test. ***Statistically significant p≤0.001.

Previous data from our research group indicated that treatment of BALB/c mice challenged with *L. (L.) amazonensis* with either MA, LAP and LAW (in three distinct dosages) led to a measurable reduction in the mean size of parasite-associated lesions, pointing to a control of the in vivo infection. The tenth week post-infection was settled as the chosen time point for lesion size measurements. Considering that no dose-response pattern was observed for any of the applied dosages of LAW and LAP and that the highest dosage of these compounds elicited significant signs of tissue toxicity (45), the intermediate dosage was elected to perform the combination treatment assays.

As previously observed, the administration of MA, LAP or LAW impacted lesion development, leading to a reduction in lesion mean size, in treated challenged-mice when compared to un-treated challenged-animals from the fourth week after treatment. Similar results were observed for animals treated with combinations of the compounds (Figure 2A and 2C). The lesions mean size measured in the untreated animals 10 weeks post-infection was 10.7 ± 1.25 mm^3. Regarding the animals treated with only one of the assayed drugs, MA presented the lowest effect on reducing lesions mean size. Leading to a 30% decrease in lesions size, whereas both LAP and LAW presented better effects, decreasing lesions size in 40% (Figure 2B and D). As for the combination mixes test, the best result were observed for MA/LAP or LAW at 3:1 ratio (75% of MA and 25% of LAP or LAW) with the reduction of lesion mean size amounting 61.7 and 54.4%, respectively. Such data indicates that oxiranes may potentiate the leishmanicidal effects of MA. Additionally, the combination of MA/LAW at a 1:3 ratio also presented statistical difference compared to MA alone (Figure 2D).

The search for new drugs to treat leishmaniasis is still incipient. One of the biggest problems faced is that new candidate drugs rarely go beyond animal assays. The few advances so far are limited to (i) the development of new formulations of currently used drugs or (ii) clinical trials evaluating medicines used to treat other diseases (46). Therefore, there are no new drugs or formulations approved for the treatment of this neglected tropical disease, and despite the high incidence of adverse effects and the increasing numbers of unresponsive strains; pentavalent antimonials remain the basis of chemotherapy (47).

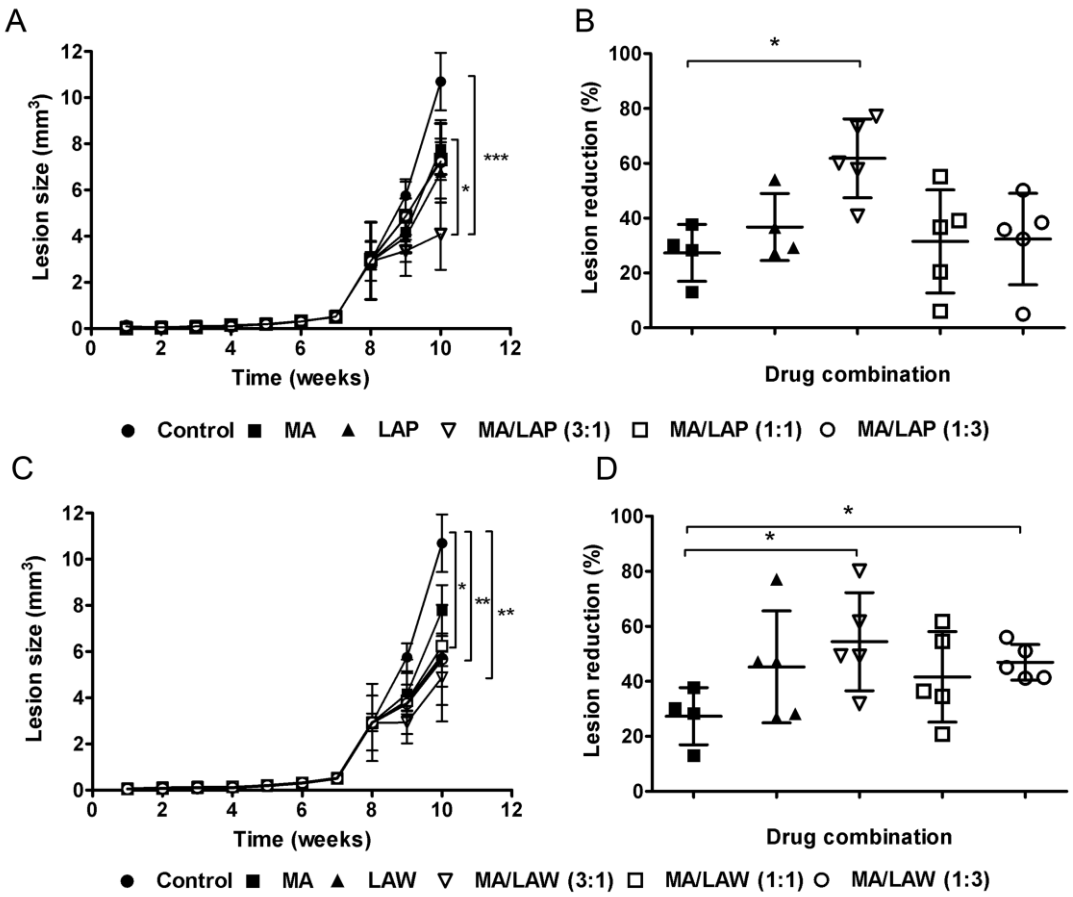

Figure 3: Treatment of mice challenged with *Leishmania (L.) amazonensis*. BALB/c mice were treated daily with meglumine antimoniate (MA), epoxy-α-lapachone (LAP) or epoxymethyl-lawsone (LAW) alone or combinations at fixed-ratio of 3:1, 1:1 and 1:3 based on the follow doses: MA = 2.27 mg of Sb5+/Kg/day; LAP = 2.27 mg/Kg/day and LAW = 1.14 mg/Kg/day. The treatments were administrated daily until 20 doses and lesion sizes (mm^3) were measured weekly (A and C). The results are represented as mean and standard deviation from three independent experiments. The endpoint was on the 10^{th} week and the differences (%) of each treatment are shown (C and D). Difference between groups was analyzed using Mann-Whitney test. *Statistically significant p≤0.05, **Statistically significant p≤0.01 and ***Statistically significant p≤0.001.

Enhancement of effects due to combination therapies can occur basically in two ways: (i) one drug may increase the activity of the other, or (ii) the effect of the two drugs may combine to produce an activity distinct to that observed for each one individually (48). The global increment in leishmancidal activity observed for treatment with MA/LAP or MA/LAW (at 3:1 ratio) may be due to a potentialization of

the activity of meglumine antimoniate by oxiranes or even be related to the marked structural differences between MA and oxiranes, which ensure distinct mechanisms of action.

The combination treatments proposed here may represent a new alternative for leishmaniasis chemotherapy, keeping the first-line drugs, but with reduction in the incidence of adverse effects and risk of emergence of antimonial-unresponsive strains.

3. Conclusions

The results presented herein indicate that the combination of meglumine antimoniate with oxiranes leads an increment of global antileishmanial potential in vitro and in vivo, as compared to the use of these compounds separately. Thereby, this strategy may be a new approach for the cutaneous leishmaniasis treatment. Finally, we emphasize the need for further studies to thoroughly understand the basis of the effects of such combinations.

4. Experimental section

4.1 Chemicals and culture reagents

Dimethyl sulfoxide (DMSO), penicillin, streptomycin, Lab-Tek chamber slides, Greiner CELLSTAR® 96 well plates, RPMI 1640 medium and Schneider's Drosophila medium were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was acquired from Cultilab S/A (Brazil). CellTiter-Glo® luminescent cell viability assay was purchased from Promega Corporation (USA). Meglumine antimoniate (Glucantime®) was kindly provided by Dr. Armando de Oliveira Schubach team (INI / Fiocruz). Propylene glycol was purchased from Vetec Quimica. Epoxy-α-lapachone and epoxymethyl-lawsone compounds were synthesized by the Department of Organic Chemistry of the Instituto de Química, Universidade Federal Fluminense.

4.2. Cell culture

Peritoneal macrophages were removed from BALB/c mice as previously described (39) and recovered in RPMI 1640 medium containing 10% FCS by centrifugation (2 ×, 1 800 × g, 10 min, 4 °C). After, cells were seeded in Lab-Tek chamber slides at a density of 5×10^5 cells/well and incubated (37 °C, 5% of CO2) for 24 hours. Adherent macrophages were used in the cytotoxicity and parasite infection assays.

4.3. Parasite cultures

Promastigotes of *Leishmania (Leishmania) amazonensis* (strain MHOM/BR/ 73/LTB0016) was obtained from the Leishmania collection (Coleção de Leishmania do Instituto Oswaldo Cruz – CLIOC) of the Instituto Oswaldo Cruz (Fiocruz). Cell cultures were maintained at 28 °C in Schneider's medium (pH 7.2) containing 1 mM L-glutamine, 10 % FCS, 100 IU/mL penicillin, and 100 µg/mL of streptomycin. Subpassages were made to maintain the parasites in the logarithmic growth phase.

4.4. Activity against intracellular amastigotes

Macrophages were co-incubated (4 h, 37 °C) with promastigotes in a proportion of 5:1 (parasite:cell) in Lab-Tek chamber slides, followed by washing with PBS and addition of RPMI medium containing 5% FCS. After, cultures were incubed (24 h, 37 °C) with meglumine antimoniate, epoxy-α-lapachone and epoxymethoxy-lawsone alone and combined in fixed ratios (3:1, 1:1, 1:3). Then, slides were fixed with 100% methanol and Giemsa-stained. The endocytic index was calculated by multiplying the percentage of infected cells by the mean number of parasites per infected cell.

4.5. Toxicity of compounds and its combinations to BALB/c mice macrophages

Macrophages seeded on 96-well plates (2 x 105 per well) were exposed (37 °C for 72 h) to meglumine antimoniate, epoxy-α-lapachone and epoxymethyl-lawsone and alone at a concentration equivalent to half of IC_{50} previously defined (data not shown) and in combinations that followed the ratio 3:1, 1:1 and 1:3. Then, macrophages viability was determined by addition of CellTiter-Glo® (20µl/well) and incubation (3 min, 25 °C) under agitation. Luminescence was measured using a FlexStation 3 reader (Molecular Devices, Sunnyvale, CA, USA).

4.6. Experimental murine infection

BALB/c mice with 5- to 7-week-old weighing approximately 22 g were inoculated in the footpad of the left hind limb with 1.0×10^5 promastigotes of *L. (L.) amazonensis* in the stationary growth phase in a total volume of 50 µl diluted in phosphate-buffered saline pH 7.2 at 10 mM (PBS).

4.7. Mice treatment schedules

To determine doses to be applied in the combination assays, BALB/c mice were previously treated with three different doses of each drug, defined here as low,

intermediate and high dose, as follow: meglumine antimoniate (0.23 mg of Sb^{5+}/Kg/day, 2.27 mg of Sb^{5+}/Kg/day and 22.7 mg of Sb^{5+}/Kg/day); epoxy-α-lapachone (0.23 mg /Kg/day, 2.27 mg/Kg/day and 22.7mg Kg/day); and epoxymethyl-lawsone (0.11 mg/Kg/day, 1.14 mg/Kg/day and 11.4 mg/Kg/day), starting four weeks after infection, as previously described (37, 38). Briefly, oxiranes were diluted in a mixture of DMSO: propylene glycol: saline (1 : 12 : 7) because of their low solubility in water, then all drugs were administrated daily by subcutaneous route (100µl per animal), from Monday to Friday until 20 doses. Negative-control group was treated with vehicle used to dissolve the oxirane compounds. The lesions were evaluated weekly by measuring the height and width of the paw, and lesion areas were obtained by multiplying these measures in mm3 with a digital caliper. After definition doses, drugs were tested alone and in combinations following fixed-proportion of 3:1; 1:1 and 1:3.

4.8. Ethical aspects

Mice experimental procedures performed here were approved by the Committee for the Ethical Use of Animals of Instituto Oswaldo Cruz (L-052/2015). The animals were obtained from the Instituto de Ciência e Tecnologia em Biomodelos – Fiocruz.

4.9. Statistical analysis

Student's test was applied to compare results, data matrices were considered statistically different when the P value was less than 0.05. Statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).

Conflict of Interest Disclosure

The authors declare no competing financial interest.

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5. DISCUSSÃO

As leishmanioses são doenças tropicais negligenciadas que afetam principalmente as populações mais pobres dos países em desenvolvimento. Representam problema de saúde pública nas Américas, devido à sua distribuição em vários países e alta prevalência (PAHO, 2013). O tratamento é uma das medidas de controle destas doenças, cuja eficácia vem sendo questionada devido ao risco eminente de resistência e toxicidade; o que alerta para novas propostas de fármacos (WHO, 2010).

Portanto, têm sido pesquisados novos fármacos antiparasitários potenciais com atividade leishmanicida, a partir de fontes vegetais, principalmente metabólitos secundários e seus derivados sintéticos (Newman e Cragg, 2012).

Este tema vem sendo alvo de ampla discussão na qual contribuímos com uma recente publicação sobre produtos naturais derivados de espécies vegetais, cujos estudos sobre efeitos leishmanicidas são considerados promissores: *Kalanchoe pinnata*, *Plumbago scandens*, *Physalis angulata*, *Piper aduncum*, *Peschiera (Tabernaemontana) australis*, *Phyllanthus amarus*, *Artemisia annua* e membros das famílias Bignoniaceae e Verbanaceae (ANEXO II). Ressaltamos neste artigo os principais compostos ativos destas espécies vegetais, com dados de ensaios de atividade leishmanicida e seus mecanismos de ação.

Mesmo com toda a informação reunida nesse trabalho, fica claro que a busca por novos fármacos para o tratamento da leishmaniose ainda é incipiente. Um dos maiores problemas enfrentados é que os novos candidatos raramente vão além dos ensaios em animais (ANEXO II). Os poucos avanços são limitados ao desenvolvimento de novas formulações com os fármacos recomendados, ou ensaios clínicos avaliando o reposicionamento de medicamentos usados para tratar outras doenças (Oliveira et al., 2013). Consequentemente, não há novos medicamentos ou novas formulações aprovadas para o tratamento da doença, e os antimoniais pentavalentes seguem constituindo a base da quimioterapia.

Os antimoniais pentavalentes têm sido usados há mais de 60 anos e permanecem como a primeira escolha para o tratamento da leishmaniose, sobretudo nos países da América Latina. No Brasil, o principal medicamento utilizado é o antimoniato de meglumina, que além dos frequentes efeitos adversos já citados, representa um alto custo de aquisição para abastecer o programa de controle das leishmanioses tegumentar e visceral. Segundo dados do Ministério da Saúde, o

governo gastou só com a compra deste medicamento nos anos de 2010, 2011 e 2012 o valor de R$ 17.307.241,00 (Oliveira et al., 2013). Há ainda a questão da dependência tecnológica em que os países da América Latina se encontram, uma vez que o Glucantime é importado e o fabricante pode, a qualquer momento, interromper a produção do medicamento por desinteresse econômico ou desabastecimento de matéria prima (Oliveira et al., 2013).

Por outro lado, o longo tempo de experiência clínica com os antimoniais pentavalentes originou uma extensa literatura contendo informações sobre seu uso, o que permitiu a elaboração de manuais internacionais e nacionais com diferentes regimes terapêuticos que podem ser aplicados, dependendo do caso, além de recomendações sobre o monitoramento clínico de pacientes em tratamento. Adicionalmente, alguns estudos têm apontado que regimes alternativos, como uso baixas doses (5 mg Sb^{5+}/kg/dia), regimes intermitentes (Costa & Marsden, 1988; de Oliveira-Neto & Mattos, 2006; Saheki et al., 2017) ou sua aplicação intralesional (Oliveira-Neto et al., 1997) podem ser tão eficazes quanto o esquema recomendado, sendo menos tóxicos e custosos.

Nos últimos anos, vários esforços têm sido realizados no sentido de melhorar a quimioterapia antimonial. Pesquisas recentes incluem novas informações sobre sua estrutura química (Frezárd et al., 2008), mecanismos de ação, além de alguns novos métodos de preparação e diferentes formulações com esses compostos (revisado por Frezárd et al., 2009). Neste sentido, uma estratégia promissora para melhorar a eficácia da quimioterapia antimonial envolve sua associação com os oxiranos, uma classe de composto com atividade leishmanicida, visando a redução da dose total aplicada, mantendo a eficácia e diminuindo o perfil de toxicidade do tratamento. Esta hipótese foi testada neste trabalho de tese em modelo murino com a linhagem BALB/c altamente susceptível à infecção por *L. (L.) amazonensis* (DOCUMENTOS 1, 2 e 3).

Com os estudos neste modelo foi possível explorar, pela primeira vez, a ação leishmanicida do epoximetoxi-lauson sobre a infecção experimental *in vitro* e *in vivo*. As infecções de macrófagos com altas taxas de multiplicação de amastigotas se mostraram apropriadas para a determinação da metade da concentração inibitória máxima. O epoximetil-lausona demonstrou eficácia similar ao fármaco de referência e com menor toxicidade. Na infecção de camundongos por *L.(L.) amazonensis*,

houve desenvolvimento de lesões progressivas bem visíveis por volta de 30 dias, sobre as quais comprovamos a efetividade da intervenção de ambos os fármacos (DOCUMENTO 2). Resultado semelhante fora previamente demonstrado para o epoxi-α-lapachona (ANEXO I). Desta forma, o modelo murino vem se mostrando válido e adequado para os experimentos propostos na avaliação dos efeitos leishmanicidas dos compostos oxiranos.

Entretanto, apontamos para evidências de efeitos tóxicos para tecidos de camundongos BALB/c não infectados com a administração dos oxiranos e antimonato de meglumina no esquema terapêutico efetivo no controle da evolução da lesão (DOCUMENTO 1). Tanto o tratamento com epóxi-α-lapachona e epoximetil-lausona quanto com o antimoniato de meglumina, nas doses de 22,7mg/kg/dia, 11,4mg/kg/dia e 22,7mg/kg/dia, respectivamente, administrados diariamente até 20 doses induziu alterações nos pulmões, coração, rins, fígado e cérebro de camundongos. Estes resultados serviram como alerta para a reavaliação dos protocolos de tratamento dos animas, e levantam a questão de que os testes em cultura de células não são suficientes para refletir a resposta fisiológica real a novas substâncias químicas. Estudos adicionais são necessários para uma análise mais acurada destes efeitos, incluindo seu mecanismo de geração e o acompanhamento dos animais nas semanas posteriores ao tratamento para a avaliação da persistência ou reversibilidade das alterações detectadas.

A dose de antimoniato de meglumina usada (DOCUMENTO 1) foi equivalente à dose recomendada pela OMS para o tratamento de pacientes com LT (WHO 2010). Como já descrito, pacientes tratados com antimoniais pentavalentes podem desenvolver cardiotoxicidade (revisado por Oliveira et al, 2011), hepatotoxicidade (Hepburn et al., 1994) e nefrotoxicidade (Rodrigues et al., 1999). Além disso, os animais expostos ao antimoniato de meglumina também apresentaram lesões no tecido pulmonar. Entretanto, o registro de efeitos adversos associados ao aparelho respiratório em pacientes é raro (revisado por Oliveira et al., 2011). Não obstante as observações terem sido realizadas em modelo murino, não se pode descartar a possibilidade de que alterações histológicas semelhantes possam ocorrer nos pacientes e serem responsáveis por parte dos efeitos adversos frequentemente relatados.

Uma possível explicação para as alterações histológicas detectadas nos tecidos dos camundongos seria, ao menos em parte, pela natureza química que os oxiranos compartilham com seus compostos precursores as 1,4-naftoquinonas, ou devida a sua alta distribuição nos tecidos. Estes compostos podem agir em várias vias metabólicas envolvidas no mecanismo de toxicidade causada por esta classe química, como proposto no ANEXO III.

As naftoquinonas são agentes oxidantes e eletrófilos, que podem iniciar um ciclo redox pela redução de um ou dois elétrons (López López et al., 2014). A redução de um elétron é catalisada principalmente pela NADPH-citocromo P450 redutase, levando a semiquinonas instáveis que transferem elétrons para o oxigênio molecular (O_2), retornando à sua estrutura original. Um radical ânion superóxido ($\bullet O_2^-$) é gerado e pode ser convertido em peróxido de hidrogênio (H_2O_2) através da reação catalisada pela superóxido dismutase, seguido pela formação de um radical hidroxila (HO•) através da redução de peróxido catalisada por ferro (Monks & Jones, 2002; Kumagai et al., 2012). Há a possibilidade de que estes compostos possam atuar como eletrófilos formando ligações covalentes com grupos tiol presentes em moléculas biológicas. Outro mecanismo de toxicidade proposto é que os oxiranos, assim como outras naftoquinonas, podem participar da iniciação e propagação de espécies reativas de oxigênio que são poderosos agentes oxidantes, sendo provavelmente os responsáveis por danos a macromoléculas, como DNA, proteínas e lipídios, levando ao estresse oxidativo e apoptose nas células (Monks & Jones, 2002; Kumagai et al., 2012).

A abordagem aplicada no DOCUMENTO 1 consiste num esforço para identificar e descrever o perfil de toxicidade dessa nova classe de compostos sintéticos através da análise histopatológica, e combinou a predição *in silico* de parâmetros farmacocinéticos relevantes. Os dados obtidos neste artigo nos fornecem informações preliminares que servirão como base para os estudos que antecedem a pré-formulação e, posterior estabelecimento de uma margem segura para o uso desses compostos, além de enfatizar a importância de uma investigação mais profunda dos efeitos tóxicos sobre órgãos vitais nos ensaios com novos compostos.

Na continuidade deste estudo, demonstramos que epoximetil-lausona tem ação direta sobre *L. (L.) amazonensis* em diferentes condições experimentais, o que está de acordo com os resultados obtidos para o epoxi-α-lapachona (ANEXO 1). Uma

explicação plausível para este achado está na similaridade estrutural entre os compostos, o que aponta que eles podem compartilhar dos mesmos mecanismos de ação. Uma vez que foi comprovado o efeito inibitório do epoxi-α-lapachona sobre a atividade de serino proteases deste parasito, é esperado que o epoximetil-lausona também possa agir como inibidor de proteases, contribuindo para o efeito leishmanicida. Estudos adicionais são necessários para comprovar esta hipótese.

Apesar das pesquisas por novos fármacos para substituir os antimoniais pentavalentes, esses medicamentos continuam a primeira linha para o tratamento de todas as formas de leishmaniose. A combinação de fármacos é uma alternativa que tem sido estudada no tratamento da doença (Omollo et al. 2011; Romero et al., 2017). Neste trabalho de tese exploramos o uso associado do antimoniato de meglumina com os oxiranos (DOCUMENTO 3).

A hipótese de que esta estratégia seria promissora foi comprovada neste trabalho que usou o método de razões fixas para combinar antimoniato de meglumina (AM) com epoxi-α-lapachona (LAP) e epoximetil-lausona (LAU). Mostramos aqui que tais fármacos e suas combinações causam marcada redução no índice endocítico da infecção experimental em macrófagos por *L. (L.) amazonensis* e, na redução da lesão de pata em camundongos. Em ambos os experimentos foi evidenciado que a razão 3:1 (AM:LAP ou AM:LAW) apresentou melhor efeito, o que pode significar uma potencialização dos efeitos leishmanicidas do antimoniato de meglumina pelos oxiranos (DOCUMENTO 3).

Os resultados aqui apresentados indicarem que os oxiranos são tão eficazes quanto o antimoniato de meglumina, porém combinados demonstraram efeito leishmacida maior, podendo representar uma nova alternativa para a quimioterapia da leishmaniose. A vantagem desta associação é atuação de duas classes químicas agindo sobre o parasito através de diferentes mecanismos.

6. CONCLUSÕES

- Na presente tese, reunimos evidências do potencial terapêutico dos oxiranos, bem como a avaliação do efeito destes compostos em associação com antimoniato de meglumina no tratamento da infecção tegumentar experimental causada por *L. (L.) amazonensis* em modelo murino.
- O curso de tratamento de 20 dias com as doses mais eficazes de ambos oxiranos acarretou alterações histopatológicas em pulmão, coração, fígado, rim, cérebro e cerebelo de camundongos BALB/c sadios. Interessantemente, o fármaco de referência induziu alterações menos intensas e não causou danos nos tecidos do sistema nervoso central. Esta abordagem enfatiza a importância da análise toxicológica em animais concomitantemente aos estudos de eficácia na busca por novos fármacos para tratar a leishmaniose.
- Embora a via de administração dos fármacos aqui estudados ter sido a subcutânea, a predição dos parâmetros farmacocinéticos dos oxiranos indica a possibilidade de uso pelas vias oral e tópica. Estes compostos apresentam características apolares que, em teoria, lhes confere boa absorção e distribuição.
- A atividade leishmanicida do epoximetil-lausona foi pela primeira vez descrita em amastigotas intracelulares e promastigotas, bem como seu efeito no controle da lesão da pata de camundongos BALB/c.
- A exposição de macrófagos em cultura aos compostos testados indicou que o antimoniato de meglumina tem efeito citotóxico ligeiramente maior (CC_{50} 1,7x) que o encontrado para o epoximetil-lausona.
- O epoximetil-lausona mostrou efeito direto sobre amastigotas de *L. (L.) amazonensis* nas lesões cutâneas dos animais, evidenciado por alterações ultraestruturais que comprometeram a integridade dos parasitos.
- Os efeitos das combinações do antimoniato de meglumina com os oxiranos nas razões 3:1 mostraram resultados significativos e promissores em relação aos controles de infecção e fármacos usados individualmente, indicando que os oxiranos talvez potencializem os efeitos leishmanicidas do antimoniato de meglumina e, por isso, podem ser explorados no intuito de melhorar o tratamento com o medicamento de primeira escolha.

7. PERSPECTIVAS

Este trabalho de tese apresenta uma nova perspectiva para o tratamento da leishmaniose tegumentar, pois aponta para o possível avanço no desenvolvimento de um (ou dois) novo (s) fármaco (s) baseado nos oxiranos, além de sua utilização associada ao antimoniato de meglumina.

Para tanto, há a necessidade de realizar estudos de formulação com estes compostos visando sua incorporação em bases para adminstração por via oral, tópica ou intravenosa para veiculá-los de maneira apropriada e retomar os estudos de eficácia e toxicidade em animais, incluindo novos parâmetros nas análises.

Adicionalmente, os estudos envolvendo a associação do fármaco de referência com os oxiranos devem ser estendidos a outras espécies de *Leishmania*, e aprofundados bioquimicamente no intuito de melhorar a compreensão dos efeitos incrementais produzidos nos diferentes modelos de infecção.

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ANEXO I

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Epoxy-α-Lapachone Has *In Vitro* and *In Vivo* Anti-*Leishmania* (*Leishmania*) *amazonensis* Effects and Inhibits Serine Proteinase Activity in This Parasite

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***Leishmania* (*Leishmania*) *amazonensis* is a protozoan that causes infections with a broad spectrum of clinical manifestations. The currently available chemotherapeutic treatments present many problems, such as several adverse side effects and the development of resistant strains. Natural compounds have been investigated as potential antileishmanial agents, and the effects of epoxy-α-lapachone on *L.* (*L.*) *amazonensis* were analyzed in the present study. This compound was able to cause measurable effects on promastigote and amastigote forms of the parasite, affecting plasma membrane organization and leading to death after 3 h of exposure. This compound also had an effect in experimentally infected BALB/c mice, causing reductions in paw lesions 6 weeks after treatment with 0.44 mM epoxy-α-lapachone (mean lesion area, 24.9 ± 2.0 mm^2), compared to untreated animals (mean lesion area, 30.8 ± 2.6 mm^2) or animals treated with Glucantime (mean lesion area, 28.3 ± 1.5 mm^2). In addition, the effects of this compound on the serine proteinase activities of the parasite were evaluated. Serine proteinase-enriched fractions were extracted from both promastigotes and amastigotes and were shown to act on specific serine proteinase substrates and to be sensitive to classic serine proteinase inhibitors (phenylmethylsulfonyl fluoride, aprotinin, and antipain). These fractions were also affected by epoxy-α-lapachone. Furthermore, *in silico* simulations indicated that epoxy-α-lapachone can bind to oligopeptidase B (OPB) of *L.* (*L.*) *amazonensis*, a serine proteinase, in a manner similar to that of antipain, interacting with an S1 binding site. This evidence suggests that OPB may be a potential target for epoxy-α-lapachone and, as such, may be related to the compound's effects on the parasite.**

An array of *Leishmania* species are able to infect humans, as well as other mammalian hosts, and cause diseases that are known under the common name of leishmaniasis. Leishmaniasis has high incidence and prevalence in tropical and subtropical regions of the world, affecting mostly populations in poor or emerging countries, and is included among the 17 neglected tropical diseases defined by the World Health Organization (http://www.who.int/neglected_diseases/diseases/en).

These parasites can affect cells in the skin, mucosa, and cartilage, causing cutaneous leishmaniasis (CL). Some species may infect internal tissues and organs, such as the liver, spleen, and bone marrow, causing visceral leishmaniasis (VL) (1). Mucosal leishmaniasis (ML) is a metastatic outcome of a CL infection, resulting in the dissemination of parasites to the oropharynx mucosa.

In Brazil, *Leishmania* (*Leishmania*) *amazonensis* is a species described to cause a wide spectrum of clinical manifestations (2), accounting for unusual clinical presentations (3). Great genetic diversity among strains isolated from patients (4) has been reported, as well as a trend toward increasing geographical distribution.

The currently available treatments for these infections are restricted to two option groups, namely, (i) the antimonials, which are the first-choice drugs (5, 6), and (ii) pentamidine and amphotericin B, the second-choice drugs (7). Both groups of drugs have many limitations regarding their use, such as (i) high cost, (ii) difficulty of administration, (iii) toxicity, and (iv) the development of resistance by parasite strains. Undoubtedly, these limitations represent obstacles for successful therapy (8), emphasizing the need to develop new drugs for the treatment of leishmaniasis.

Several natural compounds have been reported to have antileishmanial effects, but none has transitioned into an effective drug for treatment of leishmaniasis. In this context, some natural products obtained from plant extracts or their derivatives, such as quinones, alkaloids, terpenes, and phenolic derivatives, have been

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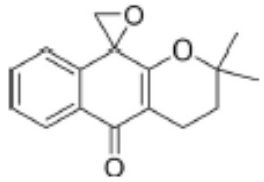

FIG 1 Structure of 2,2-dimethyl-3,4-dihydrospiro[benzo[g]chromene-10,20-oxiran]-5(2*H*)-one, also known as epoxy-α-lapachone (CID 12000280; molecular formula, $C_{16}H_{16}O_3$; molecular weight, 256.29644 g/mol) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top).

proposed for leishmaniasis chemotherapy (9). Recently, we reported evidence that quinone derivatives exhibited promising properties against protozoan parasites, such as *Trypanosoma cruzi* (10–14), *Leishmania* (*Viannia*) *braziliensis*, and *Leishmania* (*Leishmania*) *amazonensis* (15). These compounds can be isolated from *Bignoniaceae* or *Verbenaceae* trees, and their antimicrobial properties have been well established (16).

Among the naphthoquinone derivatives, epoxy-α-lapachone (Fig. 1) is a good candidate to serve as the basis for antileishmanial treatments, as it has been shown to have low cytotoxicity for mammalian cells (10, 11) while being effective against *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* (15); it was able to kill promastigotes of both species *in vitro* and affected amastigotes infecting human macrophages. We previously reported that epoxy-α-lapachone can inhibit serine and cysteine proteinase activities in *Trypanosoma cruzi* (17), but we have not yet assessed this possibility in *Leishmania* spp.

It is known that proteinases are pivotal virulence factors for *Leishmania* spp. (18). Serine proteinases, such as oligopeptidase B (OPB; clan SC, family S9), have been reported to correlate with the infection of murine macrophages by parasites and the survival of those located within infected cells (19). Therefore, in the present study, we aimed to assess the potential inhibitory effect of epoxy-α-lapachone on *L.* (*L.*) *amazonensis* serine proteinase activity, as it may be part of the antileishmanial mechanism of this compound, and we applied a molecular modeling approach to investigate how this inhibitor binds to target enzymes such as OPB. In parallel, we investigated the effects of epoxy-α-lapachone on both parasite forms and on the outcome of experimental murine infection with *L.* (*L.*) *amazonensis*.

MATERIALS AND METHODS

Chemicals and culture reagents. Coomassie brilliant blue R-250, detergents (sodium dodecyl sulfate [SDS] and Triton X-100), proteinase inhibitors (phenylmethylsulfonyl fluoride [PMSF], aprotinin, and antipain), HiTrap Benzamidine FF, Tris, glycerol, dimethyl sulfoxide (DMSO), penicillin, streptomycin, Schneider's *Drosophila* medium, and fluorogenic peptide substrates (*Z*-Phe-Arg-7-amido-4-methylcoumarin [Z-FR-AMC], Ala-Phe-Lys-7-amido-4-methylcoumarin [AFK-AMC], and *Z*-Gly-Gly-Arg-4-methoxy-β-naphthylamide [Z-GGR-MβNA]) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Amicon Centriprep YM-10 filter devices were purchased from Millipore (Billerica, MA). Fetal calf serum (FCS) was purchased from Cultilab S/A (Brazil). Brain heart infusion (BHI) medium was purchased from Oxoid Australia (West Heidelberg, Australia). The micro-bicinchoninic acid (BCA) protein assay kit was purchased from Pierce Chemical Co. (Appleton, WI). TO-PRO-3 (Invitrogen, Waltham, MA) and tetramethylrhodamine ethyl ester perchlorate (TMRE) were purchased from Molecular Probes (Eugene, OR). Meglumine antimonate (Glucantime) was purchased from Sanofi-Aventis Farmacêutica (Suzano, Brazil). The epoxy-α-lapachone compound was synthesized by the Department of Organic Chemistry of the Instituto de Química, Universidade Federal Fluminense.

Parasite cultures. *L.* (*Leishmania*) *amazonensis* (strain MHOM/BR/73/LTB0016) was obtained from the *Leishmania* collection of the Instituto Oswaldo Cruz (Fiocruz). *In vitro* promastigote cultures were maintained at 28°C in Schneider's medium (pH 7.2) containing 1 mM L-glutamine, 10% FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, with frequent subpassages to maintain the parasites in the logarithmic growth phase.

Axenic amastigote transformation. Axenic amastigotes were obtained as described previously (20–22). Briefly, promastigotes of both parasite species, in the logarithmic growth phase (5×10^5 cells/ml), were seeded in axenic medium (Schneider's medium [pH 7.2] containing 10 mM HEPES buffer, 1 mM L-glutamine, 60 IU/ml penicillin, and 60 μg/ml streptomycin) and incubated for 24 h at 26°C. The promastigotes were then reseeded in new axenic medium with the pH adjusted to 5.5 and were cultivated under the same conditions. Following 4 days of incubation, the parasites were reseeded in new axenic medium at pH 5.5 and incubated at a higher temperature (32°C). To assess the degree of successful differentiation, the morphology of the cells in the cultures was analyzed by optical microscopy.

Effects of epoxy-α-lapachone on promastigotes and amastigotes. The parasites were seeded at a density of 1.0×10^7 parasites/ml in Schneider's medium and were incubated under different conditions (1 h or 3 h at 28°C or 32°C) in the absence or presence of epoxy-α-lapachone (0.175 μM) or DMSO, which was used as a diluent for the compound and as a control. Parasite viability was then assessed by flow cytometry using specific fluorescent markers. TO-PRO-3, a membrane-impermeable DNA marker, was used (10 μM) to assess parasites' membrane integrity. The TMRE probe was used (50 nM) to verify variations in ionic pumping metabolism and transmembrane potential (plasma membrane and organelles). Parasites (1.0×10^6 cells/well) were incubated for 20 min with the markers, and the samples were immediately analyzed using a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). Data analysis was carried out using Summit version 4.3 software.

Experimental murine infections and treatment of animals with epoxy-α-lapachone. Experimental infections were conducted with 6- to 8-week-old BALB/c mice weighing approximately 22 g. The animals were obtained from the animal breeding center of Fiocruz, and all experimental procedures were performed as approved by the Committee for the Ethical Use of Animals of Fiocruz (P-40/13-2). The mice were inoculated in the footpad of the left hind limb with 1.0×10^6 promastigotes of *L.* (*L.*) *amazonensis* in 10 mM phosphate-buffered saline (PBS). The parasites were in the stationary growth phase after 5 days of culture in Schneider's medium.

The experimental treatments were performed with either Glucantime (as a comparative control for treatment efficacy) or epoxy-α-lapachone at different concentrations (0.44, 0.09, and 0.02 mM). The drugs (0.5 ml/animal) were administered subcutaneously in the dorsal region of each mouse. Treatments were carried out for 1 week with daily injections, starting 1 week after challenge infection, when the paw lesions had already become noticeable. Two negative-control groups were included, in which sterile PBS or DMSO was administered during treatment. The lesions were evaluated on a weekly basis, by measuring lesion areas (in mm^2) with a caliper.

Parasite protein extracts. Protein extracts were obtained as described previously (23). Briefly, parasites (2.0×10^7), either promastigotes in the logarithmic growth phase or amastigotes, were washed three times by centrifugation (3,000 × *g* for 10 min at 4°C) in PBS (pH 7.2) and then were subjected to 4 cycles of vortex-mixing for 30 min in the presence of lysis buffer (100 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10% glycerol, 0.6% Triton X-100). The soluble protein fraction was obtained by centrifugation of the samples (25,000 × *g* for 30 min at 4°C) and then was stored at −20°C until further use. The protein concentrations of the extract samples were determined using the micro-BCA protein assay kit.

Serine proteinase-enriched fractions from parasites. Soluble protein fraction samples of promastigotes or amastigotes (adjusted to 35 or 40 mg/ml, respectively, in 10 mM Tris-HCl [pH 7.5]) were bound in a Hi-Trap Benzamidine FF column that had been previously equilibrated with binding buffer (0.05 M Tris-HCl, 0.5 M NaCl [pH 7.4]). The column was washed with the same buffer to flush out unbound proteins; the bound proteins were retrieved using elution buffer (0.05 M glycine [pH 3.0]) and preserved in 1 M Tris-HCl (pH 9.0). The eluted proteins (here called the serine proteinase-enriched fraction) were concentrated for 30 min and dialyzed against a buffer (10 mM Tris-HCl [pH 7.5]) for further use in proteinase assays.

Zymographic assays. The serine proteinase-enriched fraction (5 μg of total protein) was subjected to electrophoresis under reductive conditions using 12% acrylamide gels copolymerized with 0.1% gelatin (24). Following electrophoresis, the gels were washed for 1 h at 4°C in 0.1 M Tris-HCl (pH 7.5) (washing buffer) containing 2.5% Triton X-100 and then were incubated for 6 h at 37°C in washing buffer without supplements. The gel was then stained with Coomassie brilliant blue R-250.

Assessments of proteinase activity and inhibitory efficacy. The proteinase activities (in solution) of the serine proteinase-enriched fraction (0.5 μg of total proteins) and trypsin, which was used as a positive activity control, were characterized in activation buffer (10 mM Tris-HCl [pH 7.5]), at a final volume of 60 μl, using specific fluorescent peptide substrates for serine proteinase (*Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA at 0.1 mM). Samples were incubated for 60 min at 37°C, and the variance in the relative fluorescence units (RFU), corresponding to enzymatic cleavage of the substrates, was monitored with a Molecular Devices SpectraMax spectrophotometer (Gemini XPS) (7). Concomitantly, the efficacy of various serine proteinase inhibitors was assessed under the same conditions. Inhibition assays were performed with 1 mM PMSF, 0.3 μM aprotinin, 5 μM antipain (all used as controls), and 1 mM epoxy-α-lapachone.

The substrate enzymatic cleavage rate was defined using the formula $v = \Delta s/\Delta t$, where v represents velocity (reaction rate), Δs represents substrate concentration variation, and Δt represents the total reaction time (20). Self-degradation of the fluorescent peptide substrate was controlled throughout the assay, to avoid incorrect readings. The enzymatic activity is expressed as $(\times 10^{-3})$ mmol · min^{-1} · mg $protein^{-1}$.

Determination of IC_{50} values for serine proteinase inhibitors. The 50% inhibitory concentration (IC_{50}) values for all tested inhibitors were obtained as described previously (25). Briefly, the tests were performed by combining a fixed *Z*-FR-AMC substrate concentration (0.1 mM) with 10 distinct concentrations (from 1.5×10^{-4} mM to 5 mM) of each inhibitor, using the same methodology as described above. These results were applied to a linear interpolation of the concentrations for each inhibitor versus the corresponding percentage of enzymatic inhibition and were analyzed using the following equation: $IC_{50} = [(50\% - \text{lower inhibition \%})/(\text{higher inhibition \%} - \text{lower inhibition \%}) \times (\text{higher concentration} - \text{lower concentration}) + \text{lower concentration}]$ For the linear interpolation analysis, the mean values for triplicate determinations in each assay were used.

Statistical analysis. To compare results, Student's *t* test was applied; data matrices were considered statistically different when the *P* value was less than 0.05. Statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).

Molecular docking of oligopeptidase B. In order to investigate the binding mode of epoxy-α-lapachone, this compound was docked into *L.* (*L.*) *amazonensis* oligopeptidase B (OPBa) using the DockThor program (27). First, the three-dimensional structures of ligand molecules were built and minimized with the Avogadro 1.1 program. The crystal structure of *Leishmania* (*Leishmania*) *major* oligopeptidase B (OPBm) complexed with antipain was obtained from the Protein Data Bank (PDB accession number 2XE4), and the OPBa model was constructed using the Modeller 9.14 program (28), which was used with the OPBm template. The model construct with the lowest value for discrete optimized protein energy (DOPE) was selected and evaluated with ProCheck (29), Errat (30), and Prosa (31) software. The molecular docking was established in a cubic grid box of 8 by 8 by 8 $Å^3$, and the parameters are referred to as defaults in DockThor. Structures with positional root mean square deviation (RMSD) of up to 2 Å were clustered together, and the results with the most favorable free energy of binding were selected as the resultant complex structures. We also performed redocking of antipain to the crystal structure of *L.* (*L.*) *major* OPB, with a success rate (RMSD of ≤2.0 Å for the interface backbone atoms) of 53%.

RESULTS

Epoxy-α-lapachone has leishmanicidal activity with promastigotes and amastigotes of *Leishmania* (*L.*) *amazonensis*. We used flow cytometry to demonstrate that epoxy-α-lapachone can affect both promastigotes and amastigotes. First, the drug causes metabolic dysfunction in ionic pumping, which can be mainly due to mitochondrial damage, and this was evidenced by a reduction in TMRE labeling. Then, it leads to parasite death through the loss of membrane integrity, as evidenced by an increase in TO-PRO-3-positive cells. Our results also indicated that the drug is capable of quickly crossing the plasma membrane (Fig. 2).

As indicated in Fig. 2A, 87.9% of control promastigotes had normal ionic pump activity, with a mean fluorescence intensity (MFI) value of 96.9. After 1 hour of incubation with 0.175 μM epoxy-α-lapachone, there was an abrupt decrease in metabolic activity, as evidenced by a reduction in TMRE staining (MFI of 49.5). Exposure for 3 h led to a further decrease in MFI to 40.6. The loss of membrane integrity was confirmed by an increase in promastigotes stained with TO-PRO-3 (control, 4.0%; 1 h of exposure, 39.9%; 3 h of exposure, 78.9%) (Fig. 2A, upper right quadrant of each graph). Amastigote forms also showed a reduction in the difference in transmembrane potential, with MFI values from 35.0 in control cells to 35.4 at 1 h and 29.4 at 3 h. Regarding membrane integrity, we observed that only 1.7% of cells were TO-PRO-3 positive in the control and there was an increase to 44.5% after 1 h of incubation. We observed no increase in this result after 3 h of incubation (Fig. 2B).

Mouse lesions caused by *Leishmania* (*L.*) *amazonensis* infection decrease after treatment with epoxy-α-lapachone. The role of epoxy-α-lapachone in the progression of lesions during the course of an experimental infection was analyzed in BALB/c mice injected with different concentrations of the compound after 1 week of infectious challenge. The results indicated that the treated animals exhibited reductions in paw lesion areas, compared to animals from the control group (Fig. 3). Although no dose-response correlation was observed in assays with Glucantime and epoxy-α-lapachone, the effects of the compounds were statically significant, compared with negative-control results (Fig. 3A and B).

Serine proteinase activity in *Leishmania* (*L.*) *amazonensis* is inhibited by epoxy-α-lapachone. The potential of epoxy-α-lapachone to act as an inhibitor of *L.* (*L.*) *amazonensis* serine proteinases from promastigotes and amastigotes was assessed in this study. To this end, assays were performed with serine proteinase-enriched fractions obtained by affinity chromatography, which were analyzed by using gelatin-SDS-PAGE and fluorogenic peptide substrates. These fractions yielded approximately 0.1 and 0.05 mg of protein, corresponding to 0.28 and 0.13% of the total applied protein, respectively, for promastigotes and amastigotes. SDS-PAGE analysis revealed a major proteinase band with an estimated molecular mass of 68 kDa, which was stained with both

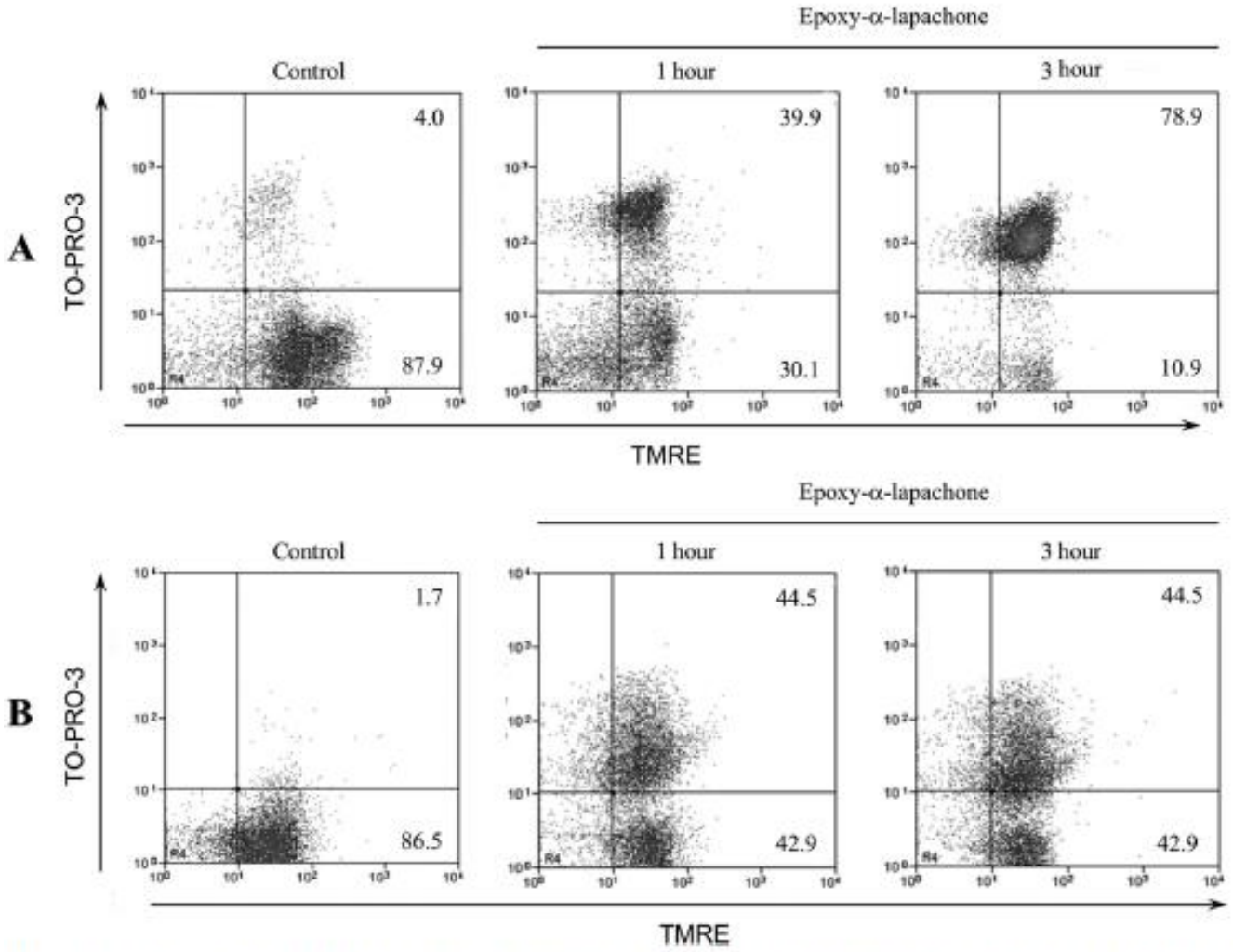

FIG 2 Flow cytometry assays demonstrating that the epoxy-α-lapachone compound can affect promastigotes and amastigotes. Dot-plot analyses of untreated (control) and epoxy-α-lapachone (0.175 μM)-treated (1 h and 3 h) promastigote (A) and amastigote (B) forms are shown. Before the acquisition of data (10^6 events), the parasites were stained with TMRE and TO-PRO-3 in Schneider's medium. The data are representative of three experiments, and values within the graphs are percentages.

silver (Fig. 4) and Coomassie brilliant blue (data not shown), presenting similar results by both methods.

Serine proteinase activity in fractions from both parasite forms was subsequently detected in assays of enzymatic activity in solution. The protein fractions from promastigotes and amastigotes were both able to hydrolyze *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA but at different velocity rates, with the amastigote fraction exhibiting higher velocity rates for substrate hydrolysis than the promastigote fraction, as follows: for the amastigote fraction, *Z*-FR-AMC, $(16 \pm 0.03) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; AFK-AMC, $(20 \pm 0.7) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and *Z*-GGR-MβNA, $(18 \pm 0.4) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and for the promastigote fraction, *Z*-FR-AMC, $(8 \pm 0.8) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; AFK-AMC, $(3 \pm 0.6) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}; and *Z*-GGR-MβNA, $(3 \pm 0.3) \times 10^{-3}$ mmol · min^{-1} · mg protein^{-1}. These fractions exhibited distinct profiles of inhibition by classic serine proteinase inhibitors (Fig. 4).

In the course of this study, we were able to verify that the epoxy-α-lapachone inhibits serine proteinase activity in protein fractions from both *L.* (*L.*) *amazonensis* promastigotes and amastigotes. This inhibitory effect was observed for parasite enzymatic activity with all tested substrates, i.e., 85%, 80%, and 93% inhibition of promastigote enzymes and 80%, 77%, and 91% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively. With the three assayed substrates, this was a better profile than those of PMSF (11%, 14%, and 2% inhibition of promastigote enzymes and 15%, 14%, and 3% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively), aprotinin (2%, 0%, and 7% inhibition of promastigote enzymes and 11%, 11%, and 4% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively), and antipain (85%, 65%, and 67% inhibition of promastigote enzymes and 66%, 92%, and 99% inhibition of amastigote enzymes with *Z*-FR-AMC, AFK-AMC, and *Z*-GGR-MβNA, respectively) (Fig. 4).

Additionally, the IC_{50} values for all of the tested inhibitors were

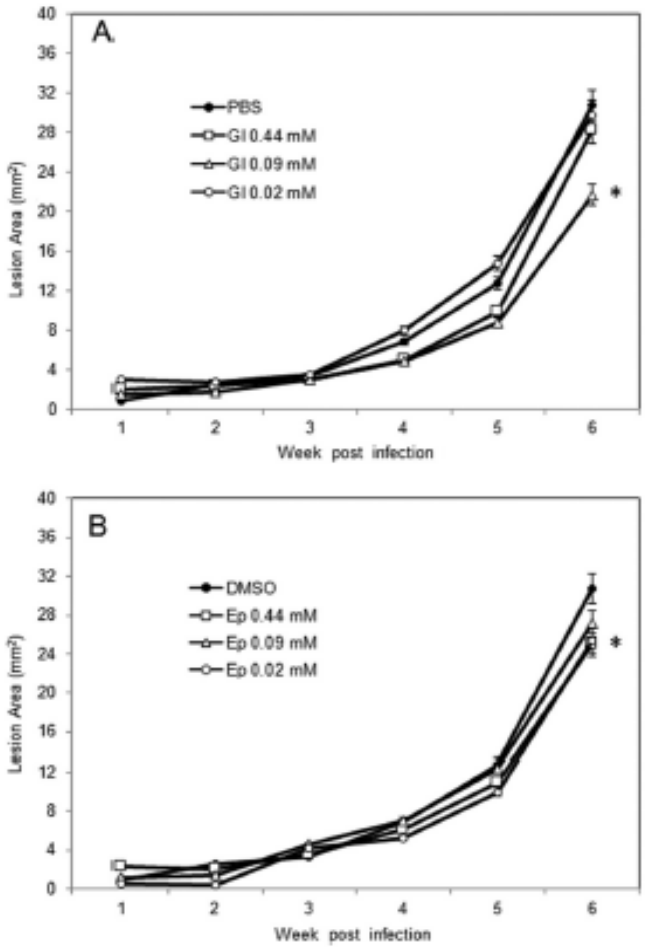

FIG 3 Experimental treatment of infections in mice caused by *Leishmania* (*L.*) *amazonensis*. BALB/c mice were inoculated subcutaneously, in the left footpad, with 1.0×10^6 promastigotes at the logarithmic phase of growth. After 1 week of infection, the mice were treated weekly with meglumine antimoniate (GI) (A) or epoxy-α-lapachone (Ep) (B) at concentrations of 0.44 mM, 0.09 mM, and 0.02 mM administered subcutaneously, with five animals per group. Controls were treated with PBS (A) or 0.44 mM DMSO (B) alone. The lesion sizes were measured, and the results represent the means ± standard deviations from three independent experiments. *, $P < 0.05$.

determined with each protein fraction (and trypsin, used as a positive control) using the *Z*-FR-AMC substrate. As shown in Table 1, the IC_{50} of epoxy-α-lapachone, although higher than those of aprotinin and antipain for both protein fractions, was lower than the IC_{50} value of PMSF, suggesting that it effectively impairs serine proteinase activities.

***In silico* simulations of epoxy-α-lapachone.** Due to the absence of crystallographic data for *L.* (*L.*) *amazonensis* OPB in data banks, it was necessary to build a three-dimensional model of this enzyme to proceed with molecular docking tests. OPBa showed a high degree of identity (90%) with OPBm. The model of OPBa with a lower DOPE value revealed an RMSD of 0.19 Å when aligned with OPBm. The stereochemical evaluation exhibited 90.2% and 85.5% of residues with most favored regions in a Ramachandran plot and G-factor values of −0.25 and −0.19 for OPBm and OPBa, respectively. In addition, analysis of non-bonded interactions showed Errat scores of 93.5% and 87.0% and *Z*-scores of −11.56 and −11.26 for OPBm and OPBa, respectively.

An analysis of the results of the redocking of antipain into OPBa showed an RMSD of 2.0 Å when aligned with the original cocrystallized conformation, demonstrating that the methodology was theoretically reliable for showing ligand-bound conformations (Fig. 5A). In order to theoretically analyze the binding mode of epoxy-α-lapachone, we docked these molecules into OPBa and compared them with antipain. The comparison of epoxy-α-lapachone docking with that of antipain revealed several differences in binding to the amino acid residues (data not shown). The OPBa-epoxy-α-lapachone complex conserved the main binding in S1 by hydrogen bonds and the hydrophobic interactions that are observed in antipain (Fig. 5B). The data showed that the main hydrogen bonds occurred with the residues Ser577, Ala578, and Try496 and the hydrophobic interactions with the residues Phe698, Arg576, Ile501, and Leu617 (Fig. 5C), between OPB and the epoxy-α-lapachone. The interaction energy value of epoxy-α-lapachone (−22.08 kcal/mol) was comparable to the energy value of antipain redocking (−26.95 kcal/mol).

DISCUSSION

American tegumentary leishmaniasis has spread across North and South America and remains without an efficient treatment (32). The current treatment, based on pentavalent antimony, is associated with severe side effects, such as pain, gastrointestinal disorders, headache, anorexia, and cardiac, hepatic, and pancreatic toxicity (33). For this reason, the development of new chemotherapeutic agents, potentially including plant-derived compounds such as the naphthoquinones, is required (10, 11). We therefore aimed to assess the potential leishmanicidal activity of epoxy-α-lapachone in the treatment of experimental murine infections and to identify the targets in the parasite affected by this compound. This compound was selected for further analysis because of its previously reported low cytotoxicity in mammalian cells (10, 15), which highlighted its usefulness to serve as a basis for the development of novel antileishmanial drugs.

With our assays, we collected evidence that epoxy-α-lapachone is in fact a potent leishmanicidal agent; it readily affected *L.* (*L.*) *amazonensis* promastigotes and axenic amastigotes *in vitro* after a short coincubation period, as well as inducing decreases in infection-related paw lesions in experimentally infected mice. The results presented show that both epoxy-α-lapachone and Glucantime had effects in the reduction of paw lesions in the treated BALB/c mice. These results, in association with the previous data on the low toxicity of epoxy-α-lapachone for mammalian cells (15, 17), are strong indicators of the potential use of this compound in the treatment of leishmaniasis. Possibly the effects of epoxy-α-lapachone in the control of lesions in mice are due to multifactorial actions on parasite physiology. The chemical structures of naphthoquinone derivatives, such as the compound in this study, contribute to the formation of reactive oxygen and accelerate intracellular hypoxic conditions, causing severe damage to the parasite cells (34, 35).

Flow cytometry results indicated that epoxy-α-lapachone was able to freely enter both parasite forms and eventually led to a loss of parasite plasma membrane integrity, as parasites exposed to this compound had DNA that was stainable by TO-PRO-3, a marker that is unable to cross intact plasma membranes (36). TMRE

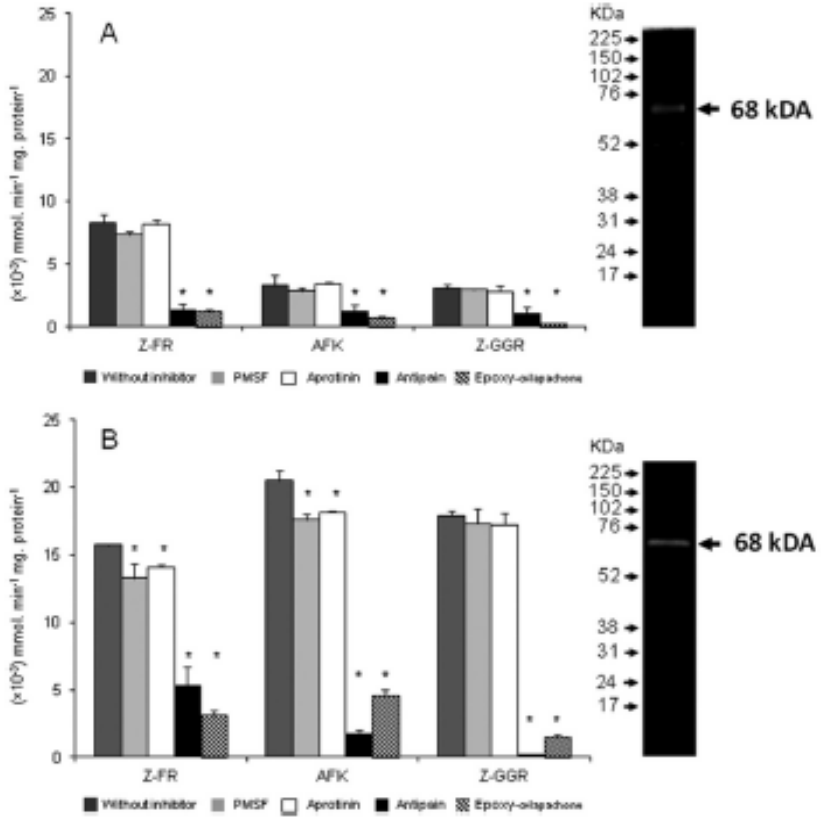

FIG 4 Proteinase activities of *Leishmania* (*L.*) *amazonensis* in solution. Fractions enriched in serine proteinase from promastigotes (A) and amastigotes (B) were obtained through benzamidine-Sepharose affinity chromatography. The enzymatic activities of fractions (10 μg) were measured with 100 μM levels of the substrates Z-FR-AMC, Z-GGR-MβNA, and AFK-AMC, in the absence (control) or presence of inhibitors (1 mM PMSF, 1 mg of aprotinin, or 1 mM antipain) or 1 mM epoxy-α-lapachone. The reaction mixtures were incubated for 60 min at 37°C in 10 mM Tris-HCl buffer (pH 7.5). The enzymatic activity of the fractions is expressed as $(\times 10^{-3})$ mmol · min^{-1} · mg $protein^{-1}$. Inset, zymographic profile of enriched serine proteinase fractions (5 μg). The molecular mass markers are indicated, and results are expressed as the means ± standard deviations from three independent experiments. *, $P < 0.05$.

staining demonstrated that this compound also possibly induced alterations in the membrane potential of parasite mitochondria, revealing yet another physiological effect of epoxy-α-lapachone on the parasites.

This effect was evidenced by fluorescent labeling indicating membrane potential ($\Delta\psi_m$) changes, mainly in mitochondria, using TMRE labeling (37). An experiment in which the organelle was reconstructed in three dimensions showed that the physical continuity of intact functional mitochondria can be determined by fluorescence from TMRE (38). Here, we propose that epoxy-α-lapachone is able to act in any metabolic pathway by compromising the $\Delta\psi_m$ of intracellular (primarily mitochondrial) organelles, as revealed by reductions in TMRE staining. The collapse of the mitochondrial transmembrane potential is related to the opening of mitochondrial permeability pores, leading to the release of cytochrome *c* into the cytosol, which then leads to other events in the apoptotic cascade (39).

Data from this study confirmed that epoxy-α-lapachone inhibited a 68-kDa proteinase from *L.* (*L.*) *amazonensis*, which was subsequently characterized as a serine proteinase, as it was isolated by benzamidine-based affinity chromatography and its hydrolytic activity was inhibited in the presence of classic serine proteinase inhibitors but was not affected by other proteinase inhibitors (data not shown). Additional data suggested that, similar to trypsin, the folding of the serine proteinase from *L.* (*L.*) *amazonensis* is

TABLE 1 IC_{50} values for inhibition of serine proteinase activity

Enzyme or fraction[a]	IC_{50} (mM)			
	Epoxy-α-lapachone	Antipain	PMSF	Aprotinin
Trypsin	0.9 ± 0.05	$(4.0 \pm 1.7) \times 10^{-3}$	1.4 ± 0.2	$(33 \pm 2.8) \times 10^{-3}$
Fraction from promastigotes	0.9 ± 0.1	$(4.1 \pm 0.2) \times 10^{-3}$	2.8 ± 0.8	$(0.9 \pm 0.04) \times 10^{-3}$
Fraction from amastigotes	1.2 ± 0.06	$(1.8 \pm 0.5) \times 10^{-3}$	9.5 ± 2.4	$(4.8 \pm 0.08) \times 10^{-3}$

[a] Serine proteinase fractions from *L.* (*L.*) *amazonensis* promastigotes and amastigotes were tested. Enzymatic assays were performed with 0.1 mM Z-FR-AMC substrate in 10 mM Tris-HCl (pH 7.5) and at least five concentrations of epoxy-α-lapachone, antipain, PMSF, and aprotinin. The data are expressed as means ± standard deviations.

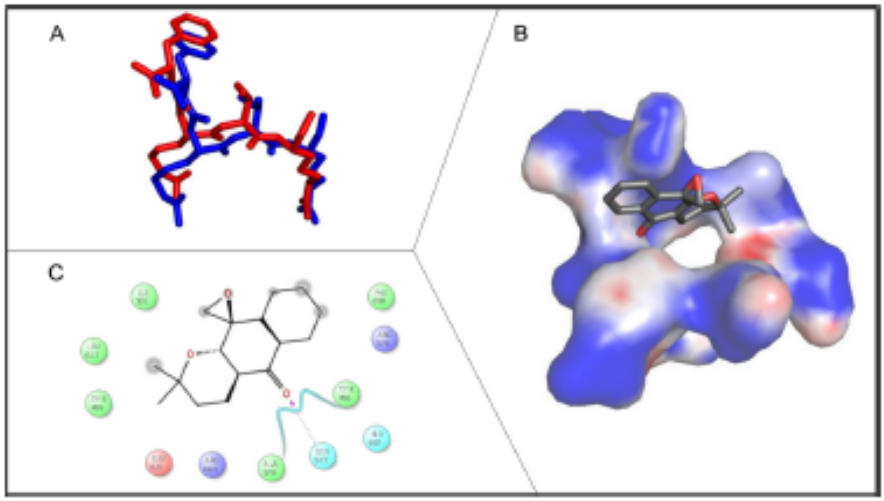

FIG 5 Docking complexes of compounds with *L.* (*L.*) *amazonensis* oligopeptidase B. (A) Structural alignment of the redocking complexes of antipain (blue) and antipain cocrystallized with oligopeptidase B (red). (B) Binding of epoxy-α-lapachone (sticks) in the active site (surface). (C) Details of amino acid residues at approximately 5 Å, showing interactions with epoxy-α-lapachone. Black, carbon atoms; red, oxygen atoms.

resistant to mild denaturing conditions, indicating that the structural stability of the isolated enzyme was maintained during the enzyme activity assays.

Other serine proteinases of various molecular sizes (i.e., 115 kDa [40], 68 kDa [41, 42], and 56 kDa [43]) were previously identified in *L.* (*L.*) *amazonensis* and may also be affected by the compound. Additionally, a serine proteinase named OPB has been described for other *Leishmania* species and has been found to play roles in many essential events for the parasites in their mammalian hosts (19, 44). These data indicate that many other potential serine proteinase targets that may be affected by epoxy-α-lapachone are present in the parasites, and they emphasize the importance of these molecules in parasite survival.

Notably, the affinity chromatography approaches applied here were able to demonstrate that, in *L.* (*L*) *amazonensis*, the amastigotes contain more serine proteinase than the promastigotes. Both parasite forms hydrolyze a selective group of substrates related to the fibrinolytic serine proteinases, i.e., Z-FR-AMC (kallikrein) (45), AFK-AMC (plasmin, urokinase, and thrombin) (46), and Z-GGR-AMC (urokinase) (47). These enzymes preferably cleave Arg and Lys residues in the P1 position and Gly and Ser (urokinase) and Pro, Ala, Gly, and Leu (thrombin) in the P2 position (48). Additionally, enzyme activity is greater at the parasite stage related to the infection of mammalian cells, which reinforces the hypothesis that serine proteinases are essential for *Leishmania* survival, are feasible targets for the development of new inhibitors such as epoxy-α-lapachone, as proposed here, and can be targeted in combined treatments for effective antileishmanial therapy, as recently suggested (49).

Generally, desired IC_{50} values for potential inhibitors are in the nanomolar or low micromolar ranges. Values for epoxy-α-lapachone were within this range, which importantly demonstrates that, in molecular docking, this compound was able to bind to the active center of a serine proteinase with inhibitory capabilities. Therefore, the mechanism of action of epoxy-α-lapachone on a *Leishmania* serine proteinase was assessed here by molecular docking.

Due to the absence of crystallography coordinates for the serine proteinase structure of *L.* (*L.*) *amazonensis*, molecular docking studies for these enzymes in *Leishmania* are constrained, because target-based ligand selection methods depend on the availability of target structural information (50). We experimentally and theoretically tested the ability of epoxy-α-lapachone to inhibit this enzyme.

Our theoretical evaluation of the docking complexes of epoxy-α-lapachone with *L.* (*L.*) *amazonensis* OPB and comparison of those complexes with complexes with a classic inhibitor (antipain) revealed that epoxy-α-lapachone underwent hydrophobic binding with residue Leu617 in the S3 pocket and formed hydrogen bonds with Ala578, Ser577, and Try496 in the S1 pocket of *L.* (*L.*) *major* OPB, with distances of 2.7 to 3.9 Å. These interactions may contribute to the stabilization and maintenance of epoxy-α-lapachone at the active site. In addition, epoxy-α-lapachone has an electrophilic moiety susceptible to nucleophilic attack by the activated catalytic serine at a distance that suggested this reaction. In summary, this study presents additional evidence that epoxy-α-lapachone can affect *L.* (*L.*) *amazonensis* parasites in mice during experimental infections and this compound can act as a serine proteinase inhibitor, making it a promising candidate to serve as a basis for the development of novel drugs for controlling leishmanial infections.

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All authors approved the final version of the manuscript.

We confirm that there are no conflicts of interest associated with this article.

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ANEXO II

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Natural products and phytotherapy: an innovative perspective in leishmaniasis treatment

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Abstract The interest in phytopharmaceutical products and herbal medicines has been a trend in recent years and this approach may be useful as basis for developing new treatment against leishmaniasis. In this review, we discuss the perspectives of leishmaniasis treatment based on natural products and phytotherapy and compare it to the advantages and disadvantages of using the current drugs of first- and second-choice against leishmaniasis. The reports gathered herein reinforce the leishmanicidal effects of medicinal plants and its derivatives, such as *Kalanchoe pinnata*, *Plumbago scandens*, *Physalis angulata*, *Piper aduncum*, *Peschiera* (*Tabernaemontana*) *australis*, *Phyllanthus amarus*, and *Artemisia annua*, and indicates their use as possible alternative or complementary treatments against leishmaniasis. The data presented here support the use of medicinal plants as safe and inexpensive treatments for leishmaniasis.

Keywords Natural products · Herbal medicines · Phytotherapy · Leishmaniasis treatment

Abbreviations

AmB	Amphotericin B
CL	Cutaneous leishmaniasis
CLF	Chloroform fraction
DCL	Diffuse cutaneous leishmaniasis
DMC	2′,6′-dihydroxy-4′-methoxychalcone
IL-2	Interleukin-2
INF-γ	Interferon gamma
Kp	*Kalanchoe pinnata*
LR	Leishmaniasis recidivans
MA	Meglumine antimoniate
ML	Mucocutaneous leishmaniasis
MOA	Mechanism of action
NO	Nitric oxide
NTDs	Neglected tropical diseases
PKDL	Post-kala-azar dermal leishmaniasis
SSG	Sodium stibogluconate
Th1	Type 1 T helper
TNF	Tumoral necrosis factor
VL	Visceral leishmaniasis

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Introduction

Leishmaniasis is classified as one of the neglected tropical diseases (NTDs) by the World Health Organization (WHO) and it is estimated that 350 million people are at risk of contracting this infection, while nearly two million new cases occur annually. Recently, some important advances have been made in the treatment, diagnosis and prevention of leishmaniasis, and the cost of key drugs has been reduced (WHO 2010). Nevertheless, current control programs are not sufficient for effective control of leishmaniasis, and mortality and morbidity worldwide show a worrying increase trend (WHO 2010). Various factors such as the epidemic of human immunodeficiency virus (HIV), increased international travel, lack of effective vaccines, ineffective vector control and the loss of efficacy due to the development of strains resistant to current chemotherapies led to increases in the number of leishmaniasis cases (Monzote 2009).

The group of diseases known as leishmaniases is caused by several species of the genus *Leishmania*. Parasite transmission occurs during the bloodmeal of phlebotomine insects (genus *Phlebotomus* in the Old World and genus *Lutzomyia* in the New World). Leishmaniases have traditionally been classified into three clinical forms, according to the parasite tropism: cutaneous leishmaniasis (CL), mucocutaneous leishmaniasis (ML) and visceral leishmaniasis (VL). These clinical features range from simple skin ulcers in CL to massive destruction of subcutaneous tissue in ML and injuries to internal organs in VL. Other cutaneous manifestations that may occur include diffuse cutaneous leishmaniasis (DCL), leishmaniasis recidivans (LR) and post-kala-azar dermal leishmaniasis (PKDL). When the infection affects liver macrophages and other organs in VL, the disease can be fatal if not treated (Croft and Coombs 2003; Health Ministry of Brazil 2006; Monzote 2009). Visceral leishmaniasis is mainly caused by *Leishmania donovani* or *Leishmania infantum* in endemic countries of the Old World, like India, Bangladesh and Sudan, and by *L. infantum* syn. *L.chagasi* in Brazil and other Latin America countries. In contrast, much higher diversity is observed for cutaneous cases as over twenty-one *Leishmania* species have already been related to dermotrophic clinical cases, which occur in ninety-eight countries worldwide and are endemic to eighty-two of those (Health Ministry of Brazil 2006; WHO 2010).

In this context, the search for novel antileishmanial drugs has been the subject of several studies. Interest in applying herbal extracts, essential oils and natural products towards this end has increased in recent years (WHO 2011). There is a growing number of public health programs designed to encourage the use of medicinal plants on the development of safe and cheap therapies for treating infectious and non infectious diseases. The focus is to promote treatments that would avoid dependency on imported reagents and that can be obtained via the development of family farming in local populations (WHO 2011; Health Ministry of Brazil 2012). Populations in rural areas rely mainly on the traditional use of plants to relief and diminish various disease symptoms. Extracts of medicinal plants or homologues of their components chemically synthesized certainly have potential as sources of specific compounds with antileishmanial activity.

Current chemotherapies of leishmaniasis and their pharmacological features and limitations

Currently, the pentavalent antimonial compounds are the drugs considered as first-line treatment in chemotherapy of leishmaniasis by the WHO. These compounds present a better therapeutic index than other antileishmanial drugs as amphotericin B and pentamidine, which are, therefore, called second-line drugs (Berman 1988). In Table 1 we summarize relevant information regarding therapeutic regimen, adverse effects and mechanism of action (MOA), when known, of each antileishmanial drug.

Pentavalent antimonials

Pentavalent antimonials [meglumine antimoniate (MA) and sodium stibogluconate (SSG)] have been used for many decades for the treatment of leishmaniasis and are still considered the 'gold standard' for CL treatment in the New World (Blum and Hatz 2009). A wide variation in the efficacy of pentavalent antimonial-based treatments has been reported and can be attributed to an array of factors: differing susceptibility to antimonial among *Leishmania* species, emergence of resistant parasite strains, variable

Table 1 Dugs currently used in leishmaniasis treatment, their most frequently adverse effects and mechanism of action

Drug	Dosage	Adverse effects		Mechanism of action
		Subjective complaints	Laboratorial alterations/toxicity	
First line				
Pentavalent antimonials (SSG/MA)	CL: 10–20 mg Sb^{5+}/kg/day, for 20 days at least or until healing the lesions. VL or ML: 20 mg Sb^{5+}/kg/day for 20–30 days (Berman 1988; Health Ministry of Brazil 2006; WHO 2010)	Myalgia, arthralgia, headache, nausea, vomiting, abdominal pain, and swelling at site of application (Herwaldt and Berman 1992; Oliveira et al. 2011)	Ventricular repolarization disorders (as alterations in T wave and ST segment); arrhythmia; mild to moderate transient increase of transaminases and/or amylases (Herwaldt and Berman 1992; Oliveira et al. 2011)	Possible inhibition of glycolytic and/or fatty acid oxidation; active efflux of thiols as glutathione and trypanotione and/or inhibition of trypanothione reductase, causing oxidative stress into parasite (Berman et al. 1985; Cunningham and Fairlamb 1995; Ameen 2007)
Second line				
Amphotericin B deoxicolate	CL: 0.5–1 mg/kg/day (total dose of 1–1.5 g); VL or ML: 1 mg/kg/day (total dose of 2.5–3 g) for 20 days in subsequent or alternate days (Monzote 2009)	Hyperpyrexia, phlebitis, headache, chills, asthenia, myalgia and arthralgia, vomiting and hypotension (Sundar 2001; Health Ministry of Brazil 2006)	Nephrotoxicity (decreasing of glomerular filtration), hypokalemia, liver disturbances and bone marrow depression (Sundar 2001; Health Ministry of Brazil 2006)	Formation of channel-like structures (pores) spanning the lipid bilayer by the interaction with membrane sterols causing altered permeability to cations, water and glucose and affect membrane-bound enzymes (Bolard 1986; Neumann et al. 2009)
Pentamidine	4–7 mg/kg of salt (isethionate) on alternate days (total dose of 2 g) (Health Ministry of Brazil 2006; WHO 2010)	Musculoskeletal pain, anorexia, abdominal pain, nausea, headache, mild to moderate pain at the site of application (Oliveira et al. 2011)	Rhabdomyolysis, hypotension, hyperglycemia or hypoglycemia and arrhythmia, insulin-dependent diabetes* (Sundar 2001; Delobel and Pradinaud 2003; Health Ministry of Brazil 2010)	Binding to the parasite kinetoplast causing profound ultrastructural alterations (Soeiro et al. 2013)
Miltefosine	Children aged 2–11 years: 2.5 mg/kg/day; people aged 12 years or more: 50 mg/day (b.w < 25 kg); 100 mg/day (b.w 25–50 kg); 150 mg/day (b.w > 50 mg) (WHO 2010)	Gastrointestinal alterations (mainly diarrhea and vomiting) (Croft and Engel 2006; Dorlo et al. 2012)	Transient abnormalities affecting kidney and liver function; teratogenicity (Croft and Engel 2006; Dorlo et al. 2012)	Inhibition of the PI3 K–Akt/PKB survival pathway in a dose-dependent manner, leading to apoptosis (Ruiter et al. 2003)
Paromomycin	VL: 15–20 mg/kg paromomycin sulfate (11–15 mg base) for 21 days; CL: ointment formulations twice daily for 20 days (Monzote 2009; WHO 2010)	Skin rashes, local pruritus and burs** (Sundar 2001)	Oto- and/or nephrotoxicity (Sundar et al. 2007)	Inhibition of protein synthesis by binding to the 30S ribosomal subunit of the 30–50S ribosomal complex at the start codon of mRNA, causing accumulation of an inactive initiation complex (Sundar and Chakravarty 2008)

* Toxic effect related to prolonged treatment and/or use of high doses, ** adverse effects related to topical formulations

host's immune response during treatment and, also, distinctions in pentavalent antimonials pharmacokinetic parameters, such as absorption and distribution, when the drug is administered in different ways (Berman 1988; Herwaldt 1999). As shown in Table 1 there is a range of treatment regimens recommended by WHO. Alternative regimens for CL, feature: intralesional administration; use of lower doses (below 10 mg Sb^{5+}/kg/day); and, combination with other drugs, such as imiquimod, allopurinol and immunotherapy (Herwaldt and Berman 1992; Arana et al. 1994; Martinez et al. 1997; Arevalo et al. 2007).

The MOA of pentavalent antimony is not well understood, but is known to be multifactorial, affecting important physiological pathways in *Leishmania*. The treatment with pentavalent antimonials can potentially elicit a wide spectrum of mild to serious adverse effects, the most severe being cardiotoxicity (Table 1).

Amphotericin B

Amphotericin B (AmB) is a polyene antibiotic, first isolated in 1955 from *Streptomyces nodosus* (Dutcher et al. 1959). It is the drug of choice for life-threatening systemic fungi infections, such as *Candida albicans* or *Aspergillus fumigatus*, and visceral leishmaniasis (Sundar and Chakravarty 2010). It is extensively used in clinical practice due the high morbidity and mortality rates caused by these fungal infections in patients with immunodeficiency, such as in cases of AIDS (Wolday et al. 2001).

However, the usefulness of AmB is limited due to severe nephrotoxicity, which can result in kidney failure (Cohen 1998). These side effects have led to extensive research for alternative formulations in the form of liposomes, emulsions and nanoparticles, all of which help to reduce the amount of free AmB in the blood stream, thereby reducing its toxicity (Brajtburg and Bolard 1996).

Generally, it is considered a second-line drug for leishmaniasis due to serious adverse effects associated with its use and drawbacks as parenteral administration, long-term treatment and need for constant clinical and laboratory monitoring (Table 1). It is noteworthy that AmB is considered the drug of first choice for treating leshmaniasis in pregnant women (Sundar 2001; Health Ministry of Brazil 2006).

Pentamidine

Pentamidine is an aromatic diamidine that was initially used as a hypoglycemic agent, but eventually was reported to present activity against a number of protozoa and fungi, including *Leishmania* spp, *Pneumocystis carinii* and African trypanosomes (Blum et al. 2004; Monzote 2009). Although being generally considered a second-line drug for the treatment leishmaniases, it is used as the first-line treatment against *L. guyanensis* infections in French Guyana, Surinam, and Brazil (Blum and Hatz 2009). In the past, two salt lyophilized formulations were manufactured, pentamidine mesylate (Lomidine) and pentamidine isethionate (Pentacarinat), and these drugs were used interchangeably according to local availability (Dorlo and Kager 2008). However, differences in the composition and used dose of these two formulations have been implicated in the trend of reduced efficacy noted in later studies of these treatments. A WHO Technical Report suggested a dose of 4 mg/kg without detailing whether the dose referred to the base or the salt formulation. In 1990, the production of Lomidine was interrupted and Pentacarinat began to be used at the dose of 4 mg/kg, which was equivalent to 2.3 mg/kg of Lomidine, resulting in the aforementioned dosage issues (WHO 1990; Delobel and Pradinaud 2003; Dorlo and Kager 2008).

Pentamidine, as well as other diamidines, must be administered parenterally, due to its poor bioavailability (Wilson et al. 2008). The most important toxic effect of pentamidine is associated with the development of insulin-dependent diabetes occurring in 5–12 % of cases (Table 1; Sundar 2001). The drug is contraindicated in cases of pregnancy, diabetes mellitus, renal or liver disease and heart diseases, and, also, for children weighing less than 8 kg (Health Ministry of Brazil 2010).

Miltefosine

Miltefosine, also known as hexadecylphosphocholine, was independently synthesized by two research groups screening for platelet-aggregating-factor analogues in the UK and in Germany (Dorlo et al. 2012). Miltefosine is indicated for the treatment of VL and CL caused by *L. mexicana*, *L. guyanensis* and *L. panamensis* under a dose regime which depends on the body weight of the patient (Table 1; WHO 2010).

Paromomycin

Paromomycin is an aminocyclitol aminoglycoside antibiotic, also known as aminosidine, produced by *Streptomyces riomosus var. paromomycinus* and first isolated in 1956 (Monzote 2009). It was initially used to treat intestinal infections, but its antileishmanial activity was demonstrated by Neal et al. (1968). This drug is poorly absorbed into systemic circulation after oral administration, but rapidly absorbed from intramuscular injection. It was reported that when paromomycin is given once daily in combination with pentavalent antimonials, the total duration of antileishmanial therapy is reduced (Murray 2001). Paromomycin is particularly useful to treat bacterial or co-amoebic infections, which are common in visceral leishmaniasis cases. Drawbacks to the aminoglycosides include the long duration of treatment and potential to cause oto- or nephrotoxicity (Table 1; Sundar and Chakravarty 2008).

Three formulations of paromomycin ointments have been used for treating CL: paromomycin 15 % plus methylbenzethonium chloride 12 %, paromomycin 15 % plus urea 10 % and paromomycin plus gentamicin 0.5 %. The success of these treatments has varied depending on the species of *Leishmania* causing the infection and the epidemiologic situation (Monzote 2009).

Other antileishmanial drugs under clinical evaluation

Other drugs already used in the therapy of other diseases have been evaluated for the treatment of leishmaniasis, however the clinical results in most cases are controversial. These includes: azoles, allopurinol and sitamaquine.

The imidazoles and triazoles are well known antifungal agents administered orally, which are well tolerated. These drugs also have antileishmanial activity against certain species as their MOA is based on the inhibition of 14α-demethylase, a key enzyme in ergosterol biosynthesis and essential for cell membrane biosynthesis in *Leishmania* spp. These agents, in addition to an easy administration method (orally), have the advantage of presenting few adverse effects. Fluconazole showed encouraging results against *L. major* infections in Iran, yet these results could not be reproduced in a subsequent study (Alrajhi et al. 2002; Morizot et al. 2007). Ketoconazole was applied in some studies with a small number of patients and showed a promising cure rate, however, these studies need further confirmation (Weinrauch et al. 1983, 1987; Saenz et al. 1990). Itraconazole was also tested in patients with CL caused by *L. major*, however no distinctive improvement could be assessed when compared to the control (placebo) group (Nassiri-Kashani et al. 2005).

The antileishmanial activity of the purine analogue allopurinol was identified more than 30 years ago (Arana et al. 2001). In spite of its advantages, such as oral administration and low toxicity, this drug was not effective in controlling infection in many reported cases. Allopurinol is an inhibitor of several enzymes in the purine pathway and has an effect on xanthine oxidase as well in the production of reactive oxygen species useful in eliminating the parasites, but has poor efficacy when used alone. It has been reported that allopurinol presents a synergistic effect when combined with antimonials for treating leishmaniasis (Martinez and Marr 1992; Martinez et al. 1997; Koutinas et al. 2001; Esfandiarpour and Alavi 2002; Momeni and Aminjavaheri 2003).

Sitamaquine is an active 8-aminoquinoline analogue. This analog of primaquine was originally developed for treating malaria, however, assays with animal models showed very encouraging results against VL (Monzote 2009). In the clinical studies (reaching phases I and II), the percentage of VL cure was up to 80 % in Kenya and 67 % in Brazil. However, adverse effects, such as nephrotoxicity and methemoglobinemia, were observed (Sherwood et al. 1994; Dietze et al. 2001; Wasunna et al. 2005; Jha et al. 2005).

The design and use of protease inhibitors have also been extensively studied as a new target since proteinases are believed to be virulence factors during *Leishmania* infection. Inside the mammalian host, cell parasites inhabit the parasitophorous vacuole characterized by an acidic environment (pH 4.7–5.2) and a great diversity of macromolecules. In this potentially hostile environment the differentiation of the metacyclic promastigotes into amastigotes occurs where the parasite must be able to avoid or subvert the host's immune responses. The proteinases become active and participate in nutrients acquisition, evolutive stage differentiation etc. (Selzer et al. 1999; Pereira et al. 2014).

Medicinal herbs, extracts and phytomedicines

Brief history and relevant concepts

Historically, the use of medicines and treatment of diseases began with the use of herbs. The term "drug", used to designate a medicinal preparation, is derived from the old Dutch word *droog*, which means "to dry", as plants were usually dried to be used as medicines (Schulz et al. 2013). Among the ancient civilizations of the Mediterranean and Asia, healing practices were divulged through the first encyclopedia related to herbs, *De Materia Medica*, written by the Greek physician and botanist Pedanius Diocorides in the first century AD (Leonti et al. 2009). Dioscorides was the first to treat Botany as a science applied to Pharmacy, a field nowadays known as Pharmacognosy. In the period between the XVIII and XIX centuries, advances in organic chemistry allowed the extraction and isolation of active constituents of medicinal plants, thus leading to the development of the research field known as natural product chemistry or phytochemistry (Allen et al. 2011).

The terms "medicinal herb" and "plant medicine" refer to plants, or their parts that contain active compounds, which have usually been dried to yield a storable product. A medicinal herb or its preparation is considered as a single active entity, whether or not its specific main active constituents are known. Phytomedicines are medicinal products whose pharmacologically active components derive exclusively from medicinal herbs. Once more than 75 % of the dry weight of vegetable material is usually made up of inert structural constituents such as cellulose and starches, which possess no relevant biological effects, most phytomedicines are produced from extracts. Extracts are concentrated preparations that present liquid, powdered or viscous consistency and are commonly obtained from dried or fresh plant parts by maceration or percolation. These preparations are complex mixtures with chemical compositions only partially known and vary in their biological components. The therapeutic dose usually expressed in mg/kg is often calculated based on the content of one or more identified active constituents or on the amount of the extract needed to produce a desired biological effect. Liquid forms of phytomedicines include tinctures, syrups, essential oils and medicinal essences, and the most common solid formulations are granules, uncoated or coated tablets and capsules (Schulz et al. 2013). The quality of a pharmaceutical formulation is a fundamental issue as the extracts present great chemical complexity. Quality assurance begins by the definition of "marker compounds" which are characteristic chemicals of the plant and which can help to determine the identity, standardization, product stability and processing of the botanical material (Yau et al. 2015).

Potential of herbal medicines and their derivates in the leishmaniasis treatment

Many research groups have searched for new potential antiparasitic drugs from vegetable sources, mainly secondary metabolites. Natural products continue to play an important role in therapy. In fact, between 1981 and 2010, 1355 new drugs were approved by the Food and Drug Administration (FDA), of which 4.7 % were natural products or their derivatives and 22 % contained pharmacophores derived from natural products (Newman and Cragg 2012).

The NTDs, including leishmaniasis, have received little to none interest from the pharmaceutical industry, as they affect mainly people in poor and developing countries and, in general, treatment is provided by government programs to control endemic diseases (Wink 2012). This lack of investment can be perceived by the fact that although the NTDs represent more than 12 % of the current global burden of diseases, only 1.3 % of the drugs used in their treatment were developed after 1975. Therefore, the search for novel therapeutic drugs for these diseases is deemed by to be of high priority by the WHO and natural products can be considered as potential sources for treating neglected diseases taking advantage of the wide range of chemical compounds present in the plants which have been little exploited in treatment of these maladies (WHO 2011).

In the last 30 years, much new evidence regarding the activity of medicinal plants and their components against *Leishmania* spp has been reported. Seven plants are particularly noteworthy: *Kalanchoe pinnata*, *Plumbago scandens*, *Physalis angulata*, *Piper aduncum*, *Peschiera* (*Tabernaemontana*) *australis*, *Phyllanthus amarus*, and *Artemisia annua*.

Plants with antileishmanial activity

Kalanchoe pinnata

(Family: Crassulaceae) is an herbaceous plant or shrub, sparsely branched and reaching 1 m in height,

especially during flowering. Its chemical composition comprises triterpenes, sterols, free and glycosylated flavonoids (quercetin and kaempferol) (Lorenzi and Matos 2008). In popular medicine it is used to treat diseases such as infections, rheumatism, gastric ulcer and inflammation in general. This plant is one of the most studied in the genus *Kalanchoe* and appears in the official list of medicinal plants of interest of Brazil's Ministry of Public Health.

The leishmanicidal effect of the aqueous extract from leaves of *K. pinnata* (Kp) was evaluated in an experimental animal model by infecting BALB/c mice with *Leishmania amazonensis* and comparing different administration routes (oral, intravenous, intraperitoneal and topical; Da Silva et al. 1995). The oral administration was shown to be most effective and reduced lesions after 30 days of treatment, in a manner similar to that of meglumine antimoniate. It was also demonstrated that Kp extract suppressed parasite growth. No mortality or evidence of toxic effects was noted in the animals during treatment. Kp is rich in flavonoids and this chemical group exhibits a wide range of biological effects including anti-allergic, antitumoral and anti-inflammatory activities (Pathak et al. 1991; Middleton and Kandaswami 1992). Additionally, it was reported that the antileishmanial activity of Kp extract is mediated by an increase in nitric oxide level in the macrophages and that it could also further enhance the NO-inducing activity of IFN-γ in a synergistic manner (Da-Silva et al. 1999). Muzitano et al. (2006) isolated quercitrin (quercetin 3-O-α-L-rhamnopyranoside) (Fig. 1a), which, with an IC_{50} of 1.0 μg/ml, showed higher activity (Table 2) and less cytotoxicity than the positive control pentostam. However, the activity of the crude extract was still higher than that of isolated compounds. Kp aqueous extract was clinically tested in a Brazilian patient (36 years old, 70 kg) who was infected with cutaneous leishmaniasis in the State of Amazonas. The patient received Kp aqueous extract in an oral dose of 215 mg/kg Kp (equivalent to 21 mg of lyophilized aqueous leaf extract) twice a day for 14 days. Results showed that the treatment significantly reduced lesion size, but after interruption of the short course of treatment, the infection returned and the patient was then submitted to conventional therapy. No alterations in serum transaminases, urea and alkaline phosphatase were observed and the patient did not report any complaints (Torres-Santos et al. 2003). The Kp extract was tested in an animal model of visceral infection by *L. infantum* **syn.** *L.chagasi*: BALB/c mice received oral daily doses of Kp extract (400 mg/kg) for 30 days and displayed significantly reduced hepatic and splenic parasite burdens that were similar to those achieved with pentostam treatment. A reduction in parasite-specific IgG serum levels and impaired capacity of spleen cells to produce IL-4 were also observed, but IFN-γ and nitric oxide levels were increased (Gomes et al. 2010).

Plumbago scandens

(Family: Plumbaginaceae) is a sub-evergreen shrub or climber, much branched, measuring 2–3 m in length and present along the borders of secondary forest in the Caatinga biome of Northeast Brazil (Lorenzi and Matos 2008). Traditionally, preparations of the roots of *P. scandens* are used for their purgative and local anesthetic properties in the form of infusion. Plumbagin (Fig. 1b), a naphthoquinone which is characteristic of most species of this genus, inhibits the growth of several types of fungus and is also effective against *L. donovani*, strongly inhibiting the growth of promastigotes (Table 2; Hazra et al. 2002). Recently, it was demonstrated that the effects of plumbagin as well as of its derivative 2-methoxy-1,4-naphthoquinone are due inhibition of trypanothione reductase and consequent subversion of its physiological role as an antioxidant, impairing redox homeostasis and causing the parasite's death (Sharma et al. 2012).

Physalis angulata

(Family: Solanaceae) is an annual herb distributed in many countries in tropical and subtropical regions and it is widely used in popular medicine for the treatment of a variety of pathologies, among them are liver diseases and malaria. It has also reported activity against tumors and *T. cruzi* (Lin et al. 1992; Chiang et al. 1992; Nagafuji et al. 2004; Abe et al. 2006). This plant produces a group of secosteroids, known as physalins, that demonstrated immunomodulatory effects in vitro and in vivo (Soares et al. 2003, 2006). Physalins B, F or G, but not D, cause a decrease in nitric oxide production in macrophages stimulated with lipopolysaccaride and interferon-γ and their effects occur by a pathway distinct of that of corticosteroids (Soares et al. 2003). Antileishmanial

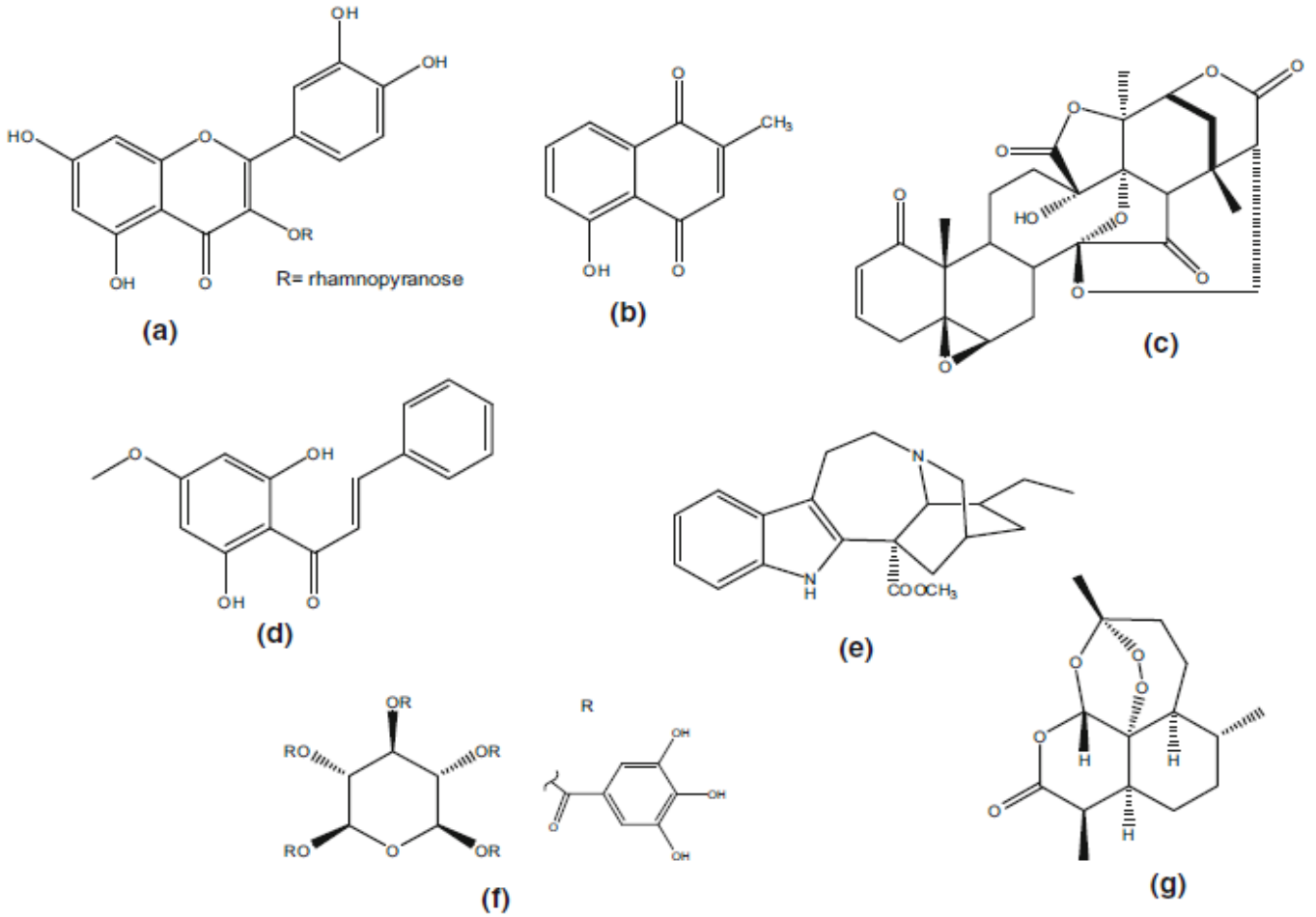

Fig. 1 Chemical structure of main active compounds. **a** Quercitrin: (CID 5359430; molecular formula, $C_{21}H_{20}O_{11}$; molecular weight, 448.3769 g/mol); **b** Plumbagin (CID 10205; molecular formula, $C_{11}H_8O_3$; molecular weight, 188.17942 g/mol); **c** Physalin F (CID 433531; molecular formula, $C_{28}H_{30}O_{10}$; molecular weight, 526.5318 g/mol); **d** 2′,6′-Dihydroxy-4′-methoxychalcone (CID 5316793; molecular formula, $C_{16}H_{14}O_4$; molecular weight, 270.27996 g/mol); **e** Coronaridine (CID 73489; molecular formula, $C_{21}H_{26}N_2O_2$; molecular weight, 338.44334 g/mol); **f** Pentagalloylglucose (CID 65238; molecular formula, $C_{41}H_{32}O_{26}$; molecular weight, 940.67718 g/mol); **g** Artemisinin (CID 68827; molecular formula, $C_{15}H_{22}O_5$; molecular weight, 282.33218 g/mol) (https://pubchem.ncbi.nlm.nih.gov/compound/12000280#section=Top)

activity of physalins B, D and F was tested in vitro against intracellular amastigotes of *L. amazonensis* and *L. major*, and in BALB/c mice infected with *L. amazonensis*. Physalins B and F, (Fig. 1c) but not D, caused a reduction in the percentage of macrophages infected at concentrations non-toxic for macrophages (Table 2). Daily topical administration of physalin F to BALB/c mice infected with *L. amazonensis* resulted in a significant reduction of the lesion size and parasite load when compared to mice treated only with vehicle. Considering that the host's control of *Leishmania* infection is dependent on macrophage activation and nitric oxide (NO) production and that the immunomodulatory activity of physalins B and F inhibits the production of NO and pro-inflammatory cytokines, the beneficial effect of *P. angulata* in the in vivo treatment of leishmaniasis seems to be due to a direct action of these secosteroids on the parasite or to its anti-inflammatory properties in the healing of lesions or to a combination of both these activities (Guimarães et al. 2009). Antileishmanial effects of physalins B, D, F and G on promastigotes of *L. amazonensis* and *L. major* were evaluated and all but not physalin D were able to induce a concentration-dependent inhibition of parasite proliferation with IC_{50} values of 6.8, 1.4, e 9.2 µM, respectively. Physalin F was the most effective one and showed an anti-parasitic activity comparable to amphotericin

Table 2 Antileishmanial activity of plant derived compounds as tested in vitro and in vivo

Plant species	Main active compounds/chemical groups	IC_{50} in amastigotes	In vivo assays	Mode of action
Kalanchoe pinnata	Quercitrin (Quercetin-3-O-α-L rhamnopyranosi-de)/ flavonoid (Muzitano et al. 2006)	1.0 μg/ml (La)	BALB/c mice infected with La (total dose of 8 mg) (Da Silva et al. 1995) BALB/c mice infected with Li (400 mg/kg) (Gomes et al. 2010)	Increased production of NO by the macrophages causing the death of the parasite (Da-Silva et al. 1999)
Plumbago scandens	Plumbagin (5-hydroxy-2-methyl-naftoquinone)	1.1 μg/ml (La) 0.4 μg/ml (Ld) (Croft et al. 1985)	BALB/c mice infected with La and Lv treated with 2.5–5 mg/kg/day of plumbagin (Fournet et al. 1992)	Inhibition of trypanothione reductase, impairing redox homeostasis of the parasite (Sharma et al. 2012)
Physalis angulata	Physalins B and F (secosteroids)	0.21 e 0.18 mM (La)	BALB/c mice infected with La, topical application 1x/day cream contain physalin F (Guimarães et al. 2009)	ND
Piper aduncum	2′,6′-Dihydroxy-4′-methoxychalcone (chalcone)	24 μg/ml (La)	ND	Destruction of the amastigotes mitochondria without affect the mitochondria of macrophages (Torres-Santos et al. 1999)
Peschiera (*Tabernaemontana*) *australis*	Coronaridine (Indole alkaloid)	12 μg/ml (La)	ND	Profound changes in the parasite without affecting the ultrastructure of the macrophage (Delorenzi et al. 2001)
Phyllanthus amarus	Pentagalloylglucose (Polyphenol)	1–8 μM (Ld)	ND	Macrophage activation probably due to release of NO, TNF or IFN-γ (Kolodziej and Kiderlen 2005)
Artemisia annua	Artemisinin (Sesquiterpene)	22 μM (Lm) (Sen et al. 2007)	Balb/c infected with Ld treated with 10 or 25 mg/kg/bw by oral route (Health Ministry of Brazil 2010)	Cell death directly induced via apoptosis accompanied by an enhanced activity by immunomodulation into macrophages (Sen et al. 2007) Additional effect of restoration of Th1 cytokines production such as INF-γ and IL-2 (Sen et al. 2010)

ND not determinated, *La L. amazonensis*, *Lm L. major*, *Ld L. donovani*, *Li L. infantum syn. L.chagasi*, *Lv L. venezuelensis*, *bw* body weight

B (IC_{50} of 3.0 μM). These effects may be due the presence of a double bond and an epoxy ring between carbons 5 and 6 which are present in both physalins B and F, suggesting its critical role in the anti-inflammatory and antileishmanial activity (Guimarães et al. 2010). Recently, the aqueous extract of *P. angulata* roots was tested against *L. amazonensis* and dose-dependent reduction on promastigotes (IC_{50} = 39.5 - μg/ml) and amastigotes (IC_{50} = 43.4 μg/ml) growth was observed. Chemical analysis detected physalins A, B, D, E, F, G and H in this extract. (da Silva et al. 2015).

Piper aduncum

(Family: Piperaceae) is an upright shrub, branched, with jointed stems, measuring 2–4 m and native throughout Brazil. Generally, tea and alcoholic extracts of the leaves, roots and fruits of *P. aduncum* are used as tonic, carminative, antispasmodic agents

and against gonorrhea and disorders of the liver, gallbladder and spleen (Lorenzi and Matos 2008). Phytochemical analysis identified the presence of 2′,6′-dihydroxy-4′-methoxychalcone (DMC) (Fig. 1d) in the leaves and inflorescences, which shows activity against promastigotes and intracellular amastigotes of *L. amazonensis* in vitro. The dichloromethane extract inhibited promastigote growth with an ED_{50} of 2.2 μg/ml, while purified DMC showed similar results at an ED_{50} of 0.5 μg/ml. When tested against intracellular amastigotes, DMC reduced by half the percentage of infected macrophages and the parasite load at 40 and 24 μg/ml, respectively (Table 2). No toxic effects on macrophages were observed up to 100 μg/ml of DMC, suggesting that it is selectively toxic to the parasites. After treatment with 50 μg/ml of DMC, promastigotes showed enlarged and more-diffuse mitochondrion profiles with a loss of matrix and cristae patterns. Treatment of infected macrophages with 80 μg/ml of DMC led to death of all parasites. The phagocytic function of macrophages was normal in the presence of DMC, however, the capacity of macrophages to produce NO in culture was inhibited at low concentrations used of this substance (Torres-Santos et al. 1999). The mechanism by which DMC destroys *Leishmania* parasites is still unknown. It is believed that it may be similar to other chalcones which inhibit glutathione reductase, perhaps through an inhibition of trypanothione reductase, the enzyme responsible for the redox balance in trypanosomatids. The effects of DMC on *L. amazonensis* promastigote's sterol biosynthesis through the mevalonate pathway were also studied. Several alterations in the sterol profile after DMC treatment were seen, such as a reduced uptake of cholesterol from the culture medium, a reduction of dehydroepisterol and episterol, which are the most abundant sterols of *L. amazonensis*. Ergosterol, ergosta-5,8-dien-3β-ol,4,14-dimethylzymosterol and lanosterol also accumulated in DMC-treated parasites. These findings indicate that DMC promotes the accumulation of intermediate sterols without inhibiting the biosynthesis of ergosterol, by an unknown mechanism, which may result in altered membrane fluidity and structure (Torres-Santos et al. 2009).

Peschiera (Tabernaemontana) australis

(Family: Apocynaceae) is a small tree found in Brazil and other countries of South America, and its extract is active against promastigotes and amastigotes of *L. amazonensis* in vitro (Lorenzi and Matos 2008). Delorenzi et al. (2001) tested a chloroform fraction (CLF), which is rich in indole alkaloids such as coronaridine (Fig. 1e), one of the most active alkaloids. An inhibition of 97 % in promastigote growth could be obtained with 12.5 μg/ml of coronaridine. Treatment with 20 μg/ml of CLF reduced parasite growth by 100 % after 72 h, suggesting irreversible damage to the ability of promastigotes to replicate. The activity of CLF and coronaridine against amastigotes was evaluated in infected macrophage cultures by adding the compounds in the first day of culture or once a day for 3 days: an exposure to 20 μg/ml of CLF induced death to 98 % of the parasites. When coronaridine and glucantime were used at 20 μg/ml, cell death rates were 79 and 70 %, respectively. Morphological alterations in the mitochondria were observed in both promatigotes and amastigotes exposed to CLF or coronaridine. The mitochondria exhibited remarkable swelling and disorganization of the internal-membrane cristae, however the kinetoplast DNA presented no changes. Also, no alterations were detected in the macrophages, suggesting that CLF has no toxic effects on these cells (Delorenzi et al. 2001). In another study, a synthetic analog of coronaridine, named 18-methoxycoronaridine (18-MCOR), was tested in murine macrophages infected with *L. amazonensis*. The effects of 18-MCOR and coronaridine were similar, however the synthetic analog was marginally more effective in killing amastigotes (IC_{90} of 16 μg/ml and IC_{90} of 22 μg/ml, respectively) (Delorenzi et al. 2002).

Phyllanthus amarus

(Family: Phyllanthaceae) is a common wild plant, erect, annual, branched horizontally and measuring 40–80 cm in height. It occurs in almost all tropical regions of the world, flourishing in the rainy seasons. The main popular use of *P. amarus* has been to eliminate kidney stones and as a diuretic. Pharmacological investigation justified its popular use by demonstrating the ability to relax the ureters and exert a simultaneous analgesic action. Phytochemical analysis of the plant's composition shows the presence of several flavonoids, lignans, triterpenoids, alkaloids and tannins (Lorenzi and Matos 2008). Tannins of *P. amarus* were shown to be highly active as

antileishmanials against *L. donovani* and *L. major*, especially ellagitannins of the geraniin type. Investigation of MOA of these tannins suggest they promote a release of TNF-α and interferons by the macrophages inducing the elimination of the amastigotes (Table 2). These tannins had no action on promastigotes of these same species but Onocha and Ali (2010) observed that the methanolic extract of *P. amarus* was active against promastigotes of *L. major* with an IC_{50} of 78 µg/ml with no signs of cytotoxity to macrophages (Kolodziej and Kiderlen 2005; Onocha and Ali 2010). The lignan niranthin isolated from aerial parts of *P. amarus* is able to elicit apoptotic events in the parasites by inducing the production of reactive oxygen species. Also, it activates nucleases leading to DNA fragmentation and can interact with topoisomerase I forming a cleavage complex. Niranthin inhibits the relaxation activity of heterodimeric type IB topoisomerase, acting as a non-competitive inhibitor (Chowdhury et al. 2012).

Artemisia annua

(Family: Asteraceae) is an annual erect, aromatic herb, from Asia and is cultivated in Brazil. The plant has been used for centuries in traditional medicine in both China and India for the treatment of fever and lupus erythematosus. More recently, with the discovery of its antimalarial properties, new studies have extended its use as an insecticide, herbicide and antiulcerogenic (Lorenzi and Matos 2008). In Brazil, there are no ethnopharmacological records of its popular use. Phytochemical analysis demonstrated a high content of essential oils (about 4 %), whose composition varies widely depending on the growing conditions and the chemotype of the plant. In *A. annua*, the oils present a variable concentration of monoterpenes and a high content of artemisia ketone (about 64 %). Oils obtained by acclimated plants in other countries have different compositions comprising cineole, beta-pinene, artemisia-alcohol, camphor and caryophyllene. Also present are fixed constituents such as triterpenoids, sterols, coumarin and a sesquiterpene lactone, known as artemisinin (Fig. 1g), discovered in 1972 and which has revolutionized antimalarial therapy (Lorenzi and Matos 2008). The activity of the methanolic extract of the leaves of *Artemisia indica*, another species rich in artemisinin, was tested against promastigotes of six *Leishmania* species, showing IC_{50} values between 210 and 580 µg/ml (Ganguly et al. 2006). In another investigation the effect of artemisinin on promastigotes and amastigotes of *L. donovani* was also studied. At concentrations between 0 and 0.5 mM tested for 48 h, dose-dependent inhibition of growth of promastigotes (with IC_{50} of 160 µM) was observed. Artemisinin also showed a dose-dependent reduction in the survival of intracellular amastigotes (Table 2). The cytotoxicity of artemisinin to murine macrophages was evaluated; their viability remained unchanged until reaching a very high concentration of artemisinin (0.25 mM). It was shown that artemisinin exerts its effects by inducing apoptosis in *Leishmania* associated with depolarization of mitochondria (Sen et al. 2007). The activity of n-hexane fractions of *A. annua* leaves and seeds was tested against *L. donovani* and the IC_{50} values for intracellular amastigotes were 6.6 and 5.0 µg/ml, respectively. The antileishmanial effect was mediated via apoptosis and active components as α-amyrinyl acetate, β-amyrine and derivatives of artemisinin were implicated as responsible for the activity (Islamuddin et al. 2012). These fractions when administered orally to BALB/c mice (200 mg/kg) reduced hepatic and splenic parasite burdens, induced significant delayed-type hypersensitivity response and elicited a Th1 immune response, characterized by increased levels of IFN-γ with generation of immunological memory (Islamuddin et al. 2015). Essential oil from *A. annua* leaves, constituted mainly by Camphor and β-caryophyllene, showed leishmanicidal effect against *L. donovani* promastigotes (IC_{50} of 14.63 ± 1.49 µg/ml) and intracellular amastigotes (IC_{50} of 7.3 ± 1.85 µg/ml). Intraperitoneal administration of 200 mg/kg (leaf essential oil) to BALB/c mice infected with *L. donovani* reduced parasite burden by almost 90 % in the liver and spleen (Islamuddin et al. 2014).

Final considerations

Leishmaniasis is still one of the most neglected tropical diseases, affecting largely the poorer populations of developing countries. Hundreds of thousands of people are under risk of contracting some form of the disease and around 2 million new cases occur yearly. Current treatment is based on the use of pentavalent antimonials for more than 60 years and, in

cases of intolerance, contraindication, or poor clinical response it may administer amphotericin B or pentamidine. However, all these drugs present a wide range of adverse effects, depend on prolonged parenteral administration and are related to increasing numbers of resistance cases. Additionally, issues such as high cost and technological dependence should be considered by the developing countries.

One of the greatest problems faced in this area is that new drug candidates rarely go beyond animal assays. In fact, the few advances are limited to development of new formulations of the drugs currently in use or in clinical trials with drugs already in use to treat other diseases. These surveys constitute progress, but cannot be considered the actual innovations.

The potential innovations in the treatment of leishmaniasis can be estimated by a search in the database of the European Patent Office, using the terms "Leishmaniasis" and "Treatment" and considering a period from 2006 to 2016. In this data bank only 13 patents directly related to the treatment of the disease were deposited within the query parameters. Among these, seven are immunomodulators, five are synthetic compounds and one is pharmaceutical composition using lipossomes. Additionally, a new search with the term "Leishmaniasis" associated to "Natural products", "Medicinal plants", "Herbal medicines" or "Phytotherapy" found no results. Currently, there is no novel medicine or formulations approved for the treatment of the disease. The approval of a novel drug or therapeutic approach by the regulatory agencies requires first its submission to clinical trials to ensure its safety and efficacy to humans.

We emphasize here the importance of researching and developing new antileishmanial drugs based on herbal medicine and natural products. The use of plants and their derivatives has a long history in humanity and some have great potential to treat leishmaniasis and other neglected diseases. This can be an important alternative to the lack of investment by large pharmaceutical companies.

Another possibility is the study of isolated active molecules in plant extracts as prototypes to produce more effective synthetic analogues and/or less toxic. An example is lapachol, a naphthoquinone extracted from various plants of the family of Bignoniaceae and Verbanaceae that showed to be effective in vitro against intracellular amastigotes of *L. braziliensis*, whereas in a hamster model, it was not able to prevent lesions provoked by the infection (Teixeira et al. 2001). However, it was previously showed that lapachol is a hemolytic agent and exhibited strong fetotoxicity and embryotoxicity (Guerra et al. 1999; Duke 2002; Lima et al. 2004). As the toxic effects of lapachone and their natural derivatives could limit their potential in the treatment of leishmaniasis, studies have applied chemical modifications in these compounds to variate the quinonoid center of α-lapachone. This approach generated the epoxy-α-lapachone (2,2-dimethyl-3,4-dihydrospiro[benzo[g]chromene-10,20-oxiran]-5(2H)-one), a derivative potentially less toxic for mammalian cells (Ferreira et al. 2006). This naphthoquinone has been studied by our group and is a good candidate to develop a new antileishmanial drug, as it has been shown to have low cytotoxicity for mammalian cells while being effective against *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* (Souza-Silva et al. 2014, 2015).

In this scenario, the development of phytomedicines with rational use of natural resources can contribute to the consolidation of public policies that may increasing the competitiveness of the pharmaceutical industry of developing countries.

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Compliance with ethical standards

Conflict of interest The authors declare no competing financial interest.

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ANEXO III

Hindawi
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Supplementary material

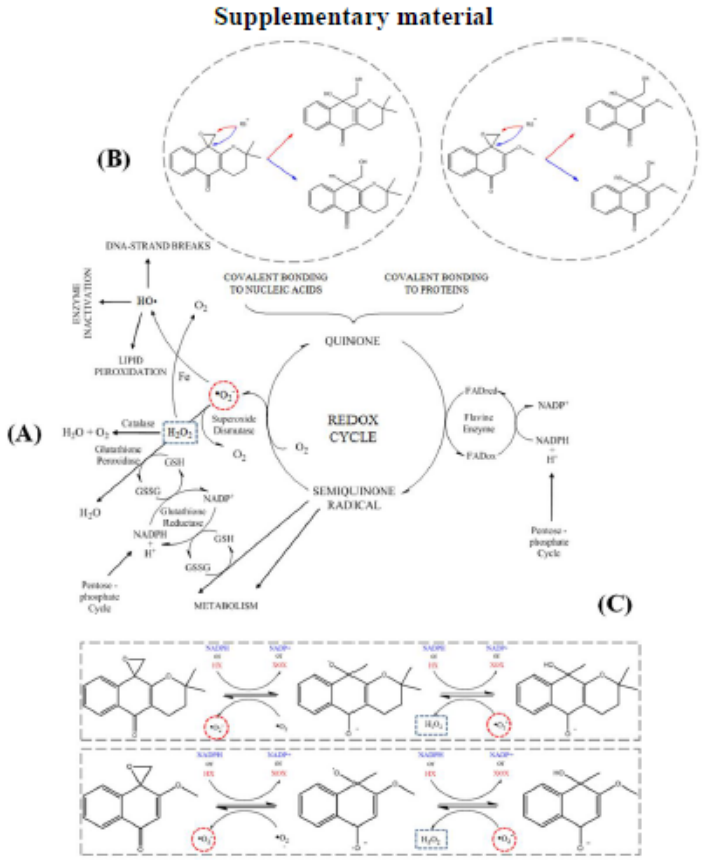

Figure - (A): Overview of redox cycling of quinonoide compounds showing oxy radical formation and inactivation (adapted from Kappus 1986). The one electron reduction of quinones is catalyzed by NADPH-cytochrome P450 reductase and other flavoprotein enzymes, leading to unstable semiquinones which transfers electrons to molecular oxygen (O_2), returning to their original quinoidal structure. A superoxide anion radical ($^{\bullet}O_2^-$) is generated and can be converted to hydrogen peroxide (H_2O_2) via a superoxide dismutase (SOD)-catalyzed reaction, followed by the formation of a hydroxyl radical ($HO^{\bullet}$) through the iron catalyzed reduction of peroxide via the Fenton reaction. **(B)**: Toxicity mechanism proposed (adapted from Kumagai 2012) is that these compounds may act as electrophiles forming covalent bonds with nucleophilic functions in biological molecules in an arylation reaction. When the nucleophile is a thiol group (represented by RS^-), the reaction generates a thioether, which is usually stable. Differently from quinones, the oxiranes not suffer the called Michael addition due to the most electronegative part of these compounds is in the oxirane moiety. The thiol of cysteine (Cys) is the major redox-active and nucleophilic functional group in biological systems. This amino acid is a component of the redox-active peptide gluttathione (GSH) and many proteins. **(C)**: Another toxicity mechanism proposed (adapted from Kumagai 2012) to explain histological changes found in healthy animal tissues treated with oxiranes. Oxiranes are synthetic derived from natural naphthoquinone and may share the toxic properties already described for these quinones and might participate in the initiation of and the propagation of free radical chain reactions. Free radicals are reactive chemical species with an unshared electron that can be transferred to other species. When oxygen is involved in these reactions, it is reduced to reactive oxygen species (ROS), a common term used to describe superoxide anion radical ($^{\bullet}O_2^-$), hydroxyl radical ($HO^{\bullet}$), and hydrogen peroxide (H_2O_2). Epoxy-α-lapachone and epoxymethoxy-lawsone were used at dose of 22.7 mg/Kg/day and 11.4 mg/Kg/day, respectively, administrated daily from Monday to Friday, until 20 doses. The dotted shapes show superoxide anion radical ($^{\bullet}O_2^-$) and hydrogen peroxide (H_2O_2) generated in both A and C. HX/XOX: Hypoxanthine/Xanthine oxidase.