MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Doutorado em Programa de Biologia Parasitária

EPOXI-α-LAPACHONA: UMA NOVA PERSPECTIVA PARA QUIMIOTERAPIA DAS LEISHMANIOSES

FRANKLIN SOUZA DA SILVA

Rio de Janeiro
Novembro de 2014

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Parasitária

Franklin Souza da Silva

Epoxi-α-lapachona: uma nova perspectiva para quimioterapia das leishmanioses

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Biologia Parasitária

Orientadores: Dr. Carlos Roberto Alves

Dr. Ernesto Raúl Caffarena

Rio de Janeiro

Novembro de 2014

Souza da Silva, Franklin.

Epoxi-α-lapachona: uma nova perspectiva para quimioterapia das leishmanioses / Franklin Souza da Silva. - Rio de Janeiro, 2014.
167 f.; il.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Parasitária, 2014.

Orientador: Carlos Roberto Alves .
Co-orientador: Ernesto Raúl Caffarena.

Bibliografia: Inclui Bibliografias.

1. Leishmania spp.. 2. Fármacos. 3. epoxi-α-lapachona. 4. Oligopeptidase B. 5. Quimioterapia. I. Título.

Elaborado pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/Icict/Fiocruz com os dados fornecidos pelo(a) autor(a), sob a responsabilidade de Igor Falce Dias de Lima - CRB-7/6930.

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz

INSTITUTO OSWALDO CRUZ

Pós-Graduação em Biologia Parasitária

AUTOR: Franklin Souza da Silva

Epoxi-α-lapachona: uma nova perspectiva para quimioterapia das leishmanioses

ORIENTADORES: Prof. Dr. Carlos Roberto Alves

Prof. Dr. Ernesto Raúl Caffarena

Aprovada em: xx / 11 / 2014

EXAMINADORES:

Prof. Drª. Veronica Figueiredo do Amaral - **Presidente** (UFF)

Profª. Drª. Rafaela Salgado Ferreira (UFMG)

Profª. Dr. Herbert Leonel de Matos Guedes (UFRJ)

Profª. Drª. Mirian Claudia de Souza Pereira (FIOCRUZ)

Profª. Dr.Mauricio Garcia de Souza Costa (FIOCRUZ)

Rio de Janeiro, x Novembro de 2014

Ata da defesa de tese de doutorado em Biologia Parasitária de **Franklin Souza da Silva**, sob orientação do Dr. Carlos Roberto Alves e co-orientação do Dr. Ernesto Raúl Caffarena. Ao vigésimo sexto dia do mês de novembro de dois mil e quatorze, realizou-se às treze horas, no Auditório Maria Deane/Fiocruz, o exame da tese de doutorado intitulada: "***Epoxi-a-lapachona: uma nova perspectiva para quimioterapia das leishmanioses***" 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: Desenvolvimento de Métodos de Diagnóstico e Controle das Doenças Infecciosas e Parasitárias. A banca examinadora foi constituída pelos Professores: Drª. Veronica Figueiredo do Amaral - UFF/RJ (Presidente), Drª. Mirian Claudia de Souza Pereira - IOC/Fiocruz e Dr. Herbert Leonel de Matos Guedes - UFRJ/RJ e como suplentes: Dr. Mauricio Garcia de Souza Costa - PROCC/Fiocruz e Drª. Rafaela Salgado Ferreira - UFMG/MG. 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 doutorado 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, 26 de novembro de 2014.

Drª. Veronica Figueiredo do Amaral (Presidente da Banca): Veronica F. Amaral.

Drª. Mirian Claudia de Souza Pereira (Membro da Banca): Mirian Claudia de Souza Pereira

Dr. Herbert Leonel de Matos Guedes (Membro da Banca): Herbert Guedes

Dr. Rafael Maciel de Freitas (Coordenador do Programa):

DEDICATÓRIA

Dedico esta tese

Aos meus pais que sempre acreditaram que a educação é o melhor caminho para o sucesso profissional;

A minha amada irmã, que acompanhou toda a minha trajetória estando ao meu lado nas idas e vindas à Fundação Oswaldo Cruz;

A minha esposa pelo carinho, amor e compreensão;

A minha filha que transformou minha vida na reta final da tese;

A todas as pessoas que acreditaram em mim, me dando força e incentivo durante a minha jornada acadêmica.

AGRADECIMENTOS

Aos meus orientadores: Carlos Roberto Alves e Ernesto Raúl Caffarena, que ao longo da minha jornada academia, formaram uma bela dupla (yin-yang), que com características distintas souberam me apoiar e incentivar nos momentos mais difíceis. Essa relação, transcendeu as características de aluno e orientador e, por isso, não os considero somente como orientadores e sim verdadeiros amigos.

A colaboração:

Lea Cysne Finkelstein (IOC/Fiocruz), que além do ensinamento de como lidar com as *Leishmanias* me mostrou como um profissional deve se portar na ciência (com dedicação, compromisso e perseverança).

Aos amigos e colaboradores do Grupo Biomarcadores:

Luzia Monteiro de Castro Côrtes, e Francisco Odêncio de Oliveira Junior, Michelle Lopes Ribeiro Guimarães, Mariana Silva Almeida, Cinthia, Carla e Luiz e Raquel Santos de Souza.

Em especial ao amigãozão, Bernardo Acácio Santini Pereira, pelo companheirismo, pela co-orientação e por ser o grande responsável pela diversão do grupo.

Aos membros do Laboratório de Biologia Molecular e Doenças Endêmicas: Constança Felícia de Paoli de Carvalho Britto, Maria Angélica, Daniela Pita, Thais, Myllena, Cristiane, Maria Carolina, Taiana, Daniela, Leticia, Pedro Cláudia Masini, Vitor Ennes, Sheila Medeiros, Bianca, Keyla, Lívia, Aline, Otacílio da Cruz, Nathalia, Paula, Ícaro, Ronaldi, José Batista, André, Geovani, Camila.

Á Coordenação do Curso de Pós-graduação em Biologia Parasitária do Instituto Oswaldo Cruz.

Ás instituições: Fundação Oswaldo Cruz; Instituto Oswaldo Cruz; Conselho Nacional de Desenvolvimento Científico e Tecnológico: pela bolsa de doutorado; Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro: projeto financiado (E-26/110.257/2010); Coordenação de Aperfeiçoamento de Pessoal de Nível Superior: projeto financiado (Pró-equipamentos Institucional/2009).

Aos meus pais, irmã e esposa Severino José da Silva; Marilena Souza da Silva, Flavia Souza da Silva e Tatiane Tavares Benvindo Cruz

INSTITUTO OSWALDO CRUZ

Epoxi-α-lapachona: uma nova perspectiva para quimioterapia das leishmanioses

RESUMO

O foco deste projeto é analisar os efeitos do epoxi-α-lapachona sobre *Leishmania (Viannia) braziliensis* e *Leishmania (Leishmania) amazonensis*. Inicialmente é proposto que promastigotas e amastigotas de ambas as espécies são afetadas pelo composto. A incubação com epoxi-α-lapachona levou a uma diminuição significativa no número de promastigotas de ambas as espécies em culturas, de forma dose e tempo dependente. A sobrevivência de amastigotas que habitam macrófagos humanos, também foi afetada pelo composto, tal como mostrado pelas variações do índice de endocitose. Na segunda fase deste trabalho, foi oportuno obter mais dados sobre a ação epoxi-α-lapachona em *L. (L.) amazonensis*. Este composto causa um drástico efeito interno em promastigotas e formas amastigotas após o contato na cultura, e em seguida, levando a morte de parasita com comprometimento da membrana plasmática como um evento tardio. Os ensaios realizados in vivo com infecção de camundongos BALB/c causada por *L. (L) amazonensis* mostram que 0,44 mM deste composto é capaz de causar diminuição da lesão de pata de 30,8 ± 2,6 mm2 para 24,9 ± 2,0 mm2, em comparação ao tratamento controle (glucantime = 28,3 ± 1,5 mm^2). Na terceira fase deste trabalho foi avaliado um possível alvo molecular do epoxi-α-lapachona. Os ensaios enzimáticos realizados mostram que este composto pode agir como um inibidor de uma serino proteinase de 68 kDa da *L. (L) amazonensis*. Em concordância com os ensaios enzimáticos, as análises teóricas realizadas mostram que o epoxi-α-lapachona pode ligar no centro ativo de oligopeptidase B da *L. (L) amazonensis*. O conjunto dos resultados aqui apresentados indica que o epoxi-α-lapachona tem uma ação leishmanicida sobre as *Leishmania* spp em ambas as fases morfológicas dos parasitos. Os dados obtidos neste projeto agregam informações relevantes sobre o potencial de epoxi-α-lapachona para o tratamento das leishmanioses.

Palavras-chave: *Leishmania* spp. *Leishmania (V.) braziliensis*, *Leishmania (L.) amazonenses;* epoxi-α-lapachona; quimioterapia, fármacos, oligopeptidase B

INSTITUTO OSWALDO CRUZ

Epoxy-α-lapachone: a new perspective for Chemotherapy of Leishmaniasis

ABSTRACT

The focus of this project is to analyze the effects of epoxy-α-lapachone on *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*. Initially was proposed that promasigotes and amastigotes from both species were affected by this compound. The incubation with epoxy-α-lapachone led to a significant decrease in the numbers of promastigotes from both species in the cultures, in a dose-and time-dependent fashion. The survival of amastigotes inhabiting human macrophages was also affected by the compound, as shown by the variations in the endocytic index. In the second phase of this work, we have the opportunity to obtaining more data of the epoxy-α-lapachone action on *L. (L.) amazonensis*. This compound cause a drastic internal effect in promastigotes and amastigotes forms after the contact in culture, and then leading the killing of parasite with impairment of the plasma membrane as a late event. The performed *in vivo* assays with BALB/c mice infection caused by *L. (L) amazonensis* show that this 0.44 mM of this compound cause a decreased of paw lesion of 30.8 ± 2.6 mm^2 to 24.9 ± 2.0 mm^2, compared to control treatment (glucantime = 28.3 ± 1.5 mm^2l). In the third phase of this work was assessed a possible molecular target of this epoxy-α-lapachone. The performed enzymatic assay show that this compound can act as an inhibitor of 68 kDa serine proteinase of the *L. (L) amazonensis*. In agreement with the enzymatic assays, the performed theoretical analysis show that epoxy-α-lapachone bind in the active center of *L. (L) amazonensis* oligopeptidase B. The set of results presented here indicate that the epoxy-α-lapachone has a leishmanicidal effect over *Leishmania spp* in both morphological stages. The data obtained in this project add relevant informations about the potential of epoxy-α-lapachone for the treatment of leishmaniasis.

Keywords: *Leishmania spp., Leishmania (V.) braziliensis*, *Leishmania (L.) amazonenses;* epoxy-α-lapachone; chemotherapy, drug, oligopeptidase B

ÍNDICE

ÍNDICE DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

AP-1	Proteína de Ativação-1
C3	Componte 3 do sistema complemento
C3b	fragmento 3b do sistema complemento
CPA	Cisteíno Proteinase A
CPB	Cisteíno Proteinase B
CPC	Cisteíno Proteinase C
GDP-Man	Guanosina Difosfato-Manose
GP63	Glicoproteína de 63 kDA
HaCaT	Queratinócitos Humanos Imortalizada Espontaneamente
iC3b	fragmento 3b inativo do sistema complemento
IFN-γ	Interferon Gamma
IL-12	Interleucina
IL-4	Interleucina
iNOS	Óxido Nítrico Sintase Induzível
IRAK-1	Interleucina-1 Associada ao Receptor Quinase-1
IκB	Inibidor de Transcrição Nuclear Kappa B
JAK/STAT	Janus Kinase/ Transdutor de Sinal e Ativador de Transcrição
kDA	Quilo Dalton
kg	Quilograma
LC	Leishmaniose Cutânea
LCAD	Leishmaniose Cutânea Anérgica Difusa
LCD	Leishmaniose Cutânea Difusa
LCDL	Leishmaniose Cutânea Disseminada Limítrofe
LCL	Leishmaniose Cutânea Localizada
LM	Leishmaniose Mucosa
LMC	Leishmaniose Mucocutânea
LPG	lipofosfoglicano
LTA	Leishmaniose Tegumentar Americana
LV	Leishmaniose Visceral
MA	Antimoniato de Meglumina

MARCKS	Substrato de Quinase C Rico em Alanina Miristoilado
mg	Miligrama
MHC	Complexo Principal de Histocompatibilidade
mTOR	Alvo da rapamicina em mamíferos
NF-kB	Fator de Transcrição Nuclear Kappa B
NO	Óxido Nítrico (NO)
OMS	Organização Mundial da Saúde
ON	Oxido Nítrico
OPB	Oligopeptidase B
PTPs	Proteína Tirosina Fosfatase
Sb^{3+}	Antimonial Trivalente
Sb^{5+}	Antimonial Trivalente
SSG	stibogluconato de sódio
STAT	Transdutor de Sinal e Ativador de Transcrição
TGF-β	Fator de Crescimento-beta
Th1	*Type 1 T helper*
Th2	*Type 2 T helper*
UDP-Glc	Uridina difosfato-Glicose
UDP-GlcNAc	Uridina difosfato-N-acetylglucosamina
VP	Vacúolo Parasitóforo

1. INTRODUÇÃO

1.1. Importância

As leishmanioses são doenças infecto-parasitárias e fazem parte do grupo das doenças tropicais endêmicas que acometem o homem. Estas doenças são causadas por mais de 20 espécies de protozoário parasito do gênero *Leishmania,* os quais são transmitidos por insetos flebotomíneos fêmeas do gênero *Lutzomyia*. É descrito casos dessas doenças em 98 países e 3 territórios nos 5 continentes do mundo (Alvar et al., 2012). A Organização Mundial da Saúde estima que cerca de 310 milhões de pessoas correm o risco de adquirir leishmaniose, com ocorrência de 1,3 milhões de novos casos por ano. No entanto, 600 mil casos são notificados, ocorrendo entre 20 e 30 mil casos fatais por ano (WHO, 2010).

Os relatados de leishmaniose no mundo estão relacionados há três principais formas de manifestação clínica:

(1) Leishmaniose Visceral (LV) é a forma mais grave da doença, que tem uma taxa de mortalidade de quase 100%, se não for tratada. Apresenta sintomas com febre irregular, perda de peso, inchaço do baço e fígado e anemia. As espécies do complexo *Leishmania (Leishmania) donovani* são as principias responsáveis por esta manifestação clínica. Outros sintomas, caracterizado como leishmaniose dérmica pós-calazar, podem aparecer vários meses (ou anos) após o tratamento de LV (Hide et al., 2007);

(2) Leishmaniose Mucocutânea (LMC) é causada principalmente por *Leishmania (Viannia) braziliensis,* e raramente por espécies do complexo *Leishmania (Viannia) guyanensis*, produz lesões mutilante que podem levar à destruição extensa dos tecidos nas mucosas do nariz, boca e corpo, incluindo o rosto, braços e pernas (Hide et al., 2007);

(3) Leishmaniose Cutânea (LC), são as mais comuns, representam 50-75% de todos os novos casos. Refere-se a lesões localizadas, podendo haver cura espontânea, ao contrário da leishmaniose cutânea difusa (LCD) e leishmaniose cutânea disseminada (Hide et al., 2007). Na LCD não há cicatrização espontânea e pode ocorrer reincidência após o tratamento. Esta forma é caracterizada por

lesões nodulares disseminadas que se assemelham a hanseníase. Os agentes etiológicos da LC no Velho Mundo são *Leishmania (Leishmania) aethiopica, Leishmania (Leishmania) tropica* e *Leishmania (Leishmania) major* assim como, *Leishmania (Leishmania) donovani*, descrito por Laveran e Mesnil; 1903, e *Leishmania (Leishmania) infantum*, descrito por Nicolle; 1908, que também já foram isoladas a partir de lesões cutâneas. As principais espécies do Novo Mundo ou estão nas espécies do complexo *Leishmania (Leishmania) mexicana* ou no subgênero *Viannia* que também é referida como espécies do complexo *Leishmania (Viania) braziliensis* (CDC, 2014).

A LV é altamente endêmica no subcontinente indiano e na África Oriental, com índice de infecção de 200 a 400 mil pessoas a cada ano e ocorrência de mais de 90% de novos casos em seis países: Bangladesh, Brasil, Etiópia, Índia, Sudão do Sul, e Sudão (WHO, 2010).

Com relação LC 95% dos casos ocorrem nas Américas, bacia do Mediterrâneo, Ásia Central e Oriente Médio. Cerca de dois terços de novos casos de LC incide seis países: Afeganistão, Argélia, Brasil, Colômbia, Irã (República Islâmica) e Síria (República Árabe). Estima-se que 0,7 a 1,3 milhões de novos casos ocorrem anualmente em todo o mundo (WHO, 2010). Além disso, LC afeta principalmente crianças com faixa etária acima de 10 anos o que corresponde 90% dos casos (Alvar et al., 2012). Já LMC ocorre em quase 90% no estado plurinacional da Bolívia, Brasil e Peru, com uma taxa de infecção menor que 5%, mas com a região, sua maior prevalência tem sido correlacionada às áreas onde LC foi recentemente introduzida. (Bedoya-Pacheco et al., 2011). Contudo, a infecção por *Leishmania* ainda pode causar uma grande variabilidade de atípicas e raras manifestações clinicas, onde a progressão ou a cura destas infecções estão relacionadas com a constituição genética e com a condição imunológica do hospedeiro, a virulência e patogenicidade das diferentes espécies e cepas de *Leishmania* (Hide et al., 2007 e Bañuls et al., 2011).

Considerando o panorama relatado da leishmaniose, a doença na sua grande maioria acomete as pessoas mais pobres, desnutridas, imunodeprimidas e também está relacionada ao deslocamento populacional e as condições

precárias de habitação. Além disso, a leishmaniose está diretamente ligada as mudanças ambientais como: desmatamento, construção de barragens, sistemas de irrigação e urbanização (WHO, 2010).

No Brasil, a leishmaniose representa um sério problema de saúde pública devido a sua ampla cobertura nacional. Relatos de LV indicam um aumento de 124% na taxa de mortalidade, que passou de 117 (3,4%) em 1994, para 262 (7,2%) em 2006. Simultaneamente, entre 1998 e 2010, um total de 32.459 casos de LV foram registrados em 21 estados, sendo mais prevalente na parte nordeste do país. Outro fator preocupante é a alta prevalência da doença em crianças, dados de 2007 revelaram que 52,8% dos casos confirmados de LV afetam crianças menores de 10 anos (Ministério da Saúde, 2010).

A LC no Brasil recebeu a denominação de leishmaniose tegumentar americana (LTA), descrito por Rabello; 1923, que abrange tanto a forma cutânea como a forma mucosa da doença. Em média, 28.000 casos de LTA são relatados por ano. Na década de 1980, apenas dezenove estados do Brasil tinham relatos de LTA posteriormente, em 2003, todos vinte e sete estados brasileiros apresentaram transmissão autóctone LTA. Adicionalmente, em 2010, 217 municípios relataram transmissões moderadas ou intensa da doença, o que demonstra a expansão geográfica contínua da doença em todo o país (Ministério da Saúde, 2010).

1.2. Leishmania Tegumentar Americana

A leishmaniose tegumentar americana (LTA) constitui um conjunto de zoonoses causadas por diferentes espécies de protozoários do gênero *Leishmania*, com diferentes padrões epidemiológicos de transmissão no continente Americano. Estas zoonoses são de difícil controle, devido a diferentes e específicas condições eco epidemiológicas de cada foco. O que *per si* ressalta a importância da caracterização e do estudo da evolução destes parasitos (de Castro et al., 2005).

A *Leishmania* é um parasita versátil, são encontrados em biomas naturais ou modificados pelo homem, podendo infectar uma grande variedade de hospedeiros e vetores. No Brasil, vários mamíferos silvestres são incriminados

como reservatórios. Os reservatórios da LTA são as espécies de animais que garantem a circulação das *Leishmania spp*. Contudo, já foram registrados como hospedeiros, e possíveis reservatórios naturais, algumas espécies de roedores, marsupiais, endentados e canídeos silvestres. Embora, os registros de infecção em animais domésticos serem numerosos, não há evidências científicas que comprovem o papel destes animais como reservatórios das *Leishmania* spp, sendo considerados hospedeiros acidentais da doença. A LTA nesses animais pode apresentar-se como uma doença crônica com manifestações semelhantes às da doença humana, ou seja, o parasitismo ocorre preferencialmente em mucosas das vias aero digestivas superiores. Além disso, as espécies de flebotomíneos vetorialmente competente (*Lutzomyia flaviscutellata, Lutzomyia whitmani, Lutzomyia umbratilis, Lutzomyia intermedia, Lutzomyia wellcome* e*, Lutzomyia migone*) também contribuem para a plasticidade da LTA (Ministério da Saúde, 2010).

Atualmente, sete espécies de *Leishmania* têm sido reportadas como agentes etiológicos de LTA, sendo seis do subgênero *Viannia* [*Leishmania (Viania) braziliensis, Leishmania (Viania) guyanensis, Leishmania Leishmania (Viania) lainsoni*, *Leishmania (Viania) naiffi*, *Leishmania (Viania) lindenberg* e *Leishmania (Viania) shawi*] e somente uma do subgênero *Leishmania* [*Leishmania (Leishmania) amazonenses*] (Shaw, 2003; Silveira et al., 2002).

No Brasil, a *Leishmania* (*Viania*) *braziliensis* é o agente etiológico que prevalece na região nordeste e *Leishmania* (*Leishmania*) *amazonensis* é a espécie mais amplamente distribuída. Ambas as espécies estão em expansão geográfica no Brasil, com uma crescente incidência de 10,5 / 100.000 habitantes em 1985 para 18,6 / 100.000 habitantes em 2005. A doença tem relatos atualmente em todas as regiões do país, mas, com uma média de 28.568 casos anuais, a região Nordeste, teve a maior incidência entre 1985 e 2005. Em 2001, por exemplo, a incidência da região foi de 93,8 casos / 100,000 habitantes (Ministério da Saúde 2010).

A *Leishmania (Leishmania) amazonensis* está presente no Norte, Nordeste, Sudeste e Centro Oeste do Brasil, causa lesões variando desde a leishmaniose

cutânea localizada (LCL), com hipersensibilidade celular moderada, leishmaniose cutânea anérgica difusa (LCAD), uma hipossensibilidade celular com marcadores da resposta imunológica do tipo *T helper type 2* (Th2). Entre a LCL moderada e a LCAD, com baixa capacidade de resposta, há uma fraca forma de hipersensibilidade celular, conhecida como leishmaniose cutânea disseminada limítrofe (LCDL), que tem se mostrado menos imunossuprimidos que LCAD. Por outro lado, a *Leishmania (Viania) braziliensis*, é amplamente distribuída na América Central e do Sul e é predominante fora da região amazônica, incluindo áreas rurais e peri-urbanas, pode causar não apenas LCL e LCDL mas também a leishmaniose mucosa (LM), uma hipersensibilidade celular da infecção com uma proeminente resposta imune do tipo *T helper type* 1 (Th1). Desta forma, a diversidade das manifestações clínicas tem sido associada principalmente com as diferenças antigênicas das diferentes espécies de parasitas, mas também pela experiência imuno-genética do hospedeiro (Carvalho et al., 2012).

A *Leishmania (Viania.) guyanensis*, causa predominantemente lesões cutâneas ulcerativas e ocorre ao norte da região amazônica. As espécies do subgênero *Viannia Leishmania (Viania.) lainsoni*, *Leishmania (Viania.) naiffi*, e *Leishmania (Viania.) shawi* são mais restritas ao ciclo silvestre na região amazônica e raramente são encontradas infectando o homem. Adicionalmente, a espécie *Leishmania (Viania.) lindenbergi* isolada de soldados brasileiros com lesões cutâneas, que praticavam manobras próximas a florestas primárias degradadas ao norte do Estado do Pará, foi incluída como agente causador de LTA (Silveira et al., 2002).

1.3. Considerações sobre o tratamento das leishmanioses

Historicamente, a LC é uma doença antiga com relatos de lesões visíveis encontradas em tabuinhas de barro cozido na biblioteca do rei Assurbanipal do século 7 a.C (Mishra et al., 2007). O primeiro relato de tratamento só ocorre no fim do século 19 em Tashkent, quando houve indício a terapia localizada como aplicação de ácido láctico puro para cauterizar as lesões (Berman, 1988). Nesta época as recaídas eram tratadas pela remoção (raspagem) da lesão com uma colher afiada. Ainda nesta época outros agentes de cauterização eram utilizados

para o tratamento como o sulfato de cobre, extratos de plantas e aquecimento das lesões por 20 horas com água. Já, LV no Velho Mundo teve a primeira epidemia registrada em 1824 no distrito de Jessore, na Índia (atual Bangladesh), matando milhares de pessoas, pois não havia nenhum tratamento. Somente, no início do século 20 o antimônio foi usado como quimioterápico no tratamento da leishmaniose (Singh & Sivakumar, 2004).

O uso terapêutico do antimônio na medicina está bem documentado, devido ao debate criado em torno da sua utilização. Paracelsus no século 16, introduziu antimônio como um remédio para todos os males (Panacea), e foi aclamado como uma das sete maravilhas do mundo por Leipzig em 1604. Em 1905, o uso do antimônio teve início quando Plimmer e Thompson mostraram a atividade do tartarato de sódio e potássio contra os tripanossomas, o qual foi utilizado no tratamento da tripanossomiase humana na África (Haldar et al., 2011).

O tártaro emético, antimonial trivalente (Sb^{3+}), foi relatado pela primeira vez para o tratamento da LC, descrito por Vianna; 1912, e depois, teve sua eficácia confirmada contra LV por Di Cristina e Caronia, na Sicília e Rogers na Índia, em 1915. Posteriormente, observações clinicas do uso do tártaro emético revelaram muitos casos de efeitos colaterais tais como tosse, dor no peito e depressão (Haldar et al., 2011). Estes fatos já vislumbravam a necessidade de novos estudos para o tratamento da leishmaniose, o que culminou com a síntese e comercialização de formulações menos tóxicas do antimônio gluconato (Solustibosan – Kikuth & Schmidt, 1937) e estibogluconato de sódio (Pentostam – Goodwin, 1995).

Atualmente, as duas formulações que usam o antimônio pentavalente (Sb^{5+}) para o tratamento da leishmaniose são: (1) stibogluconato de sódio (SSG; Pentostam™) e (2) antimoniato de meglumina (MA; Glucantime™). Para estes compostos, tanto a eficácia e toxicidade estão relacionadas com o Sb^{5+} e apesar das suas diferenças estruturais eles são considerados terapeuticamente semelhantes, embora o Pentostam^{TM} contenha cerca de 20% de Sb^{5+}/mL a mais, comparado ao Glucantime^{TM}(WHO, 2010; Ministerio da Saúde, 2010).

Hoje é bem descrito que os quimioterápicos antimoniais são caracterizados por um amplo espectro de ligeiro a moderado efeitos adversos. O mais comum desses efeitos adversos são mialgia, artralgia, náuseas, vômitos, febre, dor de cabeça, dor abdominal, dor no local da aplicação e edema. No entanto, estes efeitos secundários são raramente considerados graves o suficiente para exigir a interrupção do tratamento. O efeito colateral mais perigoso associado com antimoniais é, sem dúvida, cardiotoxicidade. Este efeito é significativamente aumentado quando Sb^{3+} é utilizado, mas pode também ocorrer com doses elevadas de Sb^{5+}. A cardiotoxicidade associada com antimoniais é caracterizado por várias alterações no sistema cardiovascular, particularmente repolarização ventricular alterada (Herwaldt & Berman, 1992; Oliveira et al., 2011).

Outro aspecto negativo ao uso dos antimoniais é a sua eficiência questionável. Neste sentido, sabe-se que um paciente que apresenta lesões resistentes ao tratamento não significa que seja portador de uma cepa de Leishmania resistente. O que parece mais provável é que os pacientes refratários ao tratamento sejam, em geral, indivíduos cujo estado imunitário é deficiente. Sendo aceito, portanto, que os casos de insucesso na quimioterapia com os antimoniais pentavalentes estejam relacionados a fatores advindos tanto do parasito quanto do hospedeiro (Saldanha et al., 2000).

Na contemporaneidade das propostas de tratamento das leishmanioses, existem aproximadamente 25 compostos e formulações que mostram efeitos anti-leishmania, mas somente alguns foram corretamente comprovados ou estão em fase de comprovação (2). Dentre estes, destaca-se que para o tratamento dos pacientes é preconizado primeiramente o uso dos compostos antimoniais pentavalente, glucantime e pentostam e como segunda linha a amfotericina B, pentamidina, aminosidina e a miltefosina (Berman, 2005). Além disso, outros compostos vêm sendo estudados quanto à sua atividade leishmanicida nas doenças tegumentar e visceral como: azoís (fluconazol, cetoconazol, itraconazol e posaconazol) (Alrajhi et al., 2002; Koutinas et al., 2001), alopurinol (Dietze et al., 2001) e sitamaquina (Sereno et al., 2001). Também, o uso dos produtos que estimulam o sistema imunitário é uma alternativa no tratamento das

leishmanioses (Murray et al., 2003; Gontijo et al., 2003). Contudo, ainda não há um fármaco que apresente um bom índice terapêutico e uma baixa toxicidade.

Em relação ao tratamento atual, a posologia recomenda pela Organização Mundial da Saúde (OMS) é uma dose de Sb^{5+}, de 10 a 20 mg/kg/dia por via intramuscular ou por via intravenosa com uma dose total máxima de 810 mg de Sb^{5+} por dia. Os tratamentos geralmente são contínuos por 20 dias, mas podem ser reduzidos a duas semanas, se a cura parasitológica antecipada for observada. Nos casos que o uso sistêmico é contraindicado, 0,2 a 1 ml de solução de antimônio pode ser administrada diretamente nas lesões (Berman, 1988; Sampaio et al.,1985; Herwaldt et al., 1992).

No Brasil, devido às diferenças regionais de sensibilidade dos parasitas a este medicamento, as dosagens devem ser ajustadas de acordo com o padrão da resposta terapêutica observada, definida pelos critérios de cura (Oliveira-Neto et al., 1996; Oliveira-Neto et al., 2000). Além disso, existem ainda esquemas alternativos para utilização dos antimoniais pentavalentes, como sua administração intralesional, o emprego de baixas doses (abaixo de 10mg/kg/dia) em combinação com outros medicamentos como imiquimode ou alopurinol e ainda em associação a imunoterapia (Arevalo et al., 2007, Martinez et al., 1997, Arana et al., 1994 e Al-Abdely et al., 1999)

1.4. Composição do degradoma de *Leishmania* spp.

As proteases são um conjunto de enzimas estruturalmente e funcionalmente diversificada que hidrolisam proteínas envolvidas na ativação ou inativação dos processos biológicos. A hidrólise da ligação peptídica pode ocorrer no grupo amino-terminal ou carboxi-terminal de uma cadeia polipeptídica (atividade exopeptidase) ou no seu interior (atividade de endopeptidase). Neste último caso, as enzimas também são denominadas proteinases (Barrett & McDonald, 1985).

O conjunto de proteases das *Leishmania* spp, degradoma, demonstra um potencial relevante como fator de virulência devido, o papel fundamental no ciclo de vida destes parasitas. Os genes de proteinases são amplamente distribuídos no genoma de *Leishmania* spp em média, 1.8% do total de genes, são

encontrados no banco de dados GenDB, compreendendo principalmente cisteína, serina, aspártico e metalloproteases que são agrupadas em distintos Clãs e Famílias com base em relações evolutivas intríncicas (Rawlings et al., 2008), que são classificadas com base nos resíduos de aminoácidos presentes no sítio catalítico (Beynon & Bond, 2001).

O genoma de *Leishmania* spp. é formado por distintos genes de cisteínas proteinases, agrupados em 4 clãs e 14 famílias, apresentando o maior número de genes em comparação as outras classes de proteases. O clã CA concentra a maior quantidade de enzimas e as famílias C1, C2 e C19 são as mais predominantes do clã (Rawlings et al., 2008).

As proteases da família C1 têm sido extensivamente caracterizadas em várias espécies de *Leishmania*, contêm enzimas catepsina L-like (CPA e CPB), assim como, enzimas do tipo catepsina B-like (CPC). A CPB possui um conjunto de genes similares com números de cópias e polimorfismo variando consideravelmente entre as espécies (Mottram et al., 2004). As calpainas são os integrantes da família C2 mais abundante entre as cisteinas proteinases. Em eucariotas superiores, calpainas desempenham papéis importantes em funções reguladas pelo cálcio, tais como, transdução de sinal, diferenciação celular e apoptose/necrose, mas sua função em *Leishmania* spp. ainda não é bem definida. Outro grupo abundante de cisteína proteinase são os membros da família C19 (ubiquitina hidrolase). As ubiquitinas são subunidades do proteossoma responsáveis pela ubiquitinação de proteínas da via de degradação citosólica (Besteiro et al., 2007).

Ao contrário das cisteíno proteinase os genes de metalloprotease estão distribuídos uniformemente em 7 clãs e 16 famílias. O clã MA apresenta a maior diversidade de família sendo a M8 a mais estudada (Rawlings et al., 2008). Os representantes dessa família são glicoproteína de 63 kDA (GP63) também nomeadas como leishmanolisin. As GP63 são enzimas dependente de zinco, que pode ser encontrada na membrana plasmática, no citosol e na bolsa flagelar do parasita. Esta proteinase pertence à classe metzincin, cujos membros incluem uma sequência motivo, HExxHxxGxxH, e um pró-peptídeo N-terminal que torna a pró-enzima inativa durante a tradução, e é removido durante a

maturação e ativação (Yao et al., 2003). Curiosamente GP63 é abundante em promastigotas, mas em amostigota é regulada negativamente (Schneider et al., 1992). No entanto, a redução da expressão de GP63 pode ser compensada pela ausência de lipofosfoglicano (LPG) na superfície das amastigotas, possibilitando a GP63 modular a resposta imune do hospedeiro, apesar da sua redução em comparação com as promastigotas. Sua atividade biológica está associada à proteção dos parasitas contra a ação de enzimas do hospedeiro no intestino médio dos insetos vetores e nos fagolisossomos de macrófagos (Pimenta et al., 1991).

Os genes de serina proteinase são representados por oito famílias onde se destacam a subitilisina-like, proteinase peptídeo sinal e as proteínas pertencentes à família S9. Em *Leishmania* spp há seis genes que codificam proteínas desta família, incluindo prolioligopeptidase, peptidil-dipeptidase IV e oligopeptidase B (OPB), que ocorre apenas em plantas, bactérias e kinetoplastídeos. Todas essas enzimas contem uma tríade catalítica com resíduos de aspártico, histidina e serina, apresentando múltiplos domínios com um domínio catalítico globular, onde o sitio ativo é formado por uma grande fenda e um domínio β-hélice que limita o acesso ao sítio ativo (Besteiro et al., 2007).

Leishmania spp. tem apenas três aspártico proteinase, uma pepsina A classificada no clã AA família A28 e outras duas no clã AD família A22 dessas duas, uma tem similaridade a sequência de presenilina 1, que é uma proteinase membrana que cliva proteínas membrana do tipo I. A segunda é uma enzima com identidade a proteinase peptídeo sinal de peptidase intramembranar que cliva peptídeos sinal dentro da sua região transmembranar. Curiosamente, aspártico proteinases semelhante as plasmepsina, que são abundantes em *Plasmodium* sp e outros parasitas apicomplexa, aparentemente são totalmente ausente em *Leishmania* spp.

1.5. Importância das proteinases na sobrevivência dos amastigotas de *Leishmania* spp

A proteinases atuam nas diferentes etapas do ciclo de vida das *Leishmania* spp, seja nos hospedeiros vertebrado o invertebrado (Silva-Almeida et al., 2012);

influenciando no processo de transmissão, infecção e doença. O parasito é transmitido ao hospedeiro vertebrado pela picada da fêmea do flebótomo infectada (família Psychodidae, subfamília Phlebotominae) e a contaminação dos flebotomíneos ocorre a partir da ingestão do sangue de vertebrados infectados (Saliba & Oumeish, 1999; Ashford, 2000).

Os flebotomíneos fêmea possui o hábito alimentar hematofágico, quando o inseto adquire os macrófagos do hospedeiro vertebrado, contendo as formas amastigotas. Esta forma do parasita é liberada no intestino médio do inseto se diferenciando na forma promastigota, dando início ao ciclo de vida extracelular do parasita no vetor. Os promastigotas migram para a parte anterior do aparelho digestivo do flebotomíneo, onde ocorre multiplicação por fissão binária. Aproximadamente, sete dias após a alimentação, os promastigotas entram em metaciclogênese e se tornam infecciosos (promastigotas metacíclicas), que são preparados para invadir as células do hospedeiro mamífero após a alimentação do inseto vetor (Saliba & Oumeish, 1999; Ashford, 2000).

Os promastigotas metacíclicas são fagocitadas geralmente por macrófago, a principal célula do hospedeiro que é infectada pelas *Leishmania* spp. No entanto, há outras células do sistema imune do hospedeiro mamífero que podem ser infectadas, como os monócitos, células dendríticas, derivadas de monócitos, células abundantes em lesões inflamatórias e linfonodose outras células destes tecidos (eosinófilos, neutrófilos e fibroblastos) também podem ser infectadas (McConville & Naderer, 2011).

Durante as primeiras horas de infecção das *Leishmania* spp.*,* um repertório de adaptações assegura a entrada e saída da célula, bem como, a modulação do mecanismo da imunidade inata. Estas adaptações incluem a invasão e destruição de tecidos do hospedeiro e a penetração dos sistemas vasculares do hospedeiro, permitindo que o parasita migre aos locais específicos ao seu desenvolvimento (Piña-Vázquez et al., 2012).

O primeiro obstáculo a ser vencido é adaptar-se a variação térmica na transição do inseto vetor para o hospedeiro mamífero, de 25-26°C para 37°C, respectivamente. Neste momento, há um aumento na secreção das proteases, tais como, a GP63 e a cisteíno protease B (CPB) (Hassani et al., 2011). A

mudança de temperatura desencadeia a formação de vesículas (exossomos) contendo CPB e GP63 ancoradas à membrana via glicosilfosfatidilinositol. Os exossomos podem se fundir a membrana plasmática dos macrófagos transportando rapidamente as proteases para o citoplasma. Esse mecanismo, representa outra via de entrada das proteases ao ambiente dos macrófagos do hospedeiro (Hassani et al., 2011).

A via de glicosilação também é requerida para aumentar a tolerância térmica, incluindo a biossíntese dos nucleotídeos GDP-Man e UDP-GlcNAc na *Leishmania* spp. Adicionalmente, o retículo endoplasmático apresentam proteínas responsável pela manutenção das glicoproteínas (UDP-Glc: glicoproteína glicosiltranferase, calreticulina e glicosidase I), onde a N-glicosilação é necessária para o enovelamento correto e/ou exportação de proteínas à temperatura elevada (33 °C – 37 °C) do hospedeiro mamífero. Da mesma forma, o LPG contribui para a adaptação térmica tanto no vetor flebotomíneo quanto nas infecções iniciadas por promastigotas no hospedeiro mamífero (McConville & Naderer, 2011).

Igualmente, a lise mediada pelo complemento tem que ser superada nas etapas iniciais da infecção. Para isso, a GP63 também atua auxiliando na evasão da lise dos promastigotas mediada pelo complemento, através da clivagem de C3 para os seus produtos de gradação, bem como a conversão de C3b para a forma inativa iC3b (Brittingham et al., 1995). Além disso, GP63 impede a ação dos peptídeos antimicrobianos, como defensinas e pexiganan (Kulkarni et al., 2008).

A abundância e diversidade das GP63, bem como a alta atividade catalítica favorecida pela mudança de temperatura no hospedeiro mamífero, propicia a disseminação do parasita, uma vez que a GP63 pode digerir componentes da matriz extra celular, tais como, colágeno tipo IV, fibronectina e laminina (McGwire et al., 2003). Da mesma forma, a CPB pode atuar na degradação de fibronectina quando associada os glicosaminoglicanos na superfície da célula hospedeira (Piña-Vázquez et al., 2012).

No decorrer da infecção do hospedeiro vertebrado a OPB é altamente ativa, sendo responsável pela regulação da endolase na superfície da célula do

parasita auxiliando a multiplicação dos amastigotas no macrófago (Swenerton et al., 2011). Dentro das células fagocitárias, a diferenciação dos promastigotas metacíclica para amastigota tem início no fagossomo, o qual é formado após a fagocitose. Esse, pode subsequentemente se fusionar ao lisossomo, endossomo, fagossomo e autofagossomo, sugerindo que uma gama de macromoléculas estejam presente, resultando na formação do vacúolo parasitóforo (VP) acidificado, pH 4,7 a 5,2 (McConville & Naderer, 2011). Esse ambiente, favorece um estado de protonação aos grupos ionizáveis na cadeia lateral dos resíduos de aminoácido das proteínas catalíticas expostas ao pH ácido. Nesse ambiente, a *Leishmania* se diferencia em amastigota que são metabolicamente mais ativas em pH ácido (Burchmore & Barrett, 2001).

Pouco se sabe sobre a composição exata do VP. Estudos recentes sugerem que amastigotas intracelulares são dependentes da absorção e utilização de glicose e outras hexoses presentes no VP. Além das hexoses, os amastigotas intracelulares podem usar outras fontes de carbono como aminoácidos gerados pela ação hidrolítica das cisteíno-, metallo- e serino-proteases (Kulkarni et al. 2008; Choudhury et al., 2010), os quais seriam absorvidas pelos parasitas por permeases (McConville & Naderer, 2011).

Além disso, as proteases intracelulares exercem importante função na aquisição de peptídeos oriundos da digestão de proteínas absorvidas pelo parasito, como por exemplo, na obtenção de ferro. Após absorção de da transferrina ou lactoferrina pelo parasito as proteases do compartimento lisossomal atuam na degradação destas proteínas (Borges et al., 1998). Ainda, as proteases intracelulares põem atuar no contínuo estado de síntese e degradação de proteínas, *turnover*, que é necessário para manter o *pool* metabólico e a capacidade de reciclagem de proteínas do parasito, direcionadas ao compartimento lisossomal através da via autofágica (Besteiro et al., 2007).

Outra forma de atuação das proteinases na persistência dos amastigotas de *Leishmania* nas células fagocitárias seria a modulação do sistema imune do hospedeiro, tendo início na resposta imune inata e subsequentemente na resposta imune adaptativa. Nesse processo é conhecida a atuação das cisteíno proteinase A (CPA), cisteíno proteinase C (CPC) e CPB; sendo a enzima CPB

com maior número de ações descritas, até o momento. A CPB está relacionada com o controle da resposta imune do hospedeiro, por promover a expressão de IL-4 (citocina que favorece a resposta Th2) (Denise et al., 2003). Adicionalmente, a CPB também pode inibir a expressão de citocinas (IL-12 e IFN-γ) associadas a resposte imune Th1 (Buxbaum et al., 2003). O mecanismo pelo qual a CPB pode inibir a expressão de IL-12, mediadas pelo lipopolissacarídeo, pode estar relacionado a clivagem do fator de transcrição nuclear kappa B (NF-kB) e seus inibidores (IκB α and β), interferindo assim, na expressão de interleucinas pelo hospedeiro (Cameron et al., 2004). Outros fatores de transcrição, com transdutor de sinal e ativador de transcrição (STAT-1 e 4) e proteína de ativação-1 (AP-1), também sofrem a ação da CPB, tendo sua translocação ao núcleo prejudicada, impedindo a produção de oxido nítrico (ON), que é induzido pelo IFN-γ. A CPB ainda está envolvida no balanço da resposta Th através da clivagem de proteínas do complexo principal de histocompatibilidade (MHC) (Abu-Dayyeh et al., 2010). Há evidencias que a CPB é capaz de clivar o MHC de classe II dentro do VP dos macrófagos (De Souza Leao et al., 1995).

As atuações das enzimas CPC e CPA na resposta imune do hospedeiro vertebrado são pouco conhecidas. Por exemplo, a CPC induz a expressão de TGF-β em culturas de células humana. O TGF-β, também limita a produção de IFN-γ durante a infecção primária em camundongos (Somanna et al., 2002). Já a CPA, pode estar envolvida no processo de autofagia, prevenindo a metaciclogênese (Lasakosvitsch et al., 2003).

Além das cisteíno proteinase as Gp63 também estão envolvidas na resistência do sistema imune do hospedeiro mamífero. Essa enzima pode interferir na cascata de sinalização e afetar os fatores de transcrição, impedindo assim, a ativação do sistema imune das células hospedeiras adequadas à infecção pelo parasita. Uma das primeiras moléculas envolvidas na sinalização, afetada pela GP63 é o substrato de quinase C rico em alanina miristoilado (MARCKS) e proteínas relacionada a MARCKS, ambos são substratos da proteína quinase C, está envolvida no sinal de transdução associado com a proliferação, diferenciação e apoptose, em diversos tipos celulares incluindo os macrófagos (Aderem, 1992).

Outro efeito da GP63 na modulação do sistema imune ocorre pela ativação da proteína tirosina fosfatase (PTPs) em macrófagos, que conduz a diminuição da produção de óxido nítrico (NO), atenuando a resposta inflamatória inata, aumentando assim, a probabilidade de sobrevivência do parasita (Shio et al., 2012). Esta ativação ocorre, pela clivagem da porção COOH-terminal. A influência da GP63 no controle das PTPs pode estar relacionada ao controle das vias de sinalização de janus kinase (JAK)/STAT afetando a estimulação do IFN-γ (Abu-Dayyeh et al., 2008). Além disso, ativação dos PTPs mediada pela GP63 inativa a IRAK-1, uma quinase fundamental para sinalização dos receptores *Toll-like*. Consequentemente, a inativação de interleucina 1 associada ao receptor quinase-1 (IRAK-1) pela PTPs reflete na incapacidade de induzir importantes funções imunitárias inatas, tais como TNF-α, NO e IL-12, devido ao estimulo do lipopolissacarídeo (Abu-Dayyeh et al., 2008).

Com relação aos fatores de transcrição a GP63 está envolvida na clivagem do c-Jun, um componente central do complexo transcricional AP-1, evitando que células hospedeiras respondam adequadamente aos parasitas durante a infecção (Contreras et al., 2010). Similarmente a CPB, a GP63 também cliva o NF-kB na subunidade p65 para uma subunidade menor p35, que entra no núcleo da célula hospedeira e desencadeia a expressão das quimiocinas. Ao subverter o funcionamento adequado da maquinaria transcricional, o parasita inibe a expressão de fatores do hospedeiro como IL-12 e óxido nítrico sintase induzível (iNOS), que ameaçam sua sobrevivência (Gregory et al., 2008).

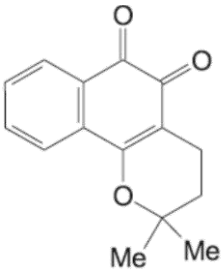
A GP63 também pode degradar a fostidil 3-quinase envolvida na via de sinalização celular do alvo da rapamicina em mamíferos (mTOR), que inibe a tradução de proteínas e aminoácidos responsáveis pela ativação da nutrição na célula hospedeira (Jaramillo et al., 2011).

1.6. Naftoquinonas, seus derivados e atividades farmacológicas

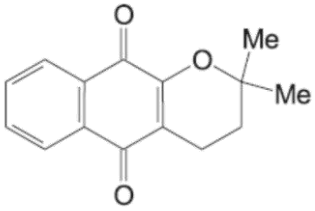
Quinones e, particularmente, naftoquinonas, estão difundidos no metabolismo dos vegetais superiores, artrópodes, fungos, liquens, bactérias, algas e vírus secundários dos vegetais. Nos vegetais, a naftoquinona é

encontrada em várias famílias (*Bignoniaceae*, *Verbenaceae*, *Proteaceae*, *Leguminosae*, *Sapotaceae*, *Scrophulariaceae* e *Malvaceae*), atuando nas vias metabólicas da cadeia transportadora de elétrons e múltiplos processos biológicos oxidativos (Hussain et al., 2007).

A estrutura molecular das quinonas é dividida em diferentes grupos considerando-se do sistema aromático que sustenta o anel quinonoídica: (i) benzoquinonas - um anel benzênico; (ii) antraquinonas - um anel antracênico linear ou angular e (iii) naftoquinonas - um anel naftalênico. Diferentes arranjos quinonoídicos (isomeria), com um mesmo tipo de anel, tendo as disposições relativas das carbonilas diferentes quinonas. Por exemplo, no arranjo de base naftalênica tem-se a forma isomérica 1,2 ou *orto*-quinonoídica, quando as carbonilas são vizinhas, ou a 1,4, *para*-quinonoídica, com as carbonilas tendo entre si dois carbonos (Figura 1) (da Silva et al, 2003).



β-Lapachona *orto*-Naftoquinona



α-Lapachona *para*-Naftoquinona

Figura 1: Classificação e formas isoméricas da naftoquinona

A estrutura química das naftoquinonas facilita a redução e, portanto, esse composto pode atuar como agente oxidante ou desidrogenante em reações químicas. A estrutura molecular das naftoquinonas são valorizadas na química medicinal devido sua ampla propriedade farmacológica**,** tais como, antibacteriana, anti-inflamatória, anti-tumoral, anticancerígena e tripanocida. Os derivados de naftoquinona também são comuns na natureza, por exemplo, o lapachol**,** β-lapachona e α-lapachona, todos extraídos de árvores da família *Bignoniaceae* (*Tabebuia sp*). Curiosamente, a casca interna de *Tabebuia avellanedae*, vulgarmente conhecido como "pau d'arco" (lapacho, taheebo), é usada como analgésico, anti-inflamatório, antineoplásico e diurético pela população local nas regiões Nordeste do Brasil (Hussain et al., 2007).

A atividade anti-inflamatória do lapachol foi comprovada experimentalmente pelo efeito inibitório sobre a liberação da histamina. Além disso, em estudos *in vitro*, o lapachol e seus análogos indicaram potencial antipsoriáticos uma vez que inibiu o crescimento de queratinócitos humanos imortalizada espontaneamente (HaCaT) e reduzindo a inflamação (Müller et al., 1999).

O lapachol, também demonstrou atividade quimioterápica altamente significativa contra tumores cancerosos em ratos. No entanto, ensaios clínicos posteriores não demonstraram efeitos terapêutico significativo (Block et al 1974). Outro estudo clínico, com nove pacientes portadores de diferentes tipos de câncer (fígado, rim, mama, próstata e colo do útero), avaliou o efeito do lapachol. Neste estudo, foi demonstrado a propriedade deste composto em reduzir o tamanho dos tumores e a sensação de dor causada por estes (da Consolação et al., 1975). Embora os estudos com lapachol indicarem possíveis efeitos benéficos, altas concentrações do composto no sangue não foram alcançadas para mostrar um efeito terapêutico. Isto levou ao encerramento do estudo clínico anticâncer (Hussain et al., 2007).

Em microrganismos o lapachol foi testado inicialmente no *Plasmodium Knowles,* atuando no mecanismo respiratório, inibindo 74% da absorção do oxigênio e 26% o succinato oxidase. No entanto, o mecanismo exato de ação ainda permanece controverso. Supõe-se que o lapachol inibe a interação entre

o citocromos “b” e “c” ou diretamente uma enzima desconhecida entre os dois citocromos (Murray & Pizzorno, 1998).

O uso tópico do lapachol foi considerado como barreira no controle de infecções de trematódeos, especificamente *Shcistosoma mansoni*. A forma larvar deste parasito, as cercarias, abandonam o caramujo do gênero *Biomphalaria* e penetram a pele dos seres humanos, causando a esquistossomose, uma parasitose grave que causa milhares de mortes por ano. O uso de uma formulação do lapachol é eficaz contra a penetração da larva na pele.

Constatou-se também que o lapachol tem atividade contra vários tipos de bactérias como *Helicobacter pylori, Staphylococcus* sp*, Streptococcus sp, Enterococcus* sp*, Bacillus* sp e *Clostridium* sp. Além disso, foi relatado que o lapachol tem um efeito significativo contra *Candida albicans, Candida tropicalis e Cryptococcus neoformans,* semelhante à Anfotericina B. Acredita-se, que a possível atividade antifúngica do lapachol seja devido à sua interação com a membrana celular (Eyong et al., 2006; Breger et al., 2007). O lapachol também apresenta atividade contra algumas cepas de vírus, como vírus herpes do tipo I e do tipo II, vírus da gripe, da poliomielite e da estomatite vesicular (da Silva et al., 2002). O mecanismo de ação suposto seria a inibição da DNA e RNA polimerases ou da enzima transcriptase reversa.

Em *Trypanosoma cruzi,* a literatura descreve significativos relatos sobre lapachol contra o parasito (Neves-Pinto et al, 2002; De Moura et al, 2001; Menna Barreto et al, 2005; Jorqueira et al., 2006; Ferreira et al., 2006). Entre estes derivados, o composto β-lapachona vem sendo amplamente estudado. Similar a outras quinonas, β-lapachona possui uma variedade de efeitos farmacológicos, incluindo a atividade tripanocida, mas com uma significativa citotoxicidade contra diferentes linhagens de célula.

Uma alternativa para reduzir o efeito tóxico do α-lapachona foi a adição de um anel oxirano, modificando o centro quinonóide deste composto, originando o epoxi-α-lapachona (2,2-dimetil-3,4-di-hidroespiro[benzo[g] cromeno-10,20-oxiran]-5(2H)-one), um derivado potencialmente menos tóxicos para células de mamíferos (Ferreira et al., 2006). Estudos recentes indicaram que o epoxi-α-

lapachona apresenta atividade similar ao β-lapachona contra a forma epimastigota da cepa Dm28c do *T. cruzi*, mas com menor perfil citotóxico (Jorqueira et al., 2006; Ferreira et al., 2006). Posteriormente, o efeito do epoxi-α-lapachona foi avaliado em todas as formas do *T. cruzi* (Bourguignon et al., 2009). Além disso, o potencial de inibição do epoxi-α-lapachona foi comparado ao α-lapachona, β-lapachona e nor-β-lapachona) sobre as proteinases do *T. cruzi*. Os resultados indicaram que os compostos β-lapachona e epoxi-α-lapachona inibem o crescimento do parasito, afetando diferentes proteinases (Bourguignon et al., 2010). Assim, estes dados indicam o potencial destes compostos como inibidores de proteinase do parasito e como protótipos de fármacos para quimioterapia da doença de Chagas.

Outros estudos apontam ainda que o lapachol e seus derivados (isolapachol e dihydrolapachol) atuam sobre os parasitos do gênero *Leishmania.* Alguns experimentos indicam que estes compostos afetam o desenvolvimento de promastigotas metacíclicas de *Leishmania (Leishmania) amazonensis* e *Leishmania (Leishmania) braziliensis in vitro* (Lima et al., 2004). Outros derivados, como 2,3-dicloro-5,8-dihidroxi-1,4-naftoquinona e 2,3-dibromo-1,4-naftoquinona, também tiveram seus efeitos comprovados em promastigotas e amastigotas intracelulares de *Leishmania (L.) donovani* (Lezama-Dávila et al., 2012).

1.7. Documento 1:

Pereira BAS, Souza-Silva F, Silva-Almeida M, Santos-de-Souza R, Oliveira LFG, Ribeiro-Guimarães ML, Alves CR. Proteinase inhibitors: a promising drug class for treating leishmaniasis. *Current Drug Targets,* 2014*, 15,* 000-000.

Neste documento, abordamos a situação e as perspectivas do tratamento atual da leishmaniose, com um foco especial no uso de inibidores de proteainases. O tratamento desta doença ainda é desafiador, pois os fármacos atuais de primeira e segunda escolha são tóxicos, apresentam baixa eficácia e graves efeitos colaterais.

Neste cenário, as proteinases de *Leishmania* surgem como possíveis alvos para o tratamento da leishmaniose e o uso de inibidores específicos de proteinase, com pouco ou nenhum efeito sobre as proteinases do hospedeiro, vem demonstrando resultado na interrupção do desenvolvimento do parasita *in vitro*. Em paralelo, outros estudos têm avaliando a toxicidade para o hospedeiro *in vivo* ou *in vitro* dos inibidores. Apesar das evidencias promissoras do uso de inibidores de proteinase ainda não há nenhum inibidor em uso na fase clinica como alternativa ou complementar ao tratamento da leishmaniose.

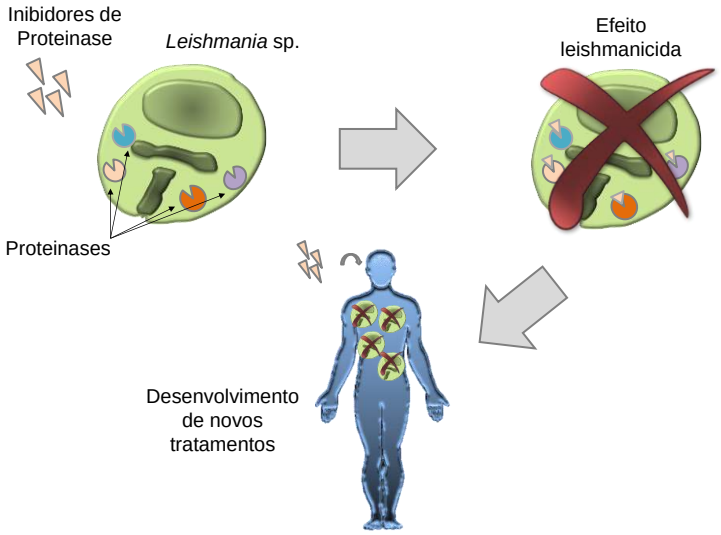


Figura 2: Resumo gráfico do DOCUMENTO 1

Send Orders for Reprints to reprints@benthamscience.net

Current Drug Targets, 2014, *15*, 000-000

Proteinase Inhibitors: A Promising Drug Class for Treating Leishmaniasis

Bernardo Acácio Santini Pereira[#], Franklin Souza-Silva[#], Mariana Silva-Almeida, Raquel Santos-de-Souza, Luiz Filipe Gonçalves de Oliveira, Michelle Lopes Ribeiro-Guimarães, and Carlos Roberto Alves[*]

Abstract: This review presents and discusses the current status and perspectives of leishmaniasis treatment, with a special focus on the use of proteinase inhibitors. The history of treatment development, the first- and second-choice modern drugs and the advantages and disadvantages of using proteinases inhibitors as leishmanicidal treatments are presented and discussed. The reports gathered herein confirm the potential usefulness of proteinases inhibitors as an alternative or complement to the current leishmaniasis treatments. They also support the hypothesis that a combined treatment with multiple proteinase inhibitors may be efficient against *Leishmania* infections in vertebrate hosts.

Keywords: Leishmaniasis, *Leishmania*, proteinases, proteinase inhibitors, treatment, virulence factors.

INTRODUCTION

Leishmaniasis is considered an endemic disease in 88 countries and is found in the Americas, Africa, Asia, Eastern Europe and Oceania [1, 2]. It is a tropical disease that primarily affects populations in poor or emerging countries. The World Health Organization estimates that the incidence of this disease, considering all clinical forms, is approximately 1.5 million new cases/year; however, this number is likely underestimated due to difficulties in identifying all cases and the occurrence of asymptomatic infections [2].

This parasitic disease is caused by species of the genus *Leishmania* and is acquired by a vertebrate host during the blood meal of phlebotomine sandflies. After entering the host as promastigotes, the parasites undergo morphological changes into amastigotes and become able to infect cells in the skin, mucosa or cartilage, thus causing the cutaneous form of the disease (CL). Depending on the parasite species and strain, the amastigotes may colonize cells in internal organs, including the liver, spleen and bone marrow, to cause the visceral form of the disease (VL) [3]. In contrast to extracellular promastigotes that possess a visible flagellum, amastigotes are intracellular round-shaped forms that preferentially inhabit cells of the mononuclear phagocyte system. This last morphological stage of the parasite is the preferred target for the development of novel chemotherapies because it is the parasite stage that inhabits vertebrate hosts.

However, despite the high incidence rate and the commitment of researchers working to develop new drugs for leishmaniasis treatment, little success has been achieved. The current drugs used in the treatment of leishmaniasis have limitations regarding their use, such as high cost, difficulty of administration, toxicity or the occasional development of resistant parasite strains, which represent obstacles to successful therapies [4]. For this reason, there is a dire need to identify novel and specific drugs to treat *Leishmania* infections.

*Address correspondence to this author at the Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz - Fiocruz, Avenida Brasil, 4365 Pavilhão Leonidas Deane, sala 209A, Manguinhos, Rio de Janeiro, RJ, Brasil - CEP: 21040-900; Tel: +55 21 38658191; Fax: +55 21 38658200; E-mail: calves@ioc.fiocruz.br

#These authors have equally contributed to this review paper.

HISTORY AND CURRENT STATUS OF LEISHMANIASIS TREATMENT WITH ANTIMONIALS

Despite the length of time that the *Leishmania* parasites have been known (the first report dates back to 1885 by David D. Cuningham) [5], leishmaniasis remains a major cause of suffering for many millions of people in the tropical and subtropical regions of the world. This situation is due to the absence of effective treatment options, inadequate control practices and little to none interest of the major pharmaceutical industries to research for novel treatment alternatives. This lack of interest characterizes leishmaniasis as a tropical neglected disease.

The current treatment for leishmaniasis is based on that proposed by Gaspar Vianna in 1912, who was able to effectively treat cutaneous leishmaniasis patients by intravenously injecting emetic tartar [6]. His approach was inspired by the then promising microbicidal effects that antimonials presented against other trypanosomatid species [7].

In 1915, Vianna's treatment has also been shown to be effective against visceral leishmaniasis, as reported by Cristina and Caronia in Italy [8] and by Rogers in India [9]. However, the treatment has disadvantages, as the drug is highly toxic to the patients and is very unstable in the tropical climate. Antimony has even been reported to have no beneficial effect in other studies [10, 11].

This controversy led to further studies and, in 1920, to the development of a new antimonial compound to treat kala-azar: urea stibamine by Upendranath Brahmachari [12]. The next developments in leishmaniasis treatment were achieved only decades later with the advent of the pentavalent antimonials: sodium antimonyl (V) gluconate in 1937

1389-4501/14 $58.00+.00

[13] and sodium stibogluconate in 1945 [14]. These drugs are associated with fewer toxic effects for the patients.

Currently, these pentavalent antimonials (Sb^{5+}) that have been in use since the 1940s and remain the first-choice drugs for the treatment of all leishmaniasis clinical forms. The longevity of this treatment course is explained by the higher risks of toxicity associated with other antileishmanial drugs (amphotericin B and pentamidine). These drugs are only recommended in cases in which there is a contraindication, intolerability or resistance to the pentavalent antimonials [15].

The following two pentavalent antimonials formulations are currently available: sodium stibogluconate (SSG; Pentostam™) and meglumine antimoniate (MA; Glucantime™). While SSG is used mainly in English-speaking countries, MA is prevalently marketed in French-, Spanish- and Portuguese-speaking countries [11]. Both the efficacy and toxicity of these compounds are related to their Sb^{5+} content and, despite their structural differences, they are considered therapeutically similar, even though the Pentostam formulation contains nearly 20% more Sb^{5+} per ml than Glucantime [16, 17].

To date, the precise mechanism of action of the pentavalent antimonials against the parasite remains largely unknown. However, it appears to be multifactorial, as observed for other heavy metal-based compounds, rather than being due a specific pathway. At the molecular level, it is believed that the antimony binds to the sulfhydryl groups of certain proteins, causing alterations in their structures and, eventually, impairing their function [18].

There is evidence suggesting that the biologically active form of the heavy metal in the compound is, in fact, the trivalent antimony (Sb^{3+}). Thus, the pentavalent antimony may be a pro-drug, as proposed by Goodwin and Page in 1943 [19]. Other authors have supported this hypothesis, as Miekeley *et al.* (2002) that observed the bioreduction of Sb^{5+} into Sb^{3+} after the intramuscular administration of MA in patients with leishmaniasis [20]. In this case, the bioreductive agent implicated in the transformation was glutathione (GSH), a thiol present in high quantities in the intracellular environment [21-23].

In the search to understand how antimonials exert their antileishmanial effect, it has been reported that these compounds inhibit certain stages of the energy metabolism cycle in amastigotes. Experimental observations indicated that *in vitro* exposure of *Leishmania (Leishmania) mexicana* to SSG resulted in a dose-dependent decrease in both cell viability and CO_2 production derived from [6-C]glucose and [C]palmitate in the parasite cultures. Thus, it can be inferred from these data that the glycolytic enzymes and the components of the fatty acid oxidation pathway are inhibited by SSG, although the hexose monophosphate pathway and the citric acid cycle are not, leading to a depletion of intracellular ATP levels [11, 24].

Wyllie *et al.* (2004) showed that Sb^{3+} inhibits trypanothione reductase (TR) activity in *Leishmania (Leishmania) donovani*, an enzyme essential for the survival of the parasite inside macrophages [25]. Later, Baiocco *et al.* (2009) demonstrated that Sb^{3+} also binds, with high affinity, to the active site of *Leishmania (Leishmania) infantum* TR and inhibits its enzymatic activity. The inhibition of TR by antimonials has been described as a pivotal step for the antileishmanial activity of these compounds [26].

The additional microbicidal effects of the antimonial compounds include the following: the induction of apoptosis in amastigotes by Sb^{3+}, as observed by DNA fragmentation and exposure of phosphatidylserines on the outer surface of plasma membrane in parasites exposed to antimonials [27]; the inhibition of topoisomerases [28]; the formation of complexes with ribonucleosides [29]; and, interference in the translocation of preformed purines [30].

Regarding the current treatment posology, the WHO recommends a dose of Sb^{5+} of 10 to 20 mg/kg/day intramuscularly or intravenously with a maximum total daily dose of 810 mg of Sb^{5+}. The treatments generally continue for 20 days but may be reduced to a minimum of two weeks if the anticipated parasitological cure is observed. In cases in which systemic use of Sb^{5+} is contraindicated, 0.2 to 1 ml of the antimonial solution may be administered directly into the lesions [11, 31, 32].

The use of antimony is characterized by a broad spectrum of mild to moderate adverse effects. The most common of these adverse effects are myalgia, arthralgia, nausea, vomiting, fever, headache, abdominal pain, pain at the site of application and edema. Nevertheless, these side effects are seldom considered severe enough to necessitate treatment discontinuation. The most hazardous side effect associated with antimonials is undoubtedly cardiotoxicity. This effect is markedly increased when Sb^{3+} is used, but it may also occur with high doses of Sb^{5+}. The cardiotoxicity associated with antimonials is characterized by several changes in the cardiovascular system, particularly altered ventricular repolarization [32, 33].

SECOND-CHOICE DRUGS FOR LEISHMANIASIS TREATMENT

In cases where there are contraindications for antimonial-based treatments, when patients present intolerance or due to emergence of resistant parasite strains, the use another set of drugs is recommended, as Amphoterecin B, Pentamidine, Miltefosine or Paromomycin. Therefore, those are classified as second-choice drugs [15].

Amphotericin B

Amphotericin B deoxycholate is a polyene antibiotic obtained from *Streptomyces nodosus*, with well-known antifungal activity and reported to be effective against *Leishmania* promastigotes and amastigotes both *in vitro* and *in vivo* [34]. This drug has been applied for the treatment of VL in India and Brazil for many years and has proven to be an effective yet difficult treatment. Amphotericin B has also been shown to be effective against the mucosal form of leishmaniasis, in which relapses are common [35-38]. Attempts to reduce the side effects of amphotericin B led to the development of lipid formulations of this drug that encapsulate it in micelles. The lipid particles are quickly removed from patient circulation by mononuclear phagocytes that then deliver large quantities of the drug inside the infected cells, thus enhancing its antiparasitic effects.

Currently, the following three lipid formulations are available: liposomal amphotericin B (AmBisome; Nexstar, USA); amphotericin B lipid complex (Abelcet, ABLC; Liposome Co., USA); and amphotericin B colloidal dispersion (Amphocil, Amphotec; Sequus, USA) [35, 37, 39]. These formulations are similar to amphotericin B deoxycholate in their efficacy but are significantly less toxic. The liposomal formulation of amphotericin B is used for the treatment of VL only in Europe due to its very high cost, which precludes its use in developing countries [40, 41].

The mechanism of action of amphotericin B is due to its reaction with sterols that contain a methyl substitution in C-24 (episterol and ergosterol) in the parasite cell membrane, thus forming pores that alter the ionic balance, cell permeability and, eventually, cause cell death. However, this drug can also bind to cholesterol molecules present in the cell membrane of host cells, thus causing toxic side effects in the patients [17, 35, 40, 42]. This drug is highly effective against *Leishmania*; in assays with hamsters or monkeys infected with *L. (L.) donovani,* it was described to be 400 times more potent than pentavalent antimonials against the parasite.

In Brazil, the recommended dose of amphotericin B for VL or CL treatment is 1 mg/kg/day on alternate days for 20 days. The recommended total dose ranges from 1.0-1.5 g for CL treatment and 2.5-3.0 g for VL [16, 40, 43].

Despite its high effectiveness, amphotericin B is used as a second-choice drug due to its serious adverse effects and many treatment drawbacks, including the need for parenteral administration, long-term therapy and constant clinical monitoring. Thus, it is usually only used in cases where treatment with pentavalent antimonials did not produce an adequate response, however it is considered the first-choice drug for the treatment of pregnant women [17, 35, 37, 40, 42].

Pentamidine

Pentamidine is an aromatic diamine used for the treatment of patients who are unresponsive to therapy with antimonials. This drug is also applied in the treatment of incipient cases of Rhodesian or Gambia trypanosomiasis. It was first introduced as an antileishmanial agent in 1952 and has been used in the treatment of various clinical forms of leishmaniasis. However, its high toxicity and low effectiveness compared with other treatment options led to a suspension of its use in several countries [44-47].

Pentamidine's mechanism of action appears to be related to its ability to bind kinetoplast DNA in the parasites and thus affect their survival. However, this hypothesis requires further investigation, and other potential effects of pentamidine on the parasites must be addressed [41, 48, 49].

Pentamidine isothionate is preferably administered by intravenous infusion or, alternatively, intramuscularly, as it is readily absorbed and exits the circulation rapidly. The recommended dose is 7 mg/kg (corresponding to 4 mg of pentamidine base) in 48-hour intervals. Alternatively, a dose of 2 mg/kg of pentamidine base may be administered in seven injections. The total dose of pentamidine base in the treatment should not exceed 2 g [16, 43].

The most common adverse effects related to the use of pentamidine isothionate are pain and sterile abscesses at the injection site, nausea, vomiting, dizziness, malaise, myalgia, arthralgia, headache, hypotension, syncope, cytolysis of pancreatic beta cells, hypoglycemia and hyperglycemia. In extreme cases, cardiotoxicity may occur, leading to fatal arrhythmia. Another major toxic effect of pentamidine is the development of insulin-dependent diabetes in treated patients; this effect has an incidence rate of 12.5% in cases in which the total dose of treatment nears 1 g [17, 35, 40, 50].

Miltefosine

Miltefosine, a hexadecylphosphocholine, was originally developed as an oral antineoplastic agent (for cutaneous cancer treatment). After a series of clinical studies between 1997 and 2000, it was approved under the commercial name Impavido™, becoming, in some countries, the first available oral treatment for leishmaniasis [51].

Its mechanism of action against the *Leishmania* parasites appears to be via the modulation of cell surface receptors that affect many relevant cell processes, including calcium homeostasis, ether-lipid remodeling mechanisms, the synthesis of phosphatidylcholine, signal transduction, inositol metabolism, phospholipase activation and protein kinase C, as well as other mitogenic and apoptotic pathways. Miltefosine also increases macrophage cytotoxicity by increasing oxidative stress and stimulating cellular glucose consumption (by the production of reactive oxygen species such as H_2O_2 and superoxide O_2), eventually leading to the death of the parasites inhabiting these cells [52-57].

Miltefosine has been used at doses of 2-2.5 mg/kg/day or 50 mg twice a day for 28 days. It is noteworthy that the efficacy of this drug in the treatment of CL in the New World is limited [16, 43, 58].

The most common adverse effects observed with miltefosine are related to the gastrointestinal tract and include diarrhea and vomiting. These effects occur in more than 30% of the treated patients, and its use is contraindicated during pregnancy because it has known teratogenic effects. Severe symptoms may occur when doses as high as 200 mg per day are used [35, 59, 60].

Paromomycin

Paromomycin, also known as aminosidine, is the only aminoglycoside with clinically important antileishmanial activity; both the visceral and cutaneous forms can be treated with this antibiotic. Due its poor oral absorption, a parenteral formulation for VL treatment and a topical formulation for CL treatment have been developed [61-63]. Paromomycin has been tested against VL at dose of 15-20 mg/kg of paromomycin sulfate for 21 days [16]. The following three topical formulations have been used for cutaneous leishmaniasis: 15% paromomycin with 12% methylbenzethonium chloride; 15% paromomycin with 10% urea; and 15% paromomycin with 0.5% gentamicin. All of these formulations are administered twice a day for up to 20 days. These formulations have shown varying results depending on the species of *Leishmania* involved [43]. A notable advantage of paromomycin is observed when it is applied in combination with antimonials; in this case, paromomycin aids in reducing the therapy duration from 30 days to 17-21 days.

The exact mechanism of action of paromomycin requires further elucidation, but it has been reported to inhibit protein synthesis in protozoans by binding to the 30S ribosomal subunit and causing an accumulation of abnormal initiation complexes [64].

Similar to other aminoglycosides, paromomycin has several adverse effects, including ototoxicity, nephrotoxicity, eighth cranial nerve disease and liver function abnormalities [62].

Azoles

The many azole compounds have been widely used as oral antifungal agents as they are well tolerated by the patients and efficient to treat these infections [65, 66]. They have been suggested for clinical treatment of leishmaniasis, as these drugs have shown antileishmanial activity *in vitro* and *in vivo*, by inhibiting ergosterol biosynthesis in the parasites and thereby affecting their cell membrane.

Fluconazole has been reported to show promising results in the treatment of cutaneous or visceral leishmaniasis caused by parasites of both subgenera [67-69], although its efficiency, applicability or required dosage are still in debate [70-73].

Itraconazole presents similar contradictory data in the literature: there are reports of clinical cases where patients have been successfully treated for cutaneous leishmaniasis with this azole [74, 75]; but, in a larger clinical trial, it was noted that the cure rates of patients with cutaneous leishmaniasis were similar between the placebo group and the group treated with itraconazole [76].

Ketoconazole has also been used in studies with small patient numbers infected with species from both the Old and New Worlds and presented an acceptable cure rate [77-79]. However, these results still require confirmation by further larger studies.

PROTEINASES AS POTENTIAL TARGETS FOR NOVEL LEISHMANIASIS TREATMENTS

Proteolysis is a common mechanism of activation or inactivation of enzymes involved in an array of biological processes, such as digestion, blood clotting, cell differentiation and apoptosis [80].

Peptide bond hydrolysis can occur at the amino or carboxyl-terminal position of a polypeptide chain (exopeptidase activity) or within the polypeptide (endopeptidase or proteinase activity). The proteinases are classified based on the amino acid residues present in their catalytic site. The most common proteinase classes are serine, cysteine, aspartyl, metallo, threonine and glutamine proteinases. Among these, four classes have already been described in *Leishmania* parasites: serine, cysteine, aspartyl and metallo proteinases [81, 82].

Serine proteinases (SPs) contain a characteristic catalytic triad (histidine, serine and aspartic acid) in their active site. The hydrolytic activity of this triad occurs when the histidine and the aspartic residues interact with a serine residue and deprotonate a hydroxyl group. The enzyme then performs a nucleophilic attack on a carbonyl carbon of the substrate and hydrolyzes it [83].

Cysteine proteinases (CPs) have a hydrolytic mechanism similar to that of SPs; however, the active nucleophile radical is the thiol group of a cysteine residue rather than a hydroxyl group of a serine residue [83].

Aspartyl proteinases contain two aspartic acid residues in their active site that can act similar to an acid/base mechanism. In these enzymes, a water molecule coordinated between the two aspartic residues is activated by deprotonation and then attacks a carbonyl group in the substrate [83].

Metalloproteinases (MPs) contain a coordinated metal atom in their structure, usually zinc, which stabilizes the oxyanion hole. In many MPs, such as thermolysin and matrix MPs, two or three histidine residues and an acidic side chain perform the coordination of the metal ion. A water molecule is deprotonated by the coordinated metal ion and serves as the agent of hydrolytic activity against the substrate [83].

Despite their distinct and specific mechanisms of action, proteinases from these classes have been reported, in different studies and to variable degrees, to be virulence factors with relevant activity during the processes of *Leishmania* infection establishment and evolution in the vertebrate hosts.

Proteinases of *Leishmania* spp. as Virulence Factors and Targets for Novel Treatments

In the mammalian host cell, especially those of the mononuclear-phagocytic system, *Leishmania* parasites inhabit the parasitophorous vacuole (PV), which is originated by the fusion of a parasite-containing phagosome with other organelles, such as lysosomes and endosomes, characterized by an acidic environment (pH 4.7-5.2) with a great diversity of macromolecules [84, 85].

This potentially hostile environment is also the site where the metacyclic promastigotes that entered the mammal host differentiate into amastigotes, and must be able to adapt to the PV conditions [86]. In addition to this pivotal adaptation to the PV, the amastigotes must also be able to avoid or subvert the host's immune responses to further infect other cells.

It is in such conditions that the proteinases of *Leishmania* develop their activities and participate in nutrients acquisition, metabolic turnover and, as previously mentioned, host-parasite interactions.

Our group has previously extensively reviewed the data currently available in the literature regarding the roles of proteinases as virulence factors in *Leishmania* infections [82].

CPs are currently the protein class with the most reports of activity as virulence factors in *Leishmania* and are prevalent in species that belong to the *L. (L.) mexicana* complex [86]. Of all of the distinct CPs of these parasites, three papain-like CPs have been most thoroughly analyzed (CPA, CPB and CPC) [87].

CP activity is higher in *L. (L.) amazonensis* amastigotes, the evolutive form that infects mammals, than in promastigotes [88], which inhabit the gut of sandflies, and there is a correlation between the levels of CP expression and the infectivity of the parasite [89]. Thus, these proteinases have potential to influence the outcome of the infection.

Studies of CP gene suppression in *Leishmania* spp. have further demonstrated the important role of these proteinases. The suppression of CP expression diminishes the infectivity of *Leishmania (Leishmania) infantum* in hamsters [90] and that of *Leishmania (Leishmania) chagasi* in human cell cultures [91]. In addition, suppressing the multiple copies of the CPB genes in *L. (L.) mexicana* reduces the capacity of the parasites to infect and induce lesions in the hosts [92, 93].

CPB has been reported to be the most relevant CP for parasites of the *L. (L.) mexicana* complex, as it exerts an extensive array of effects on the vertebrate host. This enzyme promotes interleukin (IL)-4 expression [94], inhibit IL-12 expression [95], affect the transcription factors STAT-1, AP-1 and NF-κB, impair nitric oxide (NO) production [96] and cleave major histocompatibility complex (MHC) class I proteins [97].

A specific portion of CPB, the COOH-terminal extension, is not observed in other CPs and has been implicated in the infection process in the vertebrate host. This domain of CPB influences the production of cytokines and NO by the host and affects the capacity of parasites to survive inside macrophages [98-100].

Both CPA and CPC have been reported to play roles in the parasite-host interaction; however, these enzymes are less relevant to infection than CPB. Their suppression leads to fewer significant effects. *L. (L.) mexicana* parasites with a suppressed CPC gene are more susceptible to killing by host cells [101, 102], and *L. (L.) infantum* parasites with a suppressed CPA gene are less able to infect mammalian hosts [103].

Regarding MPs, the major surface protein (MSP or gp63) is abundantly expressed on the surface of *Leishmania* parasites [104] and has known roles as a virulence factor. GP63 is required for the promastigotes to survive complement-mediated lysis in the mammalian host [105, 106], it modulates certain cytokine responses in the host [107], and it affects the proliferation of natural killer cells during the infection [108]. This MP also affects transcription factors and signal transduction cascades and can cleave c-Jun (the central component of the transcription complex AP-1) [109] and NF-κB [110], and it also activates tyrosine phosphatases in macrophages [108]. Interestingly, GP63 also affects the host immune responses by cleaving CD4 glycoprotein, as observed in assays with human T cells [111].

Some studies have also identified roles of SPs as virulence factors. In attenuated strains of *L. (L.) donovani*, the surface SP levels are decreased, and the presence of a 115 kDa SP affects the ability of parasites to infect their hosts [112]. Moreover, the expression of oligopeptidase B appears necessary for *Leishmania* parasites to remain undetected in macrophages during infection [113].

As the above data indicate, proteinases are highly relevant factors for many species of *Leishmania* and participate in pivotal processes of the parasite life cycle. Understanding the distinct mechanisms of proteinase action and their importance in the biology of the parasites are necessary to adequately define the potential of proteinase inhibitors for infection treatment.

Effects of Proteinase Inhibitors on *Leishmania* Parasites in Culture

Previous studies have reported the inhibitory effects of different classes of proteinases on the survival and/or proliferation of *Leishmania* parasites. These studies often describe the use of viral proteinase inhibitors, especially those targeted to HIV proteinases [114-117].

Certain HIV aspartyl proteinase inhibitors, including Ac-Leu-Val-Phenylalaninal, saquinavir mesylate and nelfinavir, impair *Leishmania* cell division. In addition, these drugs have been shown to decrease the number of monocytes co-infected by HIV/*Leishmania* in culture, in a dose-dependent fashion [118]. Together, these reports indicate the potential of using these drugs for leishmaniasis treatment and underline the need to develop drug design studies to increase their affinity for the *Leishmania* proteinases.

Another promising front for the search of new antileishmanial drugs is research into natural compounds obtained from plants or other organisms. Interestingly, the mechanism of action of some of these compounds is through proteinase inhibition.

The bioflavonoid fukugetin is a compound isolated from the fruits of *Garcinia brasiliensis* by ethyl-acetate extraction, and it has been shown to inhibit the activity of *L. (L.) amazonensis* cysteine and serine proteinases. However, this compound showed no activity against promastigotes or amastigotes *in vitro* [119].

A Kunitz-type serine proteinase inhibitor obtained from a sea anemone, named ShPI-I, was able to affect SP activity in promastigotes of *L. (L.) amazonensis* and, also, to reduce parasites viability in culture, inducing morphological alterations to the cells [120]. This inhibitor's effects on parasites viability and morphology were more pronounced than those of classic SP inhibitors (N-tosyl-L-phenylalanine chloromethyl ketone and benzamidine) at the same time point but in lower concentration, suggesting an effective antileishmanial acitivity.

In addition, chemically synthesized compounds with proteinase-inhibiting properties that have not been previously studied for the treatment of other diseases have been analyzed with regard to their effects on *Leishmania* parasites. MP-inhibiting synthetic compounds have been selected through *in silico* analysis from databanks, and these compounds were able to block *L. (L.) donovani* proliferation *in vitro*. Specifically, these compounds inhibit parasite dipeptidyl carboxypeptidase, which has been established as a putative target for antileishmanial chemotherapy [121].

By applying a similar strategy, the virtual screening of the ChemBridge databank for inhibitors of parasitic cysteine proteinases led to the identification of five non-peptide inhibitors with antileishmanial activity against *L. (L.) donovani* promastigotes *in vitro*. These inhibitors were selected by their potential capacity to bind to falcipain-2 and 3 from *Plasmodium* parasites, as measured through *in silico* assays, and their binding to *Leishmania* CPs can be explained by the high conservation of the CP binding pocket structure across these protozoans [122].

The small molecule thiocarbazate (PubChem SID 26681509) is another synthetic inhibitor that was discovered

by analyzing the NIH Molecular Libraries Small Molecule Repository. It is a potent inhibitor of human cathepsin L and has demonstrated toxicity against *L. (L.) major* promastigotes, although it is safe to human aortic endothelial cells even at high concentrations [123].

A potent synthetic inhibitor of calpains, carbobezoxy-valinyl-phenylalaninal (commercial name, MDL 28170), also presents antileishmanial activity. MDL 28170 induces *L. (L.) amazonensis* parasite death in culture and promotes alterations in the cell morphology. Possible targets for this inhibitor are the calpain-like molecules present on the cell surface of the promastigotes [124].

Effects of Proteinase Inhibitors in the Treatment of Experimental *Leishmania* Infection

Presently, little data are available in the literature regarding the outcome of using proteinase inhibitors targeting parasite enzymes to treat experimental *Leishmania* infections in experimental animal infections or in host cell cultures.

Nevertheless, CP inhibitors have been reported to have promising results when applied in a chemotherapeutic context in experimental animal infection models. Two derivatives of oxalic bis[(2-hydroxy-1-naphthyl) methylene]hydrazide (named ZLIII43A and ZLIII115A), both reversible CP inhibitors, and the pseudopeptide substrate analogue morpholine urea-phenylalanine-homophenylalanine-vinylsulfonyl-benzene (K11002, Arris Pharmaceuticals), an irreversible inhibitor of CP, were shown to interfere in *L. (L.) major* infections *in vitro* and *in vivo* [86]. These compounds prevented parasite replication and infection of mouse macrophages (cell line J774) challenged in culture with *L. (L.) major* promastigotes. However, none of these compounds affected the host cells (as assessed by analysis of morphological changes).

These same compounds delayed lesion progression and reduced the parasite burden in infected BALB/c mice, without toxic effects to the treated animals. Contrary to the mechanism of action previously suggested for another CP inhibitor (CA074, a cathepsin B-specific inhibitor) that was also able to cure *L. (L.) major*-infected BALB/c mice [125], these inhibitors were not able to alter the T lymphocyte response of the host and appeared to act directly on the *Leishmania* CPs.

A distinct CP inhibitor, N-benzoyloxycarbonyl-phe-ala-diazomethylketone (Z-FA-DMK, Sigma), also impaired the parasite infection of host cells [126]. In this case, it has been suggested that Z-FA-DMK affects the activity of *L. (L.) mexicana* cysteine proteinase B, a known pivotal virulence factor for this species; however, the specificity of this action was not completely defined. This inhibitor was very efficient at preventing the infection of peritoneal cells from BALB/c mice by promastigotes or amastigotes and also reduced the number of infecting parasites per host cell; however, when used in parasite cultures for extended periods, it did not alter the growth of the parasite cells and only partially inhibited their CP activities.

The treatment of *L. (L.) tropica*-infected BALB/c mice with subcutaneous or intraperitoneal injections of an irreversible CP inhibitor, the pseudopeptide substrate analogue *N*-Pip-phenylalanine-homophenylalanine-vinyl sulphone phenyl (N-Pip-F-hF-VS Phenyl or K11777), showed that this compound possesses antiparasitic activity. The treated animals showed a reduction in lesion size, and this effect persisted for as long as 2 months after the end of treatment [127].

This same CP inhibitor, K11777, was also reported to hinder the survival of *L. (L.) mexicana* parasites inside peritoneal macrophages from CD1 mice [128]. When parasites were incubated with K11777 prior to the infection assays, macrophage infection rates fell by nearly 75%. This effect is comparable to what is observed in experimental challenges using mutant parasites with cpa and cpb gene depletion. The effect of this inhibitor appears to be related to preventing autophagosome digestion in the parasites.

Cystatin, another inhibitor of CPs, has also been reported to show activity in murine models of *Leishmania* infection, specifically in BALB/c mice infected with *L. (L.) donovani* [129]. The use of cystatin in conjunction with interferon (IFN)-γ demonstrated antiparasitic effects. This combination affected amastigote growth inside macrophages *in vitro* and had curative effects for the infected animals, including the virtual elimination of parasites in the spleen after treatment. Interestingly, such treatment also led the treated mice to develop subsequent resistance to infection challenge. This evidence suggests the possibility that this CP inhibitor is not acting directly on parasite enzymes but is rather affecting the host cells in some way. This hypothesis is supported by the observations that macrophage nitrite production is enhanced, and the T lymphocyte response is altered by cystatin.

Two compounds derived from *trans*-aziridine-2,3-dicarboxylic acids (13b and 13e), which are irreversible inhibitors of cathepsin-B-like enzymes, have been reported to reduce the *L. (L.) major* infection rate in peritoneal macrophages obtained from BALB/c mice without showing toxic effects against the mammalian cells. These compounds induce parasite death with features that are similar to apoptosis, namely the accumulation of undigested debris in lysosome-like vacuoles within the parasites [130]. Similar to the pattern observed for cystatin, co-treatment with IFN-γ enhanced the antiparasitic effects of these compounds in infected macrophages. Furthermore, compounds 13b and 13e interfered with the production of interleukins and NO in infected peritoneal macrophages. These compounds changed the levels of IL-6, IL-12 and tumor necrosis factor (TNF)-α in cell culture and increased NO levels [131].

CP inhibitors have also demonstrated potential in the treatment against parasites during *Leishmania* infection of human cells or tissues. The cathepsin B-specific inhibitor CA074, which had a protective effect for BALB/c mice against *L. (L.) major* as described above, was shown to interfere in the infection of macrophages from the human myeloid cell line U937 with *L. (L.) infantum*. CA074 reduced parasite survival inside host cells [132]. Remarkably, the mechanism of this effect appears distinct from the one observed in the murine host and is possibly related to the inhibition of the parasite CPs rather than a direct effect on the host enzymes. In addition, because *Leishmania* cathepsin B has been implicated in the activation of host transforming growth factor (TGF)-β, which leads to an inefficient Th2

response, the inhibition of this enzyme by CA074 would prevent TGF-β activation and allow for the host to control the infection through an effective Th1 response.

Specific inhibitors of another class of CPs, cathepsin L, have also been shown to abrogate the parasite effect on IL-12 expression [133]. The compound cathepsin L inhibitor IV (Calbiochem) has been reported to interfere in the parasite-related cleavage of nuclear factor (NF)-κB or its endogenous inhibitors, as assayed in bone marrow-derived macrophages challenged with *L. (L.) mexicana*. This effect prevents IL-12 production by macrophages even after lipopolysaccharide (LPS) stimulation. Similar results have also been observed for K11002, which was previously mentioned due its effects on *L. (L.) major* survival in infected mice or murine cell cultures.

Inhibitors of other proteinase classes also have the potential to be used for the treatment of *Leishmania* infection. Five aspartyl proteinase inhibitors that are currently used for the treatment of HIV infection (amprenavir, indinavir, lopinavir, nelfinavir and saquinavir) have been shown to affect *L (L.) amazonensis* parasites *in vitro*, although with varying degrees of activity. These inhibitors impaired parasite growth in culture and induced changes in cell morphology. In addition, amprenavir, lopinavir and nelfinavir interfered with the parasite-macrophage association indexes if they were incubated with the promastigotes prior to the interaction assays [116].

However, other studies have reported that indinavir and ritonavir could not effectively control the infection in *L. (L.) amazonensis* experimentally infected mice; these antiretrovirals were able to reduce lesions in infected mice after 3-5 weeks of treatment, but they did not affect the parasite load [134].

Serine proteinase inhibitors present in potato tuber extract, which were effective to inhibit SP activities in log-phase promastigotes of *L. (L.) donovani*, also reduced viability of promastigotes in culture and affected amastigotes proliferation inside murine peritoneal macrophages [135]. This extract presented the potential to serve as basis for the development of new drugs against visceral leishmaniasis, as it was shown to have no adverse effects on macrophages, while enhancing the production of nitric oxide and reactive oxygen species, molecules related to parasite killing, in these cells.

REMARKS

Leishmaniasis is a neglected tropical disease and, as this term implies, there is a great need for additional research to improve the treatment options for this disease. Antimonials are the current first-choice drugs for leishmaniasis treatment and have been so for a substantial period of time. However, they are highly toxic compounds and, if they were proposed currently as a new drug, they would likely not be approved due to the rigid eligibility criteria and strict regulatory aspects for clinical trial investigations for drug approval [136, 137].

The current trends for drugs research in parasitic diseases include the application of proteinase inhibitors as chemotherapeutic agents. However, the data collected in independent reports have been unable to effectively prove that proteinase inhibitors can adequately replace antimonials in the treatment of leishmaniasis.

As previously described for *L. (L.) amazonesis* [81], *Leishmania* parasites differentially express proteinases in the various stages of their life cycle, reflecting the changes in their environment. These variations must be regarded when considering proteinase inhibitors as potential chemotherapeutic drugs. New drugs should focus primarily on the proteinases that are relevant to the parasite morphological stages that inhabit vertebrate hosts.

Another important point to consider is that certain minor structural differences in proteinases of the same type can affect their catalytic site microenvironment. These variations may make it difficult to develop proteinase inhibitors with wide activity against an array of proteinases in *Leishmania* spp.

In addition, due to the large variety of proteinases reported in these parasites, it is possible that only a combined treatment with distinct proteinase inhibitors would be an effective antileishmanial therapy.

Finally, it is important to consider that, despite the relative safety that proteinase inhibitors have been presenting when used against *Leishmania* in cell cultures or animal models, these drugs may cause some undesired side effects when applied in the treatment of leishmaniasis patients. It has been reported that proteinase inhibitors used in anti-HIV therapies may cause dyslipidemia, insulin resistance, type 2 diabetes, cardiac conduct abnormalities, nausea and diarrhea, however the more recently developed inhibitors cause these side effects less frequently [138-141]. Currently, an experimental animal model, using hamsters, is being developed to conduct studies about the side effects of antiretroviral proteinase inhibitors [141] and may, therefore, be also useful to determine the safety of potential antileishmanial proteinase inhibitors.

We firmly believe that only by understanding the modulation of distinct isoforms of the same proteinase will it be possible to propose a fine-tuned and effective strategy for controlling *Leishmania* parasites during infection of the vertebrate host. The establishment of treatments based on such variations would be a great contribution to controlling and curing this relevant parasitic disease.

Proteinases are a promising target for developing drugs to treat leishmaniasis because:	**Relevant aspects to be considered for the development of treatments based on *Leishmania* proteinase inhibitors:**
• They are pivotal factors for parasites' life cycle and interaction with host; • Drugs currently available for leishmaniasis present many toxic side effects and resistant strains are emerging;	• Parasites express distinct sets of proteinases in different phases of their life cycle; • Minor structural differences in the catalytic site of proteinases of a same type may prevent broad effect by the inhibitors;

• Reports from *in vitro* and *in vivo* studies show that specific inhibitors for *Leishmania* proteinases are safe for mammalian cells and animals; • Knowledge about characteristics and diversity of parasite's proteinases has been advancing greatly in recent years.	• Development of inhibitors should focus primarily on those proteinases known to be expressed by amastigotes (mammalian host-inhabiting evolutive stage).

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Alves CR designed this review study and Alves CR, Pereira BAS, Souza-Silva F, Silva-Almeida M, Santos-de-Souza R, Oliveira LFG and Ribeiro-Guimarães ML performed the research. All authors approved the final version of this manuscript.

This study received financial support from PAPES (CNPq/Fiocruz), CAPES and Faperj.

REFERENCES

[1] Neghina R, Neghina A. Leishmaniasis, a global concern for travel medicine. Scandinavian J Infect Dis 2010; 42(8): 563-70.
[2] Alvar J, Vélez ID, Bern C, *et al.* Leishmaniasis worldwide and global estimates of its incidence. PloS One 2011; 7(5): e35671.
[3] Kato H, Gomez E, Cáceres A, *et al.* Molecular epidemiology for vector research on leishmaniasis. Int J Environ Res Public Health 2010; 7(3): 814-26.
[4] Ameen M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. Clin Exp Dermatol 2010; 35(7): 699-705.
[5] Cox F. History of human parasitology. Clin Microbiol Rev 2002; 15(4): 595-612.
[6] Vianna G: Tratamento da leishmaniose tegumentar por injeções intravenosas de tártaro emético. In: Anais do 7º Congresso Brasileiro de Medicina e Cirurgia, Belo Horizonte, Brasil 1912; 42: pp 426-8.
[7] Rey L. Gaspar Vianna and the discovery of the treatment of leishmaniasis by antimonials. Rev Inst Med Trop Sao Paulo 1962; 4(2): 47-52.
[8] Di Cristina G. & Caronia G. Sulla terapia della leishmaniosi interna. Pathologica 1915; 7: 82-83.
[9] Cook GC: Leonard Rogers KCSI FRCP FRS (1868-1962) and the founding of the Calcutta School of Tropical Medicine. Notes Rec R Soc Lond 2006; 60(2): 171-81.
[10] Shortt HE: Recent research on kala-azar in India. Trans R Soc Trop Med Hyg 1945; 39(1): 13-31.
[11] Berman JD. Chemotherapy for leishmaniasis: biochemical mechanisms, clinical efficacy, and future strategies. Rev Infect Dis 1988; 10(3): 560-86.
[12] Brahmachari UN. A Treatise on Kala-azar. London: John Bale, Sons & Danielsson 1928.
[13] Kikuth W, Schmidt H. Contribution to the progress of antimony therapy of kala-azar. Chin Med J 1937; 52: 425-32.
[14] Goodwin LG. Pentostam (sodium stibogluconate); a 50-year personal reminiscence. Trans R Soc Trop Med Hyg 1995; 89(3): 339-41.
[15] Haldar AK, Sen P, Roy S. Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int 2011; 2011: 571242.
[16] World Health Organization (WHO). WHO Technical Report Series 949. Control of the leishmaniasis: report of a meeting of the World Health Organization Expert Committee on the Control of Leishmaniasis. Geneva; WHO 2010.
[17] Secretaria de Vigilância em Saúde (Ministério da Saúde - Brasil). Manual de Vigilância da Leishmaniose Tegumentar Americana. Série A. Normas e Manuais Técnicos 2ª edição. Brasília: Editora do Ministério da Saúde 2010.
[18] Frézard F, Demicheli C, Ribeiro RR. Pentavalent Antimonials: New Perspectives for Old Drugs. Molecules 2009; 14: 2317-36.
[19] Goodwin LG, Page JE. A study of the excretion of organic antimonials using a polarographic procedure. Biochem J 1943; 37(2): 198-209.
[20] Miekeley N, Mortari SR, Shubach AO. Monitoring of total antimony and its species by ICP-MS and online ion chromatography in biological samples from patients treated for leishmaniasis. Anal Bioanal Chem 2002; 372(3): 495-502.
[21] Frèzard F, Demicheli C, Ferreira CS, *et al.* Glutathione- induced conversion of pentavalent antimony to trivalent antimony in meglumine antimoniate. Antimicrob Agents Chemother 2001; 45(3): 913-916.
[22] Frezard F & Garnier-Suillerot A. Permeability of lipid bilayer to anthracycline derivatives. Role of the bilayer composition and of the temperature. Biochim Biophys Acta 1998; 1389(1): 13-22.
[23] Ferreira CS, Martins PS, Demicheli C, *et al.* Thiol-induced reduction of antimony(V) into antimony(III): a comparative study with trypanothione, cysteinyl-glycine, cysteine and glutathione. BioMetals 2003; 16(3): 441-6.
[24] Berman JD, Waddell D, Hanson BD. Biochemical Mechanisms of the Antileishmanial Activity of Sodium Stibogluconate. Antimicrobial Agents Chemother 1985; 27(6): 916-20.
[25] Wyllie S, Cunningham ML, Fairlamb AH. Dual action of antimonial drugs on thiol redox metabolism in the human pathogen *Leishmania donovani*. J Biol Chem 2004; 279(38): 39925-32.
[26] Baiocco P, Colotti G, Franceschini S, *et al.* Molecular Basis of Antimony Treatment in Leishmaniasis. J Med Chem 2009; 52(8): 2603-12.
[27] Sudhandiran G & Shaha C. Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular *Leishmania donovani* amastigotes. J Biol Chem 2003; 278(27): 25120-32.
[28] Lucumi A, Robledo S, Gama V, *et al.* Sensitivity of *Leishmania Viannia panamensis* to pentavalent antimony is correlated with the formation of cleavable DNA-protein complexes. Antimicrob Agents Chemother 1998; 42(8): 1990-5.
[29] Demicheli C, Frezard F, Lecouvey M, *et al.* Antimony(V) complex formation with adenine nucleosides in aqueous solution. Biochim Biophys Acta 2002; 1570(3): 192-8.
[30] Carter NS, Drew ME, Sanchez M, *et al.* Cloning of a novel inosine-guanosine transporter gene from *Leishmania donovani* by functional rescue of a transport-deficient mutant. J Biol Chem 2000; 275(27): 20935-41.
[31] Sampaio RNR, Sampaio JHD, Mardsen PD. Pentavalent antimonial treatment in mucosal leishmaniasis. Lancet 1985; 1(8437): 1097.
[32] Herwaldt B, Berman JD. Recommendations for treating Leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. Am J Trop Med Hyg 1992; 46(3): 296-306.
[33] Oliveira LF, Schubach AO, Martins MM, *et al.* Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Act Trop 2011; 118(2): 87-96.
[34] Yardley V & Croft SL. A comparison of the activities of three amphotericin B lipid formulations against experimental visceral and cutaneous leishmaniasis. Int J Antimicrob Agents 2000; 13(4): 243-8.
[35] Sundar, S. Treatment of visceral leishmaniasis. Med Microbiol Immunol 2001; 190(1-2): 89-92.
[36] Herwaldt BL. Leishmaniasis. Lancet 1999; 354(9185): 1191-9.
[37] Wolday D, Berhe N, Akuffo H, *et al.* Emerging Leishmania/HIV Co-infection in Africa. Med Microbiol Immunol 2001; 190(1-2): 65-7.
[38] Sampaio RNR, Mardsen P. Tratamento da forma mucosa de leishmaniose sem resposta a Glucantime com anfotericina B lipossomal. Rev Soc Bras Med Trop 1997; 30(2): 125-8.
[39] Sievers TM, Kubak BM, Bering AW. Safety and efficacy of Intralipid emulsions of Amphotericin B. J Antimicrob Chemother 1996; 38(3): 333-47.

[40] Secretaria de Vigilância em Saúde (Ministério da Saúde - Brasil). Manual de Vigilância e Controle da Leishmaniose Visceral. Brasília: Editora do Ministério da Saúde; 2003.
[41] Brynceton A. Current issues in the treatment of visceral leishmaniasis. Med Microbiol Immunol 2001; 190(1-2): 81-4.
[42] Murrey H W, Hariprashad J, Fich HRE. Treatment of Experimental Visceral Leishmaniasis in a T-cell-deficient Host: Response to Amphotericin B and pentamidine. Antimicrob Agents Chemother 1993; 37(7): 1504-05.
[43] Monzote L. Current Treatment of Leishmaniasis: A Review. Open Antimicrob Agents J 2009; 1: 9-19.
[44] Jha TK. Evaluation of allopurinol in the treatment of kala-azar occurring in North Bihar, India. Trans R Soc Trop Med Hyg 1983; 77(2): 204-7.
[45] Jha SN, Singh NK, Jha TK. Changing response to diamidine compounds in cases of kala-azar unresponsive to antimonial. J Assoc Physicians India 1991; 39(4): 314-6.
[46] Sundar S. Drug resistance in Indian visceral leishmaniasis. Trop Med Int Health 2001; 6(11): 849-54.
[47] Ouellette M, Drummelsmith J, Papadopoulou B. Leishmaniasis: drugs in the clinic, resistance and new developments. Drug Resist Updat 2004; 7(4-5): 257-66.
[48] Doua F, Miezan TW, Singaro JRS, *et al.* The efficacy of pentamidine in the treatment of early-late stage *Trypanosoma brucei gambiense* trypanosomiasis. Am J Trop Med Hyg 1996; 55(6): 586-8.
[49] Correia D, Macêdo VO, Carvalho EM, *et al.* Comparative study of meglumine antimoniate, pentamidine isethionate and aminosidine sulfate in the treatment of primary skin lesions caused by *Leishmania (Viannia) braziliensis*. Rev Soc Bras Med Trop 1996; 29(5): 447-53.
[50] Rang NP, Dale MM, Ritter JM. In: *Farmacologia 4th ed.;* Rang NP, Dale MM, Ritter JM, Guanabara Koogan. 2002: pp 610-21.
[51] Croft SL, Engel J. Miltefosine discovery of the antileishmanial activity of phospholipid derivatives. Trans R Soc Trop Med Hyg 2006; 100(Suppl 1): S4-8.
[52] Sundar S, Jha TK, Thakur CP, *et al.* Oral miltefosine for Indian visceral leishmaniasis. N Engl J Med 2002; 347(22): 1739-46.
[53] Croft SL, Seifert K, Duchêne M. Antiprotozoal activities of phospholipids analogues. Mol Biochem Parasitol 2003; 126(2): 165-72.
[54] Verma NK, Dey CS. Possible mechanism of miltefosine-mediated death of *Leishmania donovani*. Antimicrob Agents Chemother 2004; 48(8): 3010-5.
[55] Jha TK, Sundar S, Thakur CP, *et al.* Miltefosine, an oral agent, for the treatment of Indian visceral leishmaniasis. N Engl J Med 1999; 341(24): 1795-800.
[56] Sundar S, Rosenkaimer F, Makharia MK, *et al.* Trial of oral miltefosine for visceral leishmaniasis. Lancet 1998; 352(9143): 1821-3.
[57] Bhattacharya SK, Jha TK, Sundar S, *et al.* Efficacy and tolerability of miltefosine for childhood visceral leishmaniasis in India. Clin Infect Dis 2004; 38(2): 217-21.
[58] Minodier P & Parola P. Cutaneous leishmaniasis treatment. Travel Med Infect Dis 2007; 5(3): 150-158.
[59] Arana B, Rizzo N, Diaz A. Chemotherapy of Cutaneous leishmaniasis: a review. Med Microbiol Immunol 2001; 190(1-2): 93-5.
[60] Fisher C, Voss A, Engel J: Development status of miltefosine as first oral drug in visceral and cutaneous leishmaniasis. Med Microb Immunol 2001; 190(1-2): 85-7.
[61] Croft SL, Yardley V, Kendrick H. Drug sensitivity of *Leishmania* species: some unresolved problems. Trans R Soc Trop Med Hyg 2002; 96(Suppl 1): S127-29.
[62] Sundar S, Jha TK, Thakur CP, *et al.* Injectable paromomycin for visceral leishmaniasis in India. N Engl J Med 2007; 356(25): 2571-81.
[63] Ben Salah A, Zakraoui H, Zaatour A, *et al.* A randomized, placebo-controlled trial in Tunisia treating cutaneous leishmaniasis with paromomycin ointment. Am J Trop Med Hyg 1995; 53(2): 162-6.
[64] Sundar S, Chakravarty J. Paromomycin in the treatment of leishmaniasis. Expert Opin Invest Drugs 2008; 17(5): 787-94.
[65] Rani N, Sharma A, Gupta GK, *et al.* Imidazoles as potential antifungal agents: a review. Mini Rev Med Chem 2013; 13(11): 1626-55.
[66] Lestner J & Hope WW. Itraconazole: an update on pharmacology and clinical use for treatment of invasive and allergic fungal infections. Expert Opin Drug Metabol Toxicol 2013; 9(7): 911-26.
[67] Alrajhi AA, Ibrahim EA, De Vol EB, *et al.* Fluconazole for the treatment of cutaneous leishmaniasis caused by *Leishmania major*. N Engl J Med 2002; 346: 891-5.
[68] Jha BB. Fluconazole in Visceral Leishmaniasis. Indian Pediatrics 1998; 35: 268-9.
[69] Sousa AQ, Frutuoso MS, Moraes EA, *et al.* High-dose oral fluconazole therapy effective for cutaneous leishmaniasis due to *Leishmania (Vianna) braziliensis*. Clin Infect Dis 2011; 53: 693-5.
[70] Morizot G, Delgiudice P, Caumes E, *et al.* Healing of Old World cutaneous leishmaniasis in travelers treated with fluconazole: drug effect or spontaneous evolution? Am J Trop Med Hyg 2007; 76: 48-52.
[71] Zulunov A, Klaus S, Vardy D. Fluconazole for the treatment of cutaneous leishmaniasis (Letters to the Editor). N Engl J Med 2002; 347(5): 370.
[72] Torres JR & Suaréz JA. Fluconazole effectiveness against *Leishmania (Viannia) braziliensis*: is the evidence enough? Clin Infect Dis 2012; 55: 309.
[73] Emad M, Hayati F, Fallahzadeh MK, *et al.* Superior efficacy of oral fluconazole 400 mg daily versus oral fluconazole 200 mg daily in the treatment of cutaneous *Leishmania major* infection: a randomized clinical trial. J Am Acad Dermatol 2011; 64(3): 606-8.
[74] Consigli J, Danielo C, Gallerano V, *et al.* Cutaneous leishmaniasis: successful treatment with itraconazole. Int J Dermatol 2006; 45: 46-9.
[75] Baroni A, Aiello FS, Vozza A, *et al.* Cutaneous leishmaniasis treated with itraconazole. Dermatologic Therapy 2009; 22: S27-S29.
[76] Nassiri-Kashani M, Firooz A, Khamesipour A, *et al.* A randomized double-blind placebo-controlled clinical trial of itraconazole in the treatment of cutaneous leishmaniasis. J Eur Acad Dermatol Venereol 2005; 19: 80-3.
[77] Saenz RE, Paz H, Berman JD. Efficacy of ketoconazole against *Leishmania braziliensis panamensis* cutaneous leishmaniasis. Am J Med 1990; 89: 147-55.
[78] Weinrauch L, Livshin R, Even-Paz Z, *et al.* Efficacy of ketoconazole in cutaneous leishmaniasis. Arch Dermatol Res 1983; 275: 353-4.
[79] Weinrauch L, Livshin R, El-On J. Ketoconazole in cutaneous leishmaniasis. Br J Dermatol 1987; 117: 666-8.
[80] Beynon RJ, Bond JS. Proteolytical enzymes: a pratical approach, 2nd ed. Oxorfd: Oxford University Press 2001.
[81] Alves CR, Corte-Real S, Bourguignon SC, *et al. Leishmania amazonensis*: early proteinase activities during promastigote-amastigote differentiation *in vitro*. Exp Parasitol 2005; 109(1): 38-48.
[82] Silva-Almeida M, Pereira BAS, Ribeiro-Guimarães ML, *et al.* Proteinases as virulence factors in *Leishmania* spp. infection in mammals. Parasit Vectors 2012; 5: 160.
[83] Erez E, Fass D, Bibi E. How intramembrane proteases bury hydrolytic reactions in the membrane. Nature 2009; 459(7245): 371-8.
[84] Pulvertaft RJ, Hoyle GF. Stages in the life-cycle of *Leishmania donovani*. Trans R Soc Trop Med Hyg 1960; 54: 191-6.
[85] McConville MJ, Naderer T. Metabolic pathways required for the intracellular survival of *Leishmania*. Annu Rev Microbiol 2011; 65: 543-61.
[86] Selzer PM, Pingel S, Hsieh I, *et al.* Cysteine protease inhibitors as chemotherapy: lessons from a parasite target. Proc Natl Acad Sci USA 1999; 96(20): 11015-22.
[87] Sajid M & McKerrow JH. Cysteine proteases of parasitic organisms. Mol Biochem Parasitol 2002; 120(1): 1-21.
[88] Lasakosvitsch F, Gentil LG, dos Santos MR, *et al.* Cloning and characterization of a cysteine proteinase gene expressed in amastigotes of *Leishmania (L.) amazonensis*. Int J Parasitol 2003; 33(4): 445-54.
[89] de Araújo Soares RM, dos Santos AL, Bonaldo MC, *et al. Leishmania (Leishmania) amazonensis*: differential expression of proteinases and cell-surface polypeptides in avirulent and virulent promastigotes. Exp Parasitol 2003; 104(3-4): 104-12.
[90] Poot J, Denise H, Herrmann DC, *et al.* In: Experimental challenge models for canine leishmaniasis in hamsters and dogs, optimization and application in vaccine research; Poot J, Utrecht University press, 2006: pp 93-107.
[91] Mundodi V, Kucknoor AS, Gedamu L. Role of *Leishmania (Leishmania) chagasi* amastigote cysteine protease in intracellular parasite survival: studies by gene disruption and antisense mRNA inhibition. BMC Mol Biol 2005; 6(1): 3.

[92] Mottram JC, Coombs GH, Alexander J. Cysteine peptidases as virulence factors of *Leishmania*. Curr Opin Microbiol 2004; 7(4): 375-81.
[93] Alexander J, Coombs GH, Mottram JC. *Leishmania mexicana cysteine* proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. J Immunol 1998; 161(12): 6794-801.
[94] Denise H, McNeil K, Brooks DR, *et al.* Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence *in vivo*. Infect Immun 2003; 71(6): 3190-5.
[95] Cameron P, McGachy A, Anderson M, *et al.* Inhibition of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania mexicana* amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J Immunol 2004; 173(5): 3297-304.
[96] Abu-Dayyeh I, Hassani K, Westra ER, *et al.* Comparative study of the ability of *Leishmania mexicana* promastigotes and amastigotes to alter macrophage signaling and functions. Infect Immun 2010; 78(6): 2438-45.
[97] De Souza Leao S, Lang T, Prina E, *et al.* Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci 1995; 108(Pt 10): 3219-31.
[98] Marin-Villa M, Vargas-Inchaustegui DA, Chaves SP, *et al.* The C-terminal extension of *Leishmania pifanoi* amastigote-specific cysteine proteinase Lpcys2: a putative function in macrophage infection. Mol Biochem Parasitol 2008; 162(1): 52-9.
[99] Alves CR, Benévolo-De-Andrade TC, Alves JL, *et al.* Th1 and Th2 immunological profile induced by cysteine proteinase in murine leishmaniasis. Parasite Immunol 2004; 26(3): 127-35.
[100] Pereira BAS, Silva FS, Rebello KM, *et al. In silico* predicted epitopes from the COOH-terminal extension of cysteine proteinase B inducing distinct immune responses during *Leishmania (Leishmania) amazonensis* experimental murine infection. BMC Immunol 2011; 12: 44.
[101] Bart G, Frame MJ, Carter R, *et al.* Cathepsin B-like cysteine proteinase-deficient mutants of *Leishmania mexicana*. Mol Biochem Parasitol 1997; 88(1-2): 53-61.
[102] Mottram JC, Brooks DR, Coombs GH. Roles of cysteine proteinases of trypanosomes and *Leishmania* in host-parasite interactions. Curr Opin Microbiol 1998; 1(4): 455-60.
[103] Denise H, Poot J, Jiménez M, *et al.* Studies on the CPA cysteine peptidase in the *Leishmania infantum* genome strain JPCM5. BMC Mol Biol 2006; 13(7): 42.
[104] Etges R, Bouvier J, Bordier C. The major surface protein of *Leishmania* promastigotes is a protease. J Biol Chem 1986; 261(20): 9098-101.
[105] Yao C, Donelson JE, Wilson ME. The major surface protease (MSP or GP63) of *Leishmania* sp. Biosynthesis, regulation of expression, and function. Mol Biochem Parasitol 2003; 132(1): 1-16.
[106] Yao C. Major surface protease of trypanosomatids: one size fits all? Infect Immun 2010; 78(1): 22-31.
[107] Thiakaki M, Kolli B, Chang KP, *et al.* Down-regulation of gp63 level in *Leishmania* amazonensis promastigotes reduces their infectivity in BALB/c mice. Microbes Infect 2006; 8(6): 1455-63.
[108] Lieke T, Nylén S, Eidsmo L, *et al. Leishmania* surface protein gp63 binds directly to human natural killer cells and inhibits proliferation. Clin Exp Immunol 2008; 153(2): 221-30.
[109] Contreras I, Gómez MA, Nguyen O, *et al. Leishmania*-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog 2010; 6(1): e1001148.
[110] Gregory DJ, Godbout M, Contreras I, *et al.* A novel form of NF-kappaB is induced by *Leishmania* infection: involvement in macrophage gene expression. Eur J Immunol 2008; 38(4): 1071-81.
[11] Gomez MA, Contreras I, Hallé M, *et al. Leishmania* GP63 alters host signaling through cleavage-activated protein tyrosine phosphatases. Sci Signal 2009; 2(90): ra58.
[112] Choudhury R, Das P, Bhaumik SK, *et al. In situ* immunolocalization and stage-dependent expression of a secretory serine protease in *Leishmania donovani* and its role as a vaccine candidate. Clin Vaccine Immunol 2010; 17(4): 660-7.
[113] Swenerton RK, Zhang S, Sajid M, *et al.* The oligopeptidase B of Leishmania regulates parasite enolase and immune evasion. J Biol Chem 2011; 286(1): 429-40.
[114] Savoia D, Allice T, Tovo PA. Antileishmanial activity of HIV protease inhibitors. Int J Antimicrob Agents 2005; 26(1): 92-4.
[115] Trudel N, Garg R, Messier N, *et al.* Intracellular survival of *Leishmania* species that cause visceral leishmaniasis is significantly reduced by HIV-1 protease inhibitors. J Infect Dis 2008; 198(9): 1292-9.
[116] Santos LO, Marinho FA, Altoé EF, *et al.* HIV aspartyl peptidase inhibitors interfere with cellular proliferation, ultrastructure and macrophage infection of *Leishmania amazonensis*. PLoS One 2009; 4(3): e4918.
[117] Santos LO, Vitório BS, Branquinha MH, *et al.* Nelfinavir is effective in inhibiting the multiplication and aspartic peptidase activity of *Leishmania* species, including strains obtained from HIV-positive patients. J Antimicrob Chemother 2013; 68(2): 348-53.
[118] Valdivieso E, Rangel A, Moreno J, *et al.* Effects of HIV aspartyl-proteinase inhibitors on *Leishmania* sp. Exp Parasitol 2010; 126(4): 557-563.
[119] Pereira IO, Assis DM, Juliano MA, *et al.* Natural products from *Garcinia brasiliensis* as *Leishmania* protease inhibitors. J Med Food 2011; 14(6): 557-62.
[120] Silva-Lopez RE, Morgado-Díaz JA, Chávez MA, *et al.* Effects of serine protease inhibitors on viability and morphology of *Leishmania (Leishmania) amazonensis* promastigotes. Parasitol Res 2007; 101: 1627-35.
[121] Gangwar S, Baig MS, Shah P, *et al.* Identification of novel inhibitors of dipeptidylcarboxypeptidase of *Leishmania donovani* via ligand-based virtual screening and biological evaluation. Chem Biol Drug Des 2012; 79(2): 149-56.
[122] Desai PV, Patny A, Sabnis Y, *et al.* Identification of novel parasitic cysteine protease inhibitors using virtual screening. 1. The ChemBridge database. J Med Chem 2004; 47(26): 6609-15.
[123] Shah PP, Myers MC, Beavers MP, *et al.* Kinetic characterization and molecular docking of a novel, potent, and selective slow-binding inhibitor of human cathepsin L. Mol Pharmacol 2008; 74(1): 34-41.
[124] d'Avila-Levy CM, Marinho FA, Santos LO, *et al.* Antileishmanial activity of MDL 28170, a potent calpain inhibitor. Int J Antimicrob Agents 2006; 28(2): 138-42.
[125] Maekawa Y, Himeno K, Ishikawa H, *et al.* Switch of CD4+ T cell differentiation from Th2 to Th1 by treatment with cathepsin B inhibitor in experimental leishmaniasis. J Immunol 1998; 161(5): 2120-27.
[126] Frame MJ, Mottram JC, Coombs GH. Analysis of the roles of cysteine proteinases of *Leishmania mexicana* in the host-parasite interaction. Parasitology 2000; 121: 367-77.
[127] Mahmoudzadeh-Niknam H, McKerrow JH. *Leishmania tropica*: cysteine proteases are essential for growth and pathogenicity. Expl Parasitol 2004; 106(3-4): 158-63.
[128] Williams RA1, Tetley L, Mottram JC, Coombs GH. Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 2006; 61(3): 655-74.
[129] Das L, Datta N, Bandyopadhyay S, *et al.* Successful Therapy of Lethal Murine Visceral Leishmaniasis with Cystatin Involves Up-Regulation of Nitric Oxide and a Favorable T Cell Response. J Immunol 2001; 166(6): 4020-8.
[130] Schurigt U, Schad C, Glowa C, *et al.* Aziridine-2,3-dicarboxylate-based cysteine cathepsin inhibitors induce cell death in *Leishmania major* associated with accumulation of debris in autophagy-related lysosome-like vacuoles. Antimicrob Agents Chemother 2010; 54(12): 5028-41.
[131] Ponte-Sucre A, Vicik R, Schultheis M, *et al.* Aziridine-2,3-Dicarboxylates, Peptidomimetic Cysteine Protease Inhibitors with Antileishmanial Activity. Antimicrob Agents Chemother 2006; 50(7): 2439-47.
[132] Somanna A, Mundodi V, Gedamu L. Functional Analysis of Cathepsin B-like Cysteine Proteases from *Leishmania donovani* Complex: Evidence for the Activation of Latent Transforming Growth Factor B. J Biol Chem 2002; 277(28): 25305-12.
[133] Cameron P, McGachy A, Anderson M, *et al.* Inhibition of Lipopolysaccharide-Induced Macrophage IL-12 Production by *Leishmania mexicana* Amastigotes: The Role of Cysteine Peptidases and the NF-κB Signaling Pathway. J Immunol 2004; 173(5): 3297-304.
[134] Demarchi IG, Silveira TG, Ferreira IC, *et al.* Effect of HIV protease inhibitors on New World *Leishmania*. Parasitol Int 2012; 61(4): 538-44.
[135] Paik D, Das P, De T, Chakraborti T. *In vitro* Anti-leishmanial Efficacy of Potato Tuber Extract (PTEx): Leishmanial Serine Pro-

tease(s) as Putative Target. Exp Parasitol 2014; pii: S0014-4894(14)00199-4.
[136] Croft SL, Yardley V. Chemotherapy of Leishmaniasis. Curr Pharm Des 2002; 8(4): 319-42.
[137] Croft SL, Coombs GH. Leishmaniasis-current chemotherapy and recent advances in the search for novel drugs. Trends Parasitol 2003; 19(11): 502-8.
[138] Carr A, Samaras K, Chisholm DJ, *et al.* Pathogenesis of HIV-1-protease inhibitor-associated peripheral lipodystrophy, hyperlipidaemia, and insulin resistance. Lancet 1998; 352: 1881-3.
[139] Anastos K, Lu D, Shi Q, *et al.* Association of serum lipid levels with HIV serostatus, specific antiretroviral agents, and treatment regimens. J Acquir Immune Defic Syndr 2007; 45: 34-42.
[140] Günthard HF, Aberg JA, Eron JJ, *et al.* Antiretroviral treatment of adult HIV infection: 2014 recommendations of the International Antiviral Society-USA Panel. JAMA 2014; 312(4): 410-25.
[141] Ramos-Sanchez EM, Goto H, Rivero DHRF, *et al. In vivo* assessment of antiretroviral therapy-associated side effects. Mem Inst Oswaldo Cruz 2014; 109(4): 484-7.

Received: July 09, 2014 **Revised: August 22, 2014** **Accepted: October 07, 2014**

1.8. Documento 2:

Alves CR, Pereira BAS, Silva-Almeida M, Souza-Silva F. Why strategies to control Leishmania spp multiplication based on the use of proteinase inhibitors should consider multiple targets and not only a single enzyme. J Mol Model. 2014 Oct;20(10):2465. doi: 10.1007/s00894-014-2465-4.

Neste documento, encaminhados a ideia de que a utilização de proteinases como alvos para novos quimioterápicos contra infecções de *Leishmania* spp, não deve ser baseada em único alvo. Discutimos a hipótese do tratamento combinado com os inibidores de proteinases como uma terapia leishmanicida. Esta é uma estratégia promissora, levando em consideração a ampla distribuição das proteinases no genoma do parasita, questionado o uso da oligopeptidase B como um único alvo no tratamento da leishmaniose.

Propomos também, que para desenvolver uma estratégia de quimioterapia eficiente é necessário considerar o uso de vários inibidores de proteinase e, eventualmente, até mesmo combinar esses inibidores com os medicamentos atuais em uso. Embora estes inibidores de proteinases afetam as *Leishmania* spp, eles ainda estão em estudo. Neste contexto, outros inibidores de proteases mostraram também efeitos sobre a Leishmania em cultura e infecção experimental e podem ser potencialmente utilizados para desenvolver terapias.

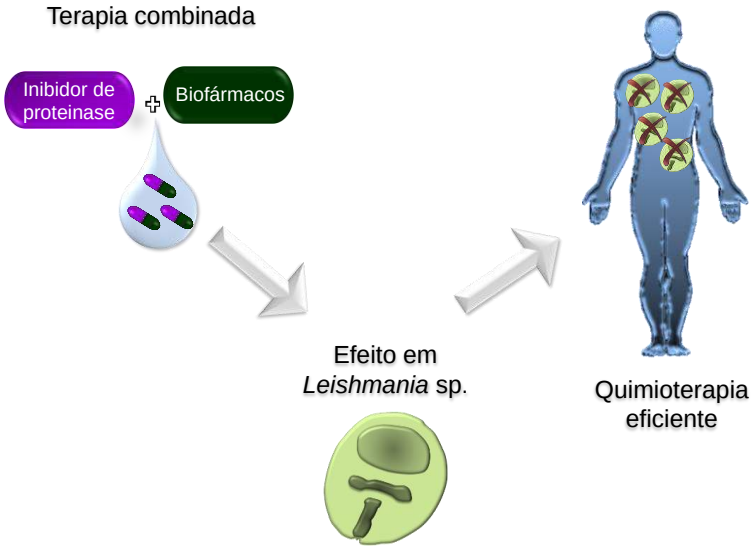


Figura 3: Resumo gráfico do DOCUMENTO 2

J Mol Model (2014) 20:2465
DOI 10.1007/s00894-014-2465-4

SHORT COMMENT

Why strategies to control *Leishmania* spp. multiplication based on the use of proteinase inhibitors should consider multiple targets and not only a single enzyme

Carlos Roberto Alves · Bernardo Acácio Santini Pereira · Mariana Silva-Almeida · Franklin Souza da Silva

Received: 3 July 2014 / Accepted: 8 September 2014

Abstract The use of proteinases as targets to develop novel chemotherapies against *Leishmania* spp. infections is a very promising strategy. Based on a previous study by Goyal et al. [J Mol Model (2014) 20:2099], we discuss herein the idea that only a combined treatment with distinct proteinase inhibitors would be an effective antileishmanial therapy.

Keywords Leishmania · Oligopeptidase B · Proteinase inhibitors · Antileishmanial therapy

Short comments

Leishmaniases are parasitic tropical diseases that affect humans. Currently, pentavalent antimony compounds (Glucantim and Pentostam) are the drugs of first choice to treat these diseases, while Amphotericin B, Pentamidine, Miltefosine or Aminosidine are used as secondary options [1]. In fact, to date, near 25 compounds or drug formulations have been shown to present antileishmanial effects; however, only a few have been tested properly, and even fewer are in the process to be considered for clinical use [2].

As the current chemotherapy options for these diseases are not entirely efficient and resistant strains are emerging, other components of the parasite have been proposed as potential targets to control the cutaneous or visceral infections; and, one such target are the proteinases [3].

A pivotal advantage of using proteinases as targets for leishmaniasis treatment derives from the fact that proteolysis is a common mechanism of activation or inactivation of enzymes involved in an array of biological processes, such as digestion, blood clotting, cell differentiation and apoptosis [4]. Thus, an increasing number of research articles have become available in the scientific literature that analyze characteristics of *Leishmania* proteinases and their roles during the course of infection, defining those that are more prominently suitable as drug targets.

In this context, serine proteinases are a topic of great interest for developing new chemotherapies, and an interesting study was published recently about the activity of oligopeptidase B (OPB) inhibitors against *Leishmania* parasites [5]. This paper reported relevant information concerning the interactions of naturally occurring compounds with *Leishmania major* OPB. The authors used a structure-based approach to perform a virtual screening of a large library of compounds and two of those, COP and TOA, were selected for further analysis by molecular dynamics simulations. A good binding affinity to OPB was shown for these compounds, as well as a low toxicity to human cells, indicating a promising new path for developing drugs against *Leishmania* spp.

Using OPB as a target allowed the development of a very relevant study, but it is important to bear in mind that other serine proteinases of *Leishmania* could also be targeted in similar approaches. At the present time, the genomes of *Leishmania* (*L.*) *infantum*, *Leishmania* (*V.*) *braziliensis*, *Leishmania* (*L.*) *mexicana* and *Leishmania* (*L.*) *major*, as

C. R. Alves (✉) · B. A. S. Pereira · M. Silva-Almeida · F. S. da Silva
Fundação Oswaldo Cruz, Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz, Av. Brasil 4365, Rio de Janeiro CEP 21040-360, Brazil
e-mail: calves@ioc.fiocruz.br

Published online: 09 October 2014

annotated in online databases, harbor 18, 17, 20 and 20 genes for serine proteinases, respectively. In comparison, only 2 genes encoding OPB have been identified in these species: in chromosome 9 (OPB) and chromosome 6 (OPB-like) [6]. Thus, these numbers reveal a great diversity of potential new targets for inhibition studies.

In addition to the importance of considering other serine proteinases as potential targets, studies by Munday et al. [7] and Swenerton et al. [8] showed that using OPB as a single target against *Leishmania* parasites may not be the most effective strategy. It was reported that, although knockout of the OPB gene does cause a defect in the differentiation of *L.* (*L.*) *major* during experimental infection assays in animals, this effect is only transient, suggesting that OPB is important in virulence processes but may play a less relevant role in pathogenesis of the disease.

As indicated in these studies, the inhibited OPB activity may be compensated by a second OPB-like enzyme. This OPB-like enzyme, although exhibiting only low sequence identity to the OPB, may compensate its activity by having a similar substrate specificity. Such specificity may be associated to a conserved amino acid residue, Glu-621, as well as other important residues composing the S1 binding site [7].

So, these observations are in agreement with the idea that targeting a single proteinase, OPB in this case, is not an appropriate strategy to treat leishmaniasis, even more so when one considers the complex profile of proteinases of *Leishmania* spp. [6]. We advocate that, to develop an efficient chemotherapy strategy, it is necessary to consider the use of multiple protease inhibitors and eventually even combine these inhibitors with drugs currently in use. However, to date, no such combination has been tested clinically and the only reported use of a combination of drugs refers to miltefosine associated with amphotericin B [9].

The concept of a chemotherapy strategy based on a combination of proteinase inhibitors has been applied in the treatment of patients with HIV-1 infection [10]. As for infections with protozoan parasites, a combined chemotherapy based on protease inhibitors is still a proposal at the experimental stage with few empirical data available.

An example of a potential chemotherapy against protozoa based on inhibition of multiple proteinase activities has been reported for *Plasmodium falciparum* [11]. It was shown that a combination of inhibitors targeting cysteine proteinases (falcipain) and aspartic proteinases (plasmepsin I and II) had synergistic effects on blocking this parasite's metabolism. Regarding treatment of leishmaniasis, a study reported the control of infection in laboratory mice by *Leishmania* (*Leishmania*) *donovani* through the administration of anticytokine antibody therapy combined with cystatin inhibitor [12]. Such studies shed light on the potential of combined chemotherapies for treating these diseases, but, at the moment, clinical evidence is still lacking.

Nevertheless, classical and novel proteinase inhibitors have been assayed continuously in parasite cultures or in experimental infection models to assess their potential antileishmanial effect [3]. Some of the assayed proteinase inhibitors that could be potentially used to develop therapies are listed in Table 1.

Among these compounds, some specific inhibitors against serine proteinases, such as antipain and leupeptin, have been assayed on *Leishmania* parasites. Antipain is an oligopeptide isolated from actinomycetes and is an inhibitor of trypsin and papain [28] while leupeptin is naturally occurring inhibitor that can affect cysteine-, serine- and threonine-proteinases [29]. Although these inhibitors are known to interfere with *Leishmania* growth in vitro, they still lack clinical validation.

Our group has been continuously publishing new data about proteinases of *Leishmania* spp. [18, 30–34] and *Trypanosoma cruzi* [35–37] and their importance in host–parasite interactions for over a decade. Collectively, these manuscripts make a relevant contribution to the search for proteinases that could serve as targets for developing future leishmaniasis treatments. As consequence, during our ongoing studies, some peculiarities of proteinases of *Leishmania* spp., in the context of potential targets for drugs, have become apparent to us that could be very useful if considered in other studies in this same research field.

One of such point of interest is the fact that *Leishmania* parasites exhibit variations and fluctuations in the levels of proteinase expression during different stages of their life cycle, and, thus, it would be important to target those proteinases expressed at the parasitic stage that inhabits the vertebrate host when considering targets for novel drugs.

Another point we observed is that even minor structural differences in proteinases in the same class can affect their catalytic site microenvironment, and these variations may account for the difficulties in developing proteinase inhibitors that have wide activity against proteinases of the same class in *Leishmania* spp.

Therefore, due to all these observations, as well as the reports that suggest a great variability and importance of proteinases in these parasites, we reinforce our statement that only treatments that combine distinct proteinase inhibitors have the possibility to deliver an effective antileishmanial therapy. To this end, a deep understanding of the expression modulation of different proteinase classes, even of distinct isoforms of the same proteinase, in *Leishmania* parasites infecting vertebrate hosts is required.

Table 1 Proteinase inhibitors tested in experimental assays against *Leishmania* spp. and against experimental infection with these parasites

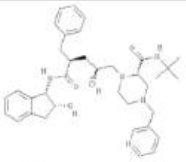
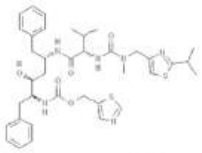
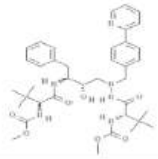
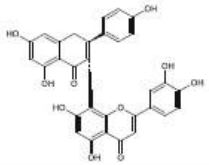
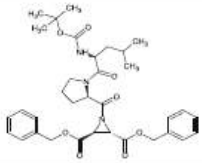
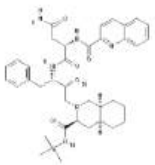
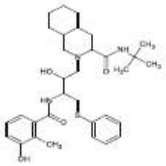
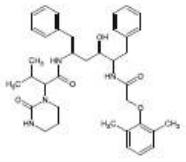
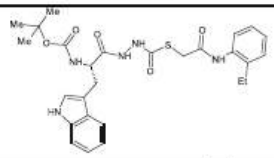
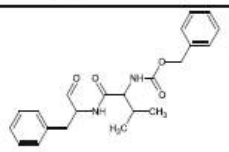
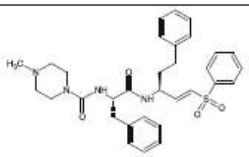
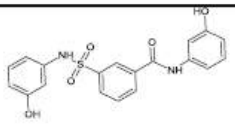
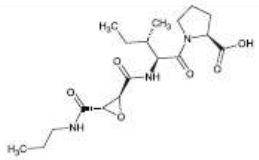
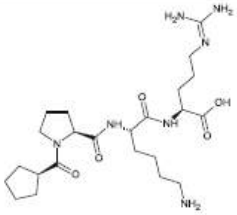
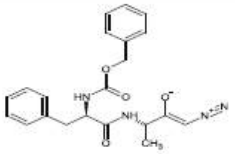
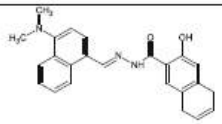
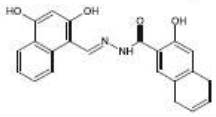
Drug	2D structure	Tested parasite	Efficiency	Target enzyme	Reference
Indinavir (CID 5362440)			IC_{50} 100 and 400 μM (promastigote)		
Ritonavir (CID 392622)		*L. (L.) amazonensis and L. (V.) braziliensis*	IC_{50} 40 and 2.3 μM (promastigote)	Aspartic proteinase	[13]
Atazanavir (CID 158550)			IC_{50} 266 and 400 μM (promastigote)		
Fukugetin		*L. (L.) amazonensis*	IC_{50} 3.2 μM (amastigote)	Cysteine proteinase and Serine proteinase	[14]
13b		*L. (L.) major*	IC_{50} 40 μM (promastigote) IC_{50} 3.0 μM (amastigote)	Cysteine proteinase	[15, 16]
Saquinavir (CID 441243)		*L. (L.) infantum, L. (L.) donovani, L. (L.) mexicana, L. (L.) amazonenses, L. (V.) brazilensis and L. (L.) major*	IC_{50} 48.04/55.21, 51.89, 40.67/24.44, 40, 36 and 46.95 μM (promastigote/amastigote)	Aspartic proteinase	[17]
Nelfinavir (CID 64142)		*L. (L.) infantum, L. (L.) donovani, L. (L.) mexicana, L. (L.) amazonenses, L. (V.) brazilensis and L. (L.) major*	IC_{50} 18.21/22.86, 14.10, 12.44/13.83, 13.36, 14.60 and 13.37 μM (promastigote/amastigote)	Aspartic proteinase	[17]
Lopinavir (CID 92727)		*L. (L.) amazonensis*	IC_{50} 15 μM (promastigote)	Aspartic proteinase	[19]

Table 1 (continued)

Drug	2D structure	Tested parasite	Efficiency	Target enzyme	Reference
CID 16725315		*L. (L.) major*	IC_{50} 12.5 μM (promastigote)	Cysteine proteinase	[19]
MDL 28170 (CID 11199915)		*L. (L.) amazonensis*	LD_{50} 23.3 μM (promastigote)	Cysteine proteinase	[20]
K11777 (CID 9851116)		*L. (L.) mexicana and L. (L.) tropica*	antiparasitic properties with a capacity of reduction in lesion size in treated animals and hinder the survival inside peritoneal macrophages of CD1 mice	Cysteine proteinase (amastigote)	[21, 22]
CID 1069242		*L. (L.) donovani*	IC_{50} 0.3 μM (promastigote)	Cysteine proteinase	[23]
CA074 (CID 9821383)		*L. (L.) donovani* *L. (L.) major*	reduction the parasite survival within the macrophages and capacity induce cure in infected BALB/c mice	Cysteine proteinase	[24, 25]
Cystatin (CID 128439)		*L. (L.) donovani*	affects amastigotes growth inside macrophages in vitro and had curative effects for infected animals	Cysteine proteinase	[12]
Z-FA-DMK (CID 5488522)		*L. (L.) mexicana*	prevent infection of peritoneal cells from BALB/c mice (promastigote and amastigote) and also reduced the number of infecting parasites per host cell	Cysteine proteinase	[26]
ZLIII115A		*L. (L.) major*	prevent parasite replication as well as infection of mouse macrophages	Cysteine proteinase	[27]
ZLIII43A					

References

1. WHO (1998) Technical Report Series. Rapport sur la Santé dans le Monde 1:59
2. Berman J (2005) Recent developments in leishmaniasis: epidemiology, diagnosis and treatment. Curr Infect Dis Repor 7:33–38
3. Pereira BAS, Silva FS, Silva-Almeida M, Santos-de-Souza R, Oliveira LFG, Ribeiro-Guimarães ML, Alves CR (2014) Proteinase inhibitors: a promising drug class for treating leishmaniasis. Curr Drug Target (in press)
4. Beynon RJ, Bond JS (2001) Proteolytical enzymes: a pratical approach, 2nd edn. Oxford University Press, Oxford
5. Goyal S, Grover S, Dhanjal JK, Goyal M, Tyagi C, Chacko S, Grover A (2014) Mechanistic insights into mode of actions of novel oligopeptidase B inhibitors for combating leishmaniasis. J Mol Model 20:2099
6. Silva-Almeida M, Souza-Silva F, Pereira BA, Ribeiro-Guimarães ML, Alves CR (2014) Overview of the organization of protease genes in the genome of *Leishmania* spp. Parasit Vectors 7(1):387
7. Munday JC, McLuskey K, Brown E, Coombs GH, Mottram JC (2011) Oligopeptidase B deficient mutants of *Leishmania major*. Mol Biochem Parasitol 175(1):49–57
8. Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, McKerrow JH (2011) The oligopeptidase B of Leishmania regulates parasite enolase and immune evasion. J Biol Chem 286(1): 429–40
9. Jha TK (2006) Drug unresponsiveness & combination therapy for kala-azar. Indian J Med Res 123:389–398
10. Hughes PJ, Cretton-Scott E, Teague A, Wensel TM (2011) Protease inhibitors for patients With HIV-1 infection: a comparative overview. P T 36(6):332–336
11. Semenov A, Olson JE, Rosenthal PJ (1998) Antimalarial synergy of cysteine and aspartic protease inhibitors. Antimicrob Agents Chemother 42(9):2254–2258
12. Das L, Datta N, Bandyopadhyay S, Das PK (2001) Successful therapy of lethal murine visceral leishmaniasis with cystatin involves up-regulation of nitric oxide and a favorable T cell response. J Immunol 166(6):4020–4028
13. Demarchi IG, Silveira TG, Ferreira IC, Lonardoni MV (2012) Effect of HIV protease inhibitors on New World *Leishmania*. Parasitol Int 61(4):538–544
14. Pereira IO, Assis DM, Juliano MA, Cunha RL, Barbieri CL, do Sacramento LV, Marques MJ, dos Santos MH (2011) Natural products from *Garcinia brasiliensis* as *Leishmania* protease inhibitors. J Med Food 14(6):557–562
15. Schurigt U, Schad C, Glowa C, Baum U, Thomale K, Schnitzer JK, Schultheis M, Schaschke N, Schirmeister T, Moll H (2010) Aziridine-2,3-dicarboxylate-based cysteine cathepsin inhibitors induce cell death in *Leishmania major* associated with accumulation of debris in autophagy-related lysosome-like vacuoles. Antimicrob Agents Chemother 54(12): 5028–5041
16. Ponte-Sucre A, Vicik R, Schultheis M, Schirmeister T, Moll H (2006) Aziridine-2,3-dicarboxylates, peptidomimetic cysteine protease inhibitors with antileishmanial activity. Antimicrob Agents Chemother 50(7):2439–2447
17. Valdivieso E, Rangel A, Moreno J, Saugar JM, Cañavate C, Alvar J, Dagger F (2010) Effects of HIV aspartyl-proteinase inhibitors on *Leishmania* sp. Exp Parasitol 26(4):557–563
18. Santos LO, Marinho FA, Altoé EF, Vitório BS, Alves CR, Britto C, Motta MC, Branquinha MH, Santos AL, d'Avila-Levy CM (2009) HIV aspartyl peptidase inhibitors interfere with cellular proliferation, ultrastructure and macrophage infection of *Leishmania amazonensis*. PLoS ONE 4(3):e4918
19. Shah PP, Myers MC, Beavers MP, Purvis JE, Jing H, Grieser HJ, Sharlow ER, Napper AD, Huryn DM, Cooperman BS, Smith AB 3rd, Diamond SL (2008) Kinetic characterization and molecular docking of a novel, potent, and selective slow-binding inhibitor of human cathepsin L. Mol Pharmacol 74(1): 34–41
20. d'Avila-Levy CM, Marinho FA, Santos LO, Martins JL, Santos AL, Branquinha MH (2006) Antileishmanial activity of MDL 28170, a potent calpain inhibitor. Int J Antimicrob Agents 28(2):138–142
21. Williams RA, Tetley L, Mottram JC, Coombs GH (2006) Cysteine peptidases CPA and CPB are vital for autophagy and differentiation in *Leishmania mexicana*. Mol Microbiol 61(3):655–674
22. Mahmoudzadeh-Niknam H, McKerrow JH (2004) *Leishmania tropica*: cysteine proteases are essential for growth and pathogenicity. Exp Parasitol 106(3–4):158–163
23. Desai PV, Patny A, Sabnis Y, Tekwani B, Gut J, Rosenthal P, Srivastava A, Avery M (2004) Identification of novel parasitic cysteine protease inhibitors using virtual screening The ChemBridge database. J Med Chem 47(26):660966–660915
24. Somanna A, Mundodi V, Gedamu L (2002) Functional analysis of cathepsin B-like cysteine proteases from *Leishmania donovani* complex. Evidence for the activation of latent transforming growth factor beta. J Biol Chem 277(28):25305–25312
25. Maekawa Y, Himeno K, Ishikawa H, Hisaeda H, Sakai T, Dainichi T, Asao T, Good RA, Katunuma N (1998) Switch of CD4+ T cell differentiation from Th2 to Th1 by treatment with cathepsin B inhibitor in experimental leishmaniasis. J Immunol 161(5):2120–2127
26. Frame MJ, Mottram JC, Coombs GH (2000) Analysis of the roles of cysteine proteinases of *Leishmania mexicana* in the host-parasite interaction. Parasitology 121(4):367–377
27. Selzer PM, Pingel S, Hsieh I, Ugele B, Chan VJ, Engel JC, Bogyo M, Russell DG, Sakanari JA, McKerrow JH (1999) Cysteine protease inhibitors as chemotherapy: lessons from a parasite target. Proc Natl Acad Sci USA 96(20):11015–11022
28. Umezawa S, Tatsuta K, Fujimoto K, Tsuchiya T, Umezawa H (1972) Structure of antipain, a new Sakaguchi-positive product of streptomyces. J Antibiot (Tokyo) 25(4):267–270
29. Maeda K, Kawamura K, Kondo S, Aoyagi T, Takeuchi T (1971) The structure and activity of leupeptins and related analogs. J Antibiot (Tokyo) 24(6):402–404
30. Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, Alves CR (2012) Proteinases as virulence factors in *Leishmania* spp. infection in mammals. Parasit Vectors 5:160
31. Pereira BA, Britto C, Alves CR (2012) Expression of infection-related genes in parasites and host during murine experimental infection with *Leishmania* (Leishmania) *amazonensis*. Microb Pathog 52(2):101–108
32. Rebello KM, Britto C, Pereira BA, Pita-Pereira DD, Moraes MO, Ferreira AB, Cysne-Finkelstein L, Otto TD, Côrtes LM, da-Silva GG, Alves CR (2010) *Leishmania (Viannia) braziliensis*: influence of successive in vitro cultivation on the expression of promastigote proteinases. Exp Parasitol 126(4):570–576
33. Rebello KM, Côrtes LM, Pereira BA, Pascarelli BM, Côrte-Real S, Finkelstein LC, Pinho RT, d'Avila-Levy CM, Alves CR (2009) Cysteine proteinases from promastigotes of *Leishmania (Viannia) braziliensis*. Parasitol Res 106(1):95–104
34. Alves CR, Corte-Real S, Bourguignon SC, Chaves CS, Saraiva EM (2005) *Leishmania amazonensis*: early proteinase activities during promastigote-amastigote differentiation in vitro. Exp Parasitol 109(1):38–48
35. Bourguignon SC, Cavalcanti DF, de Souza AM, Castro HC, Rodrigues CR, Albuquerque MG, Santos DO, da Silva GG, da Silva FC, Ferreira VF, de Pinho RT, Alves CR (2011)

Trypanosoma cruzi: insights into naphthoquinone effects on growth and proteinase activity. Exp Parasitol 127(1):160–166
36. Bourguignon SC, Castro HC, Santos DO, Alves CR, Ferreira VF, Gama IL, Silva FC, Seguins WS, Pinho RT (2009) *Trypanosoma cruzi*: in vitro activity of Epoxy-alpha-Lap, a derivative of alpha-lapachone, on trypomastigote and amastigote forms. Exp Parasitol 122(2):91–96
37. Pinho RT, Beltramini LM, Alves CR, De-Simone SG (2009) *Trypanosoma cruzi*: isolation and characterization of aspartyl proteases. Exp Parasitol 122(2):128–133

2. OBJETIVOS

2.1. Objetivo geral

O presente estudo tem como objetivo contribuir na caracterização dos efeitos biológicos e bioquímicos do epoxi-α-lapachona sobre a *Leishmania* sp.

2.2. Objetivos específicos

- Avaliar o efeito *in vitro* do epoxi-α-lapachona contra *L. (V.) braziliensis* e *L. (L.) amazonensis.*
- Avaliar o efeito *in vivo* na infecção experimental por *L. (L.) amazonensis* em camundongos.
- Verificar a ação do composto epoxi-α-lapachona sobre a serino proteinase de *L. (L.) amazonensis*.
- Avaliar o modo de ligação do composto epoxy-α-lapachone na oligopeptidase B de *L. (L.) amazonensis.*

3. MÉTODOS e RESULTADOS

3.1. Documento 3:

Souza-Silva F, Nascimento SB, Bourguignon SC, Santini Pereira BAS, Carneiro PD, Silva WS, Alves CR, Pinho, RT. Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone. Exp Parasitol. 2014 Oct 9. pii: S0014-4894(14)00225-2. doi: 10.1016/j.exppara.2014.10.002

Nesta etapa deste trabalho foi oportuno avaliar os efeitos leishmanicidas do epóxi-α-lapachona sobre *Leishmania (Viannia) braziliensis* e *Leishmania (Leishmania) amazonensi*s. A atividade do composto foi avaliada sobre promastigotas e amastigotas de ambas as espécies.

A incubação com epóxi-α-lapachona levou a uma diminuição significativa no número de promastigotas de ambas as espécies de culturas, de forma dose e tempo dependentes. A sobrevivência dos amastigotas no interior de macrófagos humanos também foi drasticamente afetada pelo composto, como mostrado pelas variações do índice de endocitose. Nossos resultados indicam que o epoxi-α-lapachona tem um efeito antiparasitário sobre o parasito em ambas os estágios morfológicos e pode potencialmente afetar uma série de espécies nos dois subgêneros distintos deste parasita.

Desta forma, com os dados desta publicação foi possível alcançar objetivo um deste trabalho tese, quando avaliamos o efeito *in vitro* do epoxi-α-lapachona sobre duas espécies de Leishmania: *L. (V.) braziliensis* e *L. (L.) amazonensis.*

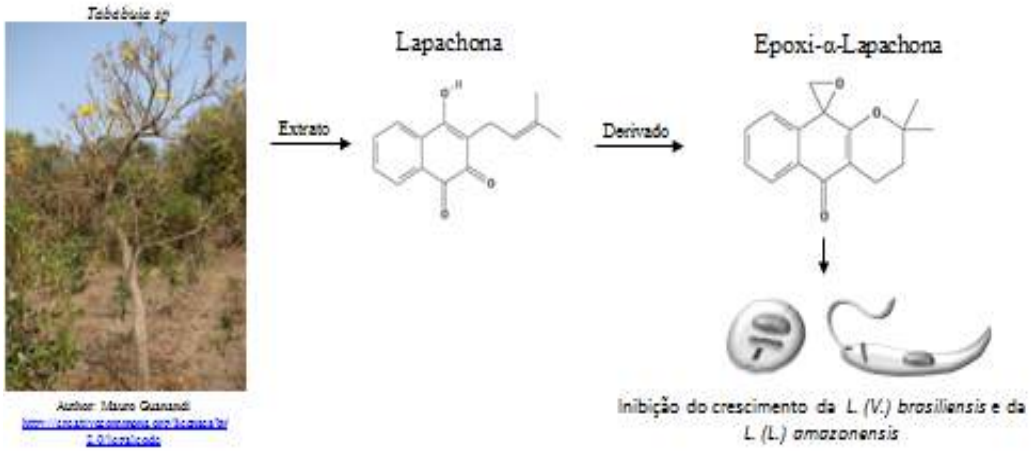


Figura 4: Resumo gráfico do DOCUMENTO 3

ARTICLE IN PRESS

Experimental Parasitology ■■ (2014) ■■–■■

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Research Brief

Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone

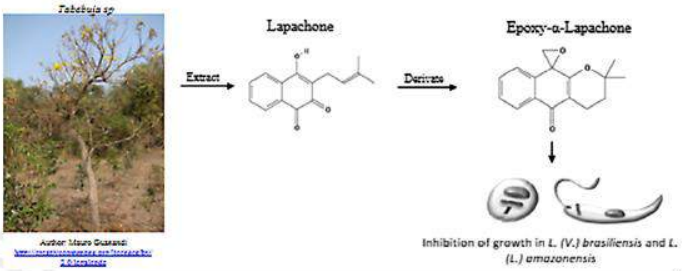
Franklin Souza-Silva [a,1,*], Samara Braga do Nascimento [b,1], Saulo Cabral Bourguignon [b], Bernardo Acácio Santini Pereira [a], Paula Fernandes Carneiro [c], Wellington Seguis da Silva [d], Rosa Teixeira de Pinho [d], Carlos Roberto Alves [a]

[a] Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Biologia Molecular e Doenças Endêmicas, Av. Brasil, 4365, Maguinhos, CEP 21040-360, Rio de Janeiro, Brazil
[b] Instituto de Biologia, Pós-Graduação em Ciências e Biotecnologia, Universidade Federal Fluminense, Outeiro de São João Baptista s/n – Centro, Niterói, CEP 24020-150 Rio de Janeiro, Brazil
[c] Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, Cidade Universitária CEP 21944-970, Rio de Janeiro, Brazil
[d] Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Imunologia Clínica, Av. Brasil, 4365, Maguinhos, CEP 21040-360 Rio de Janeiro, Brazil

HIGHLIGHTS

- Epoxy-α-lapachone has activity against *L. (V.) braziliensis* and *L. (L.) amazonensis*.
- It is able to decrease promastigotes growth in culture.
- It also drastically affected the survival of amastigotes inside human macrophages.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 17 April 2014
Received in revised form 13 August 2014
Accepted 6 October 2014
Available online

Keywords:
Leishmania (Viannia) braziliensis
Leishmania (Leishmania) amazonensis
Naphthoquinones
Epoxy-α-lapachone

ABSTRACT

In this work, we analyze the leishmanicidal effects of epoxy-α-lapachone on *Leishmania (Viannia) braziliensis* and *Leishmania (Leishmania) amazonensis*. Promasigotes and amastigotes (inhabiting human macrophages) from both species were assayed to verify the compound's activity over the distinct morphological stages. The incubation with epoxy-α-lapachone led to a significant decrease in the numbers of promastigotes from both species in the cultures, in a dose-and time-dependent fashion. The survival of amastigotes inhabiting human macrophages was also drastically affected by the compound, as shown by the variations in the endocytic index. Our results indicate that the epoxy-α-lapachone has an antiparasitic effect over *Leishmania* in both morphological stages and may potentially affect a range of species in two distinct subgenera of this parasite.

Leishmaniasis is a very relevant parasitic disease, especially in the tropical and subtropical regions of the world, with increasing importance as its geographical spreading is a reported fact (Hotez et al., 2007). Currently, the first-line treatment for leishamniasis involves antimonial, which are expensive and potentially toxic, requiring also a long therapy timeframe (Berman et al., 1997; Deps et al., 2000). Additionally, the emergence of cases of parasites

* Corresponding author. Fax: +21 2590 3495.
E-mail address: calves@ioc.fiocruz.br (Franklin Souza-Silva).
[1] These authors have equally contributed to this paper.

http://dx.doi.org/10.1016/j.exppara.2014.10.002
0014-4894/

Please cite this article in press as: Franklin Souza-Silva, et al., Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone, Experimental Parasitology (2014), doi: 10.1016/j.exppara.2014.10.002

presenting resistance to this treatment has been reported (Sundar et al., 2014).

The drugs used in cases where treatment with antimonials is ineffective or must be avoided, known as second choice drugs, e.g. Pentamidin, Amphotericin B and Miltefosine, are also toxic and present difficulties to their administration (Jha, 2006; Saldanha et al., 1999). These drugs present differences in their efficiency against distinct *Leishmania* species (Santos et al., 2008). Therefore, leishmaniasis still lacks adequate therapies and the development of new drugs is a point of great and, even, urgent interest.

The derivates of naphthoquinones, organic compounds originally described in the heartwood of *Bignoniaceae* and *Verbanaceae* trees are known by their significant anti-microbial properties (Ferreira et al., 2006; Jorqueira et al., 2006). β-lapachone (1,2-naphthoquinone) and its isomer α-lapachone (1,4-naphthoquinone) were shown to have trypanocidal activity but with a significant cytotoxicity on mammalian cells.

As they have been described as effective against *Trypanosoma cruzi*, derivatives of naphthoquinones were also analyzed as potential leishmanicidal drugs: It was reported that lapachol, isolapachol and dihydrolapachol, as well as soluble derivatives and acetate, are able to kill *in vitro* metacyclic promastigotes of *Leishmania (Leishmania) amazonensis* and *Leishmania (Leishmania) braziliensis* (Lima et al., 2004). Other derivatives, such as 2,3-dichloro-5,8-dihydroxy-1,4-naphthoquinone and 2,3-dibromo-1,4-naphthoquinone, were also proven to kill promastigotes and intracellular amastigotes of *Leishmania (L.) donovani* (Lezama-Dávila et al., 2012).

However, the toxicity of the lapachone derivatives could limit their potential to be used in the treatment of leishmaniasis and other parasite infections. Thus, studies have applied chemical modifications in these compounds to address this issue and a variation at the quinonoid center of α-lapachone 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).

When used in assays with *T. cruzi*, the compound epoxy-α-lapachone presented a trypanocidal activity equivalent to β-lapachone but, as expected, with lower cytotoxic effect against mammalian cells, such as human macrophages (Bourguignon et al., 2009; Ferreira et al., 2006; Jorqueira et al., 2006).

In the present study, the *in vitro* effects of epoxy-α-lapachone compound against two species of *Leishmania* – *L. (V.) braziliensis* and *L. (L.) amazonensis* – and their two morphological stages, is described for the first time.

Leishmania (Viannia) braziliensis (strain MCAN/BR/1998/R619) was obtained from Instituto de Pesquisa Clínica Evandro Chagas (IPEC-Fiocruz) and *Leishmania (Leishmania) amazonensis* (strain MHOM/BR/73/LTB0016) was obtained from Coleção de *Leishmania* do Instituto Oswaldo Cruz (CLIOC/IOC – Fiocruz). The conditions of promastigotes cultures are described elsewhere (Cysne-Finkelstein et al., 1998).

The epoxy-α-lapachone was obtained by addition of the oxyranes ring in the quinone, which was the 10th compound of a chemical synthesis series that was soluble in methanol at room temperature. The methanol was removed from compound samples by evaporation and the lyophilisate samples were solubilized in dimethyl sulfoxide (DMSO – Sigma-Aldrich, St. Louis, MO, USA); stock solution of 5 mM were used in the performed assays.

The compound direct effect over promastigotes *in vitro* was assessed as follows: parasites (5×10^5/mL) in Schneider's medium (containing 10% fetal calf serum) were incubated at 28 °C in 24-well plates for 4 or 48 h in the presence or absence of epoxy-α-lapachone (25, 50 or 75 μM). A control with 75 μM of DMSO was added as this compound was used as a diluent for the epoxy-α-lapachone. Samples were analyzed each day by counting in a Neubauer chamber. Promastigotes viability was determined by observing cell motility and staining with 0.1% trypan blue (Sigma-Aldrich) in a dye exclusion test.

Table 1
Cytotoxicity effects of epoxy-α-lapachone in mammalian macrophage cells.

Epoxy-α-lapachone concentration (μM)	Incubation time (% of viable macrophages)	
	24 h	48 h
25	100 ± 8.3	99 ± 6.2
50	100 ± 8.5	98 ± 8.1
75	96 ± 6.4	90 ± 8.8

The values represent the average and standard deviation (±) of three independent experiments, as measured by CBBR-250 absorbance. In these assays, the control (not treated – 100% viable) macrophages (10^5 cells) showed an optical density of 0.8 at 570 nm.

For the assays with human macrophages, peripheral blood mononuclear cells (PBMC) from healthy donors were separated by Ficoll-Hypaque (Sigma-Aldrich) gradient centrifugation and the monocyte-derived macrophages (MØ) were isolated by plastic adherence assays (Bourguignon et al., 2009). The use of human samples in the present study was approved by the Committee of Ethics in Human Research of UFF (process CEP CMM/HUAP – 162/06).

To further use in tests for assessing *in vitro* leishmanicidal activity of epoxy-α-lapachone on infected cells, the MØ were incubated with infective promastigotes (ratio of cell/parasites 1:10) for 3 h at 37 °C in 5% CO_2, following washing (three times) with RPMI medium (Sigma-Aldrich) to remove unbound parasites.

The effects of the compound over MØ and their infecting parasites were assessed as follows: infected or non-infected MØ were incubated on Lab-Tek Chamber Slides (Sigma-Aldrich), under the same conditions above, in the presence or absence of 50 μM epoxy-α-lapachone (or 50 DMSO) for different times (24, 48 or 72 h). At each time point, the macrophages were fixed with methanol, stained with May-Grünwald-Giemsa (Sigma-Aldrich) and observed in an optical microscope (Nikon Eclipse E200).

The rate of the infected human macrophages was determined by analyzing at least 100 randomly selected cells at 1000× magnification. Infection data are expressed as endocytic index, which represents the percentage of infected cells multiplied by the average number of parasites per cell.

Additionally, cytotoxic effects of both epoxy-α-lapachone and DMSO on the human MØ were assessed. The cells were incubated with the compounds, stained with 0.2% CBBR-250 and the cells viability after incubation was measured using the correlation between the cell number and the CBBR-250 absorbance (Ferreira et al., 2006; Jorqueira et al., 2006). In these experiments, the DMSO did not affect the cells, as 100% of the MØ remained viable in culture even after 48 h incubation with 75 μM DMSO. Similarly, the compound epoxy-α-lapachone, in both tested incubation time frames (24 and 48 h), showed no effects over MØ viability in the two lower concentrations (Table 1).

Our results show that co-incubation of epoxy-α-lapachone with promastigotes is able to significantly decrease the number of viable promastigotes in test cultures compared with control cultures. In addition, the compound effect on parasites was shown to be dose- and time-dependent (Fig. 1).

The tested species were both susceptible to the compound effects, but with some differences regarding their susceptibility rate. After a 24 h incubation with epoxy-α-lapachone, decreases of 44% (25 μM), 97.1% (50 μM) and 98.5% (75 μM) in the numbers of viable promastigotes of *L. (V.) braziliensis* was observed. For *L. (L.) amazonensis* parasites, similar conditions induced decreases of 61% (25 μM), 72% (50 μM) and 80% (75 μM). To both parasites species we have found an IC_{50} 37.0 ± 0.4 μM.

Please cite this article in press as: Franklin Souza-Silva, et al., Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone, Experimental Parasitology (2014), doi: 10.1016/j.exppara.2014.10.002

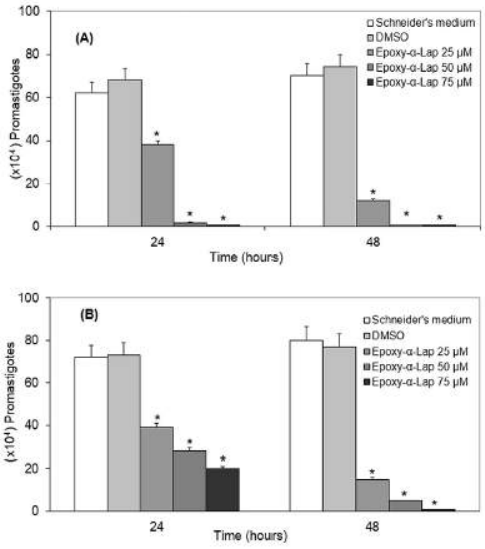


Fig. 1. Dose-response curve of epoxy-α-lapachone effects on the survival of promastigotes of *Leishmania* species in culture. Promastigotes from *L. (V.) braziliensis* (A) and *L. (L.) amazonensis* (B) were cultivated in Schneider's medium, during 24 or 48 h, in the presence (at the concentrations of 25, 50 or 75 μM) or absence of epoxy-α-lapachone. Cultures cultivated in the presence of DMSO (75 μM) were also used as controls. Data are expressed as the number of viable promastigotes (× 10^4) in the cultures. The graphics present mean and standard deviation of five independent experiments. *Indicates statistically significant difference from the controls (Student's T, $p < 0.05$).

A more extensive incubation period (48 h) potentialized the effects of the compound, especially at lower concentrations: *L. (V.) braziliensis* promastigotes numbers reduced by 83.8% (25 μM), 98.6% (50 μM) and 98.9% (75 μM), while *L. (L.) amazonensis* promastigotes numbers reduced by 80.5% (25 μM), 93.4% (50 μM) and 98.7% (75 μM).

In addition, we could observe that the compound was also able to kill intracellular parasites in a time-dependent fashion (Fig. 2). After 72 h of culture, almost no parasite of either tested species survived, as indicated by the comparison of endocytic index of samples coincubated or not with epoxy-α-lapachone: *L. (L.) braziliensis* (21.0 ± 2 and 491.1 ± 40, respectively) and *L. (L.) amazonensis* (6.0 ± 0.8 and 290.0 ± 30, respectively).

This result suggests that the compound is capable of crossing the plasma membrane of the macrophages and affect the amastigote, supporting its potential for acting as an antiparasitic agent in future treatments against leishmaniasis.

The precise mechanism of action of epoxy-α-lapachone over the parasites is not yet known, but previous reports in the literature (Bourguignon et al., 2009, 2010; Taddei, 1999) suggest it may be through inhibition of parasites proteinases activity. Preliminary zymographic assays by our group (data not shown) seem to confirm this hypothesis, but further studies are still required.

Taken together our data show a relevant effect of the compound epoxy-α-lapachone over the survival of promastigotes and amastigotes *in vitro*, including the potential to reach and affect parasites inhabiting host cells. Additionally, they suggest that this leishmanicidal activity affects a range of *Leishmania* species, as, during

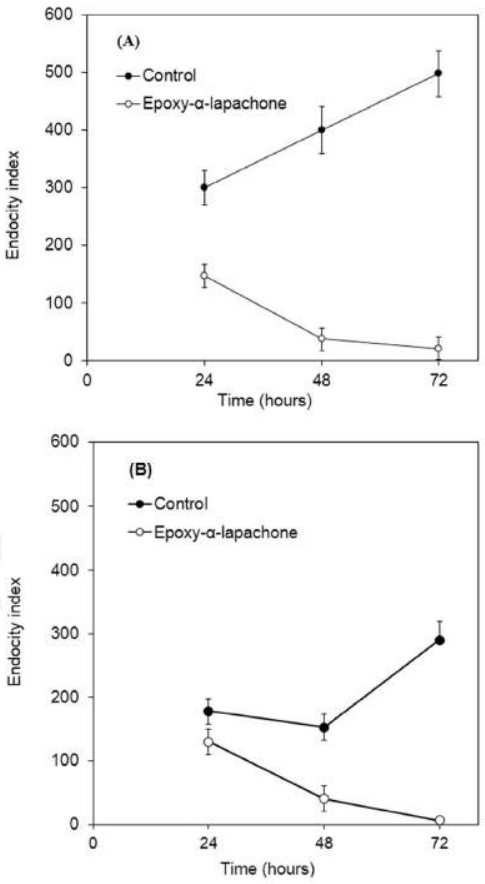


Fig. 2. Effects of the epoxy-α-lapachone on the endocytic index of the amastigotes in human macrophages. Epoxy-α-lapachone (50 μM: ○) or DMSO (50 μM: ●), used as control, were co-incubated in cultures of amastigotes-infected human macrophages, with either *L. (V.) braziliensis* (A) or *L. (L.) amazonensis* (B) parasites, for 24, 48 or 72 h. The results are expressed as the mean and standard deviation of three independent experiments, performed with macrophages from different healthy human donors. All time points analyzed presented statically significant differences from their respective controls (Student's *t*, $p < 0.05$).

our assays, two species belonging to two different subgenera were equally affected.

Thus, the evidences presented herein present for the first time the potential of using epoxy-α-lapachone as basis for the development of novel or complementary treatments against human leishmaniasis.

Acknowledgments

We thank PAPES/CNPq (403494/2008-7) and FAPERJ (E-26/111.482/2008; E-26/103.060/2008) for partial financial support of this research. Also, we thank the hemotherapy service of the

Please cite this article in press as: Franklin Souza-Silva, et al., Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone, Experimental Parasitology (2014), doi: 10.1016/j.exppara.2014.10.002

Hospital Clementino Fraga Filho (Federal University of Rio de Janeiro, Brazil), for providing buffy coats.

References

Berman, E., Wiernik, P., Vogler, R., Velez-Garcia, E., Bartolucci, A., Whaley, F.S., 1997. Long-term follow-up of three randomized trials comparing idarubicin and daunorubicin as induction therapies for patients with untreated acute myeloid leukemia. Cancer 80, 2181–2185.

Bourguignon, S.C., Castro, H.C., Santos, D.O., Alves, C.R., Ferreira, V.F., Gama, I.L., et al., 2009. *Trypanosoma cruzi*: in vitro activity of Epoxy-alpha-Lap, a derivative of alpha-lapachone, on trypomastigote and amastigote forms. Exp. Parasitol. 122, 91–96.

Bourguignon, S.C., Cavalcanti, D.F., de Souza, A.M., Castro, H.C., Rodrigues, C.R., Albuquerque, M.G., et al., 2010. *Trypanosoma cruzi*: insights into naphthoquinone effects on growth and proteinase activity. Exp. Parasitol. 127 (1), 160–166.

Cysne-Finkelstein, L., Temporal, R.M., Alves, F.A., Leon, L.L., 1998. *Leishmania amazonensis*: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. Exp. Parasitol. 9, 58–62.

Deps, P.D., Viana, M.C., Falqueto, A., Dietze, R., 2000. Comparative assessment of the efficacy and toxicity of N-methyl-glucamine and BP88 sodium stibogluconate in the treatment of localized cutaneous leishmaniasis. Rev. Soc. Bras. Med. Trop. 3, 535–543.

Ferreira, V.F., Jorqueira, A., Souza, A.M., da Silva, M.N., de Souza, M.C., Gouvêa, R.M., et al., 2006. Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorg. Med. Chem. 14, 5459–5466.

Hotez, P.J., Molyneux, D.H., Fenwick, A., Kumaresan, J., Sachs, S.E., Sachs, J.D., et al., 2007. Control of neglected tropical diseases. N. Engl. J. Med. 357, 1018–1127.

Jha, T.K., 2006. Drug unresponsiveness & combination therapy for kala-azar. Indian J. Med. Res. 123, 389–398.

Jorqueira, A., Gouvêa, R.M., Ferreira, V.F., Silva, M.N., Souza, M.C.B.V., Zuma, A.A., et al., 2006. Oxyrane derivative of α-lapachone is potent growth inhibitor of *Trypanosoma cruzi* epimastigote forms. Parasitol. Res. 99, 429–433.

Lezama-Dávila, C.M., Isaac-Márquez, A.P., Kapadia, G., Owens, K., Oghumu, S., Beverley, S., et al., 2012. Leishmanicidal activity of two naphthoquinones against Leishmania donovani. Biol. Pharm. Bull. 35 (10), 1761–1764.

Lima, N.M.F., Correia, C.S., Leon, L.L., Machado, G.M.C., Madeira, M.F., Santana, A.E.G., et al., 2004. Antileishmanial activity of lapachol analogues. Mem. Inst. Oswaldo Cruz 99, 757–761.

Saldanha, A.C., Romero, G.A., Merchan-Hamann, E., Magalhães, A.V., Macedo, V.O., 1999. A comparative study between sodium stibogluconate BP 88R and meglumine antimoniate in the treatment of cutaneous leishmaniasis. I. The efficacy and safety. Rev. Soc. Bras. Med. Trop. 32, 383–387.

Santos, D.O., Coutinho, C.E., Madeira, M.F., Bottino, C.G., Vieira, R.T., Nascimento, S.B., et al., 2008. Leishmaniasis treatment – a challenge that remains: a review. Parasitol. Res. 103, 1–10.

Sundar, S., Singh, A., Singh, O.P., 2014. Strategies to overcome antileishmanial drugs unresponsiveness. J. Trop. Med. 4, 646932.

Taddei, M., 1999. Synthesis of oligopeptides containing an oxirane ring in the place of a peptidic bond. Methods Mol. Med. 23, 189–207.

Please cite this article in press as: Franklin Souza-Silva, et al., Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone, Experimental Parasitology (2014), doi: 10.1016/j.exppara.2014.10.002

3.2. Documento 4:

Souza-Silva F, Saulo Bourguignon SC, Finkelstein LC, Oliveira Cortes LMC, Gonçalves de Oliveira LFG, Ferreira VF, Carneiro PFC, Pinho RT, Caffarena ER, Alves CR. Epoxy-α-lapachone acts as a serine proteinase inhibitor of *Leishmania (Leishmania) amazonensis.* (Submetido para publicação na revista ***Current Drug Targets***)

No primeiro grupo de ensaios *in vitro* deste trabalho aplicamos uma abordagem de citometria de fluxo com TMRE e TROP e provamos que o epoxi-α-lapachona causa um efeito interno drástico nos promastigotas e amastigotas já nas primeiras horas de contato, culminado com o comprometimento da membrana plasmática como um evento final após o efeito composto. Nos ensaios de tratamento experimental, as lesões de pata dos camundongos diminuíram após a inoculação do epoxi-α-lapachona. No segundo grupo de ensaios *in vitro* descobrimos que frações de serino proteinase de 68 kDa deste parasito, hidrolisam substratos para estas enzimas, as quais são inibidas pelo composto e por inibidores de serina proteinase. As análises in silico indicaram que o epóxi-α-lapachona pode ligar-se ao no sítio ativo da oligopeptidase B do parasito. Com o conjunto de dados desta publicação alcançamos os demais objetivos deste trabalho de tese quando descrevemos: (i) os efeitos *in vivo* e *in vivo* do epoxi-α-lapachona sobre o parasito e infecção em camundongos; (ii) a ação sobre serino proteinase de *L. (L.) amazonensis*, com os ensaios bioquímicos e de *docking* molecular.

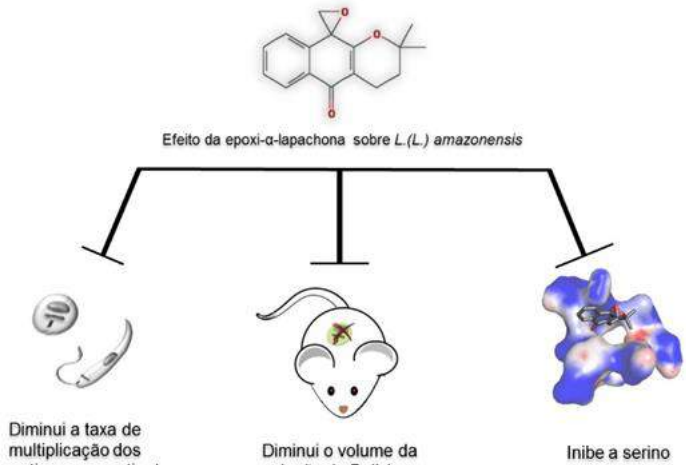


Figura 5: Resumo gráfico do DOCUMENTO 4

Title

Epoxy-α-lapachone presents *in vitro* and *in vivo* effects on *Leishmania (Leishmania) amazonensis* and inhibits serine proteinase activity in this parasite.

Running title

Epoxy-α-lapachone against *L. (L.) amazonensis*

Authors' names

Franklin Souza-Silva[a], Saulo Cabral Bourguignon[b], Bernardo Acácio Santini Pereira[a], Luzia Monteiro de Castro de Oliveira Cortes[a], Luiz Filipe Gonçalves de Oliveira[a], Andrea Henriques-Pons[c], Lea Cysne Finkelstein[d], Vitor Francisco Ferreira[e], Paula Fernandes Carneiro[ef], Rosa Teixeira de Pinho[g], Ernesto Raul Caffarena[h], Carlos Roberto Alves[a*]

Author's addres

[a]Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Biologia Molecular e Doenças Endêmicas - Av. Brasil, 4365 - Maguinhos, CEP 21040-360, Rio de Janeiro - RJ, Brasil.

[b]Universidade Federal Fluminense, Instituto de Biologia, Pós-Graduação em Ciências e Biotecnologia - Outeiro de São João Baptista s/n – Centro, CEP 24020-150, Niterói - RJ, Brasil.

[c]Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Inovações em Terapia, Ensino e Bioprodutos - Av. Brasil, 4365 - Maguinhos, CEP 21040-360, Rio de Janeiro - RJ, Brasil.

[d]Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório de Imunoparasitologia - Av. Brasil, 4365 - Maguinhos, CEP 21040-360, Rio de Janeiro - RJ, Brasil.

[e]Universidade Federal Fluminense, Instituto de Química, Departamento de Química Orgânica - Outeiro de São João Baptista s/n – Centro, CEP 24020-150, Niterói - RJ, Brasil.

[f]Universidade Federal do Rio de Janeiro, Núcleo de Pesquisas de Produtos Naturais - Cidade Universitária CEP 21944-970, Rio de Janeiro - RJ, Brasil

[g]Fundação Oswaldo Cruz, Instituto Oswaldo Cruz, Laboratório De Ultra-estrutura Celular- Av. Brasil, 4365 - Maguinhos, CEP 21040-360, Rio de Janeiro - RJ, Brasil.

[h]Fundação Oswaldo Cruz, Programa de Computação Científica (PROCC) - Av. Brasil, 4365 - Maguinhos, CEP 21040-360, Rio de Janeiro - RJ, Brasil.

*Corresponding author: calves@ioc.fiocruz.br

Abstract:

Leishmania (Leishmania) amazonensis is a protozoan parasite that causes infections with a wide spectrum of clinical manifestations and the currently available treatments present severe side effects. Herein, the potential of the epoxy-α-lapachone compound as new chemotherapeutic agent is evaluated. Using a flow cytometry approach, we were able to observe that this compound causes drastic effects on promastigotes and amastigotes forms, even parasites' death, after 3h of co-incubation. A disorganization of the parasites' plasma membrane was noted as a late event after exposure to epoxy-α-lapachone. This compound also presented effects in BALB/c mice infected with *L. (L) amazonensis* as its inoculation caused a reduction in infection-related lesions. Six weeks of treatment with 0.44 mM of epoxy-α-lapachone induced a decrease in the paw lesions of infected mice, untreated animals had 30.8 ±2.6 mm^2 lesions, epoxy-α-lapachone treated animals had 24.9 ±2.0 mm^2 lesions and control animals, treated with glucantime, had 28.3 ± 1.5 mm^2 lesions. Additionally, the effects of this compound over parasites' serine proteinase activity was assessed. A serine proteinase-enriched fraction, extracted from both amastigotes and promastigotes, able to specific serine proteinases' substrates was shown to be sensitive to both epoxy-α-lapachone and classical serine proteinase inhibitors (PMSF, aprotinin and antipain). In addition, *in silico* simulations suggested that epoxy-α-lapachone can bind to the oligopeptidase B of *L. (L) amazonensis*, in a similar manner to antipain, interacting with a binding site S1. These data indicate that proteinases like oligopeptidase B are potential targets of epoxy-α-lapachone and may contribute in controlling *L. (L) amazonensis* infection.

Keywords: epoxy-α-lapachone, serine proteinase, oligopeptidase B, *Leishmania* (*L.*) *amazonensis* and chemotherapy**.**

1. Introduction

An array of *Leishmania* species is able to infect humans, as well as other mammalian hosts, and cause diseases that are known under the common name of leishmaniasis. Leishmaniasis presents a high impact in tropical or subtropical regions of the world, affecting mostly populations in poor or emerging countries and is included among the 17 Neglected Tropical Diseases, as defined by the World Health Organization [1].

The parasites can affect cells in the skin, mucosa or cartilages, causing the cutaneous form of the disease (CL). Some species may colonize internal tissues and organs, as the liver, spleen or bone marrow, causing the visceral form of the disease (VL) [2]. Mucosal leishmaniasis (ML) is a metastatic outcome of a CL infection, resulting in the dissemination of parasites to the oropharynx mucosa.

In the Brazil, *Leishmania (Leishmania) amazonensis* is a species described to cause a wide spectrum of clinical manifestations [3], accounting for unusual clinical presentations [4]. A great genetic diversity among strains isolated from patients [5] was reported, as well as a trend to increase its geographical distribution.

The currently available treatment for these infections is restricted to two option groups: *(i)* the antimonials, which are the first choice drugs, [6, 7]; *(ii)* and, the second choice drugs, as Pentamidin and Amphotericin B [8]. Both groups of drugs present many limitations regarding their use, such as: *(i)* high cost, *(ii)* difficulty of administration, *(iii)* toxicity; and *(iv)* development resistance by parasite strains. Undoubtedly, these limitations represent obstacles for a successful therapy [9], underlining the need to develop new drugs to treatment of leishmaniasis.

Several natural compounds are reported to have antileishmanial effects but neither compounds became effective drug for leishmaniosis. In this context, some natural products, obtained from plant extracts, or their derivateshave been proposed for leishmaniasis chemotherapy, as quinones, alkaloids, terpenes and phenolic derivatives [10]. Recently, we reported evidences that quinones derivates, as the naphthoquinone, present promissing properties against protozoan parasites, as *Trypanosoma cruzi* [11-15] and, even, *Leishmania (Viannia) braziliensis* and *L. (L.) amazonensis* [16]. These compounds can be isolated from Bignoniaceae or Verbanaceae trees and their antimicrobial properties are well established [17].

Among the naphthoquinones derivates, the epoxy-α-lapachone (2,2-dimethyl-3,4-dihydrospiro[benzo[g]chromene-10,20-oxiran]-5(2H)-one) is a fine candidate for serving as basis to develop antileishmanial treatments, a once it presented low cytotoxic effect on mammalian cells [11, 12] while being effective against *L. (V.) braziliensis* and *L. (L.) amazonensis* [16]: it was able to kill promastigotes of both species *in vitro* and, also, affected amastigotes inhabiting human macrophages. Previously, we reported that epoxy-α-lapachone can inhibit serine- and cysteine-proteinase activities in *Trypanosoma cruzi* [18] and not yet assessed this possibility in *Leishmania* spp.

In addition, it is known that proteinases are pivotal virulence factors for *Leishmania* spp [19]. Specifically about serine proteinases, oligopeptidase B (OPB; Clan SC, family S9A oligopeptidase B) was reported to be related to murine macrophages invasion by the parasites and survival of those within the infected cells [20].

Therefore, in the present study, we aimed to assess the potential inhibitory effect of epoxy-α-lapachone over *L. (L.) amazonensis* serine proteinase activities, as it could be part of the antileishmanial mechanism of this compound, and applied a molecular modeling approach to investigate how this inhibitor binds to its target enzymes, as OPB. In parallel, we investigated the effects of epoxy-α-lapachone on the both parasite forms and in the outcome of a murine experimental infection with *L. (L.) amazonensis.*

2. Methods

2.1. Chemicals and culture reagents

Coomassie brilliant blue R-250 (coomassie blue), detergents [sodium dodecyl sulfate (SDS); t-octylphenoxypolyethoxyethanol (Triton X-100)], proteinase inhibitors [phenyl-methyl-sulphonyl-fluoride (PMSF); aprotinin, amastatin], HiTrap™ Benzamidine FF, tris(hidroximetil)aminometano (Tris), glycerol, dimethyl sulfoxide (DMSO), penicillin, streptomycin, Schneider's drosophila medium (Schneider's medium), fluorogenic peptide substrates [Z-Phe-Arg-7-amido-4-methylcoumarin (Z-FR-AMC), Ala-Phe-Lys-7-amido-4-methylcoumarin (AFK-AMC), Z-Gly-Gly-Arg-4-methoxy-β-naphthylamide (Z-GGR-MβNA)] were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Pre-Stained™ Plus Protein Ladder was purchased from Fermentas Life Sciences (USA). Amicon Centriprep YM-10 filter devices were purchased from Millipore (Billerica Inc, MA, USA). Fetal calf serum (FCS) was purchased from Cultilab S/A (Brasil). Brain-heart infusion (BHI) was purchased from Oxoid Australia (West Heidelberg, Vic, Australia). Micro BCA protein

assay kit was purchase from Pierce Chemical Company (Appleton, WI, U.S.A). *TO-PRO*®-V *Iodide (*TO-PRO) and tetramethylrhodamine ethylester perchlorate (TMRE) were purchased from Molecular probes (Eugene, OR, USA). *Meglumine antimoniate* - Glucantime® (Glucantime) was purchased from Sanofi - Aventis Farmacêutica Ltda. (Suzano, SP, Brasil). Epoxy-α-lapachone compound was synthesized by Departamento de Química Orgânica from Instituto de Química of Universidade Federal Fluminense (UFF).

2.2. Parasites culture

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

2.3. Axenic amastigotes transformation

Axenic amastigotes were obtained as previously described [21]. Briefly, promastigotes of both parasites species in logarithmic growth phase ($5x10^5$ cells/mL) were seeded into axenic medium (Schneider´s medium pH 7.2 containing 10mM Hepes buffer, 1mM L-glutamine, 60 IU/mL penicillin and 60µg/mL streptomycin) and incubated for 24h (26°C). The promastigotes were then reseeded into a new axenic medium with pH adjusted to 5.5 and cultivated under the same conditions. Following 4 days of incubation, the parasites was reseeded to a new axenic medium pH 5.5 and incubated at a higher temperature (32°C). To assess the successfulness of the differentiation, the morphology of the cells in the culture was analyzed by optical microscopy.

2.4. Effects of epoxy-α-lapachone on the promastigotes and amastigotes

The parasites were seeded (density of $1.0x10^6$/mL) into Schneider´s medium and incubated under assorted conditions (1 h and 3 h at 28°C or 32°C) in the presence or absence of epoxy-α-lapachone (40 µM) or DMSO, used as a diluent for the compound and, consequently, as control. Parasites viability was then assessed by flow cytometry, using specific fluorescent markers. TO-PRO, a membrane-impermeable DNA marker, was used (10µM) to assess parasites' membrane permeabilization. TMRE probe was used (50nM) to verify variations in the mitochondrial membrane potential. Parasites ($1.0x10^6$cells/well) were incubated for 20 minutes with the markers and the samples were immediately analyzed using a

FACScalibur (Becton & Dickinson, San Jose, CA, USA) flow cytometer. Data analysis was carried out using CELLQUEST software.

2.5. Experimental murine infection and treatment of animals with epoxy-α-lapachone

Experimental infections were conducted with of BALB/c, 6-8 weeks old (weighting aprox. 22 grams). The animals were obtained from Centro de Criação de Animais da Fiocruz (CECAL/Fiocruz) and all experimental procedures were performed as approved by the Committee for the Ethical Use of Animals of IOC/Fiocruz (CEUA- Lw – 48/11). The mice were inoculated in the footpad of the left hind limb with $1.0x10^6$ promastigotes of *L. (L.) amazonensis* in 10 mM phosphate buffer saline (PBS). The parasites were in stationary growth phase, after five days 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 assorted concentrations (0.44, 0.09 and 0.02 mM). The drugs (0.5ml/animal) were administered subcutaneously in the dorsal region of each mouse. Treatments were carried out for a week with daily injections, starting 30 days after challenge infection, when the paw lesions had already become noticeable. Two negative control groups were included, where sterile PBS or DMSO administered during treatment. The lesions were accompanied in weekly basis by measuring lesions area (mm^2) with a caliper.

2.6. Parasite's proteins extract

Protein extract was obtained as previously described by Gálan et al. 1992 [22]. Briefly, parasites(2.0×10^9), either promastigotes in logarithm growth phase or amastigotes, were washed three times by centrifugation (3 000 ×*g*, 10 min, 4 ºC) in PBS pH 7.2, and then submitted to 4 cycles of vortexing for 30 min in the presence of lysis buffer (100mM Tris-HCl pH 8.0, 150 mM NaCl, 10% glycerol, 0.6% Triton X-100). The soluble proteins fraction was obtained by centrifugation the samples (25 000 ×*g*, 30 min, 4 ºC) and afterwards stored at -20ºC until further user. The protein concentration in extract samples were determined using the Micro BCA protein assay kit.

2.7.Parasite's serine proteinase-enriched fraction

Soluble proteins fraction samples of promastigote or amastigote (adjusted to 35 or 40 mg/mL in 10 mM Tris-HCl pH 7.50, respectively) were binding in a HiTrap™ Benzamidine FF column previously equilibrated with binding buffer (0.05M Tris-HCl, 0.5M NaCl, pH 7.4). Subsequently, the column was washed with the same buffer, to flush out unbound proteins,

and the bound proteins were later retrieved using elution buffer (0.05M glycine, pH 3.0) and preserved in 1 M Tris-HCl, pH 9.0. The eluted proteins, forth on named as the serine proteinase-enriched fraction, was concentrated in 30 min and dialyzed against a buffer (10mM Tris-HCL pH 7.5) to further use in proteinase assays.

2.8. Zymographic assays

Serine proteinase-enriched fraction (0.5µg of proteins total) were submitted to electrophoresis under reductive conditions using 10% acrylamide gels copolymerized with 0.1% gelatin. Following electrophoresis, the gels were washed (1h, 4ºC) in 0.1 M Tris-HCL pH 7.5 (washing buffer) containing 2.5% Triton X-100 in and, then, incubated (6h, 37ºC) in washing buffer, without supplements. Then, the gel was revealed with coomassie blue R-250.

2.9.Assessment of proteinase activities and inhibitors efficacy

Proteinase activities in solution for the serine proteinase-enriched fraction (0.5 µg) of total proteins and trypsin , used as a positive activity control, were characterized, in activation buffer (10 mM Tris-HCL pH 7.5) at final volume of 60 µL, using specific fluorescent peptide substrates for serine proteinase (Z-FR-AMC, AFK-AMC and Z-GGR-MβNA; 0.1 mM). Samples and were incubated (37°C, 60 min) and the variance in the relative fluorescence units (RFU), corresponding to enzymatic cleavage of the substrates, was followed in a (Molecular Devices SpectraMax Gemini XPS)

Concomitantly, the efficacy of various serine proteinases inhibitors, under these same described conditions, was assessed. The inhibition assays were performed with 1 mM PMSF, 0.3 µM aprotinin, 5 µM antipain (all used as control) and 1mM of epoxy-α-lapachone.

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

2.10. Determination of IC50 for serine proteinase inhibitors

The half maximal inhibitory concentration (IC50) values for all the tested inhibitorts were obtained accordingly to [23]. Briefly, the tests were performed combining a fixed substrate concentration (0.1mM Z-FR-AMC) with ten distinct concentrations (from 1.5×10^{-4}mM to 5mM) of each inhibitor, using the same methodology described above. These results were applied to linear interpolation of the concentrations (conc) for each inhibitors versus the corresponding percentage of enzymatic inhibition (inh) and analyzed through the equation:

$$IC50 = \frac{50\% - lower\ inh\ \%}{higher\ inh\ \% - lower\ inh\ \%} \times (higher\ conc - lower\ conc) + lower\ conc$$

For the linear interpolation analysis, the mean values for the triplicates performed in each assay were used.

2.11. Statistical analysis

To compare results, Student's test was applied and data matrices were considered statistically distinct when p-value was lower than 0.05. Statistical analysis was performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA).

2.12. Molecular Docking of oligopeptidase B

In order to investigate the binding mode of epoxy-α-lapachone, was performed a docking of this compound into oligopeptidase B of *L. (L.) amazonensis* (OPBa) using o program DOCKTHOR [24]. Firstly, the 3D structures of ligand molecules were built in and minimized in program Avogrado 1.1. The crystal structure the oligopeptidase B *L. (L.) major* (OPBm) complexed with antipain was obtained from Protein Data Bank (PDB code 2XE4) and the model OPBa was construct with the program MODELLER 9.14 [25], used within template the OPBm. The model construct with the lowest value of the discrete optimized protein energy (DOPE) was selected and evaluated in PROCHECK [26], ERRAT [27] and PROSA [28]. The molecular docking has established in cubic grid box of 8 8 8 $Å^3$ and the parameters are referred to as defaults in page DOCKTHOR. Structures up to 2 Å in positional root-mean-square deviation (RMSD) were clustered together and the results of the most favorable free energy of binding were selected as the resultant complex structures. We also performed a redocking of the antipain with success rate (RMSD ≤ 2.0 Å for the interface backbone atoms) 53% to the crystal structure of the OPB of *L. (L.) major*.

3. Results

3.1. Epoxy-α-lapachone has leishmanicidal activity over promastigotes and amastigotes of *Leishmania (L.) amazonensis.*

Flow cytometry approach applied here was useful as to demonstrate that epoxy-α-lapachone compound can affects both promastigotes and amastigotes, first causing internal changes, probably related to metabolism, then leading the parasite killing. Results indicate that the drug is capable of crossing the plasma membrane within already the first hours of incubation (Figure 1), followed by loss of integrity of plasma membrane. Our data suggest that impairment of the plasma membrane of these parasites is a late event after epoxy-α-lapachone effect, evidenced by an increase of TO-PRO V intensity of fluorescence.

According to Figure 1, 87.9 % of the promastigotas (A) have a normal of ion pump activity with a peak of 96.9 mean intensity staining (MFI). After an hour of incubation with 0.175 μM of epoxy-α-lapachone there was an abrupt decrease in the metabolic activity to 30.1%, confirmed by reduction of TMRE staining (MFI of 49.5). The exposure of 3 h leads to 10.9 %, with MFI of 40.6. The loss of membrane integrity was confirmed by increase of promastigotes number staining by TO-PRO V: control, 1 and 3 hours of exposure (4.0, 39.9 and 78.9%), respectively. Amastigotes forms showed a reduction of ion pump activity of 86.2% (MFI of 35.0) to 42.9% after 1 hour of incubation with the compound, exhibited no difference at 3 hours of exposure.

3.2. Mice lesions caused by *Leishmania (L.) amazonensis* infection decrease after treatment with epoxy-α-lapachone

The role of epoxy-α-lapachone on the lesion progression during the course of experimental infection was analyzed in BALB/c mice injected with different concentrations of this compound one week after the infection challenge. The results indicate that the treated animals presented a reduction in paw lesions area when compared to animals from control group (Figure B). Although no dose response correlation could be observed during the performed assays, the effect of the compound on lesion reduction was statically significant for all the assayed concentrations ($p = 0.04$). Comparative control assays were also performed with glucantime, a drug in clinical use against leishmaniasis, and, similarly to epoxy-α-lapachone, no dose response correlation was observed (Figure A). The only concentration of glucantine were a statiscally significant difference to negative control was observed was 0.09 mM ($p = 0.04$).

3.3. Serine proteinase activies in *Leishmania (L.) amazonensis* are inhibited by epoxy-α-lapachone

The potential of epoxy-α-lapachone to act as an inhibitor of serine proteinases of *L. (L.) amazonensis* was assessed in this study. To this end, assays were performed with serine proteinase-enriched fractions, obtained by affinity chromatography, which were analyzed by 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 amsatigotes. SDS-PAGE analysis revealed a major proteinase band with an estimated molecular mass of 68 kDa, stained by both silver (Figure 3) and Coomassie blue (data not shown).

In subsequent step, serine proteinase activity in fractions from both evolutive forms was detected in assays of enzymatic activity in solution. The protein fractions from promastigotes and amastigotes alike were able to hydrolyze Z-FR-AMC, AFK-AMC and Z-GGR-MβNA, but at different velocity rates, with amastigotes fraction presenting higher velocity rate for substrates hydrolysis than that of promastigotes: 16 ± 0.03 (Z-FR-AMC), 20 ± 0.7 (AFK-AMC) and 18, ± 0.4 × 10^{-3} (Z-GGR-MβNA) mmol .min^{-1} mg .$protein^{-1}$, for amastigotes fraction and 8 ± 0.8, 3 ± 0.6 and 3 ± 0.3 ×10^{-3} mmol .min^{-1} mg .$protein^{-1}$, respectively, for promastigotes fraction. These fractions presented distinct profiles regarding their inhibition by classical serine proteinase inhibitors (Figure 3).

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

Additionally, the IC50 values (inhibitor concentration that halts half of total enzymatic activity) for all the tested inhibitors were determined in each protein fraction (and trypsin,

used as positive control) using the Z-FR-AMC substrate. As showed in Table 1, the IC50 of epoxy-α-lapachone, although higher than of antipain for both protein fractions, was lower than or much close to the IC50 of PMSF and aprotinin, thus, suggesting it effectively impairs serine proteinase activities.

3.4. In silico simulations of epoxy-α-lapachone

Due the absence of OPB crystallized data of *L. (L.) amazonensis* in data banks was necessary to build a three-dimensional model of this enzyme to proceed to tests of molecular docking. The OPBa showed high degree of identity with OPBm (ID = 90%). The model the OPBa with lower value DOPE showed a RMSD of 0.19 Å when aligned with OPBm. The stereo-chemical evaluation presented 90.2% and 85.5%, of residues with most favored regions in graphical the Ramachandra, and G-factor value -0.25 and -0.19 to OPBm and OPBa, respectively. In addition, the analyzing of non-bonded interactions showed an ERRAT score 93.5% and 87.0% and Z-score -11.56 and -11.26 to OPBm and OPBa, respectively.

The initial analysis of the re-docking results of antipain into OPBa showed a RMSD of 2.0 Å when aligned with its original co-crystallized conformation, pointing the methodology as theoretically reliable to show ligands bound conformations (Figure 4A). In order to theoretically analyze the binding mode of epoxy-α-lapachone, we docked these molecules into OPBa and compare with antipain. The comparison of the epoxy-α-lapachone docking with the antipain revealed several differences of the binding with the residue acid amine (data not show). The OPBa epoxy-α-lapachone complex conserved the main binding in S1 by hydrogen bonds as well as hydrophobic interactions that are observed in the antipain (Figure 4B). The data showed that main hydrogen bond occurred in the residues Ser577, Ala578 and Try496, and hydrophobic interactions in the residues Phe698, Arg576, Ile501 and Leu617 (Figure 4C). The interaction energy value of epoxy-a-lapachone (-22.08 kcal/mol) was comparable to the energy value of antipain re-docking (-26.95 kcal/mol).

4. Discussion

The American Tegumentary Leishmaniasis spreads across the American continent and remains without an efficient treatment [29]. The current treatment, based on pentavalent antimony, is associated to severe side effects, as pain, gastrointestinal disorders, headache, anorexia, and to cardiac, hepatic and pancreatic toxicity [30]. For this reason, the development of new chemotherapeutic agents is required, including the potential use of plant-derived compounds to this end, as naphthoquinones [11, 12]. In this context, we aimed

to assess a potential leishmancidal activity of epoxy-α-lapachone during treatment of experimental murine infection and to identify the targets in the parasite affected by this compound. This compound was selected for further analysis, as its previously reported low cytotoxicity on mammalian cells [11, 16] highlighted its usefulness to serve as basis in the development of novel anti-leishmania drugs.

Throughout our assays, we collected evidences that epoxy-α-lapachone is in fact a potent leishmanicidal agent: it readily affected, promastigotes and axenic amastigotes of *L. (L) amazonensis in vitro*, after a short co-incubation period and, additionally, induced a decrease in infection-related paw lesions in experimentally infected mice.

Flow cytometry results indicated that epoxy-α-lapachone is able to freely enter into both parasite forms and, eventually, lead to a loss of integrity of their plasma membrane, as it was observed that parasites exposed to this compound had their DNA stained by TO-PRO, a marker unable to cross the intact plasma membrane [31]. Also, TMRE staining showed that the compound also induced alterations in the membrane potential of parasites' mitochondria, pointing to yet another physiological effect of the epoxy-α-lapachone on the parasites.

This fact was evidenced by fluorescent labeling of membrane potential ($\Delta\psi_m$), mainly in mitochondria, using TMRE labeling [32]. Classical experiment of the organelle three-dimensional reconstruction shows that intact and functional mitochondria have its physical continuity determined by fluorescence from targeted TMRE [33]. Here is proposed that epoxy-α-lapachone is able to act in any metabolic via, compromising $\Delta\psi_m$ of intracellular organelles, mainly of mitochondrial, revealed as reduce of TMRE staining. It is very well described that the collapse of the mitochondrial transmembrane potential is related with the opening of the mitochondrial permeability pores, leading to the release of cytochrome c into the cytosol and then driving to other events in the apoptotic cascade [34].

Data of this study confirmed that epoxy-α-lapachone inhibit the 68 kDa proteinase from *L. (L.)* amazonensis, which was characterized as an serine proteinase as: it was isolated by benzamidine-based affinity chromatography and its hydrolytic activity is inhibited in the presence of classical serine proteinase inhibitors of this enzyme while remaining unaffected by other proteinases inhibitors (data not show). Additionally, the data showed here suggest that, similar to the trypsin, the folding of serine protease from *L. (L) amazonensis* is resistant to mild denaturing conditions, indicating that isolated enzyme keeps structurally stable in the enzyme activity assays.

Previously, other serine proteinases have been identified in *L. (L.) amazonensis*; 115 kDa [35], 68 kDa [36, 37] and 56 kDa [38] and may also be affected by the compound. Additionally, a serine proteinase, named OPB, has been described in other *Leishmania* species and was described to play roles involved in many essential events of the parasite in the mammalian host [20, 39]. These data indicate that many other potential serine proteinase targets that can be affected by the epoxy-α-lapachone are present in the parasite and underline the importance these molecules present to parasite's survival.

A significant fact described here is that the affinity chromatography approaches applied was able to show that in *L. (L) amazonensis,* the amastigotes contains more serine proteinase than promastigotes. Both parasites forms hydrolyze a selectivity profile of substrate relate with fibrinolytic serine proteases Z-FR-AMC (kallikrein) [40], AFK-AMC (plasmin, urokinase and thrombin) [41] and Z-GGR-AMC (urokinase) [42]. This enzymes cleave preferably Arg and Lys residue in P1 position and [Gly and Ser (urokinase) and Pro, Ala, Gly and Leu (thrombin)] in P2 position [43]. Also, this enzyme activity is more intense in parasite stage relate with infection of mammalian cells, what reinforced the hypothesis that serine proteinases are essential for *Leishmania* spp survival, are feasible targets for developing new inhibitors as proposed here to the epoxy-α-lapachone and recently suggested as one of targets in combined treatment to the effective antileishmanial therapy [44].

Generally, desired IC_{50} values for potential inhibitors is in the nanomolar or low micromolar ranges. The epoxy-α-lapachone is inside of this range, and it is important demonstrates its ability of molecular docking to find that this compound is able to biding into the active center of a serine proteinase with inhibitory capabilities. Therefore, the mechanism of action of epoxy-α-lapachone on a serine proteinase of *Leishmania* sp accessed here by molecular docking.

Due the absence of a crystal structure of serine proteinase these enzymes in Leishmania restrains the molecular docking studies since target-based virtual screening methods depend on the availability of the target structural information [45]. We experimentally and theoretically tested the ability of the epoxy-α-lapachone on inhibiting this enzyme.

Our theoretical evaluation of the docking complexes of epoxy-α-lapachone with OPB the *L. (L.) amazonensis* and their comparison with a classical inhibitor (Antipanin), revealed that epoxy-a-lapachone presented hydrophobic binding with residue Leu617 in S3 pocket as also, hydrogen bonds with Ala578, Ser577 and Try496 in S1 pocket of OPB *L.(L.) major*, with a

distance of 2.7-3.9 Å. Thus, these interactions may contribute to the stabilization and maintenance of epoxy-a-lapachone into active site. In addition, epoxy-a-lapachone presents an electrophilic moiety susceptible to a nucleophilic attack by the activated catalytic serine in a distance that suggested this reaction.

In summary, our study present additional evidence that epoxy-a-lapachone can affect the *L. (L.) amanzonensis* parasites in mice during experimental infection and that this compound can act as a serine proteinase inhibitor, therefore making it a promising candidate to serve as basis for the development of novel drugs to control infections by this parasite.

Conflict of interest

The authors confirm that this article content has no conflict of interest

Acknowledgments

Franklin S Silva doctoral fellow of the Fiocruz/Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) institution. Carlos R. Alves and Ernesto R. Caffarena are fellow researchers at the same institution. This work was supported by Brazilian research agencies: Programa Estratégico de Apoio à Pesquisa em Saúde (PAPES) CNPq/Fiocruz, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ). All authors approved the final version of this manuscript.

5. References

[1] W.H.O. - http://www.who.int/neglected_diseases/diseases/en/

[2] Ready PD. Epidemiology of visceral leishmaniasis. Clinical Epidemiology. 2014; 3(6):147-54

[3] Barral A, Pedral-Sampaio D, Grimaldi Junior G, Momen H, McMahon-Pratt D, Ribeiro de Jesus A, Almeida R, Badaro R., Barral-Netto M, Carvalho EM, Johnson JrWD Leishmaniasis in Bahia, Brazil: evidence that Leishmania amazonensis produces a wide spectrum of clinical disease. American Journal of Tropical Medicine and Hygiene 1991; 44: 536–546.

[4] Azeredo-Coutinho RGB, Conceição-Silva F, Schubach A, Cupolillo E, Quintella RP, Madeira MF, Pacheco RS, Valete-Rosalino CM, Mendoncça SCF. First report of diffuse cutaneous leishmaniasis and *Leishmania amazonensis* infection in Rio de Janeiro State, Brazil. Transactions of the Royal Society of Tropical Medicine and Hygiene 2007; 101: 735–737.

[5] De Oliveira JP, Fernandes F, Cruz AK, Trombela V, Monteiro E, Camargo AA, Barral A, de Oliveira CI. Genetic diversity of *Leishmania amazonensis* strains isolated in northeastern Brazil as revealed by DNA sequencing, PCR-based analyses and molecular karyotyping. *Kinetoplastid Biology* and Disease 2007; 6:5.

[6] Berman E, Wiernik P, Vogler R, Velez-Garcia E, Bartolucci A, Whaley FS. Long-term follow-up of three randomized trials comparing idarubicin and daunorubicin as induction therapies for patients with untreated acute myeloid leukemia. *Câncer* 1997; 80: 2181-2185.

[7] Deps PD, Viana MC, Falqueto A, Dietze R. Comparative assessment of the efficacy and toxicity of N-methyl-glucamine and BP88 sodium stibogluconate in the treatment of localized cutaneous leishmaniasis. *Revista da Sociedade Brasileira de Medicina Tropical* 2000; 3: 535-543.

[8] Saldanha AC, Romero GA, Merchan-Hamann E, Magalhães AV, Macedo VO. A comparative study between sodium stibogluconate BP 88R and meglumine antimoniate in the treatment of cutaneous leishmaniasis. I. The efficacy and safety. *Revista da Sociedade Brasileira de Medicina Tropical* 1999; 32: 383-387.

[9] Ameen, M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. Clinical and Experimental Dermatology 2010; 35: 699-705.

[10] Mishra J, Saxena A, Singh S. Chemotherapy of leishmaniasis: past, present and future. Current Medicinal Chemistry 2007;14(10):1153-69.

[11] Ferreira VF, Jorqueira A, Souza AM, da Silva MN, de Souza MC, Gouvêa RM, Rodrigues CR, Pinto AV, Castro HC, Santos DO, Araújo HP, Bourguignon SC. Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorganic & medicinal chemistry 2006; 14: 5459-5466.

[12] Jorqueira A, Gouvêa RM, Ferreira VF, Silva MN, Souza MCBV, Zuma AA, Cavalcanti DFB, Araújo HP, Santos DO, Bourguignon SC. Oxyrane derivative of α-lapachone is potent growth inhibitor of *Trypanosoma cruzi* epimastigote forms. Parasitology Research 2006; 99: 429-433.

[13] Neves-Pinto C, Malta VR, Pinto Mdo C, Santos RH, de Castro SL, Pinto AV. A trypanocidal phenazine derived from beta-lapachone. Journal of Medicinal Chemistry. 2002, 45: 2112-2115.

[14] De Moura KCG, Emery FS, Neves-Pinto C, Pinto MCFR, Dantas AP, Salomão K, de Castro SL, Pinto AV. Trypanocidal activity of isolated naphthoquinones from Tabebuia and some heterocyclic derivatives: a review from an interdisciplinary study. Journal of Brazilian Chemical Society 2001; 12: 325-338.

[15] Menna-Barreto RF, Henriques-Pons A, Pinto AV, Morgado-Diaz JA, Soares MJ, De Castro SL. Effect of a beta-lapachone derived naphthoimidazole on Trypanosoma cruzi: identification of target organelles. Journal of Antimicrobial Chemotherapy 2005; 56: 1034–1041.

[16] Souza-Silva F, do Nascimento SB, Bourguignon SC, Pereira BA, Carneiro PF, da Silva WS, de Pinho RT, Alves CR. Evidences for leishmanicidal activity of the naphthoquinone derivative epoxy-α-lapachone. Experimental Parasitology 2014; S0014-4894, (14)00225-2.

[17] Pinto AV, de Castro SL. The trypanocidal activity of naphthoquinones: a review. Molecules. 2009; 14(11):4570-4590.

[18] Bourguignon SC, Cavalcanti DF, de Souza AM, Castro HC, Rodrigues CR, Albuquerque MG, Santos DO, da Silva GG, da Silva FC, Ferreira VF, de Pinho RT, Alves CR. Trypanosoma cruzi: insights into naphthoquinone effects on growth and proteinase activity. Experimental *Parasitology; 2010,* 127(1):160-6.

[19] Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, Alves CR. Proteinases as virulence factors in Leishmania spp. infection in mammals. *Parasites* & *Vectors 2012;* 5:160.

[20] Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, McKerrow JH. The oligopeptidase B of *Leishmania* regulates parasite enolase and immune evasion. The Journal of Biological Chemistry 2011; 7, 286(1): 429-40.

[21] Alves CR, Corte-Real S, Bourguignon SC, Chaves CS, Saraiva EM. Leishmania amazonensis: early proteinase activities during promastigote-amastigote differentiation in vitro. Experimental Parasitology 2005; 109(1): 38-48.

[22] Galán JE, Pace J, Hayman MJ. Involvement of the epidermal growth factor receptor in the mammalian cells by Salmonella typhimurium. Nature (London) 1992; 357, 588-589.

[23] Pais P. Potency of a novel saw palmetto ethanol extract, SPET-085, for inhibition of 5alpha-reductase II. Advances in Therapy 2010; 27(8):555-63.

[24] Magalhães CS, Barbosa HJC, Dardenne LE. A Genetic Algorithm for the Ligand-Protein Docking Problem. Genetics and Molecular Biology 2004; 4, 605-610.

[25] Eswar N, Marti-Renom MA, Webb B, Madhusudhan MS, Eramian D, Shen M, Pieper U, Sali A. Comparative Protein Structure Modeling With MODELLER. Current Protocols in Bioinformatics 2006 15, 561-5630.

[26] Laskowski RA, MacArthur MW, Moss DS, Thornton JM. PROCHECK: A program to check the stereochemical quality of protein structures. Journal of Applied Crystallography 1993; 26, 283–291.

[27] Colovos C, Yeates TO. Verification of protein structures: Patterns of non-bonded atomic interactions. Protein Science 1993; 2, 1511-1519.

[28] Wiederstein M, Sippl M. ProSA-web: Interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Research 2007; 35, W407–W410.

[29] Reveiz L, Maia-Elkhoury AN, Nicholls RS, Romero GA, Yadon ZE. Interventions for American cutaneous and mucocutaneous leishmaniasis: a systematic review update. PLoS One 2013; 29, 8(4):e61843.

[30] Pereira BA, Souza-Silva F, Silva-Almeida M, Santos-de-Souza R, Gonçalves de Oliveira LF, Ribeiro-Guimarães ML, Alves CR. Proteinase Inhibitors: a Promising Drug Class for Treating Leishmaniasis. Current Drug Targets 2014.

[31] Milanovich N, Suh M, Jankowiak R, Small GJ, Hayes JM. Binding of TO-PRO-3 and TOTO-3 to DNA: Fluorescence and Hole-Burning Studies. The Journal of Physical Chemistry 1996; 100, 9181-9186

[32] Bernas T, Dobrucki JW. The role of plasma membrane in bioreduction of two tetrazolium salts, MTT and CTC. Archives of Biochemistry and Biophysics 2000; 380: 108–116.

[33] Collins TJ, Bootman MD. Mitochondria are morphologically heterogeneous within cells. Journal Experimental Biology. 2003; 206,1993-2000.

[34] Olszewska A, Szewczyk A. Mitochondria as a pharmacological target: magnum overview. IUBMB Life 2013; 65(3):273-81.

[35] Silva-López RE, Coelho MGP, Giovanni De Simone S. Characterization of an extracellular serine protease of *Leishmania (Leishmania) amazonensis*. Parasitol 2005; 131:85–96.

[36] Morgado-Díaz JA, Silva-Lopez RE, Alves CR, Soares MJ, Corte-Real S, De Simone SG. Subcellular localization of an intracellular serine protease of 68 kDa in Leishmania (Leishmania) amazonensis promastigotes. Memórias Instituto Oswaldo. Cruz. 2005;100(4):377-83.

[37] Silva-López RE, Giovanni De Simone S. A serine protease from a detergent soluble extract of *Leishmania (Leishmania) amazonensis*. Z Naturforsch 2004a; 59c:590–598

[38] Silva-López RE, Giovanni De Simone S. *Leishmania (Leishmania) amazonensis*: purification and characterization of a promastigote serine protease. Experimental Parasitology 2004b; 107:173–182.

[39] Munday JC, McLuskey K, Brown E, Coombs GH, Mottram JC. Oligopeptidase B deficient mutants of Leishmania major. Mol Biochem Parasitol. 2011; 175(1):49-57.

[40] Morita T, Kato H, Iwanaga S, Takada K, Kimura T. New fluorogenic substrates for alpha-thrombin, factor Xa, kallikreins, and urokinase. The Journal of Biochemistry 1977; 82(5):1495-8.

[41] Kato H, Adachi N, Ohno Y, Iwanaga S, Takada K, Sakakibara S. New fluorogenic peptide substrates for plasmin. The Journal of Biochemistry 1980; 88(1):183-90.

[42] Huseby RM, Clavin SA, Smith RE, Hull RN, Smithwick EL Jr. Studies on tissue culture plasminogen activator. II. The detection and assay of urokinase and plasminogen activator from LLC-PK cultures (porcine) by the synthetic substrate Nalpha-benzyloxycarbonyl-glycyl-glycyl-arginyl-4-methoxy-2-napthylamide. Thromb Res 1977; 10(5):679-87.

[43] Rawlings ND, Waller M, Barrett AJ, Bateman A. MEROPS: the database ofproteolytic enzymes, their substrates and inhibitors. Nucleic Acids Research 2014; 42(Database issue):D503-9.

[44] Alves CR, Pereira BA, Silva-Almeida M, da Silva FS. Why strategies to control Leishmania spp. multiplication based on the use of proteinase inhibitors should consider multiple targets and not only a single enzyme. Journal of Molecular Modeling 2014; 20(10):2465.

[45] Klebe, G. Virtual ligand screening: strategies, perspectives and limitations. Drug Discovery Today 2006; 11, 580–594.

Table 1: The IC50 values in inhibition of serine proteinase activity

	Epox-α-lapachone (mM)	Antipain (mM)	PMSF (mM)	Aprotinin (mM)
Trypsin	0.9 ± 0.05	0.004 ± 1.7	1.4 ± 0.2	$0.33 \pm 28 \times 10^{+1}$
Ser-pro	0.9 ± 0.1	0.0041 ± 0.2	2.8 ± 0.8	$0.9 \pm 4 \times 10^{-5}$
Ser-ama	1.2 ± 0.06	0.002 ± 0.5	9.5 ± 2.4	$4.8 \pm 8 \times 10^{-2}$

Serine proteinase fractions of promastigotes (Ser-pro) and amastigotes (Ser-ama) of *L. (L.) amazonensis*. Enzymatic assays were performed with 0.1mM Z-FR substrate in 10mM Tris-HCl pH 7.5, and at least five concentration of epox-α-lapachone, Antipain, PMSF and Aprotinin. The data are expressed as mean and standard deviation of the experiments.

FIGURE 1

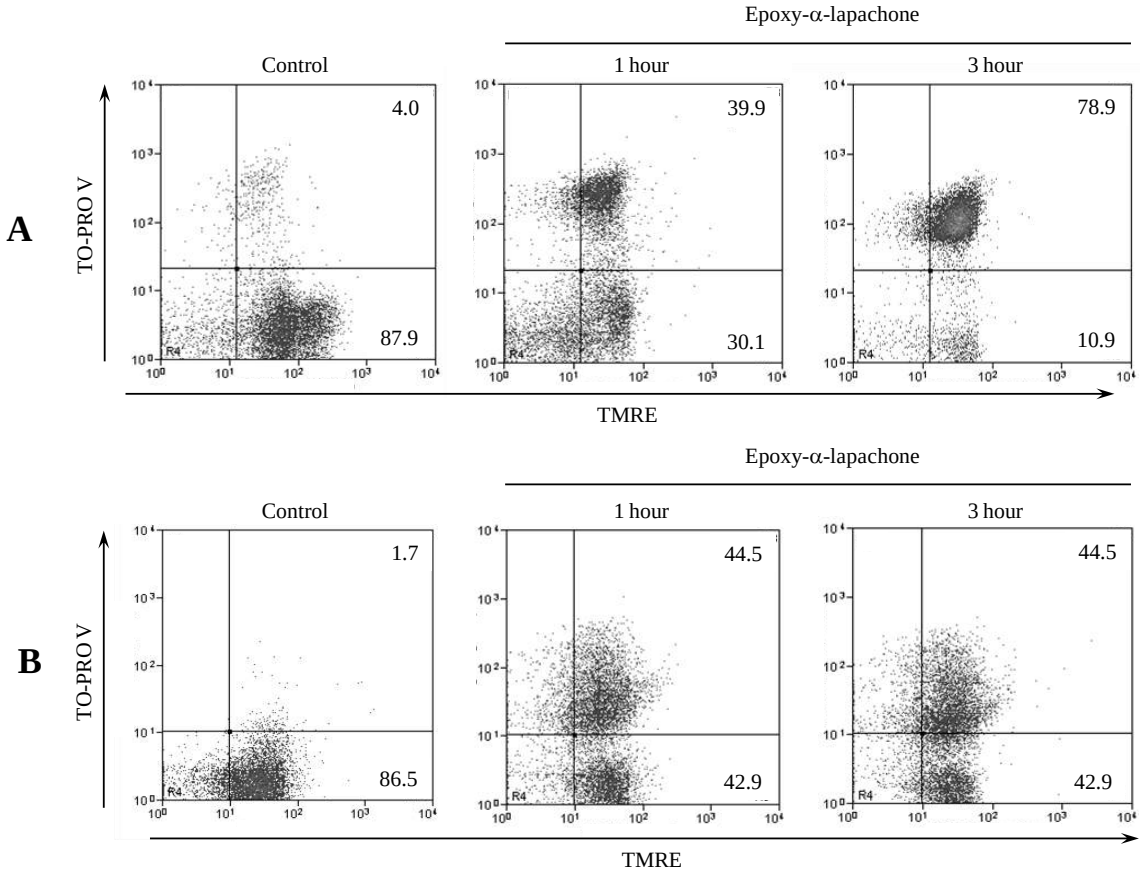


Figure 1: Figure 1: Flow cytometry assays demonstrating that epoxy-α-lapachone compound can affects promastigotes and amastigotes. Dotplot analysis of promastigotas (A) and amastigotes (B) forms of not exposed (Control) and incubated (1h and 3 h) to epoxy-α-lapachone (0.175µM). Before acquisition data (10^6 events), the parasite were staining with TMRE and TO-PRO V in Schneider's medium. The data are representative of three experiments.

FIGURE 2

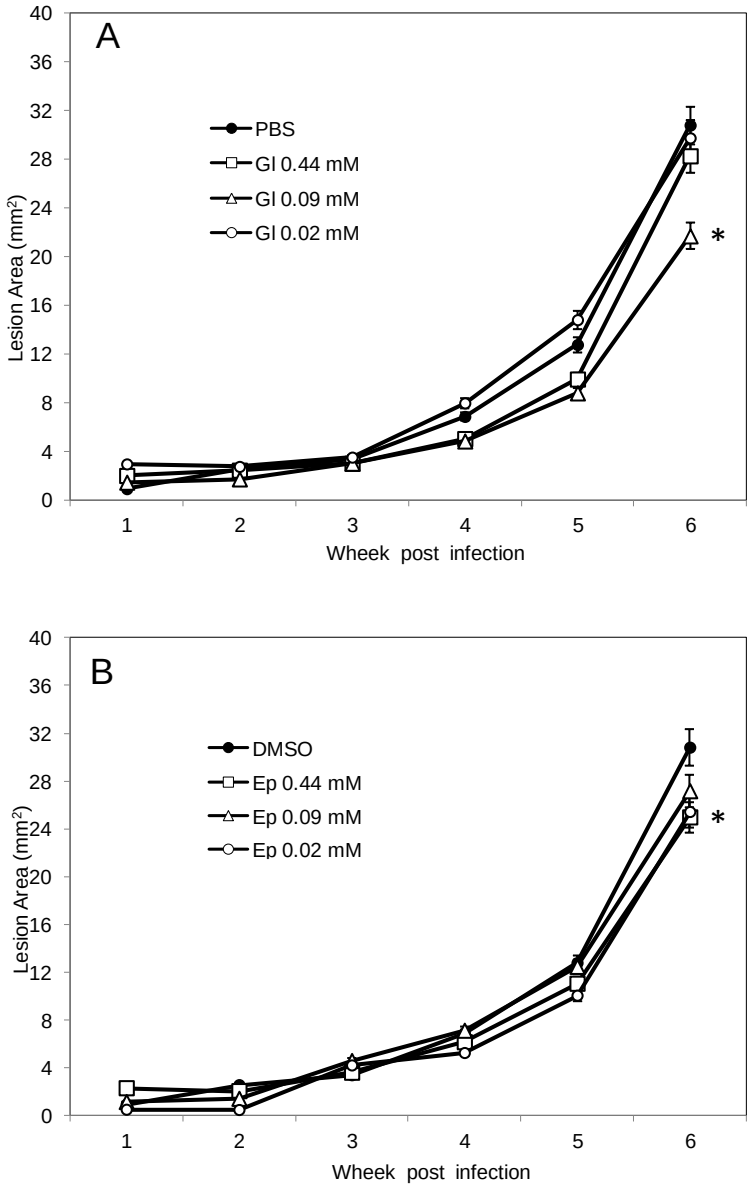


Figure 2: Experimental treatment of infections in mice caused by *Leishmania (L.) amazonensis*. The BALB/c mice were inoculated subcutaneously with 1.0×10^6 promastigotes from logarithmic phase of growth in the left foot pad. After a week of infection, BALB/c mice were treated weekly (intraperitoneally) with meglumine antimoniate (A: Gl) and epoxy-α-lapachone (B: Ep) at concentrations of 0.44 mM, 0. 09 mM and 0.02 mM, five animals in each group. Controls were treated with PBS (A) and 0,44mM of DMSO only (B). The lesion size was measured and the results represent the mean and standard deviation (±) of three independent experiments, asterisks (*) show statistical significance (p = 0.04).

FIGURE 3

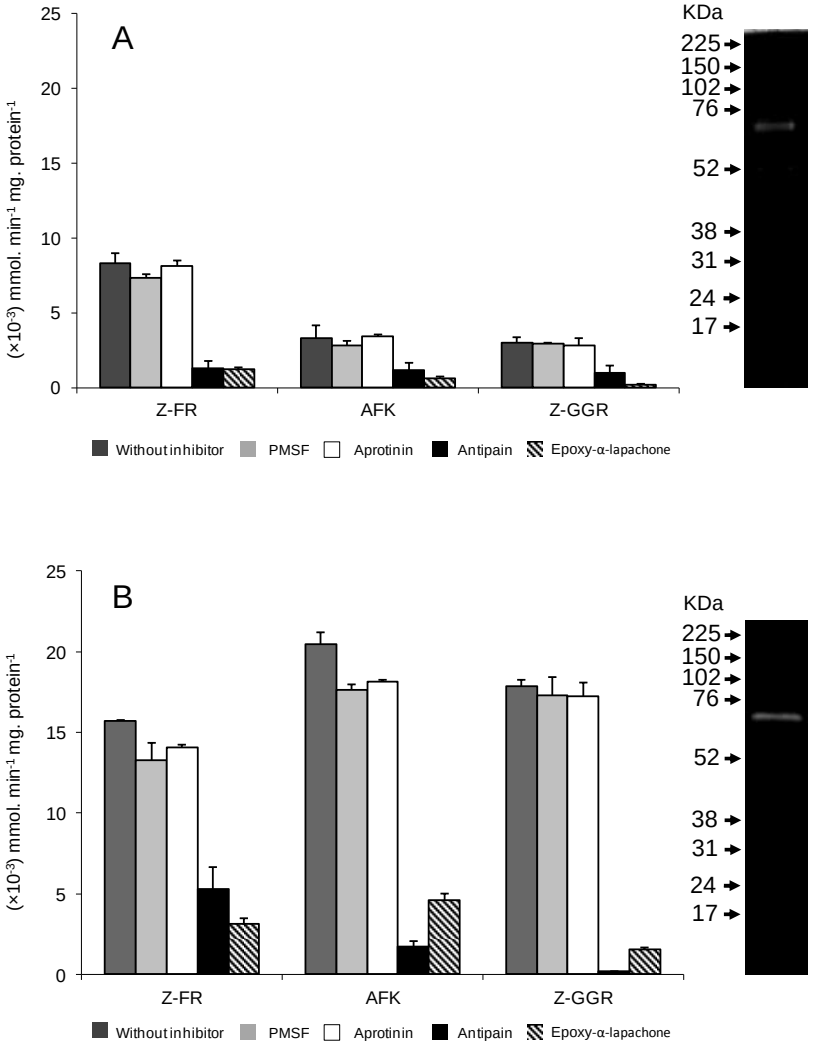


Figure 3: Proteinases activities in solution of *Leishmania (L.) amazonensis*. The fractions enriched in serine proteinase promastigote (A) and namastigotes (B) were obtained from Benzamidine-sepharose affinity chromatography. The enzymatic activities of fractions (10µg) was performed with 100µM of the substrate Z-FR, Z-GGR and AFK absence (control) and presence of inhibitors (1 mM PMSF, 1mg of aprotinin and 1 mM antipain) and 1 mM epoxy-α-lapachone. The reactions were incubated (37 °C, 60 minutes) in the buffer 10mM Tris-HCl pH 7.5. The data of enzyme activity of the fractions were expressed as ($\times 10^{-3}$) mmol .min^{-1} mg .$protein^{-1}$. In the inset, showed the zymographic profile of enriched serine proteinase fractions. Tthe molecular mass markers are indicated (kDa). Results are expressed as mean and standard deviation of three independent experiments.

FIGURE 4

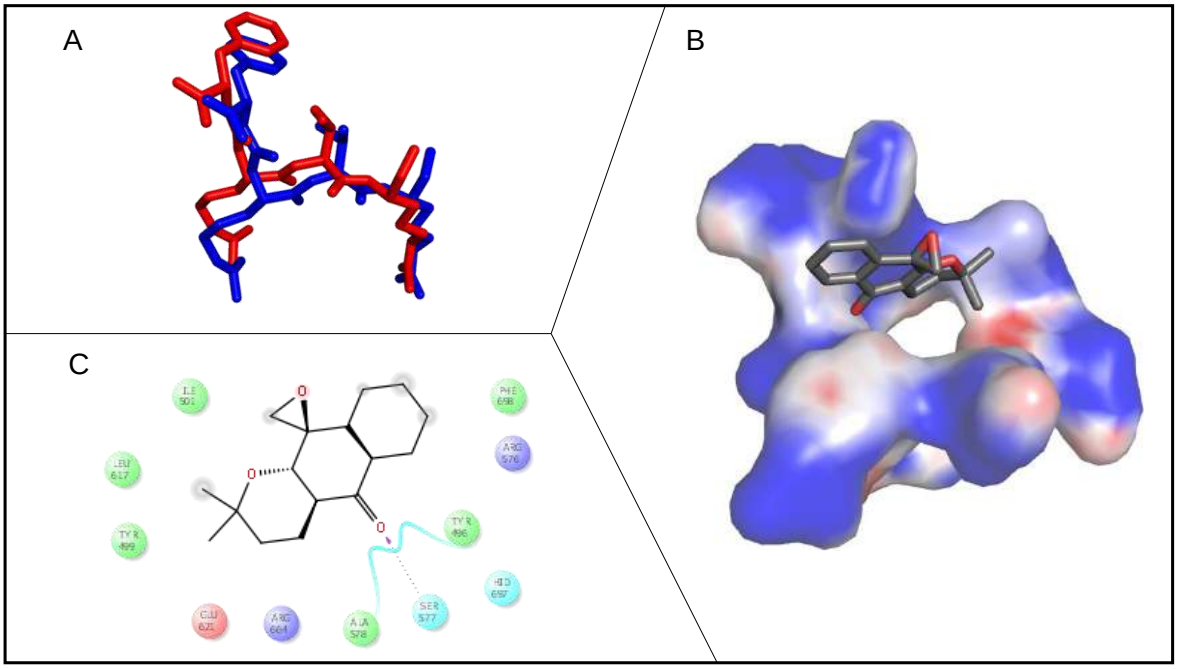


Figure 4: Docking complexes of compounds with oligopeptidase B of the *L. (L.) amazonensis*. In, (A) structural alignment of the redocking complexes of antipain (blue) and antipain co-crystallized (red) with oligopeptidase B; (B) binding epoxy-α-lapachone (gray sticks) in active site (surface); (C) Details of acid amine residues around 5 Å showed interaction with epoxy-α-lapachone (carbon atom in black, oxygen atom in red).

4. DISCUSSÃO

As leishmanioses são doenças com alta taxa de incidência nos países em desenvolvimento como Sudão, Brasil, Bangladesh, Índia/Bihar e Nepal, são endêmicas em vários outros países pobres, sendo incluída no grupo das doenças tropicais negligenciadas (Mohapatra, 2014). Apesar da importância destas doenças relatadas pelos órgãos de saúde pública mundial, ainda não há uma vacina ou fármaco que sejam efetivos.

Atualmente, os antimoniais pentavalentes têm sido utilizados como fármacos de primeira escolha para o tratamento da LC e da LV. O uso descomedido desse medicamento nos últimos 15 anos aumentou as chances do desenvolvimento de cepas resistentes. Na Índia, os isolados de LV já demonstram 80% de resistência ao tratamento com os antimoniais (Sundar, 2001). Já no Brasil, os casos de LC apresentam um cenário diferenciado, com relação a eficácia do tratamento com antimônio pentavalente. Diferentes regiões no Brasil apresentam falhas terapêuticas de 16% no Rio de Janeiro (Oliveira-Neto et al., 1997), 46,7% na Bahia (Machado et al., 2010) e 44-58% no Amazonas (Neves et al., 2011). Além disso, a incerteza da cura parasitológica da LC pode estar relacionada com distintas populações de *L. (V.) braziliensis* circulantes em várias regiões do Brasil (Torres et al., 2013) e ainda com a heterogeneidade fenotípica de cepas destes parasitos. Fato este recentemente reforçado no trabalho de tese de Silva-Almeida (Silva-Almeida, 2014), que demonstra diferentes subpopulações de *L. (V) braziliensis* em um mesmo isolado com diferenças na expressão de fatores de virulência, como as proteínas, e no comportamento biológico *in vitro*.

Ainda é preciso considerar que as limitações quanto a utilização dos medicamentos correntes são entraves ao controle da leishmaniose, tais como, alto custo, dificuldade de administração e toxicidade (Ameen, 2010). Por isso, há um incansável compromisso de pesquisadores em desenvolver novos medicamentos para o tratamento desta doença, no entanto, pouco sucesso tem sido alcançado. Desta forma, os fármacos não-convencionais e novos alvos terapêuticos estão sendo frequentemente investigados na tentativa de tornar eficaz o combate a leishmaniose (Bhargava et al., 2012). Esta necessidade premente foi a motivação deste trabalho de tese.

Um dos pontos abordados neste trabalho de tese é a importância da identificação de novos alvos para quimioterapia das leishmanioses. Dentre os diversos alvos, as enzimas relacionadas às vias metabólicas e as proteinases

são candidatos promissores a alvos devido, as funções que desempenham na sobrevivência do parasito, tais como: atuação como fatores de virulência, manutenção da infecção e proliferação do parasita intracelular, como mencionado no **ANEXO 1**.

Vale apena salientar, o quanto é complexa o processo pelo qual a informação genética é processada em um produto gênico funcional, RNA ou proteína, nos parasitos membros da família Trypanosomatidae. Estes parasitos apresentam mecanismos únicos de expressão gênica, tais como, transcrição policistronica de agrupamentos de genes, amplificação do gene, *trans-splicing* do RNAm e extensa edição de transcritos mitocondriais (Thomas et al., 2009). Em *Leishmania* spp sequências repetidas (diretas ou invertidas) envolvidos no rearranjo do DNA, alteração do número de cópias do gene (supressão ou amplificação), formação de *amplicons* circulares ou lineares extracromossômicos e cromossomos supranumerários foram descritos em *Leishmania* sp (Ubeda et al., 2008; Mukherjee et al., 2011). Além disso, há vestígio do envolvimento do retrotransposon na instabilidade do RNAm na iniciação da transcrição (Smith et al., 2009).

Possivelmente, estes mecanismos de processamento da informação do genoma destes parasitos podem proporcionar uma expressão diferencial das proteinases nas várias fases do ciclo de vida das *Leishmania* spp, refletida na adaptação do parasito aos seus ambientes, como foi anteriormente descrito para *L. (L.) amazonensis* (Alves et al., 2005) e para *L. (V.) braziliensis* (Silva- Almeida et al., 2014). Nessa linha de pensamento foi proposto uma análise da organização dos genes de proteinases para melhor compreender a distribuição destes em *Leishmania* spp. (**ANEXO 2),** e a partir de então ter uma visão ampliada das possibilidades de alvos para quimioterapia.

Uma reflexão do potencial dos inibidores de proteinases para o tratamento das leishmanioses é apresentada no **DOCUMENTO 1** deste trabalho de tese, onde é pondera a utilização desses inibidores no tratamento destas doenças. Além disso, relatamos o uso dos medicamentos atuais de primeira e segunda escolha na quimioterapia das leishmanioses e o uso dos inibidores de proteinases no controle da infecção por *Leishmania* sp. Neste documento, foram reunidas evidencias, *in vitro* e *in vivo,* que confirmam o potencial dos inibidores de

proteinases como alternativa complementar ao tratamento atual das leishmanioses.

Não obstante, este trabalho indicar a relevância das proteinases como alvos para novos fármacos, os inibidores de proteinases testados ainda não atuam plenamente na resolução da infecção e muitas vezes são tóxicos às células dos mamíferos. Este paradigma pode estar relacionado com a expressão diferenciada das proteinases que é refletida pela mudança do ambiente que o parasita sofre no seu ciclo de vida (Alves et al., 2005; Rebello et al., 2010). Estas variações devem ser consideradas para melhorar a eficácia dos inibidores de proteinase, da mesma forma, que o foco deve ser as proteinases expressas em amastigotas, as quais são responsáveis pelas formas clinicas em mamíferos, e ainda, ter a menor semelhança estrutural e funcional com as enzimas de mamíferos (Bhargava & Singh, 2012). Outro ponto importante a considerar são as diferenças estruturais em proteinases do mesmo clã ou família, que afetam o microambiente catalítico (isoformas), além das isoenzimas.

Assim, compreender os mecanismos distintos de atividade das proteinases e sua importância na biologia dos parasitas é necessário para definir adequadamente inibidores de proteinases com potencial para o tratamento da infecção. No entanto, *Leishmania* spp apresentam particularidades que agregam mais dificuldades a esta pergunta, ao lado da questão química. Tais dificuldades foram apresentadas na publicação do **DOCUMENTO 1 e DOCUMENTO 2,** quando propomos a hipótese de que as estratégias para controlar a multiplicação das *Leishmania* spp no hospedeiro vetebrado, deve ser implantada de maneira mais extensa. Desta forma, o uso de inibidores de proteinases com amplo espectro de ação no qual, o inibidor seja multivalente, atuando em mais de uma classe ou família de proteinase e, além disso, o tratamento combinado com distintos inibidores de proteinases e/ou medicamentos já utilizados no tratamento das leishmanioses, podem ser estratégias promissoras.

Na busca de novos fármacos com potencial de ação sobre os parasitos do gênero Leishmania, foi oportuno realizar ensaios experimentais com um derivado da nafatoquinona, epoxi-α-lapachona, com indicação prévia de agir como inibidor de proteinases do *Trypanosoma cruzi* (Bourguignon et al., 2010). A baixa toxicidade do composto epoxi-α-lapachona sobre células de mamíferos

foi conseguida pela epoxidação do α-lapachona (Ferreira et al 2006; Jorqueira et al 2006) mantendo ação parasiticida, o que foi comprovado no **DOCUMENTO 3** e nas publicações prévias a respeito do uso deste composto com ação sobre o *T.cruzi* (Bourguignon et al., 2009; Bourguignon et al., 2010).

Não obstante outros derivados naftoquinonas como 2,3-dichloro-5,8-dihidroxi-1,4-nafitoquinona e 2,3-dibromo-1,4-naftoquinona e triazol naftoquinona apresentarem uma boa eficiência *in vivo* contra *Leishmania* spp, com valores de IC50 < 40μM, (Croft et al., 1992; Lezama-Dávila et al., 2012; Guimarãesa et al., 2013), estes compostos demostraram significativa toxicidade para células de mamíferos. Estes achados descritos na literatura reforçam a ideia de que a eficiência *in vitro* da atividade leishmanicida do epoxi-α-lapachona é um indicativo do potencial uso de deste composto como um futuro fármaco, como descrito no **DOCUMENTO 3**.

A publicação do **DOCUMENTO 3** foi a primeira evidencia de que o composto epoxi-α-lapachona tem potencial de inibir a multiplicação de *Leishmania* dos dois subgêneros, *Viannia e Leishmania.* O composto é capaz de diminuir a taxa de crescimento dos promastigotas em cultura e também é capaz de afear drasticamente a sobrevivência de amastigotas no interior dos macrófagos humanos. O fato descrito neste trabalho que ambas as formas destes parasitos são afetadas é um indicativo de que o epoxi-α-lapachona pode agir diretamente no parasito. No entanto, não se descarta a possibilidade da sua atuação sobre os macrófagos, desencadeando as atividades microbicidas do mesmo, como descrito para outros compostos de origem vegetal (Saha et al., 2011), fato este que necessita ser investigado.

Na busca de mais evidencias sobre o potencial do epoxi-α-lapachona como agente leishmanicida, e ainda para compreender seus mecanismos de ação nestes parasitos, propomos o **DOCUMENTO 4**. Nesta etapa do nosso estudo, focamos em observações apenas na *L. (L.) amazonensis*, e os efeitos do composto sobre a infecção causada por esse parasito em camundongos BALB/c. Além disso, constatações anteriores a este trabalho de tese sobre os efeitos deste composto com inibidor de serino proteinase no *T.cruzi* (Bourguignon et al 2010), nos levou a testar esta hipótese sobre as serino proteinases de *L. (L.)*

amazonensis obtidas a partir de métodos cromatográficos, abordados no **Anexo 3.**

Ressalta-se ainda neste estudo o valor de se usar amastigotas de cultivo axênico em testes de fármacos (**DOCUMENTO 4**). Amastigotas axênicos apresentam critérios (infectividade, epítopos antigênicos, aglutinação por lectinas e expressão gênica diferencial), os quais mostram semelhanças aos amastigotas de tecidos infectados (Gupta, 2001), principalmente dos fatores de virulência envolvidos na adaptação do paradito ao hospedeiro vertebrado, como as proteinases (Silva-Almeida, 2012). Neste contexto, a expressão de proteinases em amastigotas pode apresentar alterações devido a mudança físico-química do ambiente. Em um estudo paralelo a este trabalho de tese (Silva-Almeida, 2014) foi comprovado que amastigotas de cultivo axênico *L. (V.) braziliensis* tem maior expressão de proteinases quando comparado com promastigotas e que esta expressão pode ser influenciada pela mudança de ambiente, aqui representado pelo choque de temperatura e pH (**DOCUMENTO 4**). Além disso, recentemente foi proposto que a atividade de serino proteinase é maior em amastigota axênicas do que em promastigotas de *L. (L). mexicana* e *L. (L.) donovani* (Swenerton et al., 2011), semelhante ao que foi demonstrado neste trabalho (**DOCUMENTO 4**).

As serino proteinases representam de maneira geral 16 % das proteinases presentes no genoma das *Leishmania* spp. anotado nos bancos de dados (**Anexo 2**). A expressão desta enzima é distribuída em diversas estruturas do parasita como bolsa flagelar, vesículas da via endocítica/exocítica, megassomas, superfície celular e na membrana de organelas (Silva-López et al., 2004; Morgado-Díaz et al., 2005). Esta ampla dispersão indica o envolvimento desta proteinase numa grande diversidade de processos fisiológicos essências para a sobrevivência do parasito (Silva-López, 2010). Este fato foi comprovado com a inibição de serino proteases em promastigota de *L. (L.) amazonensis* por inibidores sintéticos (N-α-tosil-L-lisina clorometil cetona e N-α-tosil-L-fenilalanina clorometil cetona), causando à morte do parasita. Outro inibidor (Kunitz-type) também foi capaz de reduzir a viabilidade dos promastigotas de cultura, causando alterações na morfologia da célula (Silva-Lopez et al., 2007). Logo, os ensaios de inibição da atividade das serino proteinases de *L. (L.) amazonensis*

pelo epoxi-α-lapachona descrita no **DOCUMENTO 4,** representa uma valiosa abordagem no desenvolvimento racional de fármacos para o tratamento das leishmanioses.

Dentro desse conjunto de serino proteinases a oligopeptidase B surge como um possível alvo quimioterápico. No entanto, a deleção do gene da oligopeptidase B causa efeitos temporários na infecção experimental *in vitro* e *in vivo* por *L. (L.) major*. Essa evidencia, indica que a inibição desta enzima não seria suficiente para causar efeitos drásticos ao parasito. (Swenerton et al., 2011). Possivelmente, o nocaute desse gene está sendo compensado por uma oligopeptidase B símile que apresenta alguns resíduos de aminoácidos conservados no sitio ativo (Guedes et al., 2007; Munday et al., 2011).

Apresentamos evidenciais de que os efeitos inibitórios do epoxi-α-lapachona sobre as serino proteinases de *L. (L.) amazonensis* (**DOCUMENTO 4**), podem ser atribuídos a capacidade do composto interagir diretamente com os resíduos de aminoácidos que compõem a tríade catalítica das serino proteinase: serino, histidina e aspártico (Erez et al, 2009). Desta forma, o epoxi-α-lapachona ocupou o mesmo sitio de ligação S1 que o inibidor antipaina cristalizado com oligopeptidase B de *L (L.) major*.

É importante destacar, que os dados apresentados neste trabalho não garantem que o efeito do epoxi-α-lapachona sobre o parasito não seja única e exclusivamente devido a sua ação inibitória sobre as serino proteinases. De maneira geral a estrutura química dos derivados dos naftoquinonas, como o composto do estudo, facilita as reações de redução atuando com aceitador de um e/ou dois elétrons, contribuindo para a formação de oxigênio reativo e acelerando as condições hipóxica intracelular (Babior, 1997), que causa danos severos a célula (Santos et al. 2004). Esta propriedade confere ao composto a potencialidade de agente oxidante ou desidrogenante em várias reações químicas, o que pode ter ocorrido com os promastigotas e amastigotas coincubadas com o composto neste trabalho. Desta forma, o efeito do epoxi-α-lapachona sobre parasitos (**DOCUMENTO 3 E DOCUMENTO 4**) e na redução das lesões de camundongos (**DOCUMENTO 4**) pode ser por múltiplos alvos, ou seja uma combinação de ações, o que necessita de confirmações posteriores.

Inibidores de serino proteinase são amplamente encontrados nos vegetais, alguns são expressos constitutivamente e outros induzidos por lesão foliar (Fosket, 1994). Há relatos que vegetais como *Solanum tuberosum* e *Solanum lycopersicum* sintetizam esses inibidores em resposta a ação de insetos (Fosket, 1994; Broadway, 1996). Recentemente foi proposto que extrato de *S. tuberosum* é capaz de inibir multiplicação de promastigotas de *L. (L.) donovani* e impedir o desenvolvimento das formas amastigotas em macrófago, sem efeito citotóxico significativo para estas células (Paik et al, 2014). Neste contexto, a exemplo da *Tabebuia* sp e do *S. tuberosum*, outras espécies vegetais podem sintetizar compostos com atividades seletivas sobre proteinases de parasitos de interesse médico, o que significa uma vasta riqueza de compostos a serem investigados (Patil et al. de 2012).

No desenvolvimento de novos fármacos, sabe-se que as indústrias de capital nacional e estrangeiro pouco têm investido em pesquisa para o desenvolvimento de novas moléculas para o tratamento da leishmaniose e outras doenças negligenciadas. Esse desinteresse comercial se fundamenta no fato da leishmaniose ser doença tropical que afeta principalmente populações pobres. Este panorama explica o baixo número de compostos e formulações, com baixa toxidade e efetividade comprovada, em uso clinico.

Ainda na vertente de novos fármacos, o Brasil é o país que detém a maior parcela da biodiversidade, em torno de 15 a 20% do total mundial, com destaque para as plantas superiores, nas quais detém aproximadamente 24% da biodiversidade. Esta biodiversidade oferece a matéria-prima para a fabricação de fitoterápicos e os seus componentes químicos ativos provém modelos para o desenvolvimento de novas drogas terapêuticas (Calixto, 1997). Estima-se que aproximadamente 40% dos medicamentos atualmente disponíveis foram desenvolvidos direta ou indiretamente a partir de fontes naturais, a maioria destas de plantas (Calixto, 2001). Das 252 drogas consideradas básicas e essenciais pela OMS, 11% são originárias de plantas e um número significativo são drogas sintéticas obtidas de precursores naturais (Rates 2001).

Os produtos naturais são fontes inesgotáveis de novas descobertas de medicamentos. Predominantemente, os fármacos sintéticos atuais originaram-se de estruturas químicas oriundas das plantas, e apenas cerca de 200 anos

atrás, a nossa farmacopeia foi dominado por medicamentos fitoterápicos (Ernst 2005). Estes compostos são reconhecidos pela indústria farmacêutica pela sua diversidade estrutural notável e ampla gama de atividade farmacológica, como os derivados do lapachol.

O potencial destes compostos vem motivando estudos de novos medicamentos para o tratamento de doenças negligenciadas (Butler, 2008). Nos últimos anos os produtos naturais já impactam na descoberta de fármacos para infecções parasitárias (Ndjonka et al, 2013). No que se refere aos resultados da pesquisa apresentada neste trabalho de tese, o epoxi-α-lapachona já cumpriu algumas das principais etapas da fase pré-clínica no processo de desenvolvimento de novos fármacos, esta etapa contempla a otimização da molécula em relação a uma série de propriedades (potência, afinidade, seletividade e toxidez). Estudos futuros precisam ser realizados para avaliar o epoxi-α-lapachona em relação a sua segurança e confirmação da eficácia em modelo animal, visando a sua introdução em fases clínicas para testes em humanos.

5. CONCLUSÕES

1. O epoxi-α-lapachona afeta o desenvolvimento e a sobrevivência de ambos os estágios morfológicos da *L. (V.) braziliensis* e *L. (L.) amazonensis*.
2. O epoxi-α-lapachona atua nos promastigotas e amastigotas de *L. (L.) amazonensis* durante primeiras horas de contato, causando um efeito interno inicial com posterior comprometimento da membrana plasmática e morte dos parasitos.
3. O tratamento dos camundongos BALB/c com epoxi-α-lapachona reduziu a lesão de pata causada pela *L. (L.) amazonensis,* semelhante ao tratamento destes animais com o glucantime.
4. A atividade de serino proteinase dos promastigotas e amastigotas de *L. (L.) amazonensis* foram melhor inibidas pelo epoxi-α-lapachona quando comparados aos inibidores clássicos de serino proteinase.
5. De maneira semelhante ao inibidor antipaína, o epoxi-α-lapachona pode ligar-se a oligopeptidase B de *L. (L) amazonensis* no sítio ativo da enzima.
6. O conjunto de evidências apresentadas neste trabalho de tese indica pela primeira vez a possibilidade de utilizar epoxi-α-lapachona como base para o desenvolvimento de tratamentos novos, ou complementares, contra leishmaniose humana.

6. REFERÊNCIAS BIBLIOGRÁFICAS

2005;19(4):405-9.

2011. 21;9(12), 4626-34.

Abu-Dayyeh I, Hassani K, Westra ER, Mottram JC, Olivier M: Comparative study of the ability of Leishmania mexicana promastigotes and amastigotes to alter macrophage signaling and functions. Infect Immun 2010; 78(6), 2438–2445.

Abu-Dayyeh I, Shio MT, Sato S, Akira S, Cousineau B, Olivier M. Leishmania-induced IRAK-1 inactivation is mediated by SHP-1 interacting with an evolutionarily conserved KTIM motif. PLoS Negl. Trop. Dis. 2008; 2, 305.

Aderem A. The MARCKS brothers: a family of protein kinase C substrates. Cell 1992; 71, 713-716.

Al-Abdely HM, Graybill JR, Loebenberg D, Melby PC. Efficacy of the triazole SCH 56592 against Leishmania amazonensis and Leishmania donovani in experimental murine cutaneous and visceral leishmaniases. 1999; 43(12):2910-4.

Alrajhi AA, Ibrahim EA, De Vol EB, Khairat M, Faris RM, Maguire JH. Fluconazole for the treatment of cutaneous leishmaniasis caused by Leishmania major. N Engl J Med. 2002; 346(12):891-5.

Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, Jannin J, den Boer M; WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012; 7(5):e35671.

Alves CR, Corte-Real S, Bourguignon SC, Chaves CS, Saraiva EM. Leishmania amazonensis: early proteinase activities during promastigote-amastigote differentiation in vitro. Experimental Parasitology 2005; 109(1), 38-48.

amastigotes. Int J Parasitol. 2001; 31(12):1311-20.

Ameen, M. Cutaneous leishmaniasis: advances in disease pathogenesis, diagnostics and therapeutics. Clinical and Experimental Dermatology 2010; 35: 699-705.

Arana BA, Navin TR, Arana FE, Berman JD, Rosenkaimer F. Efficacy of a short course (10 days) of high-dose meglumine antimonate with or without interferon-gamma in treating cutaneous leishmaniasis in Guatemala. Clin Infect Dis. 1994;18(3):381-4.

Arevalo I, Tulliano G, Quispe A, Spaeth G, Matlashewski G, Llanos-Cuentas A, Pollack H. Role of imiquimod and parenteral meglumine antimoniate in the initial treatment of cutaneous leishmaniasis. Clin Infect Dis. 2007, 44(12):1549.

Ashford RW. The leishmaniases as emerging and reemerging zoonoses. Int J Parasitol. 2000; 30(12–13):1269–81.

Babior BM. Superoxide: a two-edged sword. Braz J Med Biol Res. 1997; 30(2), 141-55. Review.

Bañuls AL, Bastien P, Pomares C, Arevalo J, Fisa R, Hide M. Clinical pleiomorphism in human leishmaniases, with special mention of asymptomatic infection. Clin Microbiol Infect. 2011; 17(10):1451-61

Barrett AJ, McDonald JK. Nomenclature: a possible solution to the peptidase anomaly. Biochem J. 1985; 1;231(3):807.

Bedoya-Pacheco SJ, Araujo-Melo MH, Valete-Rosalino CM, Pimentel MI, Conceição-Silva F, Schubach AO, Marzochi MC. Endemic tegumentary leishmaniasis in Brazil: correlation between level of endemicity and number of cases of mucosa disease. Am J Trop Med Hyg. 2011; 84(6):901-5.

Berman J. Recent developments in leishmaniasis: epidemiology, diagnosis and treatment. Curr Infect Dis Repor. 2005; 7:33-8.

Berman JD. Chemotherapy for leishmaniasis: biochemical mechanisms, clinical efficacy, and future strategies. Reviews of Infectious Diseases. 1988; 10(3): 560–586.

Besteiro S, Williams RA, Coombs GH, Mottram JC. Protein turnover and differentiation in Leishmania. Int J Parasitol. 2007; 37(10):1063-75.

Beynon RJ & Bond JS. Proteolytical enzymes: a pratical approach, 2nd ed. Oxorfd: Oxford University Press 2001

Bhargava P, Singh R. Developments in Diagnosis and Antileishmanial Drugs. Interdisciplinary Perspectives on Infectious Diseases. Interdiscip Perspect Infect. 2012; 2012/626838.

Block JB, Serpick AA, Miller W, Wiernik PH. Early clinical studies with lapachol (NSC-11905). Cancer Chemother Rep 2. 1974; 4(4), 27-8.

Borges, VM, Marcos, AV, Wanderley, dS. Subverted transferrin trafficking in Leishmania-infected macrophages. Parasitol. Res.1988; 84, 811–822.

Bourguignon SC, Castro HC, Santos DO, Alves CR, Ferreira VF, Gama IL, Silva FC, Seguins WS, Pinho RT. Trypanosoma cruzi: in vitro activity of Epoxy-alpha-Lap, a derivative of alpha-lapachone, on trypomastigote and amastigote forms. Exp Parasitol. 2009;122(2), 91-6.

Bourguignon SC, Cavalcanti DF, de Souza AM, Castro HC, Rodrigues CR, Albuquerque MG, Santos DO, da Silva GG, da Silva FC, Ferreira VF, de Pinho RT, Alves CR. Trypanosoma cruzi: insights into naphthoquinone effects on growth and proteinase activity. Experimental Parasitology; 2010, 127(1), 160-6.

Breger J, Fuchs BB, Aperis G, Moy TI, Ausubel FM, Mylonakis E. Antifungal chemical compounds identified using a C. elegans pathogenicity assay. PLoS Pathog. 2007; 3(2), e18.

Brittingham A, Morrison CJ, McMaster WR, McGwire BS, Chang KP, Mosser DM. Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complementmediated lysis. J Immunol 1995; 155: 3102-11

Broadway RM. 1996. Dietary proteinase inhibitors alter complement of midgut proteases. Archives of Insect Biochemistry and Physiology 32, 39–53.

Burchmore RJ, Barrett MP. Life in vacuoles--nutrient acquisition by Leishmania

Butler MS. Natural products to drugs: natural product-derived compounds in clinical trials, Nat. Prod. Rep. 25. 2008; 475-516.

Buxbaum LU, Denise H, Coombs GH, Alexander J, Mottram JC, Scott P: Cysteine protease B of Leishmania mexicana inhibits host Th1 responses and protective immunity. J Immunol 2003; 171(7), 3711–3717.

Calixto J. B. Biological activity of plant extracts: novem analgesic drugs. Expert Opinion Emerging Drugs. 2001; 2:261- 279.

Calixto J.B. Fitofármacos no Brasil: agora ou nunca! Ciência hoje. 1997;.21(1) 234, p. 26-30, 1997.

Cameron P, McGachy A, Anderson M, Paul A, Coombs GH, Mottram JC, Alexander J, Plevin R: Inhibition of lipopolysaccharide-induced macrophage IL-12 production by Leishmania mexicana amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. J Immunol 2004; 173(5), 3297–3304.

Carvalho AK, Silveira FT, Passero LF, Gomes CM, Corbett CE, Laurenti MD. Leishmania (V.) braziliensis and L. (L.) amazonensis promote differential expression of dendritic cells and cellular immune response in murine model. Parasite Immunol. 2012; 34(8-9):395-403.

Choudhury R, Das P, Bhaumik SK, De T, Chakraborti T. In situ immunolocalization and stage-dependent expression of a secretory serine protease in Leishmania donovani and its role as a vaccine candidate. Clin Vaccine Immunol. 2010;17(4):660-7.

Contreras I, Gómez MA, Nguyen O, Shio MT, McMaster RW, Olivier M: Leishmania-induced inactivation of the macrophage transcription factor AP-1 is mediated by the parasite metalloprotease GP63. PLoS Pathog 2010; 6(1), e1001148.

Croft SL, Hogg J, Gutteridge WE, Hudson AT, Randall AW. The activity of hydroxynaphthoquinones against Leishmania donovani. J Antimicrob Chemother. 1992; 30(6), 827-32.

da Consolação M, Linardi F, de Oliveira MM, Sampaio MR. A lapachol derivative active against mouse lymphocytic leukemia P-388. J Med Chem. 1975; 18(11):1159-61.

da Silva AJ, Buarque CD, Brito FV, Aurelian L, Macedo LF, Malkas LH, Hickey RJ, Lopes DV, Noël F, Murakami YL, Silva NM, Melo PA, Caruso RR, Castro NG, Costa PR. Synthesis and preliminary pharmacological evaluation of new (+/-) 1,4-naphthoquinones structurally related to lapachol. Bioorg Med Chem. 2002;10(8), 2731-8.

da Silva MN, Ferreira VF, de Souza MCBV. Um panorama atual da química e da farmacologia de naftoquinonas, com ênfase na β-lapachona e derivados. Quimica Nova. 2003 26(3); 407-416.

da Silva-López RE, dos Santos TR, Morgado-Díaz JA, Tanaka MN, de Simone SG. Serine protease activities in Leishmania (Leishmania) chagasi promastigotes. Parasitol Res. 2010;107(5), 1151-62.

da Silva-López RE, Giovanni De Simone S. A serine protease from a detergent soluble extract of Leishmania (Leishmania) amazonensis. Z Naturforsch 2004a; 59c, 590–598.

de Castro EA, Luz E, Telles FQ, Pandey A, Biseto A, Dinaiski M, Sbalqueiro I, Soccol VT. Eco-epidemiological survey of Leishmania (Viannia) braziliensis American cutaneous and mucocutaneous leishmaniasis in Ribeira Valley River, Paraná State, Brazil. Acta Trop. 2005; 93(2):141-9.

De Moura KCG, Emery FS, Neves-Pinto C, Pinto MCFR, Dantas AP, Salomão K, de Castro SL, Pinto AV. Trypanocidal activity of isolated naphthoquinones from Tabebuia and some heterocyclic derivatives: a review from an interdisciplinary study. Journal of Brazilian Chemical Society 2001; 12, 325-338.

De Souza Leao S, Lang T, Prina E, Hellio R, Antoine JC. Intracellular Leishmania amazonensis amastigotes internalize and degrade MHC class II molecules of their host cells. J Cell Sci. 1995; 108(10), 3219-31.

Denise H, McNeil K, Brooks DR, Alexander J, Coombs GH, Mottram JC: Expression of multiple CPB genes encoding cysteine proteases is required for Leishmania mexicana virulence in vivo. Infect Immun 2003; 71(6), 3190–3195.

Dietze R, Carvalho SF, Valli LC, Berman J, Brewer T, Milhous W, et al. Phase 2 trial of WR6026, an orally administered 8-aminoquinoline, in the treatment of visceral leishmaniasis caused by Leishmania chagasi. Am J Trop Med Hyg. 2001; 65(6):685-9.

Erez E, Fass D, Bibi E. How intramembrane proteases bury hydrolytic reactions in the membrane. Nature 2009; 459(7245), 371-378.

Ernst E. The efficacy of herbal medicine--an overview. Fundam Clin Pharmacol.

Eyong KO, Folefoc GN, Kuete V, Beng VP, Krohn K, Hussain H, Nkengfack AE, Saeftel M, Sarite SR, Hoerauf A. Newbouldiaquinone A: A naphthoquinone-anthraquinone ether coupled pigment, as a potential antimicrobial and antimalarial agent from Newbouldia laevis. Phytochemistry. 2006; 67(6), 605-9.

Ferreira VF, Jorqueira A, Souza AM, da Silva MN, de Souza MC, Gouvêa RM, Rodrigues CR, Pinto AV, Castro HC, Santos DO, Araújo HP, Bourguignon SC. Trypanocidal agents with low cytotoxicity to mammalian cell line: a comparison of the theoretical and biological features of lapachone derivatives. Bioorganic & medicinal chemistry 2006; 14, 5459-5466.

Fosket, D. E. Plant Growth and Development: a Molecular Approach.Academic Press, 1994

Fosket, D.E. 1994. Plant Growth and Development. A Molecular Approach. Academic Press, San Diego, California, USA.

Gontijo B D-CMLR. Leishmaniose tegumentar americana - American cutaneous leishmaniasis. Revista da Sociedade Brasileira de Medicina Tropical. 2003; 36(1):71-80.

Goodwin LG. Pentostam (sodium stibogluconate); a 50-year personal reminiscence. Trans R Soc Trop Med Hyg. 1995; 89(3):339–341.

Gregory DJ, Godbout M, Contreras I, Forget G, Olivier M: A novel form of NF-kappaB is induced by Leishmania infection: involvement in macrophage gene expression. Eur J Immunol 2008; 38(4), 1071–1081.

Guedes HL, Rezende JM, Fonseca MA, Salles CM, Rossi-Bergmann B, De-Simone SG. Identification of serine proteases from Leishmania braziliensis. Z Naturforsch C. 2007 62; (5-6),373-81.

Guimarães TT, Pinto Mdo C, Lanza JS, Melo MN, do Monte-Neto RL, de Melo IM, Diogo EB, Ferreira VF, Camara CA, Valença WO, de Oliveira RN, Frézard F, da Silva EN Jr. Potent naphthoquinones against antimony-sensitive and –resistant Leishmania parasites: synthesis of novel α- and nor-α-lapachone-based 1,2,3-triazoles by copper-catalyzed azide-alkyne cycloaddition. Eur J Med Chem. 2013; 63, 523-30.

Gupta N, Goyal N, Bhatia N, Kamboj KK, Rastogi AK. Identification of amastigote-specific antigens of Leishmania donovani using kala-azar patient sera. Indian J Exp Biol. 2001;39(7), 623-8.

Haldar AK, Sen P, Roy S. Use of antimony in the treatment of leishmaniasis: current status and future directions. Mol Biol Int. 2011; 571242.

Hassani K, Antoniak E, Jardim A, Olivier M. Temperature-induced protein secretion by Leishmania mexicana modulates macrophage signalling and function. PLoS One. 2011; 3;6(5):e18724.

Herwaldt B, Berman JD: Recommendations for treating Leishmaniasis with sodium stibogluconate (Pentostam) and review of pertinent clinical studies. Am. J. Trop. Med. Hyg. 1992; 46(3):296-306.

Hide M, Bucheton B, Kamhawi S, Bras-Gonçalves R, Sundar S, Lemesre J-L, Bañuls A-L. Understanding Human Leishmaniasis: The Need for an Integrated Approach “In”: Tibayrenc M. Encyclopedia of Infectious Diseases: Modern Methodologies. Hoboken (New Jersey): Wiley Online Library; 2007, 87-111.

Hussain H, Krohn K, Ahmad VU, Miana GA, Green IR. Lapachol: An overview. 2007; (2), 145-171

Infect Immun. 2008; 76(4):1738-47.

Jaramillo M, Gomez MA, Larsson O, Shio MT, Topisirovic I, Contreras I, Luxenburg R, Rosenfeld A, Colina R, McMaster RW, Olivier M, Costa-Mattioli M, Sonenberg N. Leishmania repression of host translation through mTOR cleavage is required for parasite survival and infection. Cell Host Microbe. 2011; 9(4), 331-41.

Jorqueira A, Gouvêa RM, Ferreira VF, Silva MN, Souza MCBV, Zuma AA, Cavalcanti DFB, Araújo HP, Santos DO, Bourguignon SC. Oxyrane derivative of α-lapachone is potent growth inhibitor of Trypanosoma cruzi epimastigote forms. Parasitology Research 2006; 99, 429-433.

Kikuth W & Schmidt H. Contribution to the progress of antimony therapy of kala-azar. Chin Med J. 1937; 52:425–432.

Koutinas AFS, Mylonakis ME, Leontides L, Polizopoulou Z, Billinis C, Argyriadis D, Diakou N, Paradopoulos O. A randomised, blinded, placebo-controlled clinical trial with allopurinol in canine leishmaniosis. Veterinary Parasitology. 2001; 98(4):247-61.

Kulkarni MM, Jones EA, McMaster WR, McGwire BS. Fibronectin binding and

Lasakosvitsch F, Gentil LG, dos Santos MR, da Silveira JF, Barbiéri CL. Cloning and characterisation of a cysteine proteinase gene expressed in amastigotes of Leishmania (L.) amazonensis. Int J Parasitol. 2003; 33(4), 445-54.

Lezama-Dávila CM, Isaac-Márquez AP, Kapadia G, Owens K, Oghumu S, Beverley S, Satoskar AR. Leishmanicidal activity of two naphthoquinones against Leishmania donovani. Biol Pharm Bull. 2012; 35(10), 1761-4.

Machado PR, Ampuero J, Guimarães LH, Villasboas L, Rocha AT, Schriefer A, Sousa RS, Talhari A, Penna G, Carvalho EM. Miltefosine in the treatment of cutaneous leishmaniasis caused by Leishmania braziliensis in Brazil: a randomized and controlled trial. PLoS Negl Trop Dis. 2010; 21;4(12), e912.

Martinez S, Gonzalez M, Vernaza ME. Treatment of cutaneous leishmaniasis with allopurinol and stibogluconate. Clin Infect Dis. 1997; 24(2):165-9

McConville MJ, Naderer T. Metabolic pathways required for the intracellular survival of Leishmania. Annu Rev Microbiol. 2011; 65:543-61.

McGwire, B. S., Chang, K. P., and Engman, D. M. Infect. Immun. 2003; 71, 1008–1010

Menna-Barreto RF, Henriques-Pons A, Pinto AV, Morgado-Diaz JA, Soares MJ, De Castro SL. Effect of a beta-lapachone-derived naphthoimidazole on Trypanosoma cruzi: identification of target organelles. J Antimicrob Chemother. 2005; 56(6), 1034-41.

Ministério da Saúde; Brasil; Secretaria de Vigilância em Saúde: Manual de Vigilância da Leishmaniose Tegumentar Americana. Série A. Normas e Manuais Técnicos 2ª edição. Brasília: Editora do Ministério da Saúde; 2010

Mishra J, Saxena A, Singh S. Chemotherapy of leishmaniasis: past, present and future. Current Medicinal Chemistry. 2007; 14(10): 1153–1169.

Mohapatra S. Drug resistance in leishmaniasis: Newer developments. Trop Parasitol. 2014; 4(1), 4-9.

Morgado-Díaz JA, Silva-Lopez RE, Alves CR, Soares MJ, Corte-Real S, De Simone SG. Subcellular localization of an intracellular serine protease of 68 kDa in Leishmania (Leishmania) amazonensis promastigotes. Memórias Instituto Oswaldo. Cruz. 2005;100(4), 377-83.

Mottram JC, Coombs GH, Alexander J. Cysteine peptidases as virulence factors of Leishmania. Curr Opin Microbiol. 2004; 7(4):375-81. Review

Mukherjee A, Langston LD, Ouellette M. Intrachromosomal tandem duplication and repeat expansion during attempts to inactivate the subtelomeric essential gene GSH1 in Leishmania. Nucleic Acids Res. 2011; 39, 7499–7511.

Müller K, Sellmer A, Wiegrebe W. Potential antipsoriatic agents: lapacho compounds as potent inhibitors of HaCaT cell growth. J Nat Prod. 1999; 62(8), 1134-6.

Murray HW, Brooks EB, DeVecchio JL, Heinzel FP. Immunoenhancement combined with amphotericin B as treatment for experimental visceral leishmaniasis. Antimicrob Agents Chemother. 2003; 47(8):2513-7.

Murray, M. & Pizzorno, J. The Encyclopedia of Natural Medicine. Rocklin, CA Prima Publishing. 1998; 639-641.

Ndjonka D, Rapado LN, Silber AM, Liebau E, Wrenger C. Natural products as a source for treating neglected parasitic diseases, Int. J. Mol. Sci. 14 (2013), 395-3439.

Neves LO, Talhari AC, Gadelha EP, Silva Júnior RM, Guerra JA, Ferreira LC, Talhari S. A randomized clinical trial comparing meglumine antimoniate, pentamidine and amphotericin B for the treatment of cutaneous leishmaniasis by Leishmania guyanensis. An Bras Dermatol. 2011; 86(6), 1092-101.

Neves-Pinto C, Malta VR, Pinto Mdo C, Santos RH, de Castro SL, Pinto AV. A trypanocidal phenazine derived from beta-lapachone. Journal of Medicinal Chemistry. 2002, 45, 2112-2115.

Oliveira LF, Schubach AO, Martins MM, Passos SL, Oliveira RV, Marzochi MC, Andrade CA. Systematic review of the adverse effects of cutaneous leishmaniasis treatment in the New World. Act Trop. 2011; 118(2):87-96.

Oliveira-Neto MP, Mattos M, Pirmez C, Fernandes O, Gonçalves-Costa SC, Souza CF, et al. Mucosal leishmaniasis ("espundia") responsive to low dose of N-methyl glucamine (Glucantime) in Rio de Janeiro, Brazil. Rev Inst Med Trop São Paulo. 2000; 42:321-325.

Oliveira-Neto MP, Schubach A, Araujo ML, Pirmez C. High and low doses of antimony (Sbv) in American cutaneous leishmaniasis. A five years follow-up study of 15 patients. Mem Inst Oswaldo Cruz. 1996; 91:207-209.

Oliveira-Neto MP, Schubach A, Mattos M, da Costa SC, Pirmez C. Intralesional therapy of American cutaneous leishmaniasis with pentavalent antimony in Rio de Janeiro, Brazil--an area of Leishmania (V.) braziliensis transmission. Int J Dermatol. 1997; 36(6), 463-8.

Paik D, Das P, De T, Chakraborti T. In vitro anti-leishmanial efficacy of potato tuber extract (PTEx): Leishmanial serine protease(s) as putative target. Exp Parasitol. 2014; 14 (146C), 11-19.

Patil V, Bandivadekar A, Debjani D. Inhibition of Propionibacterium acnes lipase by extracts of Indian medicinal plants. Int J Cosmet Sci. 2012; 34(3), 234-9.

Pimenta PF, Saraiva EM, Sacks DL. The comparative fine structure and surface glycoconjugate expression of three life stages of Leishmania major. Exp. Parasitol. 1991; 72, 191-204

Piña-Vázquez C, Reyes-López M, Ortíz-Estrada G, de la Garza M, Serrano-Luna J. Host-parasite interaction: parasite-derived and -induced proteases that degrade human extracellular matrix J Parasitol Res. 2012; 2012:748206.

proteolytic degradation by Leishmania and effects on macrophage activation.

RATES, S.M.K. Plants as source of drugs. Toxicon, Oxford, v. 39, p. 603-613, 2001

Rawlings ND, Morton FR, Kok CY, Kong J, Barrett AJ. MEROPS: the peptidase database. Nucleic Acids Res 2008; 36: D320-D325.

Rebello KM, Britto C, Pereira BA, Pita-Pereira Dd, Moraes MO, Ferreira AB, Cysne-Finkelstein L, Otto TD, Côrtes LM, da-Silva GG, Alves CR. Leishmania (Viannia) braziliensis: influence of successive in vitro cultivation on the expression of promastigote proteinases. Exp Parasitol. 2010; 126(4), 570-6.

Resources for Health Professionals. Centers for disease control and prevention (CDC).2014.http://www.cdc.gov/parasites/leishmaniasis/health_professionals/index.htmlHide

Saha P, Gogoi P, Saikia AK. Synthesis of oxabicyclo[3.3.1]nonenes and substituted tetrahydropyrans via (3,5)-oxonium-ene reaction. Org Biomol Chem.

Saldanha AC, Romero GA, Guerra C, Merchan-Hamann E, Macedo VO. Comparative study between sodium stibogluconate BP 88 and meglumine antimoniate in cutaneous leishmaniasis treatment. II. Biochemical and cardiac toxicity. Rev Soc Bras Med Trop. 2000; 33(4):383-8.

Saliba EK, Oumeish OY. Reservoir hosts of cutaneous leishmaniasis. Clin Dermatol. 1999; 17(3):275–7.

Sampaio RNR, Sampaio JHD, Mardsen PD. Pentavalent antimonial treatment in mucosal leishmaniasis. Lancet 1985; 1(8437):1097.

Santos LC, Honda NK, Carlos IZ, Vilegas W. Intermediate reactive oxygen and nitrogen from macrophages induced by Brazilian lichens. Fitoterapia. 2004 ;75(5), 473-9.

Schneider P, Rosat JP, Bouvier J, Louis J, Bordier C. Leishmania major: differential regulation of the surface metalloprotease in amastigote and promastigote stages. Exp. Parasitol. 1992; 75 196-206.

Sereno D, Holzmuller P, Mangot I, Cuny G, Ouaissi A, Lemesre JL. Antimonial-mediated DNA fragmentation in Leishmania infantum amastigotes. Antimicrob Agents Chemother. 2001 Jul; 45(7):2064-9.

Shaw S. Leishmaniasis. Tijdschr Diergeneeskd. 2003; 128(10):320-1.

Shio MT, Hassani K, Isnard A, Ralph B, Contreras I, Gomez MA, Abu-Dayyeh I, Olivier M. Host cell signalling and Leishmania mechanisms of evasion. J. Trop. Med. 2012; 819512.

Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, Alves CR. Proteinases as virulence factors in Leishmania spp. infection in mammals. Parasit Vectors. 2012; 7, 5:160.

Silva-Almeida M, Souza-Silva F, Pereira BA, Ribeiro-Guimarães ML, Alves CR. Overview of the organization of protease genes in the genome of Leishmania spp. Parasit Vectors. 2014; 20; 7:387.

Silva-Almeida M. Leishmania (Viannia) braziliensis, cepa MCAN/BR/1998/R619: variabilidade no perfil biológico e expressão gênica de proteases. Tese de doutorado

Silva-Lopez RE, Morgado-Díaz JA, Chávez MA, Giovanni-De-Simone S. Effects of serine protease inhibitors on viability and morphology of Leishmania (Leishmania) amazonensis promastigotes. Parasitol Res. 2007;101(6), 1627-35.

Silveira FT, Ishikawa EA, De Souza AA, Lainson R. An outbreak of cutaneous leishmaniasis among soldiers in Belém, Pará State, Brazil, caused by Leishmania (Viannia) lindenbergi n. sp. A new leishmanial parasite of man in the Amazon region. Parasite. 2002; 9(1):43-50.

Singh S, Sivakumar R. Challenges and new discoveries in the treatment of leishmaniasis. Journal of Infection and Chemotherapy. 2004; 10(6): 307–315.

Smith M, Bringaud F, Papadopoulou B Organization and evolution of two SIDER retroposon subfamilies and their impact on the Leishmania genome. BMC Genomics 2009; 10, 240.

Somanna A, Mundodi V, Gedamu L: Functional analysis of cathepsin B-like cysteine proteases from Leishmania donovani complex. Evidence for the activation of latent transforming growth factor beta. J Biol Chem 2002; 277(28), 25305–25312

Sundar S. Treatment of visceral leishmaniasis. Med Microbiol Immunol. 2001; 190(1-2), 89-92.

Swenerton RK, Zhang S, Sajid M, Medzihradszky KF, Craik CS, Kelly BL, McKerrow JH. The oligopeptidase B of Leishmania regulates parasite enolase and immune evasion. J Biol Chem. 2011; 286(1):429-40.

Thomas S, Green A, Sturm NR, Campbell DA, Myler PJ (2009) Histone acetylations mark origins of polycistronic transcription in Leishmania major. BMC Genomics 10; 152.

Torres DC, Ribeiro-Alves M, Romero GA, Dávila AM, Cupolillo E. Assessment of drug resistance related genes as candidate markers for treatment outcome prediction of cutaneous leishmaniasis in Brazil. Acta Trop. 2013;126(2), 132-41.

Ubeda JM, Legare D, Raymond F, Ouameur AA, Boisvert S, et al. Modulation of gene expression in drug resistant Leishmania is associated with gene amplification, gene deletion and chromosome aneuploidy. Genome Biol. 2008; 9, R115.

World Health Organization (WHO): Control of the leishmaniasis: report of a meeting of the World Health Organization Expert Committee on the Control of Leishmaniasis. Geneva 2010.

Yao C, Donelson JE, Wilson ME, The major surface protease (MSP or GP63) of Leishmania sp. Biosynthesis, regulation of expression, and function, Mol. Biochem. Parasitol. 20031; 32, 1e16.

7. ANEXOS

Anexo 1:

8

Componentes de Superfície do Parasito e o Papel na Interação Parasito-Hospedeiro

Carlos Roberto Alves | Mariana Silva de Almeida | Franklin Souza da Silva
Bernardo Acácio Santini Pereira | Ricardo Luiz de Azevedo Pereira | Luzia Monteiro de Castro Côrtes

Protozoários, como os parasitos pertencentes ao gênero *Leishmania*, apresentam sua superfície recoberta por uma complexa membrana celular, que é a responsável pela manutenção da integridade do meio intracelular, mantendo-o distinto do meio extracelular. Dessa forma, a membrana celular também rege a interação do protozoário com o meio extracelular, atuando na captação de nutrientes e vias de sinalizações que definem mecanismos de adaptação do protozoário.

Como a superfície celular desses protozoários representa o primeiro contato com os diferentes microambientes dos seus hospedeiros (invertebrados e vertebrados), a expressão regulada de componentes de superfície está diretamente relacionada ao sucesso da infecção. Desse modo, os componentes de superfície dos promastigotas e amastigotas são os determinantes da interação parasito/hospedeiro nas leishmanioses e podem significar alvos de ação sobre a cadeia de transmissão da doença em ambos os hospedeiros.

A membrana plasmática desses protozoários apresenta ainda uma camada mais externa, constituída por uma rede entrelaçada de carboidratos ligados covalentemente aos lipídeos e proteínas – o glicocálice ou glicocálix (*glykys* = glicídios e *calyx* = envoltório). Esse envoltório é secretado pela membrana plasmática, por vesículas secretoras, e se mantém aderido a ela, atuando nas interações entre os protozoários e as células dos hospedeiros. Uma vez que a constituição do glicocálice pode variar entre diferentes espécies e entre as formas evolutivas de uma mesma espécie, esta pode ser utilizada para a caracterização de espécies e para o acompanhamento da mudança das formas evolutivas. De maneira geral, o glicocálice dos protozoários *Leishmania* é rico em glicosilfosfatidilinositol (*glycosylphosphatidylinositol* – GPI), lipofosfoglicanos (*lipophosphoglycan* – LPG), glicoinositolfosfolipídeos (*glycoinositolphospholipids* – GIPLs), proteínas e enzimas.

Ao longo deste capítulo será relatada a composição da membrana de superfície de *Leishmania*, com enfoque na atuação desses componentes no ciclo de vida do protozoário. O capítulo pretende apresentar o estado da arte desses componentes nos subgêneros *Leishmania* e *Viannia*, principalmente nas espécies que ocorrem no continente americano.

LIPOFOSFOGLICANO

O lipofosfoglicano (LPG) é um dos principais componentes da superfície dos promastigotas de *Leishmania* sp., participando na constituição do glicocálice desses parasitos (Moody *et al.*, 1993). Foi identificado inicialmente em promastigotas por meio de anticorpos monoclonais, sendo caracterizado como um lipopolissacarídeo com propriedades anfipáticas (Handman, Greenblatt & Goding, 1984). A estrutura e a expressão do LPG variam entre as diferentes espécies e estágios evolutivos do protozoário, mostrando-se uma importante ferramenta na adaptação do parasito ante os diferentes microambientes dos hospedeiros (Pimenta, Saraiva & Sacks, 1991; Saraiva *et al.*, 1995; Olivier, Gregory & Forget, 2005; Assis *et al.*, 2012). Estudos *in vitro* utilizando *Leishmania (Leishmania) major* indicaram que a forma específica de LPG de amastigota desaparece após 48 horas da transformação amastigota-promastigota, enquanto durante a transformação promastigota-amastigota, a forma específica de LPG de amastigota foi detectada 12 horas após a infecção (Glaser *et al.*, 1991). Em outras espécies, como *Leishmania (Leishmania) donovani*, a forma específica de amastigota não é detectada (McConville & Blackwell, 1991).

O tipo de ancoramento do LPG à superfície da membrana plasmática é realizado por um grupamento GPI [1-O-alquil-2-lisofosfatidil(mio)inositol] presente em todas as espécies de *Leishmania* analisadas até o momento (McConville & Ralton, 1997; Turco & Descoteaux, 1992). O GPI apresenta um núcleo fosfossacarídico com galactofuranose (Gal*f*) interna. Uma das características do gênero *Leishmania* é o fato de o GPI dispor de um esqueleto formado por unidades repetitivas de fosfodissacarídeos e na região mais extrema, uma cobertura de oligossacarídeos (Turco *et al.*, 1987; Ilg *et al.*, 1992; McConville & Homans, 1992). Variações dessas estruturas podem ser detectadas dentro de uma mesma espécie e entre espécies de *Leishmania*, revelando um polimorfismo dos oligossacarídeos que as constituem, servindo, assim, como marcador específico de espécies ou do estágio em que se encontra o parasito, como proposto para *Leishmania (Leishmania) mexicana* e *L. (L.) major*.

A presença de resíduos de Gluβ1,3 nas ramificações das unidades repetitivas de Galβ1,4 Manα1-PO4 é um dos aspectos que distingue o LPG das formas promastigotas procíclicas de *Leishmania (Leishmania) infantum* das metacíclicas. Tais modificações são significativas na interação desse parasito com seu vetor, o flebotomíneo *Lutzomya longipalpis*, visto que, em contraste às formas procíclicas, as formas metacíclicas não são capazes de se ligar ao intestino do inseto vetor (Soares *et al.*, 2002).

O LPG das formas procíclicas de *Leishmania (Viannia) braziliensis* foi caracterizado por não conter cadeias laterais de açúcares. Diferente das espécies do subgênero *Leishmania*, as formas metacíclicas de *L. (V.) braziliensis* produzem menos LPG e adicionam resíduos de 1-2 (β1-3)Glicose nas ramificações das unidades repetitivas de Galβ1,4 Manα1-PO4 do LPG. Essa modificação foi descrita como um novo mecanismo na regulação da estrutura do LPG durante a metaciclogênese (Soares *et al.*, 2005).

No hospedeiro invertebrado, o LPG é importante para a fixação de promastigotas procíclicos ao epitélio intestinal, o que representa uma etapa essencial à manutenção do ciclo de vida do parasito no inseto vetor (Mahoney *et al.*, 1999; Sacks *et al.*, 1995; Pimenta *et al.*, 1992). A *L. (V.) braziliensis* apresenta um padrão de adesão ao epitélio intestinal de *Lutzomyia (Nyssomyia) whitmani*, que caracteriza-se como um vetor bastante competente, diferente do observado em *Lu. (N.) intermedia*, sendo que neste último, o LPG parece ter uma importância menor no processo de adesão (Soares *et al.*, 2010). Estudos recentes avaliando a permanência de uma linhagem mutante para LPG de *L. (L.) major* (*null* para o gene *lpg1* que codifica para enzima transferase da galactofuranose) (Spath *et al.*, 2000) no intestino de *Phlebotomus duboscqi* evidenciaram que a ausência da expressão de LPG reduz expressivamente a presença de parasitos 72 horas após a infecção, sendo que após 15 a 21 dias não se detecta a presença de parasitos (Secundino *et al.*, 2010). É interessante notar que a infecção com a cepa mutante em *Lu. longipalpis*, vetor não natural desse parasito, mostrou-se

mais efetiva do que com a cepa selvagem, indicando que o LPG não é necessário para infecções em vetores não naturais, fato também observado em outros estudos (Secundino *et al.*, 2010; Myskova *et al.*, 2007).

Um polipeptídeo de 65 kDa no epitélio do trato digestório de *Phlebotomus papatasi* foi descrito como ligante de LPG das formas procíclicas *L. (L.) major* (Dillon & Lane, 1999). Posteriormente, em uma série de experimentos, a PpGalec, uma galectina expressa no epitélio intestinal de *P. papatasi*, demonstrou ser crucial para sobrevivência e transmissão de *L. (L.) major* por meio de sua ligação com LPG (Kamhawi *et al.*, 2004). No inseto vetor – após o escape da matriz peritrófica –, os promastigotas necessitam aderir ao epitélio intestinal. Essa adesão evita a excreção dos promastigotas do interior da luz do intestino, assim como permite a sua multiplicação e posterior diferenciação, sendo um fenômeno essencial à manutenção do ciclo de vida.

No hospedeiro vertebrado, o LPG é um importante ligante de macrófagos, estando diretamente envolvido nas etapas iniciais da infecção. A utilização de uma cepa mutante de *L. (L.) major*, deficiente no gene *lpg1* (*lpg1*-), revelou que o parasito apresenta uma atenuação de sua virulência nas infecções de macrófagos de camundongo. Os promastigotas *lpg1*- se mostraram altamente suscetíveis à ação do sistema complemento, além de terem sido afetados por agentes oxidantes produzidos pelas células do hospedeiro e perderem a capacidade de inibir a fusão do fagolisossomo (Spath, 2000; Handman & Goding, 1985; Spath *et al.*, 2003).

Sabe-se também que o LPG de *Leishmania* spp. exerce uma ação inibitória sobre a atividade da proteína quinase C (PKC) de macrófagos, favorecendo o estabelecimento e manutenção da infecção, uma vez que essa enzima é um componente-chave do processo de ativação de tais células (Turco, 1999; Delgado-Domínguez *et al.*, 2010). Embora esse efeito do LPG seja um fato estabelecido, o mecanismo de sinalização intracelular envolvido na resposta oxidativa que lesa o parasito ainda não foi esclarecido. Um avanço na compreensão da via de ação do LPG foi a descrição de que na infecção murina por *L. (L.) donovani* há uma ação seletiva sobre a isoforma PKC β dependente de cálcio, com diminuição da expressão e atividade dessa enzima (Bhattacharyya *et al.*, 2001). Além disso, estudo avaliando ação de três tipos de LPG de *L. (L.) infantum* indicou que apenas uma variante é capaz de estimular a produção de óxido nítrico em macrófagos murinos (Coelho-Finamore *et al.*, 2011).

Em relação ao sistema complemento, o LPG de promastigotas metacíclicas de *L.(L.) major* inibe a formação do complexo de ataque à membrana em virtude de suas longas cadeias de açúcar. As modificações que ocorrem na superfície dos promastigotas durante a transição entre o estágio não infectivo (forma procíclica) ao infectivo (forma metacíclica) obstruem a inserção do componente lítico C5b-9 na membrana dos promastigotas infectivos (Puentes *et al.*, 1990; Puentes *et al.*, 1988; Sacks, 1992). Por sua vez, LPG também pode atuar na ativação do complemento, com deposição de C3b covalentemente ligado à membrana celular, o que proporciona a ligação dos promastigotas ao macrófago via receptor C3bi, com a posterior endocitose das formas metacíclicas do parasito e lise das formas não infectivas (Puentes *et al.*, 1988; Mosser, Springer & Diamond, 1992).

Além de atuar sobre macrófagos, o LPG de *L. (L.) major* promove também a ativação da resposta imune inata com a participação de linfócitos *natural killer* (NK) e de células dendríticas por intermédio do receptor *toll-like* 2 (Becker *et al.*, 2003; Brandonisio, Spinelli & Pepe, 2004; Soong, 2008). Em linfócitos NK, esta interação do LPG com o receptor *toll-like* 2 leva a um aumento da síntese de interferon gama (INF-γ) e fator de necrose tumoral alfa (*tumor necrosis factor alfa* – TNF-α), estimulando subpopulações de linfócitos T, o que conferiu certo grau de imunidade à infecção em animais de experimentação (Becker *et al.*, 2003).

Além dessas características indicarem o LPG como um dos possíveis candidatos à vacina contra as leishmanioses (Moody *et al.*, 1993; Tonui *et al.*, 2001), estudos em camundongos infectados previamente com cepas mutantes de *L. (L.) major*, deficientes nos genes *lpg*, indicaram uma resposta imune protetora contra infecção de cepas virulentas na ausência de uma forte resposta Th1 (Uzonna *et al.*, 2004).

GLICOINOSITOLFOSFOLIPÍDEOS

Os glicolipídeos majoritariamente estudados em *Leishmania* são os glicoinositolfosfolipídeos (GIPLs). Também denominados GPIs livres, são glicolipídeos de baixo peso molecular e altamente expressos na superfície do parasito tanto nas formas promastigotas como nas amastigotas. Os GILPs de *L. (L.) major* contêm de quatro a seis resíduos de açúcar ligados ao lipídeo liso-alquil-fosfatidilinositol (McConville *et al.*, 1990).

Estudos vêm demonstrando que os GIPLs auxiliam na sobrevivência de *L. (L.) major* dentro de macrófagos, inibindo a síntese de óxido nítrico principalmente pela região alquil-acil-glicerol (Proudfoot, O'Donnell & Liew, 1995; Zuffery *et al.*, 2003). Além disso, os GILPs, de modo semelhante ao LPG, provavelmente pela porção GPI comum aos dois, atuam na inibição da atividade de PKC (Zufferey *et al.*, 2003). Diferenças na estrutura do GILPs sugerem que sejam espécie-específica, cepa-específica e estágio-específico (McConville & Blacwell, 1991; McConville *et al.*, 1994).

A taxa de infecção de macrófagos por *L. (V.) braziliensis* tem sido relacionada com microdomínios de lipídeos da membrana externa (*detergent-resistant membranes* – DRMs) de promastigotas contendo GILPs. A desestruturação desses domínios é capaz de induzir a uma diminuição na taxa de infecção de macrófagos por *L. (V.) braziliensis*, sugerindo uma relação entre os microdomínios contendo GILPs e a infectividade desses parasitos (Yoneyama *et al.*, 2006).

PROTEÍNAS E ENZIMAS

Inúmeras proteínas têm sido verificadas na superfície das formas amastigotas e promastigotas de espécies de *Leishmania*, com base em estudos com marcações radioativas associadas às técnicas de imunoprecipitação, ou *Western blotting*, utilizando anticorpos monoclonais e soro imune de paciente e animais. Assim, uma faixa muito ampla de proteínas, com massa molecular relativa de 10 kDa até 120 kDa, tem sido encontrada. Poucas dessas proteínas têm sua função conhecida, sendo uma notável exceção a proteína ligante de laminina com 67 kDa, identificada na superfície de promastigota e amastigota de *L. (L.) donovani* (Bandyopadhyay *et al.*, 2001). Assim como o observado para as diferentes formas de LPG e GILPs, muitas das proteínas encontradas parecem ser estágio-específicas, como é o caso da proteína de 116 kDa presente em promastigotas infectivas de *L. (L.) major* (Sacks, Hieny & Sher, 1985) e das proteínas de 23 kDa, 52 kDa e 68 kDa em promastigotas e 38 kDa, 70 kDa e 74 kDa em amastigotas de *L. (L.) pifanoi* (Pan, 1986).

Um dos primeiros estudos sobre as proteínas de superfície de *L. (V.) braziliensis*, realizado em 1985, foi uma simples comparação entre a superfície celular dos promastigotas patogênicos e os não patogênicos. Nesse estudo foi constatado que a superfície celular desses promastigotas é diferente quanto aos receptores de lectinas e quanto às cargas (Ayesta, Arguello & Hernandez, 1985). Nesse mesmo ano foi descrita outra estratégia para a análise das proteínas de superfície desse parasito, a qual inclui a iodinação da superfície celular seguida do procedimento fracionamento com detergente não iônico e cromatografia de troca iônica, para isolar uma proteína de 65 kDa de promastigotas infectivos (Misle, Márquez & Hernandez, 1985). No ano seguinte foram isoladas duas glicoproteínas da membrana celular de promastigotas de *L. (V.) braziliensis*, uma de 53 kDa com afinidade de ligação à concanavalina A e à *Ricinus communis* e outra de 47 kDa com afinidade de ligação a *Dolichos biflorus* (Nagakura *et al.*, 1986).

A proteína de 11 kDa de cinetoplastídeos (*Kinetoplastid membrane protein of* 11 kDa –KMP-11) e as hidrofílicas aciladas (*hydrophilic acylated surface protein* – HASP) também estão localizadas na membrana plasmática de *Leishmania* spp.. Tais proteínas não apresentam domínio transmembrana, peptídeo sinal e nem âncora de GPI, e são isoladas por solventes orgânicos juntamente com o LPG. A KMP-11 apresenta fortes características hidrofóbicas e está associada ao LPG na superfície celular do parasito (Jardim *et al.*, 1995). Já foi descrita em diversos gêneros de tripanossomatídeos (Stebeck *et al.*, 1995), contudo suas propriedades imunorregulatórias foram confirmadas somente em pacientes com leishmaniose cutânea e mucosa, uma vez que induzem a expressão de interleucina-10

(Carvalho *et al.*, 2005). Um estudo recente demonstrou sua presença em amastigotas e promastigotas de *Leishmania* (*Leishmania*) *amazonensis* e também que sua expressão aumenta na superfície dos parasitos durante a metaciclogênese (Matos *et al.*, 2010).

As proteínas hidrofóbicas aciladas de superfície (*hydrophobic acylated surface proteins* – HASPs) têm características hidrofílicas além de serem distintamente reguladas nas formas evolutivas do parasito e encontradas com predominância em amastigotas e em abundância na superfície de formas infectivas de *L. (L.) major* (Flinn, Rangarajan & Smith, 1994). Estas proteínas são expressas em genes em uma mesma região cromossômica nas espécies do gênero *Leishmania* (Depledge *et al.*, 2010). Provavelmente a dupla acilação que apresentam deve mediar a associação com a membrana, agindo como uma âncora de superfície. Entre 70% a 80% das HASPs são observadas na face citosólica da membrana celular (Denny *et al.*, 2000). Já foi proposto que em pacientes com leishmaniose visceral causada pela *Leishmania (Leishmania) chagasi*, as HASPs estariam atuando na imunomodulação da infecção (Vinhas *et al.*, 1994).

A caracterização de genes por meio de métodos moleculares tem possibilitado a associação de sequências de nucleotídeos com proteínas presentes em tripanossomatídeos. É o caso de uma sequência de nucleotídeos obtida de *L. (L.) donovani*, a qual codifica para uma proteína com 198 aminoácidos, que apresentou 36% de identidade com a proteína amastina da superfície de *Trypanosoma cruzi* (Wu *et al.*, 2000). No *T. cruzi* a amastina foi caracterizada com glicoproteína e atua na invasão e proliferação intracelular desse parasito (Jackson, 2010). Em razão de ausência de estudos sobre a funcionalidade dessa proteína em *Leishmania* spp., não se pode afirmar que tenha a mesma função da amastina de *T. cruzi*. Porém, sabe-se que o gene dessa proteína, semelhante à amastina, é mais expresso na fase estacionária e principalmente em amastigotas de *L. (L.) donovani*, podendo, dessa forma, estar relacionado à sobrevivência de amastigotas dentro do fagolisossomo (Wu *et al.*, 2000).

Certas proteínas presentes na superfície do parasito parecem ter um papel na via de sinalização que leva à mudança de estágio dentro do ciclo de vida dos mesmos, como os receptores de adenilato ciclase (receptor AC). Essa enzima cataliza a conversão de adenosida trifosfato (ATP) a adenosina monofosfato cíclico (AMPc) e, assim, participam de uma via de sinalização do AMPc já descrita em protozoários kinetoplastida (Seebeck, Schaub & Johner, 2004). Esses receptores foram descritos em *L. (L.) donovani* e podem induzir a mudanças de estágio pela elevação de AMPc intracelular, fato observado em *T. cruzi* (Sanchez *et al.*, 1995; Biswas, Bhattacharya & Das, 2011).

A glicoproteína de 46 kDa, chamada de gp46/M-2, descrita na superfície de *L. (L.) amazonensis* (Kahl & McMahon-Pratt, 1987), apresenta notável resistência à digestão proteolítica. Sua estrutura contém uma sequência repetitiva de 24 aminoácidos, a qual corresponde a 22% do total da proteína madura, e um teor glicídico de 7%, correspondente a 3 kDa, além de ser ligada à membrana pela âncora de GPI (Lohhman, Langer & McMahon-Pratt, 1990). A gp46/M-2 é codificada pela mesma família de genes polimórficos do complexo de antígenos-2 de superfície de promastigotas, descrito previamente em *L. (L.) major*, e observa-se em sua estrutura regiões conservadas com esses antígenos (Murray, Spithill & Handman, 1989).

Dentre todas as espécies de *Leishmania* já estudadas, os genes que codificam gp46/M-2 não foram encontrados em somente duas espécies: *L. (V.) braziliensis* e *Leishmania (Leishmania) enrittii* (Hanekamp & Langer, 1991; McMahon-Pratt *et al.*, 1992). Além disso, a expressão de RNA mensageiro (mRNA) da gp46/M-2 de *L. (L.) chagasi* apresenta indícios de ser mais elevada em promastigotas de fase estacionária de cultura, levando a crer que seria um fator de virulência desse estágio evolutivo do parasito (Beetham *et al.*, 1997). Outro fato importante é que gp46/M-2 de *L. (L.) amazonensis* proporciona uma proteção contra a infecção dessa espécie em camundongos susceptíveis (Champsi & McMahon-Pratt, 1988; McMahon-Pratt *et al.*, 1993). Tal observação sugere que a gp46/M-2 seria outro antígeno candidato a vacina contra a leishmaniose (Liew & O'Donnell, 1993).

As espécies de *Leishmania* têm várias proteínas reconhecidas como membros de famílias conservadas durante a evolução (Requena, Alonso & Soto, 2000). Dentre essas, se destacam os proteofosfoglicanos (*proteophosphoglycan* – PPG), caracterizados como polipeptídeos altamente glicosilados. São proteínas que apresentam ligação de fosfossacarídeos à cadeia peptídica por *O*-glicosilação. Os PPGs podem ser encontrados na membrana celular do parasito ancoradas por GPI, sendo dessa forma denominadas mPPGs (Ilg *et al.*, 1999). A função dos mPPGs ainda não está totalmente clara, porém especula-se que, em razão da sua longa cadeia cobrindo a membrana plasmática dos parasitos, tenha papel importante como ligante a receptores de macrófagos e de células do trato digestório do inseto vetor (Ilg, 2000).

Em promastigotas, um PPG secretado e filamentoso (fPPG) forma um agregado viscoso, estabelecendo uma rede de filamentos fibrosos como um gel. O fPPG é secretado pela bolsa flagelar (Stierhof *et al.*, 1994) com 95% de sua composição de fosfoglicanos e a porção peptídica apresentando abundância de serina, alanina e prolina. Sua extensiva glicosilação potencialmente confere capacidade de resistir à ação de proteinases, embora ainda não existam claras evidências nesse sentido. Além disso, sua participação parece ser importante no bloqueio do intestino anterior do inseto vetor, o que impede a ingestão de alimento e leva o inseto a sucessivas tentativas de se alimentar, aumentando as chances de infecção dos hospedeiros vertebrados por *Leishmania* spp. (Ilg, 2000; Walters *et al.*, 1989).

Amastigotas secretam dentro do vacúolo parasitófaro outro tipo de PPG, que estruturalmente são definidos como uma cadeia polipeptídica modificada com fosfoglicanos ligados a resíduos de serina (Ilg, 2000) e contêm açúcares semelhantes aos encontrados em LPG e nas fosfatases ácidas. A secreção deste PPG modificado dentro de macrófagos parece contribuir para manutenção do vacúolo parasitóforo (Peters, Stierhof & Ilg, 1997). Adicionalmente, essa molécula também é capaz de ativar o complemento via proteína ligadora de manose (Peters *et al.*, 1997), podendo assim contribuir para ligação da *Leishmania* spp. à célula hospedeira.

Várias espécies de *Leishmania* estudadas revelaram a expressão de fosfatases ácidas (*acid phosphatase* – AcP) durante seu ciclo biológico (Shakarian & Dwyer, 2000). Três destas AcP foram descritas na membrana de *L. (L.) donovani* com 128, 132 e 108 kDa e pH ótimo de atividade próximo a 5,5 (Remaley *et al.*, 1985). O genes de *Leishmania* para as fosfatases ácidas secretadas (SAcP) e de membrana (MAcP) mostraram-se homólogos tanto em espécies viscerotrópicas como dermotrópicas (Shakarian & Dwyer, 2000; Shakarian *et al.*, 2002).

Tanto as MAcPs (que apresentam peso molecular 120 kDa e 134 kDa) quanto as SAcPs podem ser apontadas como uma das estratégias de sobrevivência do parasito no trato alimentar do inseto vetor, e também no interior do macrófago, uma vez que interferem na produção de metabólitos oxidativos (H_2O_2, OH^-, O^{2-}) tóxicos ao protozoário (Buchmuller-Rouiller & Mauel, 1987; Glew *et al.*, 1988). Essas ectoenzimas são monoesterases capazes de hidrolisar uma variedade de substratos fosforilados, principalmente fosfolipídios e fosfoproteínas e, dessa forma, interferem na produção de radicais livres pelos macrófagos (Glew *et al.*, 1988). Contudo, seu papel na resposta imunológica ainda não está completamente elucidado. Estudos com amostras de soro de pacientes com leishmaniose visceral (Ellis, Shakarian & Dwyer, 1998) detectaram anticorpos contra SAcPs de *L. (L.) donovani*, sugerindo que sua expressão em amastigotas induz a um estímulo da resposta imune humoral.

Outras classes de enzimas presentes na membrana plasmática, no flagelo e na bolsa flagelar de diversas espécies de *Leishmania* são as 3'-nucleotidases (~43 kDa) (Côrte-Real *et al.*, 1993) e 5'-nucleotidases (~70 kDa) (Campbell *et al.*, 1991). Essas enzimas hidrolisam nucleotídeos a ácidos nucléicos 3'-AMP e 5'-AMP, respectivamente (Farajnia *et al.*, 2004; Gottlieb & Dwyer, 1983; Hansen *et al.*, 1982). As nucleotidases teriam papel nutricional importante, já que esses parasitos não são capazes de sintetizar purina via síntese *de novo* (Debrabrant, Gottlieb & Dwyer, 1995). A identificação de genes homólogos dessa enzima em várias espécies de *Leishmania* confirma que a 3'-nucleotidase é conservada nesse gênero (Debrabant, Gottlieb & Dwyer, 1995), corroborando o seu papel crítico na captação de purinas. A remoção de porção C-terminal da enzima causa sua liberação da membrana plasmática de *L. (L.) donovani*, demonstrando que

esse domínio é responsável por seu ancoramento. Ainda assim, sua atividade enzimática não sofre interferência com a remoção desse domínio e nem com a remoção de suas *N*-glicosilações (Debrabant, Ghedin & Dwyer, 2000). Como a 3'-nucleotidase não foi detectada em células de mamíferos, ela surge como outra possibilidade de alvo para ação de quimioterápios mais seletivos (Farajnia *et al.*, 2004; Gbenle & Dwyer, 1992).

As enzimas transportadoras de íons Ca^{2+} (Ca^{2+}-ATPase) também foram descritas na membrana de superfície da *Leishmania* spp. e atuam como uma bomba de Ca^{2+}. Tais enzimas apresentam alta afinidade com esse íon e sua atividade está estritamente relacionada com a calmodulina, visto que antagonistas da calmodulina bloqueiam completamente sua atividade (Banerjee, Sarkar & Bhaduri, 1999; Benaim *et al.*, 1993). Com duas subunidades (51 kDa e 57 kDa) fortemente associadas à membrana plasmática (Ghosh *et al.*, 1990), conta com um sítio aloestérico ao Mg^{2+} capaz de modular sua atividade cinética (Mazumder *et al.*, 1992).

A homeostase da concentração submicromolar de Ca^{2+} intracelular de *L. (V.) braziliensis*, *L. (L.) mexicana*, *T. cruzi* e *Trypanosoma brucei* é mantida pela atividade da Ca^{2+}-ATPase presente na membrana plasmática desses parasitos (Benaim & Romero, 1990). Também foi proposto que o aumento da atividade da Ca^{2+} ATPase presente na membrana plasmática de *L. (L.) amazonensis* pode determinar a regulação dos níveis de cálcio no interior do fagossomo (Côrte-Real, Santos & Meirelles, 1995).

Enzimas transportadoras de íons Mg^{2+} também foram descritas na membrana celular de *Leishmania* spp., sendo denominadas Mg^{2+}-ATPases. As Mg^{2+}-ATPases de *L. (L) donovani* têm sua atividade modulada pela fluidez da membrana (Dutta *et al.*, 1990). Tais enzimas parecem ter uma função de bomba de extrusão de H^+, além de ter um possível papel na acumulação de glicose e potássio no meio intracelular (Dutta *et al.*, 1990; Mukherjee, Mandal & Bhaduri, 2001). Alterações na atividade dessas proteínas podem estar relacionadas com a virulência do parasito, participando da pré-adaptação dos promastigotas para sobrevivência dentro dos macrófagos (Berredo-Pinho *et al.*, 2001; Lu *et al.*, 1997).

As proteinases consistem em outra categoria de proteínas descrita na membrana celular de *Leishmania* spp. Entre as quatro classes de proteinase descritas nesse parasito (Alves *et al.*, 2005; Silva-Lopez *et al.*, 2004; Bouvier, Etges & Bordier, 1985; Silva-Lopez & Giovanni-De-Simone, 2004), somente as metaloproteinases e as cisteína-proteinases foram localizadas na membrana plasmática de várias espécies de *Leishmania*, até o momento.

A gp63 é uma das principais glicoproteínas encontradas na superfície de promastigotas de *Leishmania* spp., com massa molecular aproximada de 63 kDa. É uma zinco-metaloproteinase e está fixada à membrana plasmática por meio de âncora de GPI, evidenciando polimorfismo entre espécies, além de ter uma expressão reduzida em amastigotas (Olivier & Hassani, 2010; Davies *et al.*, 1990; Muskus & Marin, 2002). Nos últimos anos, tem sido demonstrado que a massa molecular das metaloproteinases de *Leishmania* spp. não é muito homogênea, podendo ser detectadas enzimas desde 50 kDa e mesmo acima de 63 kDa (Alves, Mendonça-Lima & Alves, 2004; Yao *et al.*, 2004; Cuervo *et al.*, 2006).

Não obstante as metaloproteinases terem sido descritas como proteinases de membrana em espécies de *Leishmania*, estudos recentes indicam que isoformas dessas enzimas têm localização intracelular em *L. (L.) mexicana* e em *L. (L.) chagasi* (Weise *et al.*, 2000; Yao *et al.*, 2005). Também uma análise por microscopia confocal sobre a distribuição dessas enzimas em *L. (V.) braziliensis* demonstrou a existência intracelular de metaloproteinases com domínios homólogos a gp63, localizados próximos à bolsa flagelar (Cuervo *et al.*, 2008).

A importância das metaloproteinases no ciclo biológico da *Leishmania* spp. ainda é alvo de muitos estudos. As metaloproteinases têm atividade proteolítica sobre numerosos substratos, podendo estar envolvidas na degradação de macromoléculas do hospedeiro. Essas enzimas estão associadas à hidrólise e inativação de imunoglobulinas G (Etges, Bouvier & Bordier, 1986; Mendonça-Lima & Atta, 1992) e têm propriedade de inativar o fator C3b, em C3bi, do complemento (Bogdan & Rollinghoff, 1998; Chaudhuri & Chang, 1988). Essa última atividade pode auxiliar a

internalização dos promastigotas no macrófago, tendo como consequência C3bi atuar como opsonina, ligando ao LPG ou à própria gp63 (Bogdan & Rollinghoff, 1998).

Protótipos de vacina oral usando *Salmonella typhimurium* atenuada contendo o gene da gp63 induziram um fenótipo Th1 de proteção, com linfócitos T $CD4^+$, para infecção por *L. (L.) major* em camundongos BALB/c (Xu *et al.*, 1995). O êxito de vacinas de cDNa também foi observado no controle da infecção de BALB/c por *L. (L.) mexicana* com indução de linfócitos T citotóxicos (Ali *et al.*, 2009). Também camundongos BALB/c vacinados com gp63 em lipossomos catiônicos induz proteção contra infecção por *L. (L.) donovani* (Bhowmick, Ravindran & Ali, 2008). Por sua vez, a vacinação de cães com plasmídeos de DNA codificante da gp63, e outras proteínas de *Leishmania*, não induziu proteção desses animais contra a infecção experimental por *L. (L.) infantum* (Rodriguez-Cortes *et al.*, 2007). Mesmo sem uma clara definição do perfil de proteção que a gp63 pode induzir, ainda é plausível o uso dessa proteína na construção de vacinas para as leishmanioses.

Estudos *in silico*, com sequência da *L. (V.) braziliensis*, indicaram que regiões do sítio catalítico da metaloproteinases apresentam sequências conservadas e que regiões que codificam os domínios de superfície estão possivelmente envolvidas nas interações parasito-hospedeiro: sítios de adesão a macrófago e epitopos de linfócitos T e B imunodominantes (Victoir *et al.*, 2005).

Além disso, já foi proposta uma função protetora da gp63 contra a ação de tripsina e quimiotripsina do inseto vetor, enzimas cuja expressão aumente após o repasto sanguíneo e, dessa forma, podem interferir no desenvolvimento de parasitos no intestino do inseto. Observou-se que a atividade de tripsina e quimiotripsina acarreta a diminuição da subpopulação de promastigotas com expressão menor de gp63 no intestino do vetor, quando este se encontra repleto de sangue (Pimenta *et al.*, 1997).

Várias cisteína-proteinases (CPs) foram descritas em espécies de *Leishmania* que causam a forma tegumentar e visceral da doença. Inicialmente essas enzimas foram descritas em amastigotas de *L. (L.) mexicana*, que são particularmente ricas em CPs, principalmente encontrada em megassomas, com possível papel na virulência no hospedeiro vertebrado (Pupkis & Coombs, 1984; Robertson & Coombs, 1990).

Posteriormente verificou-se que essa enzima poderia ser detectada em outras espécies de *Leishmania* e que a mesma não estaria exclusivamente relacionada às formas infectivas do parasito (Alves, Marzochi & Giovanni-de-Simone, 1993; Omara-Opyene & Gedamu, 1997). Com a descrição do genoma de *L. (L.) major*, foi observada a existência de genes que codificam um total de 65 CPs, agrupadas em quatro clãs e 13 famílias (Mottram, Coombs & Alexander, 2004).

A possibilidade de CPs na superfície celular foi inicialmente demonstrada utilizando-se o método de extração com o detergente Triton X-114 (Alves, Marzochi & Giovanni-de-Simone, 1993). Nesse trabalho, os autores demonstraram que promastigotas não infectivos de *L. (L.) major* apresentam uma proteína hidrofóbica (20 kDa) com atividade enzimática sensível à presença de um inibidor de cisteína-proteinases [L-trans-epoxisuccinil-leucilamida-(4-guanidino) butano]. Estudos posteriores, nos quais foi utilizado soro de coelho antipapaína, uma CP de *Caryca papaia*, demonstraram a presença de proteínas reconhecidas por esse antissoro na superfície da membrana celular e na bolsa flagelar de *L. (L.) amazonensis* (Alves *et al.*, 2005; Mottram, Coombs & Alexander, 2004). Hoje, sabe-se que algumas isoformas de CP estão presentes na membrana de superfície de promastigotas de *L. (V.) braziliensis* associadas a âncoras GPI (Rebello *et al.*, 2009).

A maioria dos estudos sobre a atividade das CPs está centrada nos grupos das enzimas denominadas CPA, CPB e CPC, e cada um deles apresenta características que lhes são próprias: CPA – constituído por enzimas da subfamília das catepsinas L. Esse grupo caracteriza-se por ser codificado por um gene de cópia única e pela ausência de uma longa extensão COOH-terminal antes de seu processamento final (Mottram *et al.*, 1992; Mottram & Coombs, 1998); CPB – constituído por enzimas da subfamília das catepsinas L. Esse grupo caracteriza-se por uma longa extensão

COOH-terminal nas formas das enzimas antes de seu processamento final. Além disso, as enzimas CPB são expressas por genes com múltiplas cópias, organizados em sequências em *tandem*. Essas isoformas apresentam variações entre suas especificidades por substratos e propriedades catalíticas (Brooks *et al.*, 2001); CPC – constituído por enzimas da subfamília das catepsinas B. Esse grupo, de forma similar ao grupo CPA, é codificado por um gene em cópia única e não apresenta uma extensão COOH-terminal como as enzimas CPB (Bart, Coombs & Mottram, 1995).

As CPs de *Leishmania* spp. podem atuar como moduladores da resposta imune nas infecções tegumentares. Nesse particular, é válido ressaltar que sequências peptídicas da região COOH-terminal das CPBs têm potencial imunorregulador na resposta imune celular para casos humanos de LTA causados por *L. (V.) braziliensis* (Alves *et al.*, 2001) e no desenvolvimento da infecção experimental murina por *L. (L.) amazonensis* (Alves *et al.*, 2004, Pereira *et al.*, 2011).

Existem evidências da ação de CPs de *Leishmania* atuando na regulação do sistema imune do hospedeiro, tanto induzindo este a apresentar uma resposta tipo Th2 quanto inibindo uma resposta do tipo Th1. As CPs de *L. (L.) mexicana* e de *L. (L.) amazonesis* são capazes inibir a apresentação de antígenos pela degradação da molécula do complexo principal de histocompatibilidade classe II no vacúolo parasitóforo do hospedeiro (Souza Leão *et al.*, 1995).

A geração de mutantes deficientes nos genes das CPB (Δ*cpb*) permitiu a obtenção de informações sobre a importância dessas CPs na interação parasito-hospedeiro. Mutantes de *L. (L.) mexicana* Δ*cpb* apresentaram virulência reduzida e pouca capacidade de promover lesões em camundongos BALB/c (Alexander, Coombs & Mottram, 1998) e somente a reinserção de múltiplos genes CPB nesses mutantes por um cosmídeo foi capaz de efetivamente restaurar sua virulência, sugerindo que tais genes têm funções complementares (Denise *et al.*, 2003).

Além disso, existem indícios de que a enzima CPB de *L. (L.) mexicana* ativa é capaz de clivar os receptores de IL-2 e de IgE e de induzir à expressão de IL-4 em camundongos, promovendo a resposta Th2. Camundongos infectados com parasitos mutantes Δ*cpb* foram capazes de apresentar uma resposta do tipo Th1 e, dessa forma, debelar a infecção (Pollock *et al.*, 2003). Também já foi descrita a capacidade de diferentes espécies de *Leishmania*, inclusive da *L. (L.) mexicana*, de inibir a produção de IL-12 em macrófagos e células dentríticas (Weinheber *et al.*, 1998). Esse processo é, ao menos em parte, dependente da CPB, uma vez que parasitos do tipo selvagem contam com uma capacidade maior de inibir a produção de IL-12 do que mutantes Δ*cpb*, e inibidores de CPB são capazes de impedir essa ação dos parasitos. É possível que tal capacidade seja resultado da clivagem proteolítica tanto do fator de transcrição kappa B do hospedeiro quanto de seu inibidor IκB pela CPB (Cameron *et al.*, 2004).

Não obstante homólogos de CPB terem sido detectados na membrana celular de promastigotas de *L. (V.) braziliensis* (Rebello *et al.*, 2009), assim como outras cisteína-proteinases em *L. (L.) amazonensis* (Alves *et al.*, 2000), essas enzimas estão presentes nos grandes lisossomos do estágio amastigota e também podem ser liberadas na matriz extracelular, o que pode facilitar alguns de seus efeitos (Mottram, Brooks & Coombs, 1998).

Proteínas que ligam à heparina (PLHs) também foram descritas na superfície de *Leishmania* spp. (Butcher *et al.*, 1990; Azevedo-Pereira *et al.*, 2007; Mukhopadhyay *et al.*, 1989) e outros tripanossomatídeos (Kock *et al.*, 1997; Ortega-Barria & Pereira, 1991). A maior parte dos estudos com PLHs de *Leishmania* spp. tem sido desenvolvida com *L. (L.) donovani*, *L. (L.) major* e *L. (L.) amazonensis*, e só recentemente foi proposto o estudo dessa proteína em *L. (V.) braziliensis*. Nesta espécie, a PLH sinalizou uma possível participação na interação com proteínas do intestino de *Lu. intermedia* e de *Lu. Whitmani* (Azevedo-Pereira *et al.*, 2007).

Nos parasitos nos quais as PLHs foram estudadas, verificou-se que essas moléculas agem como proteínas de adesão e podem promover a interiorização dos mesmos com as células hospedeiras. Experimentos realizados com promastigotas de *L. (L.) donovani* demonstraram que em torno de 860 mil moléculas de PLHs são encontradas na

superfície do parasito, mais precisamente na porção flagelar dos promastigotas, e que por meio de ligação com heparina pudesse induzir a inibição da atividade de PKCs sobre a superfície celular do parasito (Butcher *et al.*, 1990; Mukhopadhyay *et al.*, 1989).

As PLHs estariam relacionadas às formas infectivas de *L. (L.) donovani*, já que estas predominam em promastigotas de fase estacionária de cultivo e que sucessivas passagens desses parasitos em meio de cultivo levam à perda da capacidade de os mesmos se ligarem à heparina (Kock *et al.*, 1997; Butcher *et al.*, 1992). Amastigotas de *L. (L.) amazonensis* e de *L. (L.) major* têm maior capacidade de se ligar à heparina do que promastigotas das mesmas espécies (Volf, Svobodova & Dvorakova, 2001). Além disso, glicosaminoglicanos como heparina influenciam o desenvolvimento de *L. (L.) major* no intestino do inseto vetor, aumentando a carga parasitária dos insetos experimentalmente infectados.

Uma recente discussão alerta para o fato de que as PLHs, assim como o LPG, estariam participando dos fenômenos moleculares de ligação entre promastigotas de *L. (V.) braziliensis* e o trato digestório de espécies de *Lutzomyia* que atuam como vetores na leishmaniose tegumentar americana (Alves, Côrtes & Brazil, 2010). Assim, outros componentes da superfície dos promastigotas de *L. (V.) braziliensis*, como as proteínas, podem estar envolvidos nas etapas de ligação desse parasito ao intestino do vetor. A habilidade de os promastigotas estarem aderidos às microvilosidades do trato digestório dos flebotomíneos é um passo essencial para manutenção do ciclo de vida do parasito, sendo um fator que distingue parasitos infectivos dos não infectivos.

Demonstrou-se, em ensaios *in vitro*, que a heparina e o sulfato de heparan são os principais GAGs envolvidos no fenômeno de inibição da ligação de promastigotas e amastigotas de *Leishmania* spp. às células hospedeiras (Butcher *et al.*, 1992; Volf, Svobodova & Dvorakova *et al.*, 2001). Curiosamente, também demonstrou-se que a heparina pode aumentar a ligação de promastigotas aos macrófagos (Butcher *et al.*, 1992). Tal fato é um indicativo de que a heparina poderia influenciar duas atividades distintas sobre a infecção celular por *Leishmania* spp., sendo também uma sugestão para heterogeneidade funcional das proteínas que ligam a heparina presente na superfície do parasito.

Embora certas proteínas e proteinases de *Leishmania* spp. sejam ancoradas por GPI e têm ações definidas no ciclo de vida do parasito, ressalta-se que nem todas as proteínas ancoradas por GPI são essenciais ao crescimento ou infectividade no hospedeiro vertebrado. Mutantes Δ*GPI8* de *L. (L.) mexicana* foram capazes de promover a infecção em camundongos (Hilley *et al.*, 2000). Esses mutantes não têm o gene GPI8 que codifica a enzima transamidase, a qual é responsável pela adição da porção proteica à ancora de GPI.

CONCLUSÕES

Os parasitos do gênero *Leishmania* apresentam uma série de peculiaridades adaptativas nas diferentes fases de seu ciclo biológico. Como as superfícies celulares dos promastigotas e amastigotas estão em contato direto com tecidos e células dos hospedeiros, é razoável que a organização destas seja uma das estratégias para aumentar as chances de sucesso do parasitismo.

Ao fazer uma síntese do conhecimento atual sobre o papel funcional dos componentes do parasito é possível indicar o envolvimento desses componentes nos eventos iniciais da dinâmica de interação finas e vitais que se estabelecem entre os protozoários *Leishmania* e os seus hospedeiros (Tabela 1). Assim, quando os promastigotas ou amastigotas mudam de hospedeiro ocorre ativação de receptores presentes na superfície celular, que sinalizam para transformação do parasito (receptor de adenilciclase). Nessa mudança de hospedeiros, permanecem ativas nos parasitos enzimas que favorecem o equilíbrio e a adaptação às condições iônicas (Ca^{2+} - e Mg^{2+}- ATPases) e a captação de nutrientes essenciais (3'-nucleotidase e 5'-nucleotidase).

Tabela 1 – Principais componentes da membrana de superfície das espécies de *Leishmania* e suas atuações nos hospedeiros

Componentes	Função	
	Hospedeiro Vertebrado	Hospedeiro Invertebrado
LPG	Ligante de macrófagos; Inibe a atividade da PKC de macrófagos; Em metacíclicos: (a) inibe a formação do complexo de ataque à membrana; e (b) ativa complemento proporcionando a ligação ao macrófago via C3bi; Atua na resposta imune inata via células NK e célula dendríticas por meio de receptor *toll-like* 2 e pelo aumento de INF-γ e TNF-α	Fixa promastigotas procíclicos ao epitélio intestinal
GIPLs	Inibe síntese de óxido nítrico em macrófagos; Inibe atividade de PKC; Influencia a taxa de infecção de macrófagos;	FNd
Proteínas e enzimas:		
KMP-11	Imunomodulação	FNd
HASP	Imunomodulação	FNd
Amastina	Invasão e multiplicação intracelular	FNd
Receptor de adenilciclase	Induz a mudança de estágio de promastigota a amastigota	Induz à mudança de estágio amastigota a promastigota
gp46/M-2	FNd	FNd
PPG	mPPG – ligante de receptores de macrófagos	mPPG – ligante de células do tubo digestório
	aPPG – contribuir para manutenção do vacúolo parasitóforo	FNd
	aPPG – ativa complemento via proteína ligadora de manose	FNd
	FNd	fPPG – dificulta ingestão de alimento do flebótomo
AcPs	MAcPs/SAcPs – interferem na produção de metabólitos oxidativos (H_2O_2, OH^-, O^{2-})	Idem
3'-nucleotidase e 5'-nucleotidase	Papel nutricional – hidrólise de nucleotídeos a ácidos nucleicos (3'-AMP e 5'-AMP)	Idem
Ca^{2+}- ATPase	Manutenção dos níveis de Ca^{2+} citoplasmático e do fagossomo	Manutenção dos níveis de Ca^{2+} citoplasmático
Mg^{2+}- ATPases	Função de bomba de extrusão de H^+ Acumulação de glicose e potássio Pré-adaptação dos promastigotas no macrófago	FNd
Metaloproteinases	Hidrólise e inativação de imunoglobulinas G; Inativação do fator C3b em C3bi do complemento; Adesão interiorização nos macrófagos	Protege os promastigotas da ação da tripsina e quimiotripsina do intestino do inseto;
Cisteína-proteinases	Imunorregulador na leishmaniose cutânea: com atividade enzimática sobre componentes do sistema imune e pela atuação da região C-terminal na indução resposta Th2	FNd
PLH	Ligação dos promastigotas aos macrófagos	Ligação dos promastigotas ao trato digestório de flebótomos

Obs: FNd (função não determinada).

A superfície celular desses protozoários é capaz de controlar atividades específicas para viverem em um ou outro hospedeiro. Até o presente momento, a maioria dos componentes de superfície dos parasitos, já descritos anteriormente, tem o papel biológico relacionado ao hospedeiro mamífero, enquanto apenas um escasso número voltado à interação com o inseto vetor foi estudado. Nesse cenário, o LPG é o componente de superfície mais diversificado em funções direcionadas à manutenção do ciclo de vida do parasito, com atuação no vertebrado (influenciando na resposta imune inata e adquirida e subvertendo as funções do macrófago) e no inseto vetor (realizando a fixação do promastigota ao trato digestório).

No hospedeiro vertebrado, o parasito tem a capacidade de controlar ou subverter a ação de diversos componentes do sistema imune. A superfície celular reúne fatores como proteínas, enzimas e/ou glicoconjugados voltados à imunomodulação da resposta imune do hospedeiro vertebrado, favorecendo assim a sobrevivência do parasito.

Também as proteínas de superfície, como metaloproteinases e proteínas que ligam à heparina, têm ações no vertebrado (nas funções do macrófago), mas somente as metaloproteinases atuam no inseto vetor (protege os promastigotas da ação de proteinase do inseto); e é nítido o papel das cisteína-proteinases na resposta imune da leishmaniose cutânea do hospedeiro vertebrado, como enzima e como indutor de resposta imune celular.

Mesmo com os conhecimentos aqui reunidos sobre a os componentes de *Leishmania* spp., para uma real compreensão dos fenômenos de interação entre os parasitos e seus hospedeiros faz-se necessário levar em consideração a ação de todos os componentes de superfície – aqueles já descritos e os que ainda estão em estudo, uma vez que uma categorização artificial da importância destes pode acarretar uma interpretação incorreta de tais fenômenos.

REFERÊNCIAS

ALEXANDER, J.; COOMBS, G. H. & MOTTRAM, J. C. *Leishmania mexicana* cysteine proteinase-deficient mutants have attenuated virulence for mice and potentiate a Th1 response. *The Journal of Immunology*, 161(12): 6.794-6.801, 1998.

ALI, S. A. *et al*. CTL responses to *Leishmania mexicana* gp63-cDNA vaccine in a murine model. *Parasite Immunology*, 31(7): 373-383, 2009.

ALVES, C. R., CÔRTES, L. M. & BRAZIL, R. P. The vectorial potential of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (N.) whitmani* in the transmission of *Leishmania (V.) braziliensis* can also be related to proteins attaching. *Journal of Biomedicine & Biotechnology*, 2010.

ALVES, C. R.; MARZOCHI, M. C. & GIOVANNI-DE-SIMONE, S. Heterogeneity of cysteine proteinases in *Leishmania braziliensis* and *Leishmania major*. *Brazilian Journal of Medical and Biological Research*, 26(2): 167-171, 1993.

ALVES, C. R. *et al*. Detection of cysteine-proteinases in *Leishmania amazonensis* promastigotes using a cross-reactive antiserum. *FEMS Microbiology Letters*, 186(2): 263-267, 2000.

ALVES, C. R. *et al*. A strategy for the identification of T-cell epitopes on *Leishmania* cysteine proteinases. *Cytobios*, 104(405): 33-41, 2001.

ALVES, C. R. *et al*. Th1 and Th2 immunological profile induced by cysteine proteinase in murine Leishmaniasis. *Parasite Immunology*, 26(3): 127-135, 2004.

ALVES, C. R. *et al*. *Leishmania amazonensis*: early proteinase activities during promastigote-amastigote differentiation in vitro. *Experimental Parasitology*, 109(1): 38-48, 2005.

ALVES, J. L.; MENDONÇA-LIMA, F. W. & ALVES, C. R. The use of metal chelate affinity chromatography on the isolation of *Leishmania* chagasi promastigote hydrophobic proteinases. *Veterinary Parasitology*, 119(2-3): 137-145, 2004.

ASSIS, R. R. *et al*. Glycoconjugates in New World species of *Leishmania*: polymorphisms in lipophosphoglycan and glycoinositolphospholipids and interaction with hosts. *Biochimica et Biophysica Acta*, 1820(9):1.354-1.365, 2012.

AYESTA, C.; ARGUELLO, C. & HERNANDEZ, A. G. *Leishmania braziliensis*: cell surface differences in promastigotes of pathogenic and nonpathogenic strains. *Experimental Parasitology*, 59(2): 185-191, 1985.

AZEVEDO-PEREIRA, R. L. *et al*. Heparin binding proteins from *Leishmania* (*Viannia*) *braziliensis* promastigotes. *Veterinary Parasitology*, 145(3-4): 234-239, 2007.

BANDYOPADHYAY, K. *et al*. Role of 67 kDa cell surface laminin binding protein of *Leishmania donovani* in pathogenesis. *Journal of Biochemistry*, 130(1): 141-148, 2001.

BANERJEE, C.; SARKAR, D. & BHADURI, A. Ca2+ and calmodulin-dependent protein phosphatase from *Leishmania donovani*. *Parasitology*, 118(6): 567-573, 1999.

BART, G.; COOMBS, G. H. & MOTTRAM, J. C. Isolation of lmcpc, a gene encoding a *Leishmania mexicana* cathepsin-B-like cysteine proteinase. *Molecular and Biochemical Parasitology*, 73(1-2): 271-274, 1995.

BECKER, I. *et al*. *Leishmania* lipophosphoglycan (LPG) activates NK cells through toll-like receptor-2. *Molecular and Biochemical Parasitology*, 130(2): 65-74, 2003.

BEETHAM, J. K. *et al*. Glycoprotein 46 mRNA abundance is post-transcriptionally regulated during development of *Leishmania chagasi* promastigotes to an infectious form. *The Journal of Biological Chemistry*, 272(28): 17.360-17.366, 1997.

BENAIM, G. & ROMERO, P. J. A calcium pump in plasma membrane vesicles from *Leishmania braziliensis*. *Biochimica et Biophysica Acta*, 1.027(1): 79-84, 1990.

BENAIM, G. *et al*. Intracellular calcium homeostasis in *Leishmania mexicana*. Identification and characterization of a plasma membrane calmodulin-dependent Ca(2+)-ATPase. *Biological Research*, 26(1-2): 141-150, 1993.

BERREDO-PINHO, M. *et al*. A Mg-dependent ecto-ATPase in *Leishmania amazonensis* and its possible role in adenosine acquisition and virulence. *Archives of Biochemistry and Biophysics*, 391(1): 16-24, 2001.

BHATTACHARYYA, S. *et al*. Selective impairment of protein kinase C isotypes in murine macrophage by *Leishmania donovani*. *Molecular and Cellular Biochemistry*, 216(1-2): 47-57, 2001.

BHOWMICK, S.; RAVINDRAN, R. & ALI, N. gp63 in stable cationic liposomes confers sustained vaccine immunity to susceptible BALB/c mice infected with *Leishmania donovani*. *Infection and Immunity*, 76(3): 1.003-1.015, 2008.

BISWAS, A.; BHATTACHARYA, A. & DAS, P. K. Role of cAMP Signaling in the Survival and Infectivity of the Protozoan Parasite, *Leishmania donovani*. *Molecular Biology International*, 2.011: 1-9, 2011.

BOGDAN, C. & ROLLINGHOFF, M. The immune response to *Leishmania*: mechanisms of parasite control and evasion. *International Journal for Parasitology*, 28(1): 121-134, 1998.

BOUVIER, J.; ETGES, R. J. & BORDIER, C. Identification and purification of membrane and soluble forms of the *major* surface protein of *Leishmania* promastigotes. *The Journal of Biological Chemistry*, 260(29): 15.504-15.509, 1985.

BRANDONISIO, O.; SPINELLI, R. & PEPE, M. Dendritic cells in *Leishmania* infection. *Microbes and infection*, 6(15): 1.402-1.409, 2004.

BROOKS, D. R. *et al*. The stage-regulated expression of *Leishmania mexicana* CPB cysteine proteases is mediated by an intercistronic sequence element. *The Journal of Biological Chemistry*, 276(50): 47.061-47.069, 2001.

BUCHMULLER-ROUILLER, Y. & MAUEL, J. Impairment of the oxidative metabolism of mouse peritoneal macrophages by intracellular *Leishmania* spp. *Infection and Immunity*, 55(3): 587-593, 1987.

BUTCHER, B. A. *et al*. *Leishmania donovani*: cell-surface heparin receptors of promastigotes are recruited from an internal pool after trypsinization. *Experimental Parasitology*, 71(1): 49-59, 1990.

BUTCHER, B. A. *et al*. Heparin enhances the interaction of infective *Leishmania donovani* promastigotes with mouse peritoneal macrophages. A fluorescence flow cytometric analysis. *The Journal of Immunology*, 148(9): 2.879-2.886, 1992.

CAMERON, P. *et al*. Inhibition of lipopolysaccharide-induced macrophage IL-12 production by *Leishmania mexicana* amastigotes: the role of cysteine peptidases and the NF-kappaB signaling pathway. *The Journal of Immunology*, 173(5): 3.297-3.304, 2004.

CAMPBELL, T. A. *et al*. Purification and characterization of the 3'-nucleotidase/nuclease from promastigotes of *Leishmania donovani*. *Molecular and Biochemical Parasitology*, 47(1): 109-117, 1991.

CARVALHO, L. P. *et al*. Effect of LACK and KMP11 on IFN-gamma production by peripheral blood mononuclear cells from cutaneous and mucosal Leishmaniasis patients. *Scandinavian Journal of Immunology*, 61(4): 337-342, 2005.

CHAMPSI, J. & MCMAHON-PRATT, D. Membrane glycoprotein M-2 protects against *Leishmania amazonensis* infection. *Infection and Immunity*, 56(12): 3.272-3.279, 1988.

CHAUDHURI, G. & CHANG, K. P. Acid protease activity of a *major* surface membrane glycoprotein (gp63) from *Leishmania mexicana* promastigotes. *Molecular and Biochemical Parasitology*, 27(1): 43-52, 1988.

COELHO-FINAMORE, J. M. *et al*. *Leishmania infantum*: Lipophosphoglycan intraspecific variation and interaction with vertebrate and invertebrate hosts. *The Journal of Parasitology*, 41(3-4): 333-342, 2011.

CÔRTE-REAL, S. *et al*. Immunogold labeling and cerium cytochemistry of the enzyme ecto-5'-nucleotidase in promastigote forms of *Leishmania* species. *Memórias do Instituto Oswaldo Cruz*, 88(3): 407-412, 1993.

CÔRTE-REAL, S.; SANTOS, C. B. & MEIRELLES, M. N. Differential expression of the plasma membrane Mg2+ ATPase and Ca2+ ATPase activity during adhesion and interiorization of *Leishmania amazonensis* in fibroblasts in vitro. *Journal of Submicroscopic Cytology and Pathology*, 27(3): 359-366, 1995.

CUERVO, P. *et al*. A zymographic study of metalloprotease activities in extracts and extracellular secretions of *Leishmania* (*Viannia*) *braziliensis* strains. *Parasitology*, 132(2): 177-185, 2006.

CUERVO, P. *et al*. Cellular localization and expression of gp63 homologous metalloproteases in *Leishmania* (*Viannia*) *braziliensis* strains. *Acta Tropica*, 106(3): 143-148, 2008.

DAVIES, C. R. *et al*. Expression of LPG and GP63 by different developmental stages of *Leishmania major* in the sandfly *Phlebotomus papatasi*. *Parasitology*, 101(3): 337-343, 1990.

DEBRABANT, A.; GHEDIN, E. & DWYER, D. M. Dissection of the functional domains of the *Leishmania* surface membrane 3'-nucleotidase/nuclease, a unique member of the class I nuclease family. *The Journal of Biological Chemistry*, 275(21): 16.366-16.372, 2000.

DEBRABANT, A.; GOTTLIEB, M. & DWYER, D. M. Isolation and characterization of the gene encoding the surface membrane 3'-nucleotidase/nuclease of *Leishmania donovani*. *Molecular and Biochemical Parasitology*, 71(1): 51-63, 1995.

DELGADO-DOMÍNGUEZ, J. *et al*. *Leishmania mexicana* lipophosphoglycan differentially regulates PKCalpha-induced oxidative burst in macrophages of BALB/c and C57BL/6 mice. *Parasite Immunology*, 32(6): 440-449, 2010.

DENISE, H. *et al*. Expression of multiple CPB genes encoding cysteine proteases is required for *Leishmania mexicana* virulence in vivo. *Infection and Immunity*, 71(6): 3.190-3.195, 2003.

DENNY, P. W. *et al*. Acylation-dependent protein export in *Leishmania*. *The Journal of Biological Chemistry*, 275(15): 11.017-11.025, 2000.

DEPLEDGE, D. P. *et al*. *Leishmania*-specific surface antigens show sub-genus sequence variation and immune recognition. *PLoS Neglected Tropical Diseases*, 4(9): e829, 2010.

DILLON, R. J. & LANE, R. P. Detection of *Leishmania* lipophosphoglycan binding proteins in the gut of the sandfly vector. *Parasitology*, 118(1): 27-32, 1999.

DUTTA, M. *et al*. Fluidity-dependent Mg2(+)-ATPase activity in membranes from *Leishmania donovani* promastigotes. *The Biochemical Journal*, 265(3): 923-926, 1990.

ELLIS, S. L.; SHAKARIAN, A. M. & DWYER, D. M. *Leishmania*: amastigotes synthesize conserved secretory acid phosphatases during human infection. *Experimental Parasitology*, 89(2): 161-168, 1998.

ETGES, R.; BOUVIER, J. & BORDIER, C. The *major* surface protein of *Leishmania* promastigotes is a protease. *The Journal of Biological Chemistry*, 261(20): 9.098-9.101, 1986.

FARAJNIA, S. *et al*. Molecular characterization of a novel amastigote stage specific Class I nuclease from *Leishmania major*. *International Journal for Parasitology*, 34(8): 899-908, 2004.

FLINN, H. M.; RANGARAJAN, D. & SMITH, D. F. Expression of a hydrophilic surface protein in infective stages of *Leishmania major*. *Molecular and Biochemical Parasitology*, 65(2): 259-270, 1994.

GBENLE, G. O. & DWYER, D. M. Purification and properties of 3'-nucleotidase of *Leishmania donovani*. *The Biochemical Journal*, 285(1): 41-46, 1992.

GHOSH, J. *et al*. A high affinity Ca2(+)-ATPase on the surface membrane of *Leishmania donovani* promastigote. *The Journal of Biological Chemistry*, 265(19): 11.345-11.351, 1990.

GLASER, T. A. *et al*. An antigenically distinct lipophosphoglycan on amastigotes of *Leishmania major*. *Molecular and Biochemical Parasitology*, 45(2): 337-344, 1991.

GLEW, R. H. *et al*. Biochemistry of the *Leishmania* species. *Microbiological Reviews*, 52(4): 412-432, 1988.

GOTTLIEB, M. & DWYER, D. M. Evidence for distinct 5'- and 3'-nucleotidase activities in the surface membrane fraction of *Leishmania donovani* promastigotes. *Molecular and Biochemical Parasitology*, 7(4): 303-317, 1983.

HANDMAN, E. & GODING, J. W. The *Leishmania* receptor for macrophages is a lipid-containing glycoconjugate. *The Embo Journal*, 4(2): 329-336, 1985.

HANDMAN, E.; GREENBLATT, C. L. & GODING, J. W. An amphipathic sulphated glycoconjugate of *Leishmania*: characterization with monoclonal antibodies. *The Embo Journal*, 3(10): 2.301-2.306, 1984.

HANEKAMP, T. & LANGER, P. J. Molecular karyotype and chromosomal localization of genes encoding two *major* surface glycoproteins, gp63 and gp46/M2, hsp70, and beta-tubulin in cloned strains of several *Leishmania* species. *Molecular and Biochemical Parasitology*, 48(1): 27-37, 1991.

HANSEN, B. D. *et al*. The specificity of purine base and nucleoside uptake in promastigotes of *Leishmania braziliensis* panamensis. *Parasitology*, 85(2): 271-282, 1982.

HILLEY *et al*. *Leishmania mexicana* mutants lacking glycosylphosphatidylinositol (GPI): protein transamidase provide insights into the biosynthesis and functions of GPI-anchored proteins. *Molecular Biology* of the *Cell*, 11(4): 1.183-1.195, 2000.

ILG, T. Proteophosphoglycans of *Leishmania*. *Parasitology Today*, 16(11): 489-497, 2000.

ILG, T. *et al*. Structure of *Leishmania mexicana* lipophosphoglycan. *The Journal of Biological Chemistry*, 267(10): 6.834-6.840, 1992.

ILG, T. *et al*. Molecular cloning and characterization of a novel repeat-containing *Leishmania major* gene, ppg1, that encodes a membrane-associated form of proteophosphoglycan with a putative glycosylphosphatidylinositol anchor. *The Journal of Biological Chemistry*, 274(44): 31.410-31.420, 1999.

JACKSON, A. P. The evolution of amastin surface glycoproteins in trypanosomatid parasites. *Molecular Biology and Evolution*, 27(1): 33-45, 2010.

JARDIM, A. *et al*. Isolation and structural characterization of the *Leishmania donovani* kinetoplastid membrane protein-11, a major immunoreactive membrane glycoprotein. *The Biochemical Journal*, 305(1): 307-313, 1995.

KAHL, L. P. & MCMAHON-PRATT, D. Structural and antigenic characterization of a species- and promastigote-specific *Leishmania mexicana amazonensis* membrane protein. *The Journal of Immunology*, 138(5): 1.587-1.595, 1987.

KAMHAWI, S. *et al*. A role for insect galectins in parasite survival. *Cell*, 119(3): 329-341, 2004.

KOCK, N. P. *et al*. Receptors for carbohydrate ligands including heparin on the cell surface of *Leishmania* and other trypanosomatids. *Tropical Medicine & International Health*, 2(9): 863-874, 1997.

LIEW, F. Y. & O'DONNELL, C. A. Immunology of Leishmaniasis. *Advances in Parasitology*, 32: 161-259, 1993.

LU, H. G. *et al*. Intracellular Ca2+ pool content and signaling and expression of a calcium pump are linked to virulence in *Leishmania mexicana* amazonesis amastigotes. *The Journal of Biological Chemistry*, 272(14): 9.464-9.473, 1997.

MAHONEY, A. B. *et al*. Intra-species and stage-specific polymorphisms in lipophosphoglycan structure control *Leishmania donovani*-sand fly interactions. *Biochemistry*, 38(31): 9.813-9.823, 1999.

MATOS, D. C. et *al*. Kinetoplastid membrane protein-11 is present in promastigotes and amastigotes of *Leishmania amazonensis* and its surface expression increases during metacyclogenesis. *Memórias do Instituto Oswaldo Cruz*, 105(3): 341-347, 2010.

MAZUMDER, S. *et al*. Allosteric modulation of *Leishmania donovani* plasma membrane Ca(2+)-ATPase by endogenous calmodulin. *The Journal of Biological Chemistry*, 267(26): 18.440-18.446, 1992.

McCONVILLE, M. J. & BLACKWELL, J. M. Developmental changes in the glycosylated phosphatidylinositols of *Leishmania donovani*. Characterization of the promastigote and amastigote glycolipids. *The Journal of Biological Chemistry*, 266(23): 15.170-15.179, 1991.

McCONVILLE, M. J. & HOMANS, S. W. Identification of the defect in lipophosphoglycan biosynthesis in a non-pathogenic strain of *Leishmania major*. *The Journal of Biological Chemistry*, 267(9): 5.855-5.861, 1992.

McCONVILLE, M. J. & RALTON, J. E. Developmentally regulated changes in the cell surface architecture of *Leishmania* parasites. *Behring Institute Mitteilungen*, 99: 34-43, 1997.

McCONVILLE, M. J. *et al*. Structures of the glycoinositolphospholipids from *Leishmania major*. A family of novel galactofuranose-containing glycolipids. *The Journal of Biological Chemistry*, 265(13): 7.385-7.394, 1990.

McCONVILLE, M. J. *et al*. The developmental regulation and biosynthesis of GPI-related structures in *Leishmania* parasites. *Brazilian Journal of Medical and Biological Research*, 27(2): 139-144, 1994.

McMAHON-PRATT, D. *et al*. Loss of the GP46/M-2 surface membrane glycoprotein gene family in the *Leishmania braziliensis* complex. *Molecular and Biochemical Parasitology*, 50(1): 151-160, 1992.

McMAHON-PRATT, D. *et al*. Recombinant vaccinia viruses expressing GP46/M-2 protect against *Leishmania* infection. *Infection and Immunity*, 61(8): 3.351-3.359, 1993.

MENDONCA-LIMA, F. W. & ATTA, A. M. Partial characterization of *Leishmania* chagasi promastigote peptidases. *Memórias do Instituto Oswaldo Cruz*, 87(2): 307-308, 1992.

MISLE, J. A.; MARQUEZ, M. E. & HERNANDEZ, A. G. Solubilization and partial purification of a cell surface component of *Leishmania braziliensis*. *Zeitschrift für Parasitenkunde*, 71(4): 419-428, 1985.

MOODY, S. F. *et al*. The structure of *Leishmania major* amastigote lipophosphoglycan. *The Journal of Biological Chemistry*, 268(25): 18.457-18.466, 1993.

MOSSER, D. M.; SPRINGER, T. A. & DIAMOND, M. S. *Leishmania* promastigotes require opsonic complement to bind to the human leukocyte integrin Mac-1 (CD11b/CD18). *The Journal of Cell Biology*, 116(2): 511-520, 1992.

MOTTRAM, J. C. & COOMBS, G. H. *Leishmania* cysteine proteinases: virulence factors in quest of a function-reply. *Parasitology Today*, 14(6): 251-252, 1998.

MOTTRAM, J. C.; BROOKS, D. R. & COOMBS, G. H. Roles of cysteine proteinases of trypanosomes and *Leishmania* in host-parasite interactions. *Current Opinion in Microbiology*, 1(4): 455-460, 1998.

MOTTRAM, J. C.; COOMBS, G. H. & ALEXANDER, J. Cysteine peptidases as virulence factors of *Leishmania*. *Current Opinion in Microbiology*, 7(4): 375-381, 2004.

MOTTRAM, J. C. *et al*. A developmentally regulated cysteine proteinase gene of *Leishmania mexicana*. *Molecular Microbiology*, 6(14): 1.925-1.932, 1992.

MUKHERJEE, T.; MANDAL, D. & BHADURI, A. *Leishmania* plasma membrane Mg2+-ATPase is a H+/K+-antiporter involved in glucose symport. Studies with sealed ghosts and vesicles of opposite polarity. *The Journal of Biological Chemistry*, 276(8): 5.563-5.569, 2001.

MUKHOPADHYAY, N. K. *et al*. Heparin binds to *Leishmania donovani* promastigotes and inhibits protein phosphorylation. *The Biochemical Journal*, 264(2): 517-525, 1989.

MURRAY, P. J.; SPITHILL, T. W. & HANDMAN, E. The PSA-2 glycoprotein complex of *Leishmania major* is a glycosylphosphatidylinositol-linked promastigote surface antigen. *The Journal of Immunology*, 143(12): 4.221-4.226, 1989.

MUSKUS, C. E. & MARIN VILLA, M. Metacyclogenesis: a basic process in the biology of *Leishmania*. *Biomedica*, 22(2): 167-177, 2002.

MYSKOVA, J. *et al*. A lipophosphoglycan-independent development of *Leishmania* in permissive sand flies. *Microbes and Infection*, 9(3): 317-324, 2007.

NAGAKURA, K. *et al*. *Leishmania braziliensis*: localization of glycoproteins in promastigotes. *Experimental Parasitology*, 61(3): 335-342, 1986.

OLIVIER, M. & HASSANI, K. Protease inhibitors as prophylaxis against Leishmaniasis: new hope from the *major* surface protease gp63. *Future Medicinal Chemistry*, 2(4): 539-542, 2010.

OLIVIER, M.; GREGORY, D. J. & FORGET, G. Subversion mechanisms by which *Leishmania* parasites can escape the host immune response: a signaling point of view. *Clinical Microbiology Reviews*, 18(2): 293-305, 2005.

OMARA-OPYENE, A. L. & GEDAMU, L. Molecular cloning, characterization and overexpression of two distinct cysteine protease cDNAs from *Leishmania donovani* chagasi. *Molecular and Biochemical Parasitology*, 90(1): 247-267, 1997.

ORTEGA-BARRIA, E. & PEREIRA, M. E. A novel *T. cruzi* heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. *Cell*, 67: 411-421, 1991.

PAN, A. A. *Leishmania mexicana pifanoi*: analysis of the antigenic relationships between promastigotes and amastigotes by gel diffusion, immunoelectrophoresis, and immunoprecipitation. *The Journal of Protozoology*, 33(2): 192-197, 1986.

PEREIRA, B. A. *et al*. *In silico* predicted epitopes from the COOH-terminal extension of cysteine proteinase B inducing distinct immune responses during *Leishmania (Leishmania) amazonensis* experimental murine infection. *BMC Immunology*, 8(12): 44, 2011.]

PETERS, C.; STIERHOF, Y. D. & ILG, T. Proteophosphoglycan secreted by *Leishmania mexicana* amastigotes causes vacuole formation in macrophages. *Infection and Immunity*, 65(2): 783-786, 1997.

PETERS, C. *et al*. Secreted proteophosphoglycan of *Leishmania mexicana* amastigotes activates complement by triggering the mannan binding lectin pathway. *European Journal of Immunology*, 27(10): 2.666-2.672, 1997.

PIMENTA, P. F.; SARAIVA, E. M. & SACKS, D. L. The comparative fine structure and surface glycoconjugate expression of three life stages of *Leishmania major*. *Experimental Parasitology*, 72(2): 191-204, 1991.

PIMENTA, P. F. *et al*. Stage-specific adhesion of *Leishmania* promastigotes to the sandfly midgut. *Science*, 256(5.065): 1.812-1.815, 1992.

PIMENTA, P. F. *et al*. A novel role for the peritrophic matrix in protecting *Leishmania* from the hydrolytic activities of the sand fly midgut. *Parasitology*, 115(4): 359-369, 1997.

POLLOCK, K. G. *et al*. The *Leishmania mexicana* cysteine protease, CPB2.8, induces potent Th2 responses. *The Journal of Immunology*, 170(4): 1.746-1.753, 2003.

PROUDFOOT, L.; O'DONNELL, C. A. & LIEW, F. Y. Glycoinositolphospholipids of *Leishmania major* inhibit nitric oxide synthesis and reduce leishmanicidal activity in murine macrophages. *European Journal of Immunology*, 25(3): 745-750, 1995.

PUENTES, S. M. *et al*. Complement binding by two developmental stages of *Leishmania major* promastigotes varying in expression of a surface lipophosphoglycan. *The Journal of Experimental Medicine*, 167(3): 887-902, 1988.

PUENTES, S. M. *et al*. Serum resistance of metacyclic stage *Leishmania major* promastigotes is due to release of C5b-9. *The Journal of Immunology*, 145(12): 4.311-4.316, 1990.

PUPKIS, M. F. & COOMBS, G. H. Purification and characterization of proteolytic enzymes of *Leishmania mexicana mexicana* amastigotes and promastigotes. *Journal of General Microbiology*, 130(9): 2.375-2.383, 1984.

REBELLO, K. M. *et al*. Cysteine proteinases from promastigotes of *Leishmania* (*Viannia*) *braziliensis*. *Parasitology Research*, 106(1): 95-104, 2009.

REMALEY, A. T. *et al*. Characterization of *Leishmania donovani* acid phosphatases. *The Journal of Biological Chemistry*, 260(2): 880-886, 1985.

REQUENA, J. M.; ALONSO, C. & SOTO, M. Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. *Parasitology Today*, 16(6): 246-250, 2000.

ROBERTSON, C. D. & COOMBS, G. H. Characterisation of three groups of cysteine proteinases in the amastigotes of *Leishmania mexicana mexicana*. *Molecular and Biochemical Parasitology*, 42(2): 269-276, 1990.

RODRIGUEZ-CORTES, A. *et al*. Vaccination with plasmid DNA encoding KMPII, TRYP, LACK and GP63 does not protect dogs against *Leishmania infantum* experimental challenge. *Vaccine*, 25(46): 7.962-7.971, 2007.

SACKS, D. L. The structure and function of the surface lipophosphoglycan on different developmental stages of *Leishmania* promastigotes. *Infectious Agents and Disease*, 1(4): 200-206, 1992.

SACKS, D. L.; HIENY, S. & SHER, A. Identification of cell surface carbohydrate and antigenic changes between noninfective and infective developmental stages of *Leishmania major* promastigotes. *The Journal of Immunology*, 135(1): 564-569, 1985.

SACKS, D. L. *et al*. Stage-specific binding of *Leishmania donovani* to the sand fly vector midgut is regulated by conformational changes in the abundant surface lipophosphoglycan. *The Journal of Experimental Medicine*, 181(2): 685-697, 1995.

SANCHEZ, M. A. *et al*. A family of putative receptor-adenylate cyclases from *Leishmania donovani*. *The Journal of Biological Chemistry*, 270(29): 17.551-17.558, 1995.

SARAIVA, E. M. *et al*. Changes in lipophosphoglycan and gene expression associated with the development of *Leishmania major* in *Phlebotomus papatasi*. *Parasitology*, 111(3): 275-287, 1995.

SECUNDINO, N. *et al*. Proteophosphoglycan confers resistance of *Leishmania major* to midgut digestive enzymes induced by blood feeding in vector sand flies. *Cellular Microbiology*, 12(7): 906-918, 2010.

SEEBECK, T.; SCHAUB, R. & JOHNER, A. cAMP signalling in the kinetoplastid protozoa. *Current Molecular Medicine*, 4(6): 585-599, 2004.

SHAKARIAN, A. M. & DWYER, D. M. Structurally conserved soluble acid phosphatases are synthesized and released by *Leishmania major* promastigotes. *Experimental Parasitology*, 95(2): 79-84, 2000.

SHAKARIAN, A. M. *et al*. Molecular dissection of the functional domains of a unique, tartrate-resistant, surface membrane acid phosphatase in the primitive human pathogen *Leishmania donovani*. *The Journal of Biological Chemistry*, 277(20): 17.994-18.001, 2002.

SILVA-LOPEZ, R. E. & GIOVANNI-DE-SIMONE, S. *Leishmania* (*Leishmania*) *amazonensis*: purification and characterization of a promastigote serine protease. *Experimental Parasitology*, 107(3-4): 173-182, 2004.

SILVA-LOPEZ, R. E. *et al*. Subcellular localization of an extracellular serine protease in *Leishmania* (*Leishmania*) *amazonensis*. *Parasitology Research*, 93(4): 328-331, 2004.

SOARES, R. P. *et al*. *Leishmania* chagasi: lipophosphoglycan characterization and binding to the midgut of the sand fly vector *Lutzomyia longipalpis*. *Molecular and Biochemical Parasitology*, 121(2): 213-224, 2002.

SOARES, R. P. *et al*. *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. *International Journal for Parasitology*, 35(3): 245-253, 2005.

SOARES, R. P. *et al*. Differential midgut attachment of *Leishmania* (*Viannia*) *braziliensis* in the sand flies *Lutzomyia (Nyssomyia) whitmani* and *Lutzomyia (Nyssomyia) intermedia*. *Journal of Biomedicine & Biotechnology*, 2.010: 1-7, 2010.

SOONG, L. Modulation of dendritic cell function by *Leishmania* parasites. *The Journal of Immunology*, 180(7): 4.355-4.360, 2008.

SOUZA LEÃO, S. *et al*. Intracellular *Leishmania amazonensis* amastigotes internalize and degrade MHC class II molecules of their host cells. *Journal of Cell Science*, 108(10): 3.219-3.231, 1995.

SPATH, G. F. *et al*. Lipophosphoglycan is a virulence factor distinct from related glycoconjugates in the protozoan parasite *Leishmania major*. *Proceedings of the National Academy of Sciences of the USA*, 97(16): 9.258-9.263, 2000.

SPATH, G. F. *et al*. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. *Proceedings of the National Academy of Sciences of the USA*, 100(16): 9.536-9.541, 2003.

STEBECK, C. E. *et al*. Kinetoplastid membrane protein-11 (KMP-11) is differentially expressed during the life cycle of African trypanosomes and is found in a wide variety of kinetoplastid parasites. *Molecular and Biochemical Parasitology*, 71(1): 1-13, 1995.

STIERHOF, Y. D. *et al*. Characterization of polymer release from the flagellar pocket of *Leishmania mexicana* promastigotes. *The Journal of Cell Biology*, 125(2): 321-331, 1994.

TONUI, W. K. *et al*. Transmission blocking vaccine studies in Leishmaniasis: II. Effect of immunization using *Leishmania major* derived 63 kilodalton glycoprotein, lipophosphoglycan and whole parasite antigens on the course of *L. major* infection in BALB/c mice. *East African Medical Journal*, 78(2): 90-92, 2001.

TURCO, S. J. Adversarial relationship between the *Leishmania* lipophosphoglycan and protein kinase C of host macrophages. *Parasite Immunology*, 21(12): 597-600, 1999.

TURCO, S. J. & DESCOTEAUX, A. The lipophosphoglycan of *Leishmania* parasites. *Annual Review of Microbiology*, 46: 65-94, 1992.

TURCO, S. J. *et al*. Structure of the major carbohydrate fragment of the *Leishmania donovani* lipophosphoglycan. *Biochemistry*, 26(19): 6.233-6.238, 1987.

UZONNA, J. E. *et al*. Vaccination with phosphoglycan-deficient *Leishmania major* protects highly susceptible mice from virulent challenge without inducing a strong Th1 response. *The Journal of Immunology*, 172(6): 3.793-3.797, 2004.

VICTOIR, K. *et al*. Complexity of the major surface protease (msp) gene organization in *Leishmania* (*Viannia*) *braziliensis*: evolutionary and functional implications. *Parasitology*, 131(2): 207-214, 2005.

VINHAS, V. *et al*. Characterization of T cell responses to purified *Leishmania* antigens in subjects infected with *Leishmania chagasi*. *Brazilian Journal of Medical and Biological Research*, 27(5): 1.199-1.205, 1994.

VOLF, P. *et al*. Bloodmeal digestion and *Leishmania major* infections in Phlebotomus duboscqi: effect of carbohydrates inhibiting midgut lectin activity. *Medical and Veterinary Entomology*, 15(3): 281-286, 2001.

WALTERS, L. L. *et al*. Ultrastructural development of *Leishmania chagasi* in its vector, *Lutzomyia longipalpis* (Diptera: *Psychodidae*). *The American Journal of Tropical Medicine and Hygiene*, 41(3): 295-317, 1989.

WEINHEBER, N. *et al*. Phagocytosis of *Leishmania mexicana* amastigotes by macrophages leads to a sustained suppression of IL-12 production. *European Journal of Immunology*, 28(8): 2.467-2.477, 1998.

WEISE, F. *et al*. Distribution of GPI-anchored proteins in the protozoan parasite *Leishmania*, based on an improved ultrastructural description using high-pressure frozen cells. *Journal of Cell Science*, 113(24): 4.587-4.603, 2000.

WU, Y. *et al*. A new developmentally regulated gene family in *Leishmania* amastigotes encoding a homolog of amastin surface proteins. *Molecular and Biochemical Parasitology*, 110(2): 345-357, 2000.

XU, D. *et al*. Protection against *Leishmania major* infection in genetically susceptible BALB/c mice by gp63 delivered orally in attenuated *Salmonella typhimurium* (AroA- AroD-). *Immunology*, 85(1): 1-7, 1995.

YAO, C. *et al*. Multiple products of the *Leishmania chagasi major* surface protease (MSP or GP63) gene family. *Molecular and Biochemical Parasitology*, 135(2): 171-183, 2004.

YAO, C. *et al*. Internal and surface subpopulations of the *major* surface protease (MSP) of *Leishmania chagasi*. *Molecular and Biochemical Parasitology*, 139(2): 173-183, 2005.

YONEYAMA, K. A. *et al*. Characterization of *Leishmania* (*Viannia*) *braziliensis* membrane microdomains, and their role in macrophage infectivity. *Journal of Lipid Research*, 47(10): 2.171-2.178, 2006.

ZUFFEREY, R. *et al*. Ether phospholipids and glycosylinositolphospholipids are not required for amastigote virulence or for inhibition of macrophage activation by *Leishmania major*. *The Journal of Biological Chemistry*, 278(45): 44.708-44.718, 2003.

Anexo 2:

Silva-Almeida et al. Parasites & Vectors 2014, 7:387
http://www.parasitesandvectors.com/content/7/1/387

Parasites &Vectors

SHORT REPORT Open Access

Overview of the organization of protease genes in the genome of *Leishmania* spp

Mariana Silva-Almeida†, Franklin Souza-Silva†, Bernardo Acácio Santini Pereira, Michelle Lopes Ribeiro-Guimarães and Carlos Roberto Alves*

Abstract

Background: The genus *Leishmania* includes protozoan parasites that are able to infect an array of phlebotomine and vertebrate species. Proteases are related to the capacity of these parasites to infect and survive in their hosts and are therefore classified as virulence factors.

Findings: By analyzing protease genes annotated in the genomes of four *Leishmania* spp [*Leishmania (Leishmania) infantum*, *L. (L.) major*, *L. (L.) mexicana* and *L. (Viannia) braziliensis*], these genes were found on every chromosome of these protozoa. Four protease classes were studied: metallo-, serine, cysteine and aspartic proteases. Metalloprotease genes predominate in the *L. (V.) braziliensis* genome, while in the other three species studied, cysteine protease genes prevail. Notably, cysteine and serine protease genes were found to be very abundant, as they were found on all chromosomes of the four studied species. In contrast, only three aspartic protease genes could be detected in these four species. Regarding gene conservation, a higher number of conserved alleles was observed for cysteine proteases (42 alleles), followed by metalloproteases (35 alleles) and serine proteases (15 alleles).

Conclusions: The present study highlights substantial differences in the organization of protease genes among *L. (L.) infantum*, *L. (L.) major*, *L. (L.) mexicana* and *L. (V.) braziliensis*. We observed significant distinctions in many protease features, such as occurrence, quantity and conservation. These data indicate a great diversity of protease genes among *Leishmania* species, an aspect that may be related to their adaptations to the peculiarities of each microenvironment they inhabit, such as the gut of phlebotomines and the immune cells of vertebrate hosts.

Keywords: *Leishmania*, *Leishmania (Viannia) braziliensis*, *Leishmania (Leishmania) infantum*, *Leishmania (Leishmania) major*, *Leishmania (Leishmania) mexicana*, Proteases

Background

The World Health Organization classifies the leishmaniases, infections caused by parasites of the genus *Leishmania*, among emerging diseases that lack effective control. Annually, an estimated 1.3 million new cases occur and 20,000 to 30,000 deaths are attributed to these diseases [1]. The clinical forms range in severity and are classified as follows: punctuate skin lesions to oronasal disfigurement are classified as cutaneous leishmaniasis (CL), whereas fatal systemic infections are classified as visceral leishmaniasis (VL). *Leishmania* spp are distributed worldwide and are organized into subgenera and species complexes. Their transmission to mammalian hosts occurs during the blood meal of infected sandflies, which in turn acquire the parasites when feeding on an infected host, thus maintaining the cycle of the disease. The species grouped into the *Leishmania* (*Leishmania*) *donovani* complex, including *L.* (*L.*) *infantum*, are the agents of VL. As for the species commonly associated with CL, *L.* (*L.*) *major* is reported in the Old World, whereas *L.* (*L.*) *mexicana* and *L.* (*Viannia*) *braziliensis* are the main species reported in the New World. This latter species is also associated with the mucocutaneous form of the disease.

In a recent review study, we have highlighted the pivotal roles of proteases as virulence factors for *Leishmania* spp [2]. Such enzymes have been implicated in many parasitic activities, such as tissue invasion, survival in macrophages and host immune response modulation.

* Correspondence: calves@ioc.fiocruz.br
†Equal contributors
Laboratório de Biologia Molecular e Doenças Endêmicas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Av. Brasil 4365, Rio de, Janeiro CEP 21040-900, Brasil

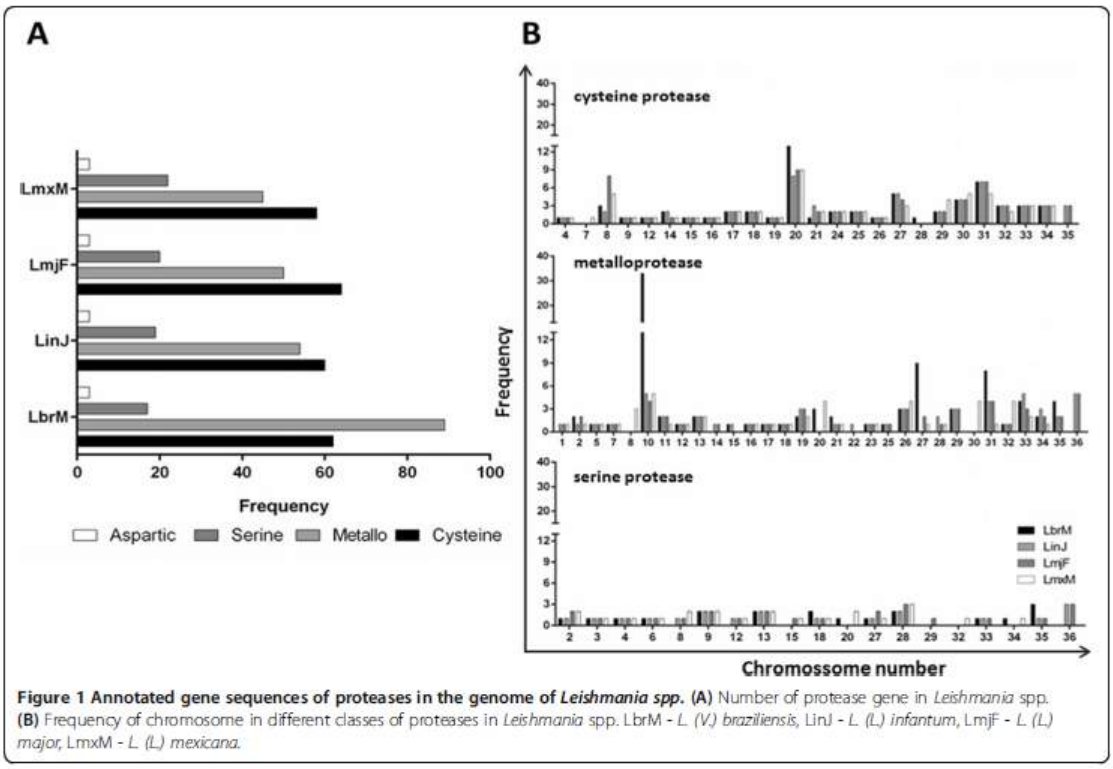


Figure 1 Annotated gene sequences of proteases in the genome of *Leishmania spp.* (A) Number of protease gene in *Leishmania* spp. **(B)** Frequency of chromosome in different classes of proteases in *Leishmania* spp. LbrM - *L. (V.) braziliensis*, LinJ - *L. (L.) infantum*, LmjF - *L. (L.) major*, LmxM - *L. (L.) mexicana*.

are common to all studied species. These genes are located on chromosomes 1, 15 and 29 (Figure 1).

Regarding genes for different protease classes that occur on the same chromosome, most of the studied chromosomes were found to contain genes for multiple protease classes. The exceptions were chromosomes 3 and 6, which were found to contain only serine protease genes and chromosomes 5, 11 and 22, which were found to contain only metalloprotease genes.

Due to fusion events that occurred in *Leishmania* chromosomes, we observed an interesting pattern of organization of protease genes where the same arrangement of alleles is maintained across different species but is located on different chromosomes. Graphical representations of such fusion events were developed using the Artemis and ACT software [10] (Additional file 1: Figure S1 to S8).

Nevertheless, there is a trend of conservation of some alleles in the same chromosomes across the studied *Leishmania* species. We observed 42 conserved alleles of cysteine proteases, 35 of metalloproteases and 15 of serine proteases (Figure 2). The conserved alleles are predominantly grouped on chromosome 10 for cysteine proteases, chromosome 30 for metalloproteases and chromosome 28 for serine proteases.

Among all the analysed protease genes, only two alleles were found to be conserved on the same chromosome for all four species: alleles of cysteine protease genes coding for ubiquitin carboxyl-terminal hydrolases (Clan CA, family C12) located on chromosomes 24 and 25 (alleles 0420 and 0190, respectively) of all species.

Notably, *L.* (*L.*) *major* and *L.* (*L.*) *mexicana* were found to show more synteny than the other species, containing 23, 15 and 13 conserved alleles for cysteine, metallo- and serine proteases, respectively. Conversely, *L. (V.) braziliensis* was not found to show synteny for serine protease genes of any other species. Although this absence of synteny was observed in the only species in our analysis classified into a different subgenus, it has been proposed by Peacock *et al.* [11] that such absence would not necessarily indicate a lineage-specific diversity in *Leishmania* spp.

One of the first comparative genomic studies of *Leishmania* showed that despite phenotypic variations among species, only a few genes are truly species-specific [11]. In agreement with such reports, we also observed few genes that do not show similarity to any others. They show sequence identities lower than 80% to other genes (Additional file 2: Table S1). This is an important finding, as these exclusive genes can help explain why these

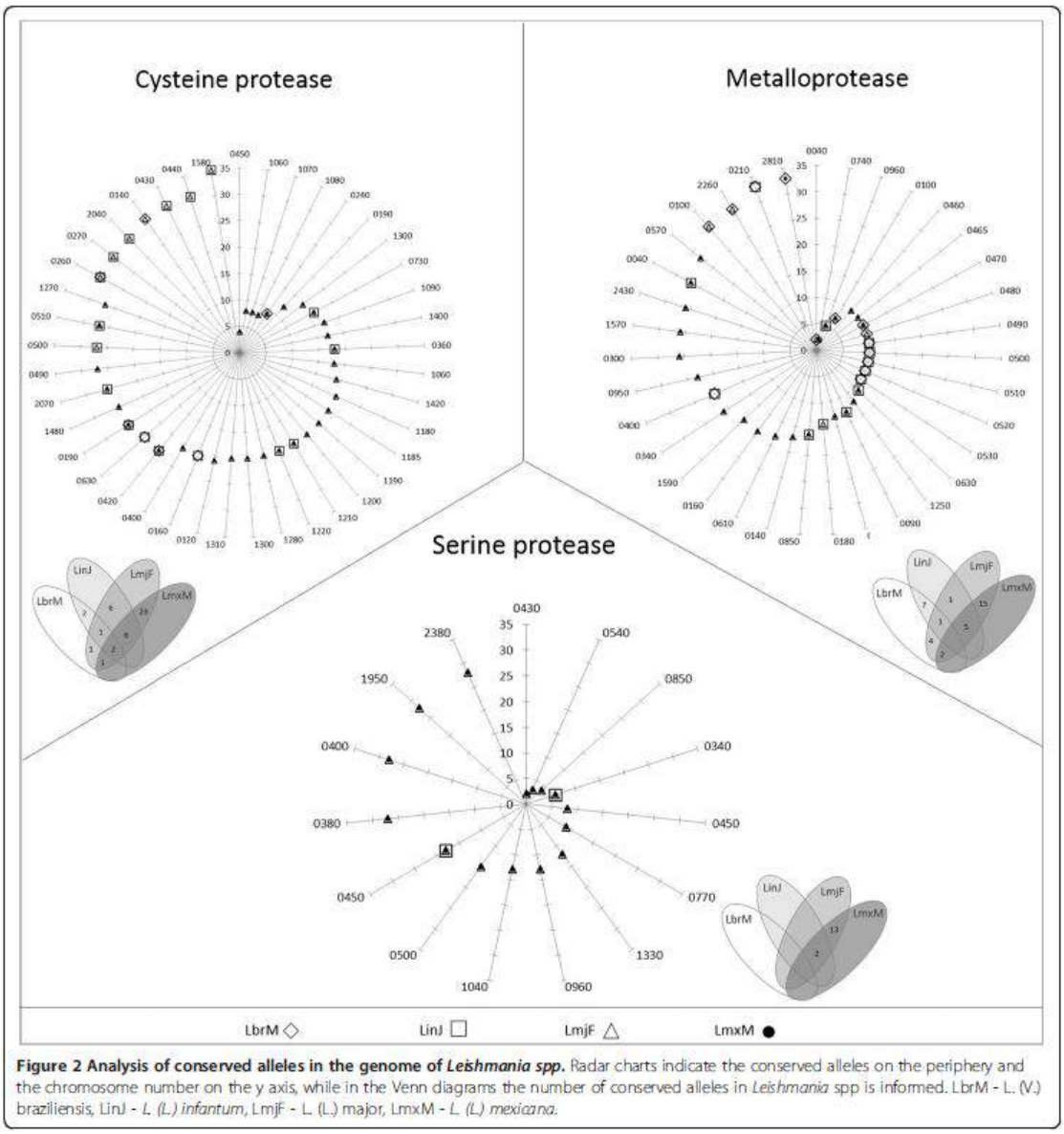


Figure 2 Analysis of conserved alleles in the genome of *Leishmania spp.* Radar charts indicate the conserved alleles on the periphery and the chromosome number on the y axis, while in the Venn diagrams the number of conserved alleles in *Leishmania* spp is informed. LbrM - L. (V.) braziliensis, LinJ - *L. (L.) infantum*, LmjF - L. (L.) major, LmxM - *L. (L.) mexicana*.

species cause different forms of diseases and are present in specific vectors and hosts. Previously, it was reported that more than 99% of genes are conserved between *L. (V.) braziliensis*, *L. (L.) infantum* and *L. (L.) major*, revealing a high degree of synteny for genomes of different *Leishmania* species [11]. Our data indicates that, when analysing strictly protease genes, this same scenario holds up, as we also observed high synteny between the studied species.

When contemplating the usefulness of parasite proteases as new targets for chemotherapies, it is very important to consider the hypothesis that these enzymes are unique to the *Leishmania* species and quite different from corresponding enzymes in their mammalian hosts, such as humans and dogs. Thus, to verify this hypothesis, we conducted a BLAST (Basic Local Alignment Search Tool) analysis to compare the genes that show synteny among the greater

number of the four species (represented in the intersection of the Venn diagram – Figure 2) with mammalian protease genes (taxid:40674). The genes 05.0960 and 11.0630 of *L. (L.) major*, *L. (L.) mexicana*, *L. (L.) major* show the highest degree of relational similarity with mammalian genes, with approximately 69% sequence identity and a query coverage of up to 39%. However, in general, the query coverage was very low, with a mean value of 2%. In addition, to perform a similar study with other proteases that did not show synteny among all the studied species, we used a different approach.

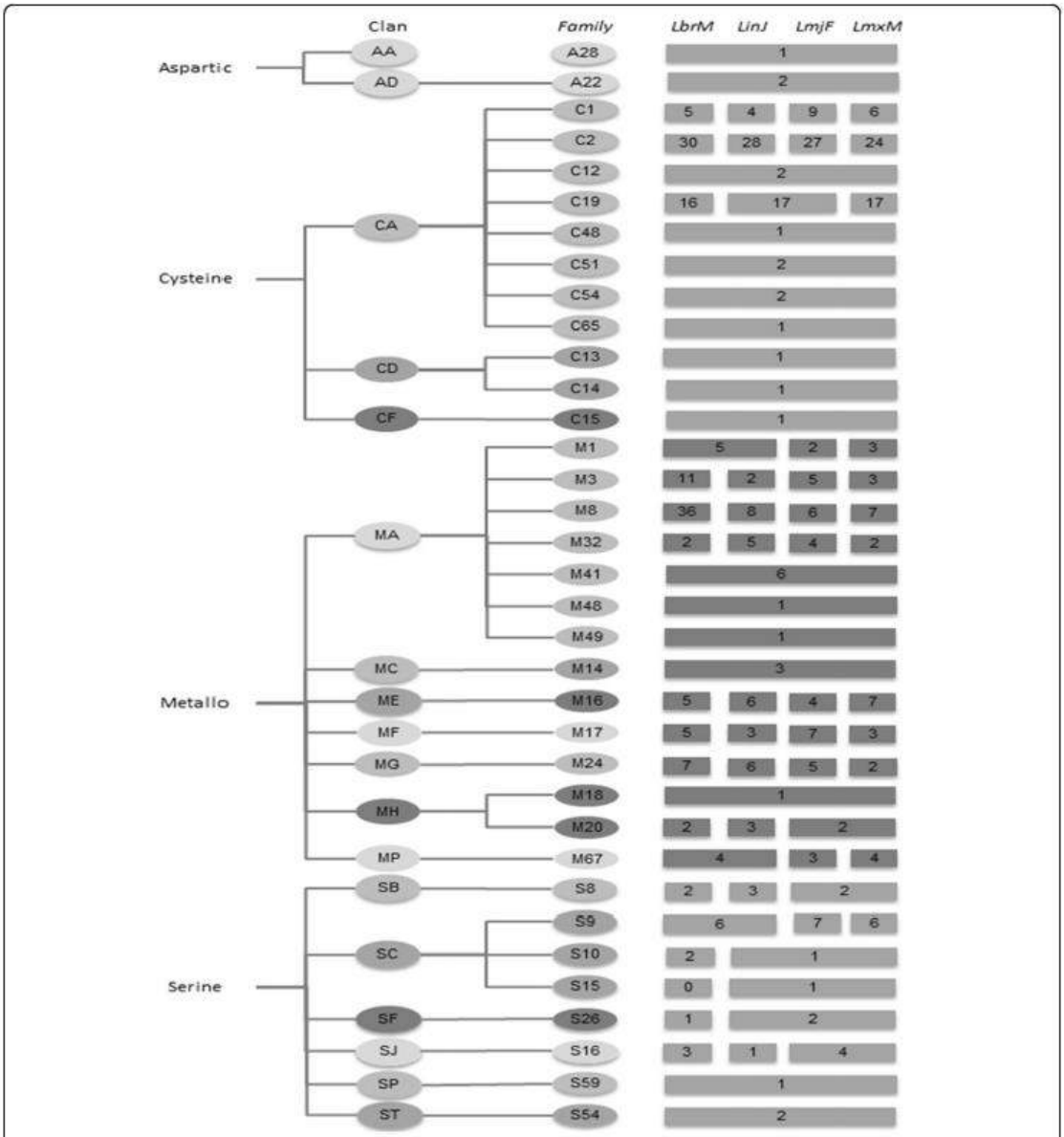


Figure 3 Clans and families of *Leishmania* spp. proteases. Classification of proteases sequence researched. Nomenclature is based on the MEROPS database (December 20013, release 9.10 - http://merops.sanger.ac.uk/). Numbers in squares represent the estimated number of protease gene in each family for each species, as number in rectangle corresponds to the same number of protease per specie. LbrM - *L. (V.) braziliensis*, LinJ - *L. (L.) infantum*, LmjF - *L. (L.) major*, LmxM - *L. (L.) mexicana*.

Initially, a multiple alignment analysis was carried out on the sequences of protease genes of the four species (software *CD-HIT* [12]), using a cutoff of 80% sequence identity to cluster them. As result, we were able to establish 28 clusters of metalloprotease genes, 27 of cysteine protease genes, 11 of serine protease genes and 1 of aspartic protease genes.

The consensus sequences (Additional file 2: Table S2) of each cluster were then used in the BLAST analysis to find similarity with mammalian genes. We identified sequences of O-sialoglycoprotein endopeptidase genes of hamster, dog, wolf and mice with 69% sequence identity to a consensus sequence of *Leishmania* metalloprotease genes LbrM.31.0100, LinJ.31.0110, LmjF.31.0100 and LmxM.31.0100. Sequences of 26S subunit ATPase genes of a lagomorph *Ochotona princeps* and of mice showed 65% sequence identity to a consensus sequence of serine protease genes LbrM.03.0450, LinJ.03.0520, LmjF.03.0540 and LmxM.03.0540. Additionally, we could not find any similarity among sequences of cysteine and aspartic protease genes of mammals and *Leishmania* spp.

As proteases can be grouped into different families and clans depending on intrinsic evolutionary relationships, we classified and organized the protease genes surveyed in this study applying criteria from MEROPS [13] (up to December 2013) (Figure 3). This classification is based on structural and functional similarities between these proteolytic enzymes. Clans contain enzymes with related structures and families contain enzymes with related sequences [4]. This classification is highly relevant to understanding the organization of these parasites' degradomes.

Cysteine proteases and metalloproteases are the major representative classes of proteases in this study, corresponding to 43% and 42%, respectively of the protease genes in the studied *Leishmania* spp. In this survey, three clans of cysteine proteases were observed in the studied species: clan CA, CD and CF. These cysteine proteases are distributed among 11 families from which C1, C2 and C19 have more members. The MPs observed in the study belong to the clans MA, MC, ME, MF, MG, MH and MP and are further distributed among 14 families (Figure 3). The diversity of protease genes observed in the analysis reinforces the idea that this class of enzyme is crucial to the parasite lifecycle, although until now the role of most of these proteases can only be predicted based on current knowledge of homologous enzymes, therefore pointing to the necessity of more studies characterising proteases [14].

The high number of metalloprotease genes in *L. (V.) braziliensis* relates to the 36 distinct genes of the zinc metalloprotease gp63. This metalloprotease is a very well-characterised virulence factor for *L. (L.) braziliensis* and has several reported functions in the interactions of this parasite with its hosts [15]. In *L. (L.) major, L. (L.) mexicana* and *L. (L.) infantum*, the diversity of gp63 genes is much lower: only 6, 7 and 8 genes, respectively, of this protease could be found (Figure 3). The organization of metalloprotease genes in species of the subgenus *Viannia* is rather different than that of species of the subgenus *Leishmania* [16]. The predominance of metalloprotease genes in *L. (V.) braziliensis*, a peculiarity also observed in *L. (V.) guyanensis* [17], has a biological significance not completely understood [8,18]. Amplification of genes is a common phenomenon in *Leishmania* [19-21] and is a likely source of the differences between the two subgenera. Such interesting variation might have fundamental implications for the way each species interacts with its hosts.

Our study highlights the informative potential of analysing genome databases for understanding the gene organization of parasites. However, one should be aware that not all annotated proteases have described roles in the *Leishmania* life cycle. Thus, the picture observed here is not yet complete.

It is still unclear how the current organization of *Leishmania* spp genomes evolved, but the set of results gathered here emphasises the capacity of *Leishmania* species to use the plasticity of their genomes to modulate their phenotypes and increase their odds of survival within hosts, among other biological processes. The diversity of protease genes described by our present study points to their potential importance as survival and adaptation tools and, consequently, as important targets in vaccination and therapy strategies.

Additional files

Additional file 1: Figure S1. Representation of fusion events between chromosomes 29 and 8 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S2.** Representation of allelic transpositions between chromosomes 30 and 29 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S3.** Representation of allelic transpositions between chromosomes 31 and 30 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S4.** Representation of allelic transpositions between chromosomes 32 and 31 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S5.** Representation of allelic transpositions between chromosomes 33 and 32 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S6.** Representation of allelic transpositions between chromosomes 34 and 33 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S7.** Representation of allelic transpositions between chromosomes 35 and 34 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively. **Figure S8.** Representation of fusion events between chromosomes 36 and 20 of *L. (L.) major* (LmjF) and *L. (L.) mexicana* (LmxM), respectively.

Additional file 2: Table S1. Protease genes exclusive to each *Leishmania* sp. amongst the four studied species. **Table S2.** Cluster of genes.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
MSA, FSS and CRA formulated the idea and wrote the manuscript; MSA, FSS, and MFM performed the analysis processes. MSA, FSS, CRA, BASP, provided critical comments to the methods and the discussion. All authors approved the final version of this manuscript.

Acknowledgements
We thank FAPERJ (E-26/102.413/2010, E-26/110.592/2012) and CAPES (23038.007057-98) for partial financial support of this research. Mariana Silva-Almeida and Franklin Souza-Silva are doctoral students of Fiocruz institution; Dr. Bernardo A. S. Pereira and Dr Michelle Lopes Ribeiro-Guimarães are postdoctoral researcher's fellow of CAPES/FAPERJ; and Dr. Carlos R. Alves is fellow researcher of CNPq institution.

Received: 7 May 2014 Accepted: 14 August 2014
Published: 20 August 2014

References

1. WHO: *Fact sheet n° 375*. 2014. updated January 2014 [http://www.who.int/mediacentre/factsheets/fs375/en/]
2. Silva-Almeida M, Pereira BA, Ribeiro-Guimarães ML, Alves CR: **Proteinases as virulence factors in *Leishmania* spp. infection in mammals.** *Parasit Vectors* 2012, **7**(5):160.
3. Barrett AJ: **Classification of peptidases.** *Methods Enzymol* 1994, **244**:1–15.
4. Rawlings ND, Waller M, Barrett AJ, Bateman A: **MEROPS: the database of proteolytic enzymes, their substrates and inhibitors.** *Nucleic Acids Res* 2014, **42**(Database issue):D503–509.
5. El-Sayed NM, Myler PJ, Blandin G, Berriman M, Crabtree J, Aggarwal G, Caler E, Renauld H, Worthey EA, Hertz-Fowler C, Ghedin E, Peacock C, Bartholomeu DC, Haas BJ, Tran AN, Wortman JR, Alsmark UC, Angiuoli S, Anupama A, Badger J, Bringaud F, Cadag E, Carlton JM, Cerqueira GC, Creasy T, Delcher AL, Djikeng A, Embley TM, Hauser C, Ivens AC, *et al*: **Comparative genomics of trypanosomatid parasitic protozoa.** *Science* 2005, **309**:404–409.
6. López-Otín C, Overall CM: **Protease degradomics: a new challenge for proteomics.** *Nat Rev Mol Cell Biol* 2002, **3**(7):509–519.
7. *GeneDB*. [www.genedb.org]
8. Victoir K, Dujardin JC: **How to succeed in parasitic life without sex? Asking Leishmania.** *Trends Parasitol* 2002, **18**(2):81–85.
9. Rogers MB, Hilley JD, Dickens NJ, Wilkes J, Bates PA, Depledge DP, Harris D, Her Y, Herzyk P, Imamura H, Otto TD, Sanders M, Seeger K, Dujardin JC, Berriman M, Smith DF, Hertz-Fowler C, Mottram JC: **Chromosome and gene copy number variation allow major structural change between species and strains of *Leishmania*.** *Genome Res* 2011, **21**(12):2129–42.
10. Carver T, Berriman M, Tivey A, Patel C, Bohme U, Barrell BG, Parkhill J, Rajandream MA: **Artemis and ACT: viewing, annotating and comparing sequences stored in a relational database.** *Bioinformatics* 2008, **24**:2672–2676.
11. Peacock CS, Seeger K, Harris D, Murphy L, Ruiz JC, Quail MA, Peters N, Adlem E, Tivey A, Aslett M, Kerhornou A, Ivens A, Fraser A, Rajandream MA, Carver T, Norbertczak H, Chillingworth T, Hance Z, Jagels K, Moule S, Ormond D, Rutter S, Squares R, Whitehead S, Rabbinowitsch E, Arrowsmith C, White B, Thurston S, Bringaud F, Baldauf SL, *et al*: **Comparative genomic analysis of three *Leishmania* species that cause diverse human disease.** *Nat Genet* 2007, **7**:839–847.
12. Li W, Jaroszewski L, Godzik A: **Clustering of highly homologous sequences to reduce the size of large protein databases.** *Bioinformatics* 2001, **17**(3):282–283.
13. *MEROPS*. [http://merops.sanger.ac.uk/]
14. Mottram JC, Coombs GH, Alexander J: **Cysteine peptidases as virulence factors of *Leishmania*.** *Curr Opin Microbiol* 2004, **7**:375–381.
15. Olivier M, Atayde VD, Isnard A, Hassani K, Shio MT: ***Leishmania* virulence factors: focus on the metalloprotease GP63.** *Microbes Infect* 2012, **14**(15):1377–1389.
16. Voth BR, Kelly BL, Joshi PB, Ivens AC, McMaster WR: **Differentially expressed *Leishmania major* gp63 genes encode cell surface leishmanolysin with distinct signals for glycosylphosphatidylinositol attachment.** *Mol Biochem Parasitol* 1998, **93**(1):31–41.
17. Steinkraus HB, Greer JM, Stephenson DC, Langer PJ: **Sequence heterogeneity and polymorphic gene arrangements of the *Leishmania guyanensis* gp63 genes.** *Mol Biochem Parasitol* 1993, **62**:173–185.
18. Victoir K, Arevalo J, De Doncker S, Barker DC, Laurent T, Godfroid E, Bollen A, Le Ray D, Dujardin JC: **Complexity of the major surface protease (msp) gene organization in *Leishmania (Viannia) braziliensis*: evolutionary and functional implications.** *Parasitology* 2005, **131**:207–214.
19. Iovannisci DM, Beverley SM: **Structural alterations of chromosome 2 in *Leishmania major* as evidence for diploidy, including spontaneous amplification of the mini-exon array.** *Mol Biochem Parasitol* 1989, **34**:177–188.
20. Inga R, De Doncker S, Gomez J, Lopez M, Garcia R, Le Ray D, Arevalo J, Dujardin JC: **Relation between variation in copy number of ribosomal RNA encoding genes and size of harbouring chromosomes in *Leishmania* of subgenus *Viannia*.** *Mol Biochem Parasitol* 1998, **92**:219–228.
21. Kebede A, De Doncker S, Arevalo J, Le Ray D, Dujardin JC: **Size-polymorphism of mini-exon gene-bearing chromosomes among natural populations of *Leishmania*, subgenus *Viannia*.** *Int J Parasitol* 1999, **29**:549–557.

doi:10.1186/1756-3305-7-387
Cite this article as: Silva-Almeida *et al.*: Overview of the organization of protease genes in the genome of *Leishmania* spp. *Parasites & Vectors* 2014 **7**:387.

Anexo 3:

16

Affinity-Based Methods for the Separation of Parasite Proteins

C.R. Alves, F.S. Silva, F.O. Oliveira Jr, B.A.S. Pereira,
F.A. Pires and M.C.S. Pereira
Instituto Oswaldo Cruz – Fundação Oswaldo Cruz,
Rio de Janeiro, RJ,
Brasil

1. Introduction

Affinity chromatography-based techniques have been developed to purify parasite proteins and improve our understanding of the parasite life cycle. These advances can be translated into concrete proposals for new drugs, diagnostic methods and vaccines for parasite diseases and help to reduce social inequality.

Affinity chromatography has been demonstrated to be a powerful tool for the isolation and purification of parasite proteins and has potential applications for diagnosis and therapy. Many studies have focused on parasite proteins that modulate host cell defense, as gp63, a glycoprotein from *Leishmania spp.*, that is involved in the cleavage of the complement factor C3b to iC3b, which promotes adhesion of promastigotes to macrophages via complement receptor 3 (Brittgham et al., 1995). This route of internalization does not lead to production of oxygen radicals or NO and favors parasite subsistence within the host cell. Another example is the cysteine protease B (CPB), an important virulence factor of the *Leishmania (L.) mexicana* complex, that inhibits lymphocytes Th1 and/or promotes the Th2 response either through proteolytic activity or through epitopes derived from its COOH-terminal extension (Pereira et al., 2011).

Due to the important role of these molecules, many researchers seek to develop specific and potent inhibitors for therapeutic strategies. Aspartic protease, a potential target for antiparasitic therapies, has been isolated from *Trypanosoma cruzi* by affinity chromatography using a specific inhibitor of this enzyme (Pinho et al., 2009); this enzyme is target for treatment of infections caused by HIV (Wlodawer & Vondrasek, 1998) and Candida (Hoegl et al., 1999). This enzyme has also been reported in *Plasmodium* spp. and *Schistosoma mansoni*, where it plays an important role in host hemoglobin degradation (Klemba & Goldberg, 2002). Additionally, specific inhibitors of plasmepsins and renin are viable drugs for the treatment of patients with malaria and high blood pressure.

These parasite proteins, along with others, have been tested as new targets for chemo- and immunotherapies for parasite diseases. They have been assessed by lectins or protease inhibitor affinity chromatography. The separation of sugars based on lectin affinity is one of main procedure that has been used. This technique is based on the ability of lectins to bind

specifically to certain oligosaccharide structures on glycoconjugates isolated from parasites. Parasite proteins are processed through a multi-lectin affinity column, and they bind to the immobilized lectins through their sugar chains. Certain glycoconjugates are important for the parasite life cycle, and lectin affinity chromatography can help to reveal their roles (Guha-Niyogi et al., 2001).

The use of protease inhibitors in affinity chromatography is another important approach for assessing parasite proteins. Proteases hydrolyze peptide bonds and can therefore degrade proteins and peptides that influence a broad range of biological functions, including the process of parasite infection (Mackeron et al., 2006). The specificity of the protease inhibitor used is an important aspect of this methodology; L- trans-epoxy-succinylleucylamido-(4-guanidino) butane (specific to cysteine-protease), pepstatin A (to aspartyl-protease) and aprotinin (to serine-protease) are frequently immobilized on a solid matrix for this technique.

Glycosaminoglycan (GAG) affinity is the only affinity chromatography method that is based on the sugar chains of lectin-like proteins. Some of these molecules (such as heparin sulfate, heparan sulfate, dermatan sulfate, keratan sulfate and chondroitin sulfate) contain complex oligosaccharide structures, which may be displayed on cell surfaces, incorporated into the extracellular matrix or attached to secreted glycoproteins, suggesting that they play structural roles (Dreyfuss et al., 2010). GAGs have been reported as potential candidates for therapeutic intervention against parasitic infections, such as leishmaniasis and Chagas diseases (Azevedo-Pereira et al., 2007; Oliveira-Jr et al., 2008).

According to the general principle of affinity chromatography (Fig. 1), a protein of interest is recovered based on its capacity to bind a specific functional group (ligand) that is immobilized on a bead material (matrix) that has been packed into a solid support (column). Although many ligands (enzymatic substrates, inhibitors of an enzyme, lectin, sugar residues, vitamins, enzyme cofactors, monoclonal antibodies) have been used to isolate proteins based on affinity, only lectin, an enzyme inhibitor and glycosaminoglycans have been used to obtain parasite proteins. The most commonly used matrix materials for the attachment of the ligand are polysaccharide derivatives (cellulose, dextran and agarose) and polyacrylamide.

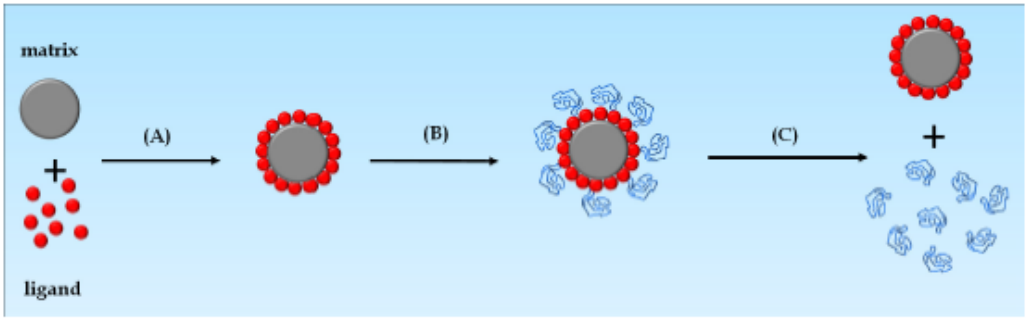


Fig. 1. The principle of affinity chromatography. The ligand is covalently bound to a matrix (A). The functionalized matrix is then able to bind to a target protein aided by a binding buffer (B). Afterwards, the bound proteins are eluted with a different buffer (C).

In these procedures, the soluble proteins are prepared from crude parasite lysates (or sub-cellular fractions) and loaded onto a column under chemical (buffer) and physical (temperature and pressure) conditions that promote the specific binding of the protein to the immobilized ligand (affinity) in what is known as the binding phase. Proteins that do not bind to the immobilized ligand under these conditions are removed from the solid phase by application of a constant liquid phase, which is referred to as the wash phase. Then, the bound protein can be recovered by changing the buffer conditions to favor desorption during the elution phase.

In this chapter, we describe the use of affinity chromatography to assess parasite proteins and the importance of these methods for public health. Several affinity chromatography protocols are considered. Additionally, we discuss our experience using affinity chromatography to obtain parasite proteins, and we include some unpublished results related to *Dermatobia hominis* third (L3) instar larvae proteases.

2. The use of affinity chromatography in parasite protein studies

2.1 Lectin affinity-based separation of parasite proteins

There are relatively few studies available in the current literature describing the use of lectins to affinity-purify glycosylated proteins from parasites. However, the reports on this subject demonstrate that this technique is useful for the retrieval of putative virulence factors or potential protective immunogens from a large array of parasites, including apicomplexan, trypanosomatids and nematodes (e.g., Fauquenoy et al., 2008, Gardiner et al., 1996, Smith et al., 2000). In addition to its utility in the isolation of parasite factors, lectin-based affinity chromatography is also a valuable resource for characterization of the structure of carbohydrates bound to proteins from these organisms due to the distinct specificities of the lectins that are available for this type of analysis.

Lectins are proteins that specifically bind to sugars, and they have been used for many types of studies, ranging from blood typing to immune regulation analysis (Rüdiger & Gabius, 2001). These proteins are generally isolated from plants (mostly legume seeds), where they can be found in abundance. Their usage is determined by the particular sugar structures that they are able to bind (Rüdiger & Gabius, 2001). The surveyed literature the use of six plant lectins [concanavalin A (Con A), ricin, jacalin, peanut agglutinin (PNA), wheat germ agglutinin (WGA) and Wisteria floribunda agglutinin (WFA)] in studies of parasites glycoproteins. Furthermore, one report described the use of Biomphalaria alexandrina lectin (BaSII), which in contrast to the others is a lectin obtained from an animal.

Con A is a lectin that can be extracted from jack beans of the species *Canavalia ensiformis* (family Fabaceae). It binds to mannose or glucose residues and is thus characterized as a mannose-binding lectin. This lectin presents a high affinity for the oligosaccharide GlcNAcβ2Manα6(GlcNAcβ2Manα3)-Manβ4GlcNAc. It is also known to be a potent mitogen (Beckert & Sharkey, 1970; Rüdiger & Gabius, 2001).

Ricin, along with jacalin and PNA, is a lectin that binds to galactose. Specifically, it binds with high affinity to the motif Galβ4GlcNAcβ2Manα6 (Galβ4-GlcNAcβ2Manα3) Manβ4GlcNAc. Ricin is highly toxic because it can impair ribosome activity through cleavage of the nucleobases of ribosomal RNA, and it has potential to be used as a biological

weapon. This lectin is extracted from *Ricinus communis* (Family Euphorbiaceae) (Rüdiger & Gabius, 2001; Lord et al., 2003).

Jacalin binds to galactose and N-acetylgalactosamine, and presents a high affinity for the motif Galβ3GalNAcα. It is obtained from *Artocarpus integrifolia* (Family Moraceae). It is commonly used to isolate IgA from human plasma (Kabir, 1998, André et al., 2007).

Like Con A, PNA is a legume lectin and is isolated from plants that belong to the family Fabaceae. It is extracted from *Arachis hypogea* and binds specifically to the monosaccharide galactose and to the motif Galβ3GalNAcα, similarly to the binding motif of jacalin. PNA is used as a marker of T-cell subpopulations and to differentiate between the stages of the Leishmania parasites life cycle (Dumont & Nardelli, 1979, Wilson & Pearson, 1984, Rüdiger & Gabius, 2001).

WGA is obtained from the species *Triticum vulgare*. It presents a low affinity for N-acetylgalactosamine, but it binds to the sialic acid N-acetylneuraminic and to the motif GlcNAcβ4GlcNAcβ4GlcNAcβ4-GlcNAcβ4GlcNAc. This lectin has been shown to bind more avidly to activated human T lymphocytes (Hellström *et al.*, 1976, Rüdiger & Gabius, 2001).

WFA is isolated from *Wisteria floribunda*, a woody liana of the family Fabaceae. Although some uncertainty regarding its binding specificity remains, it seems that this agglutinin binds preferentially to the monosaccharide N-acetylgalactosamine and to the motif GalNAcα6GalNAc. WFA is used to fractionate lymphocyte populations, and although it is not mitogenic like Con A, it can induce lymphokine production in murine splenocytes (Jacobs & Poretz, 1980; Rüdiger & Gabius, 2001).

BaSII is a lectin that can be isolated from the snail *B. alexandrina*, an intermediate host of the trematoda parasite *Schistosoma mansoni*, the causative agent of schistosomiasis. It specifically binds to the motif Fucα1,2Galβ1,4Glc (Mansour, 1996).

2.1.1 General procedures for the isolation of parasite proteins by lectin affinity

The rational for lectin-based affinity chromatography is the same as for other types of affinity-based fractionation: a sample is exposed to a solid phase that has been coupled to an affinity separation molecule (a lectin, in this case) under conditions that are adequate for binding (Fig. 2A). The unbound material from the sample is washed away (generally using the same buffer applied to equilibrate the solid-phase), and in the final step, the affinity-bound fraction is recovered by altering the equilibrium conditions of the solid phase (by changing the system pH or salt concentration) or by adding a molecules that competes for the binding site of the ligand.

To provide several practical examples, a collection of lectin affinity-based methodologies used to isolate and/or characterize glycoproteins from distinct parasites is listed in the Table 1.

It is important to note that some techniques, such as metabolic radioactive labeling (by [3H]-myristic acid or [3H]-glucosamine, for example) and cell disruption (by Triton X-100, dioxane or hypotonic solution), must be applied prior to lectin chromatography to allow for the identification of molecules eluted from the column or the preparation of suitable samples for the chromatography column, respectively.

Ligand	Organism	Isolated protein	First phase Matrix	Bind	Wash	Elution	Second phase Methods	References
WGA	*Trypanosoma cruzi*	85 kDa surface glycoprotein	Sepharose	10mM Tris-Hcl (pH 7.2), 150 mM NaCl	10mM Tris-Hcl (pH 7.2), 150 mM NaCl	0.1 M N-acetyl-D-glucosamine in 10mM Tris-Hcl (pH 7.2), 150 mM NaCl	None	Couto et al., 1990
Ricin	*Trypanosoma brucei rhodesiense*	Membrane Glycoprotein	Agarose	10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	0.2 M acetic acid in 10 mM Mops buffer (pH 6.9), 1mM $MgSO_4$, 1 mM EGTA, 0.2% (v/v) Triton X-100, 10 µg/ml DNase I, 0.05 mM leupeptin, 2.5 mM PMSF, 5 mM iodoacetamide, 0.05 mM TPCK	None	Brickman & Balber, 1993
BaSII	*Schistosoma mansoni*	37 kDa glycoprotein	Sepharose	20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM PMSF, 1% (v/v) Triton X-100, 1 mM PCMB, 1 mM o-pheri, 1 mM iodoacetamide	20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM $CaCl_2$ 1mM $MgCl_2$	300 mM L-fucose in 20 mM Tris-HCl (pH 7.3), 0.1% (v/v) Triton X-100, 150 mM NaCl, 1mM $CaCl_2$, 1mM $MgCl_2$	HPLC	Mansour, 1996
Concanavalin A or ricin	*Trypanosoma brucei*	Small Variable Surface Glycoprotein	Sepharose (Con A) or agarose (ricin)	10 mM Tris-HC (pH 7.4) ,150 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 2% NP-40, 100 µg/ml of antipain, leupeptin and E-64	10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM $MgCl_2$, 5 mM $CaCl_2$, 2% NP-40, 100 µg/ml of antipain, leupeptin and E-64	0.5 M alpha-methyl mannoside (Con A) or 0.5 M galactose (ricin) in 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% NP-40, and 50 µg/ml antipain, leupeptin and E-64	Dylasis	Gardiner *et al.*, 1996
Concanavalin A	*Trypanosoma congolense*	Variant Surface Glycoprotein	Sepharose	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM $CaCl_2$, 1mM $MnCl_2$	10mM Tris-HCl (pH 7.5), 150 mMNaCl, 1mM $CaCl_2$, 1mM $MnCl_2$	Isoelectric focusing	Gel filtration (Biogel P 30)	Gerold *et al.*, 1996
WFA	*Trypanosoma congolense*	Variant Surface Glycoprotein	Agarose	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide	50 mM Tris-HCl (pH 7.4), 0.02% sodium azide, 100 mM GalNAc	None	Gerold *et al.*, 1996
Concanavalin A	*Trypanosoma brucei*	Invariant surface glycoprotein heavily N-glycosylated	Sepharose	50 mM Tris buffer (pH 7.5), 150 mM NaCl, 1% (w/v) Triton X-100	10mM Tris buffer (pH 6.5), 0.1% (w/v) Triton X-100	0.5 M α-methylmannoside in 10mM Tris buffer (pH 6.5) containing 0.1% (w/v) Triton X-100	Ion exchange chromatography (DEAE-52)	Nolan *et al.*, 1997

Table 1. Lectin affinity-based

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
PNA or jacalin	*Haemonchus contortus*	Glycoprotein fractions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca^{2+}, 10 mM Mg^{2+}	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca_2, 10 mM Mg^{2+}	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q – jacalin-binding material only)	Smith *et al.*, 2000
Concanavalin A	*Caenorhabditis elegans*	Glycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$ 0.25% CHAPS	None	Redmond *et al.*, 2004
Ricin	*Trypanosoma brucei*	Glycoproteins presenting giant poly-N-acetyllactosamine carbohydrate chains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	30 mg/ml lactose and 30 mg/ml galactose in 12.5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X-100, 25 mM TLCK, 0.25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih *et al.*, 2005
Concanavalin A	*Toxoplasma gondii*	N-linked glycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M α-methyl-D-mannopyranoside	None	Fauquenoy et al., 2008
Concanavalin A	*Leishmania (V.) braziliensis*	Cysteine proteinases	Sheparose	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM α-Dmannose in 20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009

Table 1. (continued)

During the affinity chromatography procedure, other methods, such as isoelectric focusing, may be used instead of the application of competing carbohydrates to elude the column-bound material. Furthermore, distinct affinity columns can be used in sequence to purify fractions with specific characteristics from a single sample.

As for the handling of the material that is eluted from an affinity column, many options for further purification are available, depending on the analysis method chosen for the study. Some of these options include: anion exchange chromatography, size exclusion chromatography and dialysis.

The combination of these accessible approaches allows for a vast array of study possibilities. Several examples of the results obtained by applying lectin-affinity chromatography in association with other techniques are described in the following paragraphs.

2.1.2 Parasite proteins isolated by lectin affinity chromatography

The structure of an N-linked oligosaccharide from a surface glycoprotein of *Trypanosoma cruzi*, an important human parasite that causes Chagas disease, was defined in a study using lectin chromatography (Couto et al., 1990). It was determined that the structure of this oligosaccharide is comprised of complex carbohydrate chains that possess a terminal sialic acid, α-L-fucose and a galactosyl(α1,3)galactose unit.

The cellular localization of glycoproteins of *Trypanosoma brucei rodhesiense*, a subspecies of the parasite responsible for the African sleeping sickness, was analyzed using ricin-based chromatrography (Brickman & Balber, 1993). It was observed that the ricin-binding proteins were primarily located in the vesicles of the lysosomal /endosomal system.

Gardiner et al., (1996) characterized small glycoproteins isolated from the surface of *Trypanosoma vivax*, which causes bovine trypanosomiasis. That study was the first to detail the characteristics of a *T. vivax* Variable Surface Glycoprotein (VSG). The isolated protein, designated ILDat 2.1 VSG, presented a molecular mass of 40 kDa and contained mannose (or a derivative sugar) in small quantities, and it was poorly retained by the lectin affinity column. It is possible that carbohydrates comprise only the C-terminal anchoring structure of this protein.

The characteristics of a fucosyllactose determinant of a *S. mansoni* glycoprotein were identified using affinity chromatography based on a lectin that was isolated from a host of this parasite, *B. alexandrina*. This determinant is expressed in the outer chain of a single unit of complex type N-linked oligosaccharides (Mansour, 1996).

Additionally, the VSG glycosyl-phosphatidylinositol membrane anchors of *Trypanosoma congolense*, another trypanosomatide species that causes bovine trypanosomiasis, were studied by lectin affinity (Gerold et al., 1996) using a modification of the technique in which the bound proteins are electrophoretically desorbed (Reinwald et al., 1981). This analysis allowed for description of the VSG GPI-anchor in this parasite: it contains a β1,6-linked galactose as the terminal hexose of the branch and an N-acetyl-glucosamine residue. Also, it was observed that *T. congolense* synthesizes two potential GPI-anchor precursors, one of which is insensitive to phospholipase C activity.

Nolan et al., (1997) identified a new invariant surface glycoprotein that is heavily N-glycosylated in the bloodstream forms of *Trypanosoma brucei* and designated it as ISG_{100}. This glycoprotein presents a large internal domain composed of a serine-rich repetitive motif, which was previously undescribed, and N-glycosylation sites on the N-terminal domain. Additionally, ISG_{100} is encoded by a single gene, whereas the trypanosomal plasma membrane proteins are commonly encoded by tandemly repeated genes that are part of a multigene family.

Potentially protective glycoprotein fractions from *Haemonchus contortus*, a parasitic nematode in ruminants, were also obtained by lectin chromatography (Smith *et al.*, 2000). The findings from that study confirmed the potential of the *H. contortus* PNA-binding glycoprotein fraction as an efficacious antigen against this parasite infection in sheep. Furthermore, this study identified another highly protective fraction that binds to jacalin. This second protective fraction presents sialyted versions of the same oligosaccharides that bound to the PNA column.

Another study on the protective properties of the glycoproteins of *H. contortus* was performed by the same group (Smith et al., 2003). The results showed that the four purified glycosylated zinc metalloproteinases from this parasite were such an efficacious antigen that, to an extent, they could account for most of the protection conferred by the urea-dissociated whole glycoproteins fraction. However, the role for the glycan moieties of these enzymes in the protection process was not clear.

The capacity of glycoproteins from *Caenorhabditis elegans*, a free living nematode, to induce protection from a challenge with *H. contortus* in sheep was assayed by Redmond et al. (2004). The lectin affinity methodology was able to identify glycoproteins with molecular masses between 25 and 200 kDa in extracts prepared from *C. elegans*, but the fractionated glycoproteins were not able to confer protection against an *H. contortus* challenge. These findings suggest that the conserved glycan moieties between these two species of worm are not solely responsible for the protections levels observed when native *H. contortus* antigens are used.

Trypanosoma brucei glycoproteins were shown to present distinctive structural features, such as the presence of giant poly-N-acetyllactosamine carbohydrate chains (Atrih et al., 2005). The recovered affinity-bound molecules were predominantly, but not exclusively, from the flagellar pocket. These glycoproteins carry massive glycans, representing the largest poly-LacNAc structures reported to that date, and they may produce a gel-like matrix in the lumen of the flagellar pocket and/or the endosomal/lysosomal system. Despite their remarkable size, these glycans present a very simple neutral structure, containing only mannose, galactose and N-acetylglycosamine.

Important glycoproteins from the apicomplexan parasite *Toxoplasma gondii* have also been analyzed by lectin affinity methods. It was shown that these components are pivotal factors for host invasion and intracellular development of parasites (Fauquenoy et al., *2008*).

Cysteine proteinases from promastigostes of *Leishmania (Viannia) braziliensis* were shown to be anchored to the membrane by glysoylphosphatidylinositol structures in an analysis of the hydrophobic fraction of promastigote forms. These enzymes are suggested to play a role in the process of parasite survival inside its hosts (Rebello et al., 2009).

2.1.3 Remarks on the isolation of proteins by lectin affinity chromatography

These reports provide examples of the uses of lectin affinity chromatography to identify potentially antigenic fractions of parasites that could be used for vaccine development. Also, they point to the potential of this method to characterize glyconjugates, such as the glycoproteins that are present on the parasite surface or secreted by these organisms. However, apart from these purely structural or clinically oriented applications, this method may also be relevant in other investigations, including studies of host-parasite interactions. This hypothesis is reinforced by reports indicating that lectin-glycan binding is important for the infection and virulence processes of some parasites, *e.g. Acanthamoeba castellanii* (Garate et al., 2006), *H. contortus* (Turner et al., 2008), *L. (V.) braziliensis* (Rebello et al., 2009) and *T. gondii* (Fauquenoy et al., 2008)

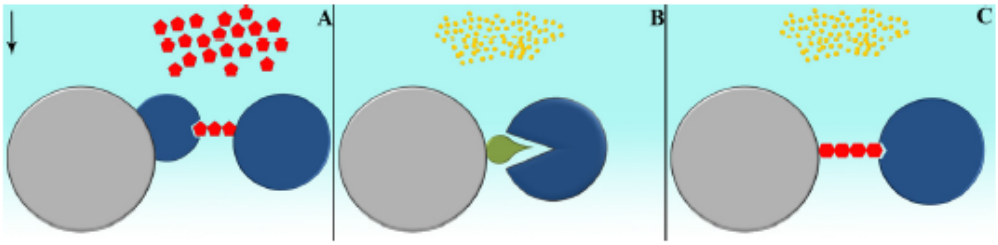


Fig. 2. Illustration of the affinity chromatography methodologies. The target molecules are bound to their ligands immobilized on a solid phase matrix. (A) Lectin affinity chromatography, (B) Protease inhibitor affinity chromatography and (C) Glycosaminoglycan affinity chromatography. Proteins = blue circle; carbohydrates = red pentagon and hexagon; protease inhibitors = green drop-like form; ions =yellow circles; and solid phase matrix beads = gray circle.

2.2 Protease inhibitors affinity-based separation of parasite proteins

Methodologies for the purification of parasite proteases have been applied in studies investigating the biological roles of these enzymes in parasite, including their participation in the infection process and in the survival of the parasites inside their hosts (McKerrow et al, 2006). Inhibitor affinity chromatography consists of the fractionation of parasite samples based on the reversible interactions between proteases and their specific inhibitors while the latter are covalently attached to a matrix (Fig. 2B). This technique can also be performed using irreversible inhibitors under particular conditions that will be described further in this section.

It is also interesting to note that, based on the specificity of the inhibitor used in the affinity chromatography, it is possible to suggest the enzyme class of the isolated protein. However, complementary analyses, such as characterization of the proteolytic activity, are often necessary to confirm these findings. Nevertheless, this purification strategy presents an initial advantage when compared to other methodologies.

In this section, fractionation approaches for serine-, aspartic acid- and cysteine proteases in specific parasites will be described. These approaches must take the class of the studied enzyme into consideration, as well as the inhibitor to be used and the characteristics of the mobile phase used for chromatography.

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
PNA or jacalin	*Haemonchus contortus*	Glycoprotein fractions	Agarose	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02% NaN3, 100 mM Ca^{2+}, 10 mM Mg^{2+}	10mM Tris-HCl (pH7.4, 0.5 M NaCl, 0.02 % NaN3, 100 mM Ca_2, 10 mM Mg^{2+}	0.5 M galactose (PNA) or 0.8 M galactose (jacalin)	Gel filtration (Sephadex G-25); Anion Exchange chromatography (Mono Q – jacalin-binding material only)	Smith *et al.*, 2000
Concanavalin A	*Caenorhabditis elegans*	Glycoproteins	Agarose	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.5% Triton X-100	10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.25% CHAPS	0.2M methylmannopyranoside and 0.2 M methylglucopyranoside in 10 mM Tris (pH 7.4) 0.5 M NaCl, 10 mM $CaCl_2$, 100 mM $MnCl_2$, 0.25% CHAPS	None	Redmond *et al.*, 2004
Ricin	*Trypanosoma brucei*	Glycoproteins presenting giant poly-N-acetyllactosamine carbohydrate chains	Agarose	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	50 mM Tris-HCl (pH 6.8), 400 mM NaCl, 0.8% Triton X-100, 0.1 M TLCK, 1 µg/ml leupeptin, 0.1% sodium azide	30 mg/ml lactose and 30 mg/ml galactose in 12,5 mM Tris-HCl (pH 6.8), 100 mM NaCl, 0.2% Triton X-100, 25 mM TLCK, 25 µg/ml leupeptin, 0.025% sodium azide	None	Atrih *et al.*, 2005
Concanavalin A	*Toxoplasma gondii*	N-linked glycoproteins	Agarose	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100, protease inhibitor mixture	10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM CaCl2, 1 mM MnCl2, 1% (v/v) Triton X-100	1% (w/v) SDS in 100 mM Tris-HCl (pH 7.4) or 0.5 M α-methyl-D-mannopyranoside	None	Fauquenoy et al., 2008
Concanavalin A	*Leishmania (V.) braziliensis*	Cysteine proteinases	Sheparose	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	50 mM α-Dmannose in 20 mM Tris-HCl (pH 7.2), 5% (v/v) glycerol, 0.5% (v/v) CHAPS	Anion exchange chromatography (DEAE-Sepahcel)	Rebello et al., 2009

Table 2. Protease Inhibitors affinity-based

Ligand	Organism	Isolated protein	First phase				Second phase	References
			Matrix	Bind	Wash	Elution	Methods	
Pepstatin A	*Neospora caninum*	52 kDa	Agarose	5 mM NaOAc, (pH 5.5)	5 mM NaOAc, (pH 5.5)	0.1 M Tris–HCl, 0.5 M NaCl, (pH 8.5)	None	Nagueleswaran *et al*, 2005
Aprotinin	*Leishmania amazonensis*	115 and 56 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez et al; 2005
Bacitracin	*Trichomonas vaginalis*	60 and 30 kDa	Agarose	20 mM NaOAc, (pH 4.0)	20 mM NaOAc, (pH 4.0)	0.1 M Tris-HCl, 1.0 M NaCl 25% 2-propanol, (pH 7.0)	Bio-Gel P-60	Sommer *et al*; 2005
Aprotinin	*Leishmania braziliensis*	60 and45 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Guedes HL *et al*; 2007
Aprotinin	*Trypanosoma cruzi*	75 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	None	Silva-Lopez *et al*; 2008
Aprotinin	*Leishmania donovani*	115 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH 7.5)	10 mM Tris–HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris–HCl, 1.5 M NaCl, (pH7,5)	None	Choudhury *et al*; 2009
Pepstatin A	*Trypanosoma cruzi*	120, 48 and 56 kDa	Agarose	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M NaOAc, 1.0 M NaCl, (pH 3.5)	0.1 M Tris–HCl, 1.0 M NaCl, (pH 8.6)	HPLC gel filtration	Pinho *et al*; 2009
Aprotinin	*Leishmania donovani*	58 kDa	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,4)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,4)	10 mM Tris-HCl, 1 M NaCl, (pH7,5)	DEAE	Choudhury *et al*; 2010
Pepstatin A	*Rhipicephalus (B.) microplus*	42 kDa	Agarose	20 mM NaOAc, 1 M NaCl, (pH 5.3)	20 mM NaOAc, 1 M NaCl, (pH 5.3)	100 mM Tris-HCl, 1 M NaCl, (pH 8.6)	Mono-Q	Cruz *et al; 2010*
Benzamidine	*Plasmodium ookinete*	37 kDa	Sepharose	0.1 % Triton X-100 in PBS	0.1 % Triton X-100 in PBS	PBS	None	Li *et al;* 2010
Aprotinin	*Leishmania chagasi*	LCSII (105, 66, 60 kDa); LCSI (60, 58 kDa) and LCSIII (76, 68 kDa)	Agarose	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 5 mM $CaCl_2$, (pH7,5)	10 mM Tris-HCl, 1,5 mM NaCl, (pH7,5)	HPLC	Silva-Lopez *et al*; 2010
E-64	*Trypanosoma cruzi*	60 kDa	Sepharose	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris-HCl, 150 mM NaCl, 100 M PMSF, (pH 7.4)	20 mM Tris–HCl, 150 mM NaCl, 100 M PMSF, 2.0 M NaCl, (pH 7.4)	None	Bourguignon *et al* 2011

Table 2. (continued)

Aprotinin and pepstatin A are examples of inhibitors that are frequently used in the isolation of serine- and aspartic acid proteases, respectively, from many parasite species (Bond & Beynon). Other inhibitors that have been previously described in the isolation of serine proteases include soybean trypsin inhibitor (SBTI) and chloromethylketone (CMK). As for the purification of cysteine proteases, the use of three other inhibitors has been reported: L-transepoxysuccinyl-leucylamido-[4-guanidino]butane (E-64), bacitracin and glycyl-phenylalanyl-glycyl-semicarbazone (Table 2). It must be emphasized that these inhibitors cannot be used to isolate all of the proteases classes from parasites, as they present distinct affinities for members of different groups and families within these enzyme classes. Therefore, investigation of the possible variations present in the active site of these enzymes may prove useful.

The features of the buffer (temperature, pH and ionic strength) to be used may vary according to the ligand's physicochemical characteristics, the chemical environment of the parasite enzyme and the analyzed species of parasite. For example, distinct buffers were used for the purification of serine proteases from *S. mansoni* and *Trichinella spiralis* using benzamidine. It is also noteworthy that for each organism, a different matrix was used to immobilize the inhibitor, sepharose for *S. mansoni* and celite for *T. spiralis*. The use of distinct buffers in studies that are based on the same inhibitor is also noted in reports of SBTI, E-64, bacitracin and glycyl-phenyalanyl-glycyl-semicarbazone, all of which are cysteine protease inhibitors.

Affinity chromatography with an irreversible inhibitor has also been described previously; the cysteine-protease inhibitor is an example of this strong binding. In the interaction between E-64 and cysteine-protease, a covalent bond is established (Matsumoto, 1989). Therefore, a reaction between the epoxy groups of the inhibitor and the thiopropyl group of the sepharose matrix is necessary to bind E-64 to a solid support. This reaction prevents the reaction of E-64 with the cysteine residue at the protease catalytic center. However, this does not affect the bond between the inhibitor and cysteine-protease; instead, it only results in inhibition of the proteolytic activity (Govrin, 1999).

2.2.1 Parasite proteins isolated by cysteine-protease inhibitors affinity chromatography

There is only one published example of the use of E-64 affinity chromatography to assess cysteine-protease isolated from a parasite, and this study was conducted with the *T. cruzi* epimastigote. In this study, chromatography was useful for assessing the effects of β-Lapachone naphthoquinones on a 60 kDa cysteine-protease activity present in *T. cruzi*. The results demonstrated the potential of this protease inhibitor as a new antichagasic compound (Bourguignon et al., 2011). Another example of a cysteine-protease isolated by inhibitor affinity chromatography in parasites was described for *Plasmodium falciparum*. In this case, a glycyl-phenyalanyl-glycyl-semicarbazone-based column was used to isolate a protease with a molecular weight of 27 kDa, as determined by SDS-PAGE (Shenai et al, 2000).

2.2.2 Parasite proteins isolated by serine-protease inhibitors affinity chromatography

Aprotinin affinity-based chromatography was useful for the isolation of a serine-protease of 115 kDa (Silva-Lopez et al., 2005), a 68 kDa (Morgado- Diaz et al., 2004; Silva-Lopez et al., 2004) and a 56kDa (Silva-Lopez et al., 2004) from *L.(L.) amazonensis* compared to other

purification procedures that were used to isolate parasite serine peptidase enzymes (Kong et al., 2000; Ribeiro de Andrade et al., 1998). In *Leishmania (V) braziliensis* promastigotes, 60 kDa and 45 kDa enzymes were purified using the aprotinin affinity-based and activity esterase assessed against N-alpha-benzoyl-L-arginine ethyl ester hydrochloride and Nalpha-p-tosyl-L-arginine methyl ester hydrochloride (Guedes et al., 2007). Furthermore, three protein profiles were isolated from *Leishmania chagasi* promastigotes, including LCSI (58 and 60 kDa), LCSII (60, 66, 105 and kDa) and LCSIII (68 and 76 kDa), which were characterized as serine-protease enzymes based on their activity toward α-N-p-tosyl-L-arginine methyl ester substrate (Silva-Lopez et al., 2010). Furthermore, serine proteases with molecular weights of 75 kDa (Silva-Lopez et al., 2008) and 115 kDa (Choudhury et al., 2009) were identified as excretory products of *T. cruzi* and components of the sub-cellular environment in *Leishmania donovani*, respectively, although the chromatography step was not able to produce a homogeneous fraction. Furthermore, a intracellular serine protease of 58 kDa was were purified from *Leishmania donovani* (Choudhury et al., 2010).

In addition, the aprotinin affinity-based chromatography was useful for the isolation of serine-proteases of 35 kDa and 26 kDa from *Anisakis simpZex* (Morris et al, 1994), 43 kDa from *Candida albicans* (Morrison et al, 1993), 15 kDa from *Schistosoma mansoni (*Salter et al, 2000*)*, 42 kDa from *Rhipicephalus (B.) microplus* (Cruz et al, *2010*), 60 kDa and 30 kDa from *Trichomonas vaginalis* (Sommer *et al*; 2005) and 35 to 52 from *Caenorhabditis elegans* (Geier et al; 1999).

Benzamidine-celite was applied in the isolation of serine proteases among the excreted or secreted proteins of *T. spirali*. The recovered proteases were not purified to homogeneity, and they showed molecular masses of 18 kDa, 40 kDa and 50 kDa (Todorova & Stoyanov). A similar finding was reported for the serine-proteases of *Chrysomya bezziana* larvae by using an SBTI-based column to purify four proteins with molecular masses of 13 kDa, 16 kDa, 26 kDa and 28 kDa (Muharsini et al., 2000).

Because it is possible to isolate heterogeneous products using inhibitors for affinity-based chromatography, we assessed a serine-protease from the third (L3) instar larvae of *D. hominis*. This ectoparasite causes dermatobiose in vertebrates, including humans, and it is particularly relevant in cattle, where it can cause a drop in production of meat and milk, leather as well depreciation (Maia & Guimarães, 1985).

Due to the association of DEAE-Sephacel with aprotinin agarose, it was possible to assess a serine protease from L3 larvae (Fig. 3). The fractions obtained by ion change chromatography containing estearasic activity were pooled and then fractionated on an aprotinin-agarose column. This fraction showed a profile with multiple bands by SDS-PAGE and silver staining, and only one band of enzyme activity (50 kDa) was detected by gelatin-SDS-PAGE at pH 7.5 (Fig. 3). Interestingly, this band of 50 kDa was not initially detected in the extracts from L3 by gelatin-SDS-PAGE. The expression of this enzyme is likely low in these larvae, and it can only be detected after concentration by chromatographic methods. The proposed strategy to isolate a serine protease allowed for the detection of a band of 50 kDa in extracts of L3 larvae, and this band had not been previously detected in the direct analysis of the total extract by gelatin-SDS-PAGE. Additionally, this fraction was found to have esterase activity (data not shown).

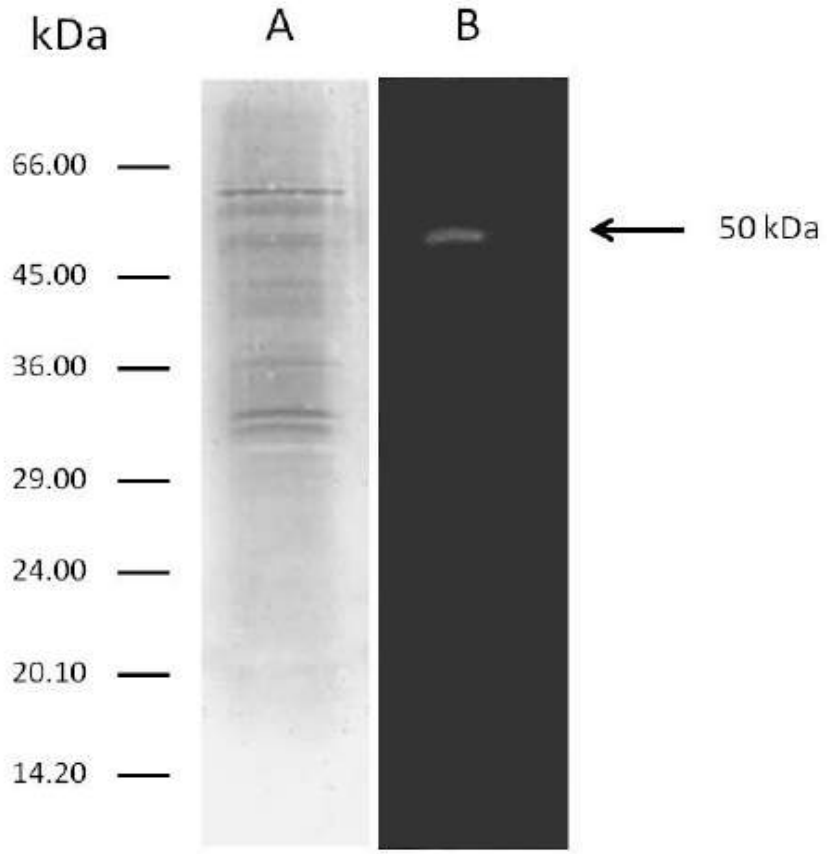


Fig. 3. Electrophoresis of proteins from L3 instar larvae of *Dermatobia hominis* eluted from a column of aprotinin-agarose. A total of 20 µg of protein from each fraction was resolved by SDS-PAGE (A) and gelatin-SDS-PAGE (B) and the bands were detected by silver staining and negative coloration, respectively. The arrow indicates a serine protease of 50 kDa. The molecular mass markers are indicated (kDa). These results are representative of two independent assays

2.2.3 Parasite proteins isolated by aspartyl-protease inhibitors affinity chromatography

Affinity-based chromatography based on pepstatine A was used to isolate a 52 kDa aspartyl protease from *Neospora caninum* tachyzoites (Naguleswaran et al., 2005) and a 45 kDa enzyme from *S.mansoni* (Valdivieso et al., 2003). In *Trypanosoma cruzi* epimastigotes, two aspartyl proteases were isolated (cruzipsin-I and cruzipsin-II). The molecular mass was estimated to be 120kDa by high performance liquid chromatography gel filtration, and the activities of these enzymes were detected in a doublet of bands (56 kDa and 48 kDa). These findings demonstrate that both proteases are novel *T. cruzi* acidic proteases. The physiological function of these enzymes in *T. cruzi* is not completely defined (Pinho et al., 2009).

An aspartyl protease with molecular mass of 37 kDa (plasmepsin) was isolated from the surface of *Plasmodium ookinete*, and its sequence was determined by mass spectrometry (Li et al., 2010). This protease was purified by using a benzamidine affinity-based column, which is typically used for the isolation of serine proteases. Structural similarity between the active site residues of the serine- and aspartyl proteases is possible, as some hydrogen-bonded residues can are arranged without any strain, such as in the formation of an oxyanion hole, in a manner that resembles the active site of a serine protease (Andreeva et al., 2004)

2.2.4 Remarks on the isolation of proteins by protease inhibitors affinity chromatography

Although the studies that have been conducted to isolate parasite proteases are of great medical interest, no parasiticide drug has been proposed thus far. In general, the chromatography methods involving inhibitor-based affinity-capture have been useful only to describe these enzymes in parasites and to establish their biochemical properties, their functions and their application in drugs tests.

Furthermore, the heterogeneous material obtained from affinity-based chromatography may require additional procedures for purification of the enzyme. Thus, other techniques must be applied to obtain proteases with greater purity, including molecular exclusion and ion exchange chromatography.

2.3 Glycosaminoglycans affinity-based separation of parasite proteins

Microbes have developed different strategies to gain access into mammalian cells (Bermúdez et al., 2010; Caradonna & Burleigh 2011; Soong et al., 2011). The first step involves the recognition of molecules at the surface of the target cell, which triggers the activation of signaling pathways that are implicated in the parasite internalization (Abban & Meneses 2010; Epting et al., 2011). Host cell surface sulfated proteoglycans have been implicated as key molecules at the host cell-parasite interface, mediating the adhesion and invasion of numerous parasitic microorganisms (Jacquet et al., 2001; Kobayashi et al., 2010; O'Donnell & Shukla 2010).

2.3.1 Structure of glycosaminoglycans

Proteoglycans (PGs) are composed of core proteins that are covalently linked to glycosaminoglycan (GAG) chains. As components of the extracellular matrix, the structural diversity of PGs depends on the identity of the core protein and the GAG composition. GAGs are linear polysaccharides comprised of disaccharide repeats containing uronic acid and hexosamine. GAGs vary in the type of hexosamine, hexose or hexuronic acid unit. The sulfated GAGs are classified as heparin [2-O-sulfo-β-D-glucuronic acid (GlcUA-2S) or 2-O-sulfo-α-L-iduronic acid (IdoUA-2S) and N-acetylglucosamine (GlcNAc) or N-sulfoglucosamine (GlcNS)], heparan sulfate [GlcUA, IdoUA or IdoUA-2S and GlcNAc or GlcNS], chondroitin sulfate [GlcUA and N-acetylgalactosamine (GalNAc)], dermatan sulfate [GlcUA or IdoUA and GalNAc] and keratan sulfate [galactose (Gal) and GlcNAc]. In fact, the structural diversity of PGs may provide sites of affinity for different ligands and, therefore, function as co-receptors or receptors for GAG-binding proteins (Dreyfuss et al., 2009; Ly et al., 2010). Although heparin is not found on the cell surface, this GAG has being

commonly used as tool for pathogen-host cell interaction assays. Heparins are negatively charged structures and native heparin presents molecular weights in the range of 5 to 30 KDa, whereas commercial heparin preparations are in the range of 12 kDa to 15 kDa.

2.3.2 Role of heparin-binding proteins in pathogen-host cell

Many pathogens express surface proteins that interact with GAGs in different stages of their life cycle. Although some parasites can bind to multiple GAGs (Coppi et al., 2007; Fallgren et al., 2001), heparan sulfate proteoglycan (HSPG) has been implicated in the recognition and/or invasion process of a wide range of pathogens, including viruses, bacteria and protozoan parasites (Bambino-Medeiros et al., 2011; Dalrymple & Mackow 2011; Yan et al., 2006;). Despite the role of heparin-binding proteins in many physiological and pathological processes, the basis of the heparin-protein interaction at the molecular level is still unclear.

Thus, efforts have been concentrated to enhance methods for the isolation and characterization of heparin-binding proteins, and, in parallel, to determine the role of this GAG in pathogen-host cell interaction. Currently, heparin affinity chromatography has been applied to the purification of GAG-binding proteins from different pathogens (Table 3). In these chromatography assays, the heparin is covalently coupled to agarose or sepharose beads and its sulfates and carboxylates chains are able to bind many proteins by basic amino acids (Fig. 2C).

This technique has been used to isolate heparin-binding proteins without loss of their biological activity, leading to a better understanding of the mechanism involved in the parasite invasion process. For example, chlamydial outer membrane complex (OmcB), a 60 kDa cysteine-rich protein, displays a protein motif (50-70OmcB peptide) that acts as an acceptor molecule to bind heparan sulfate (HS) and promote Chlamydia invasion in eukaryotic cells (Stephens et al., 2001). Attachment of *Helicobacter pylori* to gastric epithelial cells also involves HS recognition. Two major proteins, one with a molecular mass of 71.5 kDa and pI 5.0 (HSBP50) and the other with a molecular mass of 66.2 kDa and pI 5.4 (HSBP54), have been identified on the surface of bacterial cells that are able to bind HS. The amino acid sequences of these proteins (HSBP50 – VPERAVRAHT; HSBP54 - VHLPADKTNV) are not homologous with bacterial adhesins or other HS-binding proteins (Ruzi-Bustos et al., 2001). Other proteins with the ability to bind heparin (66 and 60 kDa) have been identified in *Staphylococcus aureus*. The partial characterization of the amino acid sequences, which consist of DWTGWLAAA for the 66 kDa protein and MLVT for the 60 kDa protein, revealed no identity with HBPs from Chlamydia or *Helicobacter pylori*. HBPs from *S. aureus* have been demonstrated to be sensitive to heat and proteases, such as pronase E, proteinase K, pepsin and chymotrypsin (Liang et al., 1992). Interestingly, a 17-kDa heparin-binding protein with pI 4.6 has also been isolated from *S. epidermis* and *S. haemolyticus*, but the amino acid sequence similarity is low between these two organisms (MXTAHSYTXKYNGYTAN and MATQTKGYYYSYNGYV, respectively) and other bacterial HBPs (Fallgren et al., 2001).

Trypanosomatidaes also exploit HS for successful parasite attachment to and/or invasion of the mammalian and vector hosts. The adhesion of Leishmania amastigotes to macrophages is mediated by HS, but not other sulfated polysaccharides (Love et al., 1993). Two heparin-

binding proteins, (65 and 54.5 kDa) from *L. (V.) braziliensis* promastigotes (HBP-Lb) recognize several molecules in the gut of *Lutzomyia intermedia* and *Lutzomyia whitmani* (Azevedo-Pereira et al., 2007). The biochemical characterization of these proteins revealed that only the 65-kDa HBP-Lb has metallo-proteinase activity, and this protein is primarily localized at the flagellar domain of the promastigotes. Surface plasmon resonance (spr) also demonstrated high-affinity binding at the flagellar domain, which forms a stable binding complex (Côrtes et al., 2011). In *T. cruzi*, HBPs also mediate parasite adhesion by recognition of PGHS on the surface of the target cells (Bambino-Medeiros et al., 2011; Calvet et al., 2003; Oliveira-Jr et al., 2008; Ortega-Barria & Pereira, 1991). Currently, three HBPs have been described in this parasite: a 60-kDa protein named penetrin (Ortega-Barria & Pereira, 1991) and two other proteins of 65.8 and 59 kDa that bind heparin, HS and chondroitin sulfate (CS). These proteins have been identified in both trypomastigotes and amastigotes (Oliveira-Jr et al., 2008). Interestingly, the HBP-HS binding is related to a specific region of the HS chain, the N-acetylated/N-sulfated HS domain, which promotes parasite attachment and invasion (Oliveira-Jr et al., 2008). Although only HS binding triggers *T. cruzi* invasion of mammalian cells (Ortega-Barria & Pereira, 1991; Calvet et al., 2003; Oliveira-Jr et al., 2008; Bambino-Medeiros et al., 2003), the multiple GAG recognition may provide an efficient association with other GAGs within the parasite life cycle. Recently, it has been demonstrated that sulfated proteoglycans are involved in the adhesion of epimastigotes to the luminal midgut epithelial cells of Rhodnius prolixus (Gonzalez et al., 2011).

2.3.3 Remarks on the isolation of proteins by glycosaminoglycans affinity chromatography

While the application of affinity chromatography has provided advances in our understanding of heparin-binding proteins, a large number of studies have focused on the parasite-host cell interface to improve our comprehension of the mechanisms that are activated by the receptor-ligand interaction (reviewed by Chen et al., 2008). The binding of Dengue virus to HS, for example, seems to result in the accumulation of virions at the surface of the human hepatoma cell line HuH-7 and elicit clathrin-dependent endocytosis (Hilgard & Stockert 2000). In addition to promote attachment and parasite invasion, HSPG also seems to be involved in the tropism of pathogen to specific tissues. The degree of HSPG sulfation guides the migration of Plasmodium sporozoites and the invasion of hepatocytes. Highly sulfated heparan sulfate at the surface of hepatocytes seems to regulate the proteolytic activity of the calcium-dependent protein kinase-6 on the CSP, which triggers the invasion of the parasite (Coppi et al., 2007).

Another interesting phenomenon is the release of syndecan-1, a transmembrane PGHS, as a mechanism of host defense inhibition. *Pseudomonas aeruginosa* induces syndecan-1 shedding through the enzymatic activity of LasA, leading to an enhancement of bacterial virulence (Park et al., 2001). A similar mechanism has been described for *Staphylococcus aureus* in which β-toxin, a secreted virulence factor, also induces syndecan-1 shedding by activating a metallo-proteinase involved in the host cell shedding mechanism, leading to enhancement of bacterial virulence due to the recruitment of inflammatory cells (Hayashida et al., 2009). Because heparan sulfate has been shown to be a receptor for a variety of pathogens, HS-binding polypeptides have been the subject of intense research and provide possibilities for drug intervention.

Organism	Isolated proteins	First phase				Second phase	References
		Matrix	Bind	Wash	Elution	Methods	
Trypanosoma cruzi	Penetrin – 60 kDa	Sepharose	PBS, pH 7.2	PBS containing 0.05% Triton X-100, pH7.2	1.5M guanidine-HCl 2.0M potassium thiocyanate and NaCl gradient (0.25-3.0M)	None	Ortega-Barria and Pereira 1991
Staphylococcus aureus	*S. aureus*-HBP – 66 kDa and 60 kDa	Sepharose	PBS containing 100mM PMSF, pH6.0	PBS containing 100mM PMSF, pH6.0	NaCl gradient (0-1M), followed by 2M NaCl in 200mM Tris-HCl pH 8.0	None	Liang et al., 1992
Trypanosoma cruzi	Penetrin – 60 kDa	Sepharose	PBS, pH 7.2	PBS containing 0.05% Triton X-100, pH7.2	1.5M guanidine-HCl 2.0M potassium thiocyanate and NaCl gradient (0.25-3.0M)	None	Ortega-Barria and Pereira 1991
Chlamydial	Outer membrane complex (COMC) - 60 kDa	Agarose	50 mM DTT and 2% Triton X-100	PBS	2% SDS in PBS	None	Stephens et al., 2001
Helicobacter pylori	Outer membrane protein (OMP) - 71.5 kDa and 66.2 kDa	Sepharose	0.05M sodium acetate pH5.0	0.1M Sodium acetate, pH5.0	NaCl gradient (0-2M)	None	Ruzi-Bustos et al., 2001
Staphylococcal	HBP – 17 kDa	Sepharose	PBS	None	NaCl gradient (0-2M)	None	Fallgren et al., 2001
Leishmania (V.) braziliensis	HBP-Lb – 65 kDa and 54.5 kDa	Sepharose	PBS containing 0.5% glycerol and 0.5% Chaps, pH7.2	PBS containing, 0.5% glycerol and 0.5% Chaps, pH7.2	PBS containing 0.5% glycerol, 0.5% Chaps and 2M NaCl, pH7.2	None	Azevedo-Pereira et al., 2007
Trypanosoma cruzi	HBP-Tc – 65.8 kDa and 59 kDa	Sepharose	PBS and 0.5% glycerol, pH 7.2	PBS, pH 7.2	NaCl gradient (0-1M)	None	Oliveira-Jr et al., 2008

Table 3. Heparin affinity-based

3. Conclusion

The chromatographic procedures described here maintain the minimal amount of native folding necessary for proteins to retain their biological and biochemical activities. Thus, the materials used as supports for packed affinity columns, including agarose, sepharose and celite (from diatomaceous earth), to immobilize ligands, such as lectins, protease inhibitors and glycosaminoglycans, do not interfere with the functional properties of these proteins.

Furthermore, proteins obtained by affinity-based procedure have been useful in understanding the biological processes related to the life cycles of parasites and in the interaction with hosts. These studies are essential to developing strategies, such as the use of vaccines and drugs, to control the parasite diseases.

4. Acknowledgements

We acknowledge the financial support Brazilian funding agencies, including CAPES, CNPq, FAPERJ and PAPES (CNPq/Fiocruz). Dr. Carlos Roberto Alves and Dr. Mirian Claudia de Souza Pereira are research fellows of CNPq

5. References

Abban CY, & Meneses PI. 2010. Usage of heparan sulfate, integrins, and FAK in HPV16 infection. Virology. 403(1):1-16.

André S, Sanchez-Ruderisch H, Nakagawa H, Buchholz M, Kopitz J, Forberich P, Kemmner W, Böck C, Deguchi K, Detjen KM, Wiedenmann B, von Knebel Doeberitz M, Gress TM, Nishimura S, Rosewicz S, & Gabius HJ. 2007. Tumor suppressor p16INK4a--modulator of glycomic profile and galectin-1 expression to increase susceptibility to carbohydrate-dependent induction of anoikis in pancreatic carcinoma cells. FEBS J. 274(13):3233-56.

Andreeva N, Bogdanovich P, Kashparov I, Popov M, & Stengach M. 2004. Is Histoaspartic Protease A Serine Protease With A Pepsin-Like Fold? Proteins. 55(3):705-10.

Atrih A, Richardson JM, Prescott AR, & Ferguson MA. 2005. *Trypanosoma brucei* glycoproteins contain novel giant poly-N-acetyllactosamine carbohydrate chains. J Biol Chem. 280(2):865-71.

Azevedo-Pereira RL, Pereira MC, Oliveria-Junior FO, Brazil RP, Côrtes LM, Madeira MF, Santos AL, Toma L, & Alves CR. Vet Parasitol. 2007. Heparin binding proteins from *Leishmania (Viannia) braziliensis* promastigotes. 145(3-4):234-9.

Bambino-Medeiros R, Oliveira FO, Calvet CM, Vicente D, Toma L, Krieger MA, Meirelles MN, & Pereira MC. 2011. Involvement of host cell heparan sulfate proteoglycan in *Trypanosoma cruzi* amastigote attachment and invasion. Parasitology. 138(5):593-601.

Beckert WH, & Sharkey MM. 1970. Mitogenic activity of the jack bean (*Canavalia ensiformis*) with rabbit peripheral blood lymphocytes. Int Arch Allergy Appl Immunol. (4):337-41.

Bermúdez A, Alba MP, Vanegas M, & Patarroyo ME. 2010. 3D structure determination of STARP peptides implicated in *P. falciparum* invasion of hepatic cells. Vaccine. 28(31):4989-96.

Bond JS, & Beynon RJ., eds. 1989. Proteolytic enzymes: A practical approach. IRL Press/Oxford University Press, Oxford, United Kingdom

Bourguignon SC, Cavalcanti DF, de Souza AM, Castro HC, Rodrigues CR, Albuquerque MG, Santos DO, da Silva GG, da Silva FC, Ferreira VF, de Pinho RT, & Alves CR. 2011. *Trypanosoma cruzi*: insights into naphthoquinone effects on growth and protease activity. Exp Parasitol. 127(1):160-6.

Brickman MJ, & Balber AE. 1993. *Trypanosoma brucei rhodesiense*: membrane glycoproteins localized primarily in endosomes and lysosomes of bloodstream forms. Exp Parasitol. 76(4):329-44.

Brittingham, A, Morrison, CJ, McMaster, WR, McGwire, BS, Chang, KP, & Mosser, DM. (1995). Role of the Leishmania surface protease gp63 in complement fixation, cell adhesion, and resistance to complement-mediated lysis. J of Immunol 155 (6) :3102-11.

Calvet CM, Toma L, Souza FR, Meirelles MNL, & Pereira, MCP. 2003. Heparan Sulfate Proteoglycans mediate the invasion of cardiomyocytes by *Trypanosoma cruzi*. J Eukariot Microbiol. 50: 97-103.

Caradonna KL, & Burleigh BA. 2011. Mechanisms of Host Cell Invasion by Trypanosoma cruzi. Adv Parasitol. 76:33-61.

Chen Y, Götte M, Liu J, & Park PW. 2008. Microbial subversion of heparan sulfate proteoglycans. Mol Cells. 26(5):415-26.

Choudhury R, Bhaumik Sk, De T, Chakraborti, T. 2009. Identification, Purification & Characterization of a secretory serine protease in an indian strain of *Leishmania donovani*. Mol Cell Biochem. 320(1-2):1-14

Choudhury R, Das P, De T, Chakraborti T. 2010. Immunolocalization and characterization of two novel proteases in *Leishmania donovani*: putative roles in host invasion and parasite development. Biochimie. 92(10):1274-86.

Coppi A, Tewari R, Bishop JR, Bennett BL, Lawrence R, Esko JD, Billker O, & Sinnis P. 2007. Heparan sulfate proteoglycans provide a signal to *Plasmodium* sporozoites to stop migrating and productively invade host cells. Cell Host Microbe. 2(5):316-27.

Côrtes LMC, Pereira MCS, Olivera-Jr FOR, Suzana Corte-Real, Silva FS, Pereira BAS, Madeira MF, Moraes MTB, Brazil RP, & Alves RA. 2011. *Leishmania (Viannia) braziliensis*: insights on subcellular distribution and biochemical properties of heparin-binding proteins. Parasitology. 7: 1-8.

Couto AS, Gonçalves MF, Colli W, & de Lederkremer RM. 1990. The N-linked carbohydrate chain of the 85-kilodalton glycoprotein from *Trypanosoma cruzi* tryponastigotes contains sialyl, fucosyl and galactosyl (alpha 1-3)galactose units. Mol Biochem Parasitol. 39(1):101-7.

Dalrymple N, & Mackow ER. 2011. Productive dengue virus infection of human endothelial cells is directed by heparan sulfate-containing proteoglycan receptors. J Virol. 85(18):9478-85.

Ribeiro de Andrade AS, Santoro MM, De Melo MN, & Mares-Guia M. 1998. *Leishmania (Leishmania) amazonensis*: purification and enzymatic characterization of a soluble serine oligopeptidase from promastigotes. Exp Parasitol. 89(2):153-60.

Dreyfuss JL, Regatieri CV, Jarrouge TR, Cavalheiro RP, Sampaio LO, & Nader HB. 2009. Heparan sulfate proteoglycans: structure, protein interactions and cell signaling. An Acad Bras Cienc. 81(3):409-29.

Dreyfuss JL, Veiga SS, Coulson-Thomas VJ, Santos IA, Toma L, Coletta RD, & Nader HB. 2010. Differences in the expression of glycosaminoglycans in human fibroblasts

derived from gingival overgrowths is related to TGF-beta up-regulation. Growth Factors. 28(1):24-33.

Dumont F, & Nardelli J. 1979. Peanut agglutinin (PNA)-binding properties of murine thymocyte subpopulation. Immunology. 37(1):217-24.

Epting CL, Coates BM, & Engman DM. 2011. Molecular mechanisms of host cell invasion by *Trypanosoma cruzi*. Exp Parasitol. 127(2):607.

Fallgren C, Utt M, & Ljungh A. 2001. Isolation and characterisation of a 17-kDa staphylococcal heparin-binding protein with broad specificity. J Med Microbiol. 50(6):547-57.

Fauquenoy S, Morelle W, Hovasse A, Bednarczyk A, Slomianny C, Schaeffer C, Van Dorsselaer A, & Tomavo S. 2008. Proteomics and glycomics analyses of N-glycosylated structures involved in *Toxoplasma gondii*-host cell interactions. Mol Cell Proteomics. 7(5):891-910.

Garate M, Marchant J, Cubillos I, Cao Z, Khan NA, & Panjwani N. 2006. In vitro pathogenicity of Acanthamoeba is associated with the expression of the mannose-binding protein. Invest Ophthalmol Vis Sci. 47(3):1056-62.

Gardiner PR, Nene V, Barry MM, Thatthi R, Burleigh B, & Clarke MW. 1996. Characterization of a small variable surface glycoprotein from *Trypanosoma vivax*. Mol Biochem Parasitol. 82(1):1-11.

Geier G, Banaj HJ, Heid H, Bini L, Pallini V, & Zwilling R. 1999. Aspartyl proteases in *Caenorhabditis elegans*. Isolation, identification and characterization by a combined use of affinity chromatography, two-dimensional gel electrophoresis, microsequencing and databank analysis. Eur J Biochem. 264(3):872-9.

Gerold P, Striepen B, Reitter B, Geyer H, Geyer R, Reinwald E, Risse HJ, & Schwarz RT. 1996. Glycosyl-phosphatidylinositols of *Trypanosoma congolense*: two common precursors but a new protein-anchor. J Mol Biol. 261(2):181-94.

Gonzalez MS, Silva LC, Albuquerque-Cunha JM, Nogueira NF, Mattos DP, Castro DP, Azambuja P, & Garcia ES. 2011. Involvement of sulfated glycosaminoglycans on the development and attachment of *Trypanosoma cruzi* to the luminal midgut surface in the vector, *Rhodnius prolixus*. Parasitology. 9:1-8.

Govrin E, & Levine A. 1999. Purification of active cysteine proteases by affinity chromatography with attached E-64 inhibitor. Protein Expr Purif. 15(3):247-50.

Guedes HL, Rezende JM, Fonseca MA, Salles CM, Rossi-Bergmann B, & De-Simone SG. 2007. Identification of serine proteases from *Leishmania braziliensis*. Z Naturforsch C. 62(5-6):373-81.

Guha-Niyogi A, Sullivan DR, &Turco SJ. 2001. Glycoconjugate structures of parasitic protozoa. Glycobiology. 11: 45R-59R.

Hayashida A, Bartlett AH, Foster TJ, & Park PW. 2009. *Staphylococcus aureus* beta-toxin induces lung injury through syndecan-1. Am J Pathol. 174(2):509-18.

Hellström U, Dillner ML, Hammarström S, & Perlmann P. 1976. Fractionation of human T lymphocytes on wheat germ agglutinin-sepharose. J Exp Med. 144(5):1381-5.

Hilgard P, & Stockert R. 2000. Heparan sulfate proteoglycans initiate dengue virus infection of hepatocytes. Hepatology. 32(5):1069-77.

Hoegl L, Korting HC, & Klebe G. 1999. Inhibitors of aspartic proteases in human diseases: molecular modeling comes of age. Pharmazie. 54(5):319-29.

Jacobs DB, & Poretz RD. 1980. Lectin induction of lymphokines in cultured murine leukocytes. Cell Immunol. 51(2):424-9.

Jacquet A, Coulon L, De Nève J, Daminet V, Haumont M, Garcia L, Bollen A, Jurado M, & Biemans R. 2001. The surface antigen SAG3 mediates the attachment of *Toxoplasma gondii* to cell-surface proteoglycans. Mol Biochem Parasitol. 116(1):35-4

Kabir S. 1998. Jacalin: a jackfruit (*Artocarpus heterophyllus*) seed-derived lectin of versatile applications in immunobiological research. J Immunol Methods. 212(2):193-211.

Klemba M, & Goldberg DE. 2002. Biological roles of proteases in parasitic protozoa. Annu Rev Biochem. 71:275-305

Kobayashi K, Kato K, Sugi T, Takemae H, Pandey K , Gong H, Tohya Y, & Akashi H. 2010. *Plasmodium falciparum* BAEBL Binds to Heparan Sulfate Proteoglycans on the Human Erythrocyte Surface. J Biol Chem. 285(3): 1716–1725.

Kong, H H, Kim, T H & Chung, D-Ii. 2000. Purification And Characterization of A Secretory serine protease of *Acanthamoeba Healyi* isolated from gae. Journal of Parasitology. 86, 12–17.

Li F, Patra KP, Yowell CA, Dame JB, Chin K, & Vinetz JM. 2010. Apical surface expression of aspartic protease plasmepsin 4, A Potential. J Biol Chem. 285(11):8076-83.

Liang OD, Ascencio F, Fransson LA, & Wadström T. 1992. Binding of heparan sulfate to *Staphylococcus aureus*. Infect Immun. 60(3):899-906.

Lord MJ, Jolliffe NA, Marsden CJ, Pateman CS, Smith DC, Spooner RA, Watson PD, & Roberts LM. 2003. Ricin. Mechanisms of cytotoxicity. Toxicol Rev. 22(1):53-64.

Love DC, Esko JD, & Mosser DM. 1993. A heparin-binding activity on Leishmania amastigotes which mediates adhesion to cellular proteoglycans. J Cell Biol. 123(3):759-66.

Ly M, Laremore TN, & Linhardt RJ. 2010. Proteoglycomics: recent progress and future challenges. OMICS. 14(4):389-99.

Maia, A.A.M. & Guimarães, M.P. Distribuição Sazonal De Larvas De Dermatobia Hominis (Linnaeus Jr., 1781) (Diptera: Cuterebridae) Em Bovinos De Corte Da Região De Governador Valadares - Minas Gerais. Arquivo Brasileiro De Medicina Veterinária E Zootecnia, Brasil. , 1985. V. 37, N. 5, P. 469-475.

Mansour MH. 1996. Purification and characterization of SM 37: a fucosyllactose determinant-bearing glycoprotein probed by a *Biomphalaria alexandrina* lectin on adult male shistosomes. J Parasitol. 82(4):586-93.

Matsumoto K, Yamamoto D, Ohishi H, Tomoo K, Ishida T, Inoue M, Sadatome T, Kitamura K, & Mizuno H. 1989. Mode of binding of E-64-c, a potent thiol protease inhibitor, to papain as determined by X-ray crystal analysis of the complex. FEBS. Lett 245, 177–180.

McKerrow JH, Caffrey C, Kelly B, Loke P, & Sajid M. 2006. Proteases in parasitic diseases. Annu Rev Pathol. 1:497-536.

Morgado-Díaz JA, Silva-Lopez RE, Alves CR, Soares MJ, Corte-Real S, & De Simone SG. 2005. An intracellular serine protease of 68 kDa in *Leishmania (Leishmania) amazonensis* promastigotes. Mem Inst Oswaldo Cruz. 100(4):377-83

Morris SR, & Sakanari JA. 1994. Characterization of The serine protease and serine protease inhibitor from the tissue-penetrating nematode *Anisakis Simplex*. J Biol Chem. 269(44):27650-6.

Morrison CJ, Hurst SF, Bragg SL, Kuykendall RJ, Diaz H, McLaughlin DW, & Reiss E. 1993. Purification and characterization of the extracellular aspartyl proteinase of *Candida albicans*: removal of extraneous proteins and cell wall mannoprotein and evidence for lack of glycosylation. J Gen Microbiol. 139 Pt 6:1177-86.

Muharsini S, Sukarsih, Riding G, Partoutomo S, Hamilton S, Willadsen P, & Wijffels G. 2000. Identification and characterisation of the excreted/secreted serine proteases of larvae of the old world screwworm fly, *Chrysomya Bezziana*. Int J Parasitol. 30(6):705-14.

Naguleswaran A, Alaeddine F, Guionaud C, Vonlaufen N, Sonda S, Jenoe P, Mevissen M, & Hemphill A. 2005. *Neospora caninum* protein disulfide isomerase is involved in tachyzoite-host cell interaction. Int J Parasitol. 35(13):1459-72.

Nolan DP, Jackson DG, Windle HJ, Pays A, Geuskens M, Michel A, Voorheis HP, & Pays E. 1997. Characterization of a novel, stage-specific, invariant surface protein in *Trypanosoma brucei* containing an internal, serine-rich, repetitive motif. J Biol Chem. 272(46):29212-21.

O'Donnell CD, & Shukla D. 2008. The importance of separan sulfate in herpesvirus infection. Virol Sin. 23(6):383-393.

Oliveira FO Jr, Alves CR, Calvet CM, Toma L, Bouças RI, Nader HB, Côrtes LMC, Krieger MA, Meirelles MN, & Souza Pereira MC. 2008. *Trypanosoma cruzi* heparin-binding proteins and the nature of the host cell heparan sulfate-binding domain. Microb Pathog. 44(4):329-38

Ortega-Barria E, & Pereira EA. 1991. A novel T. cruzi heparin-binding protein promotes fibroblast adhesion and penetration of engineered bacteria and trypanosomes into mammalian cells. Cell. 67: 411-421.

Park PW, Pier GB, Hinkes MT, & Bernfield M. 2001. Exploitation of syndecan-1 shedding by *Pseudomonas aeruginosa* enhances virulence. Nature. 411(6833):98-102.

Pereira BA, Silva PS, Rebello KM, Marín-Villa M, Traub-Cseko YM, Andrade TC, Bertho ÁL, Caffarena ER, & Alves CR. 2011. In silico predicted epitopes from the COOH-terminal extension of cysteine proteinase B inducing distinct immune responses during *Leishmania (Leishmania) amazonensis* experimental murine infection. BMC Immunol. 2011,12: 44.

Pinho RT, Beltramini LM, Alves CR, & De-Simone SG. 2009. *Trypanosoma cruzi*: isolation and characterization of aspartyl proteases. Exp Parasitol. 122(2):128-33.

Rebello KM, Côrtes LM, Pereira BA, Pascarelli BM, Côrte-Real S, Finkelstein LC, Pinho RT, d'Avila-Levy CM, & Alves CR. 2009. Cysteine proteases from promastigotes of *Leishmania (Viannia) braziliensis*. Parasitol Res. 106(1):95-104.

Redmond DL, Geldhof P, & Knox DP. 2004. Evaluation of *Caenorhabditis elegans* glycoproteins as protective immunogens against *Haemonchus contortus* challenge in sheep. Int J Parasitol. 34(12):1347-53.

Reinwald E, Rautenberg P, & Risse HJ. 1981. Purification of the variant antigens of *Trypanosoma congolense*: a new approach to the isolation of glycoproteins. Biochim Biophys Acta. 668(1):119-31.

Rüdiger H, & Gabius HJ. 2001. Plant lectins: occurrence, biochemistry, functions and applications. Glycoconj J.18(8):589-613.

Ruiz-Bustos E, Ochoa JL, Wadström T, & Ascencio F. 2001. Isolation and characterisation of putative adhesins from Helicobacter pylori with affinity for heparan sulphate proteoglycan. J Med Microbiol. 50(3):215-22.

Shenai BR, Sijwali PS, Singh A, & Rosenthal PJ. 2000 Characterization of native and recombinant falcipain-2, a principal trophozoite cysteine protease and essential hemoglobinase of *Plasmodium falciparum*. J Biol Chem. 275(37):29000-10.

Silva-Lopez RE, & Giovanni-De-Simone S. 2004. *Leishmania (Leishmania) amazonensis:* Purification and characterization of a promastigote serine protease. Exp Parasitol. 107(3-4):173-82.

Silva-Lopez RE, Coelho MG, & De Simone SG. 2005. Characterization of an extracellular serine protease of *Leishmania (Leishmania) amazonensis*. Parasitology. 131(Pt 1):85-96.

Silva-López RE, Dos Santos TR, Morgado-Díaz JA, Tanaka MN, & De Simone SG. 2010. Serine protease activities in *Leishmania (Leishmania) chagasi* promastigotes. Parasitol Res. 107(5):1151-62

Silva-Lopez RE, Morgado-Díaz JA, Alves CR, Côrte-Real S, & Giovanni-De-Simone S. 2004. Subcellular localization if an extracellular serine protease in *Leishmania Leishmania) amazonensis*. Parasitol Res. 93(4):328-31

Silva-Lopez RE, Morgado-Díaz JA, Dos Santos PT, & Giovanni-De-Simone S. 2008. Purification and subcellular localization of a secreted 75 KDa *Trypanosoma cruzi* serine oligopeptidase. Acta Trop. 07(2):159-67.

Smith WD, Newlands GF, Smith SK, Pettit D, & Skuce PJ. 2003. Metalloendopeptidases from the intestinal brush border of *Haemonchus contortus* as protective antigens for sheep. Parasite Immunol. 25(6):313-23.

Smith WD, Smith SK, Pettit D, Newlands GF, & Skuce PJ. 2000. Relative protective properties of three membrane glycoprotein fractions from *Haemonchus contortus*. Parasite Immunol. 22(2):63-71.

Soong G, Martin FJ, Chun J, Cohen TS, Ahn DS, & Prince A. 2011. *Staphylococcus aureus* Protein A mediates invasion across airway epithelial Cells through activation of RhoA GTPase signaling and proteolytic activity. J Biol Chem. 286(41):35891-8.

Stephens RS, Koshiyama K, Lewis E, & Kubo A. 2001. Heparin-binding outer membrane protein of chlamydiae. Mol Microbiol. 40(3):691-9.

Todorova VK, & Stoyanov DI. 2000. Partial Characterization of Serine proteinases secreted By Adult *Trichinella Spiralis*. Parasitol Res. 86(8):684-7.

Turner DG, Wildblood LA, Inglis NF, & Jones DG. 2008. Characterization of a galectin-like activity from the parasitic nematode, *Haemonchus contortus*, which modulates ovine eosinophil migration in vitro. Vet Immunol Immunopathol. 122(1-2):138-45.

Valdivieso E, Bermudez H, Hoebeke J, Noya O, & Cesari IM. 2003. Immunological similarity between Schistosoma and bovine cathepsin D. Immunol Lett. 89(1):81-8.

Wlodawer A, & Vondrasek J. 1998. Inhibitors of HIV-1 protease: a major success of structure-assisted drug design. Annu Rev Biophys Biomol Struct.27:249-84.

Wilson ME, & Pearson RD. 1984. Infect Immun. Stage-specific variations in lectin binding to *Leishmania donovani*. 46(1):128-34.

Yan Y, Silvennoinen-Kassinen S, Leinonen M, & Saikku P. 2006. Inhibitory effect of heparin 41 sulfate-like glycosaminoglycans on the infectivity of Chlamydia pneumoniae in HL 42 cells varies between strains. Microbes Infect. 8:866-872