UNIVERSIDADE FEDERAL DA BAHIA

FACULDADE DE MEDICINA

FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS GONÇALO MONIZ

UFBA **FIOCRUZ**

Curso de Pós-Graduação em Patologia Humana

TESE DE DOUTORADO

AVALIAÇÃO DO 17-AAG COMO AGENTE LEISHMANICIDA E SEU MECANISMO DE AÇÃO NA INDUÇÃO DA MORTE DE PARASITOS DO GÊNERO *Leishmania* spp.

ANTONIO LUIS DE OLIVEIRA ALMEIDA PETERSEN

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ANTÔNIO LUIS DE OLIVEIRA ALMEIDA PETERSEN

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“Vivemos em um momento especial, o único momento onde podemos verificar observacionalmente que vivemos em um momento especial.”

Lawrence M. Krauss

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RESUMO

A leishmaniose é uma doença endêmica no Brasil causada por parasitos protozoários do gênero *Leishmania*. A quimioterapia continua sendo a forma mais efetiva de tratamento com os antimoniais pentavalentes sendo usados há mais de 70 anos como a primeira linha de tratamento. O uso deste e de outros fármacos apresenta efeitos adversos graves, os esquemas terapêuticos empregados são desconfortáveis, além de relatos do aumento de casos de resistência. A proteína de choque térmico 90 (HSP90) é um membro da família das chaperonas presente em células eucarióticas e bactérias. Essa proteína é fundamental para o dobramento e estabilização de diferentes proteínas, chamadas genericamente de proteínas cliente. Essa chaperona vem sendo considerada um importante alvo molecular para o tratamento de diferentes doenças parasitárias. Nessa tese, o inibidor específico da atividade ATPásica da HSP90, o 17-allilamino-17-demethoxigeldanamicina (17-AAG) foi testado em parasitos do gênero *Leishmania*. Inicialmente, avaliamos o efeito em cultura axênica e observamos que o 17-AAG causa a morte desses parasitos em concentrações inferiores às necessárias para causar a morte de macrófagos. Observamos também que o tratamento com 17-AAG promove a morte intracelular dos parasitos em concentrações que variam de 25 a 500 nM nos tempos de 24 e 48 h, sendo também eficaz contra a forma amastigota em tempos mais tardios como 96 h de infecção. Os parasitos morrem independentemente da produção de moléculas microbicidas pelo macrófago, como superóxido e óxido nítrico, que tiveram sua produção reduzida em 61 e 58 %, respectivamente. O tratamento com 17-AAG também reduziu a produção de mediadores pró-inflamatórios como TNF-α, IL-6 e MCP-1 em 35, 35 e 92 %, respectivamente. Utilizando o modelo de camundongos BALB/c infectados por *Leishmania braziliensis* na orelha demonstramos que o tratamento com 17-AAG causou redução do tamanho da lesão cutânea em 0,5 mm e da carga parasitaria no local da infecção em 25 %, no entanto, não foi capaz de reduzir a carga parasitaria no linfonodo drenante. Análise por microscopia eletrônica de transmissão de macrófagos infectados e tratados com 17-AAG revelou alterações características de um processo autofágico com vacuolização do citoplasma e formação de vacúolos com dupla membrana, além da presença de figuras de mielina. Utilizando parasitos transgênicos observamos que 17-AAG induz um aumento de 30 % na formação de autofagossomos, que tem a sua capacidade de fusão com glicossomos e lisossomos reduzida. Além disso, parasitos ATG5 *knockout*, incapazes de formar autofagossomos foram cerca de 90% mais resistentes à morte induzida pelo 17-AAG em relação a parasitos selvagens. Observamos, também, que o tratamento com MG132, um inibidor da atividade do proteassoma, assim como o 17-AAG induziu o acúmulo de proteínas ubiquitinadas de parasitos, especialmente em

parasitos incapazes de formar autofagossomos, sugerindo um papel da autofagia na degradação de proteínas ubiquitinadas. Por último, observamos que o MG132 foi capaz de induzir a formação de autofagossomos sugerindo uma ligação entre o acúmulo de proteínas ubiquitinadas e a indução da via autofágica. Em conjunto, nossos resultados indicam que o HSP90 é um alvo molecular que dever ser explorado no tratamento das leishmanioses.

Palavras-chave: Quimioterapia. HSP90. Leishmaniose. 17-AAG. Autofagia. Ubiquitina.

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ABSTRACT

Leishmaniases are endemic disease in Brazil caused by protozoan parasites from the genus *Leishmania*. Chemotherapy remains the most effective way of treatment and pentavalent antimonials, used for more than 70 years, remaining as first choice drugs for leishmaniasis treatment. The use of this and other drugs causes severe side effects, therapeutic regimens employed for leishmaniasis treatment are unpleasant, besides an increase number of resistance cases. The Heat Shock Protein 90 (HSP90) is a member of the chaperone family present in bacteria and eukaryotic cells. This protein is essential for the folding and stabilization of different proteins, known as client proteins. This chaperone has been considered an important molecular target for the treatment of different parasitic diseases. In this thesis, the specific inhibitors of the ATPase activity from the HSP90, 17-allylamino-17-demethoxygeldanamycin (17-AAG), were tested against parasites from the genus *Leishmania*. First we evaluated its effect on axenic culture and observed that 17-AAG induces parasite cell death in lowerconcentrations than those needed to induce macrophage cell death. We also observed that 17-AAG intracellular parasite death in concentrations ranging from 25 to 500 nM after 24 or 48 h, being also able to kill amastigotes in latter times of infection, such as 96 h. The parasites die independently of the production of microbicide molecules, such as superoxide and nitric oxide, which had their production reduced by 61 and 58 %, respectively. 17-AAG treatment also reduced the production of pro-inflammatory molecules such as TNF-α, IL-6 e MCP-1 in 35, 35 and 98 %, respectively. Using the murine model of BALB/c mice infected with *Leishmania braziliensis* in the ear showed that treatment of 17-AAG reduces the size of the lesion in 0,5 mm and the parasite load in the ear in 25 %, however, the treatment wasn't able to reduce the parasite load in the draining lymph node. Transmission electron microscopy analysis of infected macrophages treated with 17-AAG revealed alterations typical of autophagic process with cytoplasm vacuolization, formation of vacuoles with double membranes, besides the presence of myelin figures. Using transgenic parasites we observed that 17-AAG induces an increase of 35 % in the autophagosome formation witch have their ability to fuse with glycosome and lisosome reduced. However, in comparison to wild type parasites, ATG5 knockout parasites that are unable to form autophagosome were 90% more resistant to 17-AAG-induced cell death. We also observed that MG132 treatment, a proteasome inhibitor, like 17-AAG induced ubiquitined proteins accumulation in parasites, especially in parasites unable to form autophagosome, suggesting a role of autophagy in degradation of ubiquitinated proteins. Lastly, we observed that MG132 induced autophagosome formation, suggesting a link between ubiquitinated and induction of the autophagic pathway. In sum, our results indicate that HSP90 is a molecular target that should be explored as a treatment for leishmaniasis.

Key-Words: Chemotherapy. HSP90. Leishmaniasis. 17-AAG. Autophagy. Ubiquitin

LISTA DE FIGURAS

LISTA DE ABREVIATURAS

17-AAG	17-allilamino-17-demethoxigeldanamicina
17-DMAG	17-Dimetilaminoetilamino-17-demethoxigeldanamicina
AHA1	Ativador da ATPase da HSP90 (do inglês: Activator of HSP90 ATPase)
AIDS	Síndrome da imunodeficiência adquirida (do inglês: Acquired immune deficiency syndrome)
ATG	Gene Relacionado a autofagia (do inglês: Autophagy-related gene)
ATP	Adenosina trifosfato
CR	Receptor de complemento
Cvt	Via citoplasma para vacúolo (do inglês: Cytoplasm-to-vacuole pathway)
GA	Geldanamicina
GFP	Proteína verde fluorescente (do inglês: Green fluorescent protein)
GM-CSF	Fator Estimulador de Colônias de Granulócitos e Macrófagos – do inglês: Granulocyte-macrophage colony-stimulating factor
GRP94	Proteína regulada pela glicose 94 (do inglês: Glucose-regulated protein 94)
HIV	Virus da immunodeficiency humana
HOP	Proteína organizadora da HSP70/HSP90
HIP	Proteína que interage com HSP70
HSP40	Proteína do choque térmico 40
HSP70	Proteína do choque térmico 70
HSP90	Proteína do choque térmico 90
IFN	Interferon

IL	Interleucina
iNOS	Óxido nítrico-sintase induzida (do inglês: Inducible nitric oxide synthase)
LCD	Leishmaniose Cutânea Difusa
LCL	Leishmaniose Cutânea Localizada
LMC	Leishmaniose Mucocutânea
LTA	Leishmaniose Tegumentar Americana
LT	Leishmaniose Tegumentar
TRAP1	Proteína 1 associada ao receptor de TNF (do inglês: TNF receptor-associated protein 1)
LV	Leishmaniose Visceral
LPG	Lipofosfoglicano
MCP	Proteína quimioatratora do monócito (do inglês: Monocyte chemoattractant protein)
NADPH	Nicotinamida adenina dinucleotíedo fosfato
NF-κB	Factor nuclear kappa B
NO	Óxido nítrico
O_2^-	Superóxido
PSG	Gel secretório de promastigotas
RNA	Ácido ribonucleico
TGF	Fator de transformação do crescimento (do inglês: transforming growth factor)
Th	T auxiliar
TLR	Receptor do tipo Toll (do inglês: Toll-like receptor)
TNF	Fator de necrose tumoral

mTOR	Alvo da rapamicina em mamíferos (do inglês: Mammalian target of rapamycin)
VP	Vacúolo parasitóforo
WHO	Organização mundial de saúde
WT	Selvagem (do inglês: Wild-type)

SUMÁRIO

1 INTRODUÇÃO

1.1 LEISHMANIOSE E O SEU AGENTE CAUSADOR

A leishmaniose é uma doença tropical que possui um grande espectro de manifestações clinicas tanto em humanos quanto em outros hospedeiros mamíferos. As diferentes formas clínicas de leishmanioses são causadas por diferentes espécies de parasitos protozoários do gênero *Leishmania* que são transmitidos ao hospedeiro vertebrado pela picada de insetos vetores flebotomínicos que se infectam durante o repasto sanguíneo (Herwaldt, 1999). A propagação da doença entre os hospedeiros vertebrados depende desses vetores que são insetos pertencentes ao gênero *Phlebotomus* no velho mundo e *Lutzomyia* no novo mundo (Banuls et al., 2007).

1.1.1 A leishmaniose

As leishmanioses ocorrem em regiões tropicais, subtropicais e temperadas do mundo, sendo endêmica em 98 países (Fig. 1). A maioria dos casos da doença ocorre em países em desenvolvimento, estando associada à pobreza, desnutrição, más condições de moradia e falta de recursos. Além disso, a doença também está associada ao desflorestamento, avanço da agricultura e urbanização de regiões endêmicas para leishmaniose, condições que facilitam a proliferação do inseto vetor dessa doença (Alvar, Yactayo, et al., 2006). A mortalidade causada pela leishmaniose varia entre 20.000 a 40.000 mortes por ano (Alvar et al., 2012). Esses números são baixos se comparados com os números de mortes atribuído a outras doenças infecciosas como AIDS, tuberculose e malária o que leva a leishmaniose a receber menos investimentos para pesquisa e inovação, quando comparada aos

investimentos destinados a outras doenças infecciosas (Alvar et al., 2012). É importante assinalar que a morbidade é mais associada à leishmaniose do que a mortalidade, sendo comum a presença de pacientes crônicos com infecções persistentes de longa duração. Essa morbidade associada à leishmaniose leva pacientes a terem uma redução na qualidade de vida, afastamento do trabalho ou morte prematura, além do estigma social e psicológico quando ocorre o aparecimento de cicatrizes desfigurantes na face dos pacientes acometidos pela forma mucosa da leishmaniose tegumentar (Who, 2010).

Mais de 20 espécies agrupadas em dois grandes subgêneros: *Leishmania* e *Viannia* podem causar doença em humanos (Tabela 1), resultando em uma variedade de manifestações clinicas a depender da espécie de *Leishmania*, uma vez que as espécies possuem tropismo distintos por derme, mucosa ou vísceras (Lainson et al., 1987; Yurchenko et al., 2000; Banuls et al., 2007). Além disso, o tipo da resposta imunológica do hospedeiro, particularmente o balanço entre uma resposta celular e humoral, desempenha um papel importante no desfecho da doença.

A leishmaniose tegumentar (LT) pode apresentar-se em quatro principais formas clínicas, leishmaniose cutânea localizada (LCL), leishmaniose cutânea difusa (LCD) e leishmaniose muco-cutânea (LMC) e a leishmaniose disseminada (LD). A LCL é a forma mais comum da doença, responsável por cerca de 95% dos casos de LT, causa úlceras em partes expostas do corpo, principalmente rosto e membros. Uma resposta imunológica eficiente mediada principalmente por células T favorece

a cura espontânea que pode ocorrer após alguns meses. A maioria dos casos de LCL ocorre no Brasil, Iraã, Peru, Síria e Arábia Saudíta (Fig. 1).

A LCD, forma clínica mais rara da LT, afeta principalmente pacientes que possuem uma resposta imunológica celular ineficiente, resultando o aparecimento de lesões cutâneas disseminadas pelo corpo do paciente que não curam espontaneamente. Nessas lesões é frequente a presença de macrófagos vacuolizados com intenso parasitismo. Além disso, pacientes com LCD e tratados com antimoniais podem apresentar reincidências das lesões em até 100% dos casos (Costa et al., 1992; Barral et al., 1995; Bomfim et al., 1996; Banuls et al., 2007).

A leishmaniose muco-cutânea (LMC) é particularmente importante na América do Sul onde cerca de 2-5% dos casos primários de LCL evoluem para LMC após a cura da lesão inicial (De Oliveira e Brodskyn, 2012). No Brasil cerca de 4% dos casos de LCL evoluem para LMC, sendo que esse percentual é de 68% em pacientes coinfectados com HIV (Strazzulla et al., 2013). Mais de 90% dos casos de LMC ocorrem em apenas três países, Brasil, Peru, Bolívia (Fig. 1). Pacientes com essa doença apresentam lesões que evoluem com destruição intensa das membranas das mucosas oro-nasal e da faringe, causando, também, dano aos tecidos vizinhos, podendo resultar em desfiguração facial (Banuls et al., 2007; Da Costa et al., 2014; Mcgwire e Satoskar, 2014).

A leishmaniose disseminada (LD) é uma forma rara de apresentação da doença sendo relacionada a casos de infecção por *L. amazonensis* e *L. braziliensis*. Pacientes com LD apresentam inúmeras lesões papulosas que podem se distribuir

por todo o seu corpo, afetando principalmente face e tronco (Carvalho EM et al; 1994). Essa forma da doença está em expansão na região Nordeste do Brasil, sendo que na década de 80 a LD representava cerca de 0,2% dos casos de LT na área endêmica de Corte de Pedra/BA, com registro de aumento para 2% dos casos na década de 90. O tratamento de pacientes com LD induz uma rápida regressão da maioria das lesões cutâneas, mas, raramente a cura é obtida após o tratamento. Um dos agravantes da LD é o comprometimento das mucosas que pode acontecer em até 38% dos casos (Turetz ML et al; 2002).

A forma visceral da leishmaniose (LV) é a forma mais grave da doença com aproximadamente trezentos mil novos casos por ano sendo quase sempre fatal se não tratada. O paciente apresenta febre, perda de peso, hepatomegalia, esplenomegalia e anemia grave. Além disso, cerca de 5-15% dos pacientes tratados para LV desenvolvem LC em até dois anos após cura clinica da LV. A maioria dos casos de LV ocorre em cinco países: Índia, Nepal, Bangladesh, Sudão e Brasil (Fig. 1) (Banuls et al., 2007; Mcgwire e Satoskar, 2014).

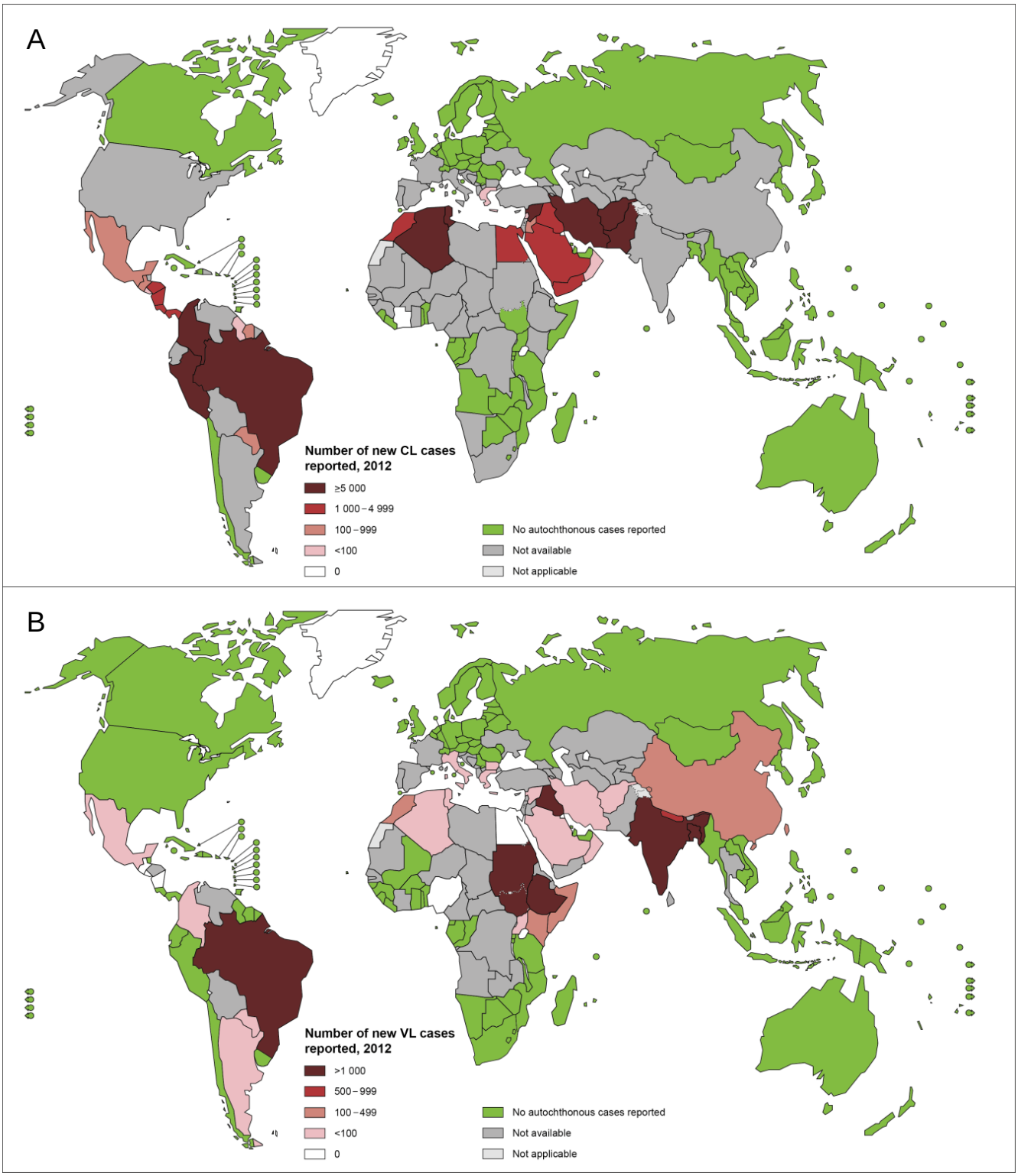

Figura 1. Distribuição geográfica da leishmaniose cutânea (A) e leishmaniose visceral (B). Fonte: World Health Organization (WHO) Global Health Observatory Map Gallery (http://gamapserver.who.int/mapLibrary/app/searchResults.aspx)**Tabela 1. Espécies de *Leishmania* causadoras de doenças em humanos e suas respectivas formas clinicas**. (Who, 2010)

Forma Clínica

	LCL	LCD	LMC	LV
Espécies do Velho Mundo	*L. (L.) major*		*L. (L.) major*	
	L. (L.) tropica		*L. (L.) tropica*	
	L. (L.) aethiopica	*L. (L.) aethiopica*		
	L. (L.) infantum		*L. (L.) infantum*	*L. (L.) infantum*
			L. (L.) donovani	*L. (L.) donovani*
Espécies do Novo Mundo	*L. (L.) venezuelensis*			
	L. (L.) mexicana	*L. (L.) mexicana*		
	L. (L.) amazonensis	*L.(L.) amazonensis*		
	L. (L.) infantum			*L. (L.) infantum*
	L. (V.) lainsoni			
	L. (V.) naïffi			
	L. (V.) peruviana			
	L. (V.) shawi			
	L. (V.) braziliensis		*L. (V.) braziliensis*	
	L. (V.) guyanensis		*L. (V.) guyanensis*	
	L. (V.) panamensis		*L. (V.) panamensis*	

LCL, leishmaniose cutânea localizada; **LCD**, leishmaniose cutânea difusa; **LMC**, leishmaniose mucocutânea; **LV**, leishmaniose visceral

1.1.2 O ciclo de vida da *Leishmania*

1.1.2.1 Inseto vetor

O ciclo de vida da *Leishmania* é complexo e envolve o desenvolvimento no interior do intestino do inseto vetor antes de sua transmissão para o hospedeiro

mamífero definitivo. Fêmeas do inseto vetor ingerem parasitos durante o repasto sanguíneo em mamíferos infectados (Bates, 2007). Nesse momento, ocorre a formação da de uma estrutura em forma de filme chamada de matriz peritrófica que confina os parasitos ingeridos juntamente com o sangue do repasto sanguíneo (Fig. 2A) (Bates, 2007). Após cerca de 16h, o parasito encontra, no trato digestório do inseto vetor, um ambiente com temperatura reduzida e pH alto que estimula a diferenciação da forma amastigota imóvel para a forma promastigota procíclica, células alongadas que se movem com auxílio de um flagelo (Fig. 2B). Nesse estágio, as promastigotas aderem à superfície do intestino através de uma molécula de superfície chamada lipofosfoglicano (LPG) e multiplicam-se, enquanto o restante do alimento é digerido (Descoteaux e Turco, 1999). Após dois a três dias, ocorre a degradação da matriz peritrófica com ajuda das quitinases secretadas pela *Leishmania*. Os parasitos migram e colonizam a parte anterior do intestino do inseto vetor onde se ligam às células epiteliais através do LPG (Fig. 2C) (Descoteaux e Turco, 1999; Kamhawi, 2006). O polimorfismo estrutural do LPG das diferentes espécies de *Leishmania* define as espécies de inseto vetor que cada parasita pode colonizar (Sacks, 2001; Kamhawi, 2006). Uma vez que a forma promastigota procíclica alcança a válvula estomacal, replica-se, produz um gel secretório de promastigotas (PSG) e diferencia-se na forma infectiva para os hospedeiros vertebrados, chamada de promastigota metacíclica, em um processo chamado de metaciclogênese (Fig. 2D). O tampão composto por PSG impede que o inseto vetor realize um repasto sanguíneo adequado, o que força que o inseto vetor regurgite o PSG, juntamente com as promastigotas metacíclicas que perdem a aderência à membrana do intestino do inseto vetor durante um novo repasto sanguíneo (Fig. 2D)

(Kamhawi, 2006; Rogers e Bates, 2007). A obstrução causada pelo PSG também induz o inseto vetor a aumentar a quantidade de tentativas de se alimentar e o tempo que leva alimentando-se, ampliando assim, as chances de transmissão dos parasitos (Kamhawi, 2006; Rogers e Bates, 2007). A saliva do inseto vetor também é conhecida por auxiliar na transmissão da *Leishmania*, uma vez que a presença de vasodilatadores, anti-coagulantes e moléculas imuno-moduladoras facilitam e favorecem o parasito na etapa de interação inicial com as células imunes do hospedeiro (Kamhawi, 2000; Sacks e Kamhawi, 2001).

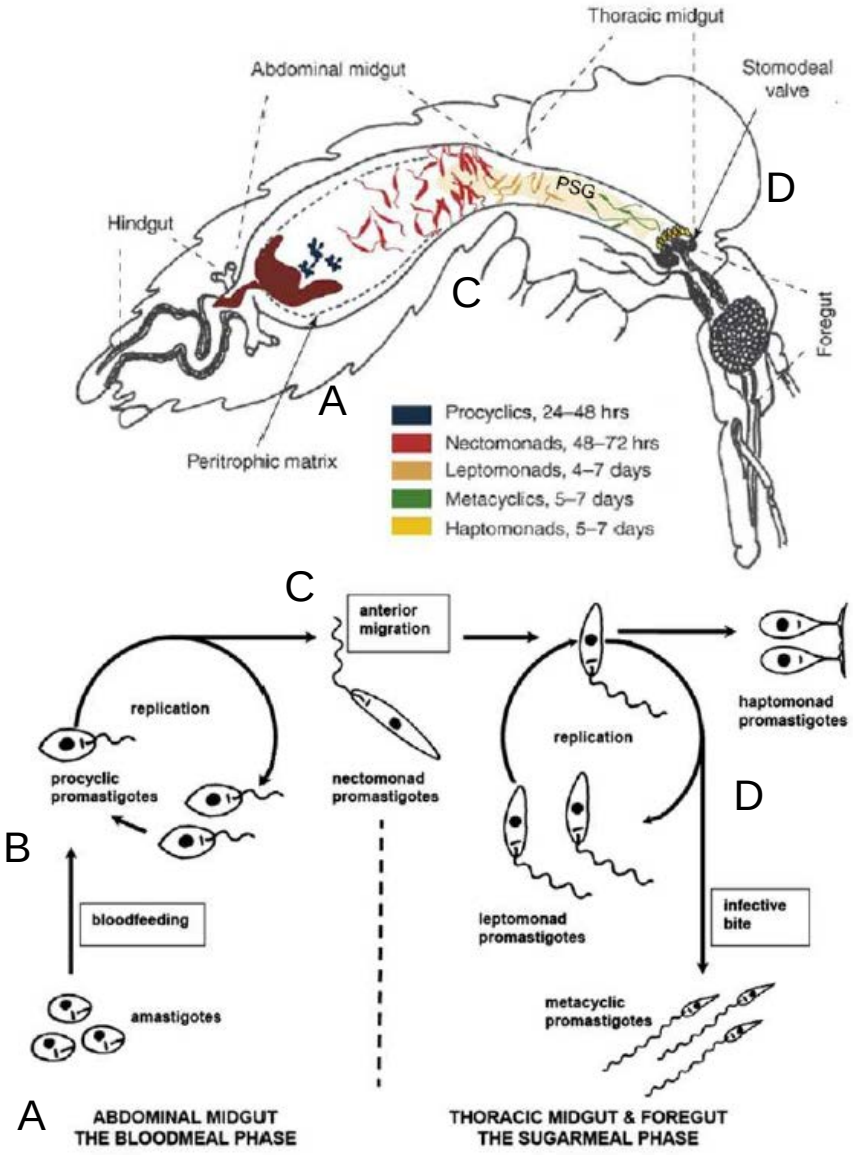

Figura 2. O ciclo de vida da *Leishmania* **no interior do inseto vetor.** Os parasitos sofrem modificações durante os diferentes estágios de desenvolvimento enquanto migram pelas diferentes partes do intestino do inseto até a válvula estomacal, onde se diferenciam na forma infectiva de promastigota metacíclica e são transmitidas para o hospedeiro mamífero enquanto o inseto vetor realiza o repasto sanguíneo. *Adaptado de Kamhawi (2006) e Bates (2007)*

1.1.2.2 Hospedeiro mamífero

As promastigotas metacíclicas, após serem inoculadas na pele do hospedeiro vertebrado através da picada do inseto vetor infectado, são capturadas por uma variedade de células do sistema imune do hospedeiro. A inserção da probóscide do inseto na pele do hospedeiro causa uma forte reação inflamatória localizada, que resulta em um rápido recrutamento de neutrófilos para o local do repasto sanguíneo. Cerca de 2 horas após ser inoculado na pele do hospedeiro, o parasito *Leishmania* é fagocitado por neutrófilos (Peters et al., 2008). Após 18 h de infecção, a maioria dos parasitos permanece no interior de neutrófilos apesar de alguns aparecerem no interior de células dendríticas ou macrófagos. Após cerca de seis dias após a infecção, a maioria dos parasitos encontra-se no interior de macrófagos (Peters et al., 2008). Neutrófilos são células que possuem uma meia vida curta, de cerca de seis a dez horas; no entanto, estas células ao serem infectadas por *Leishmania* apresentam um retardo na apoptose e permanecem vivas por até 3 dias. Após esse período, as células sofrem apoptose liberando os parasitos que serão fagocitados por macrófagos (Aga et al., 2002). Esse mecanismo parece favorecer a persistência da infecção, mantendo a viabilidade dos neutrófilos até os macrófagos chegarem ao local de infecção. Nesse contexto, neutrófilos apoptóticos contendo parasitos viáveis poderiam funcionar como “Cavalos de Tróia” uma vez que quando fagocitados por macrófagos passariam os parasitos de forma silenciosa para a sua célula hospedeira final inativando mecanismos microbicidas da resposta imune (Laskay et al., 2003). Outra possibilidade é que *Leishmania* escaparia dos neutrófilos quando esses entram em apoptose para, então, infectar macrófagos (Laskay et al., 2008). Os macrófagos parecem ser a principal célula hospedeira da *Leishmania*, porque

apesar do parasito conseguir sobreviver no interior de neutrófilos, eles não se replicam ou se diferenciam, esses processos somente ocorrem no interior de macrófagos (Alexander e Russell, 1992; Ritter et al., 2009). Além disso, camundongos que não possuem neutrófilos contem o mesmo número de parasitos no interior de seus macrófagos quando comparados com animais selvagem (Peters et al., 2008). Os parasitos na forma promastigota são fagocitados por macrófagos *in vitro* (Antoine et al., 1998; Courret et al., 2002; Forestier et al., 2011; Wenzel et al., 2012). As promastigotas interagem com o macrófago, ativando mecanismos de fagocitose e sendo, assim, internalizadas e onde permanecem albergadas no interior de estruturas membranosas chamadas de vacúolos parasitóforos (VP) (Antoine et al., 1998; Courret et al., 2002). No interior do VP, as promastigotas metacíclicas diferenciam-se em amastigotas, um processo conhecido como amastigogênese que pode levar de 2 a 5 dias (Fig. 3) e, uma vez diferenciada, as amastigotas se replicam sendo capazes de escapar do sistema imune do hospedeiro (Alexander et al., 1999; Courret et al., 2002; Peters et al., 2008). Eventualmente, as amastigotas escapam dos macrófagos e reinfectam novas células hospedeiras (Fig. 3). O ciclo de vida do parasito é finalizado quando macrófagos infectados são ingeridos durante o repasto sanguíneo do inseto vetor (Alexander e Russell, 1992).

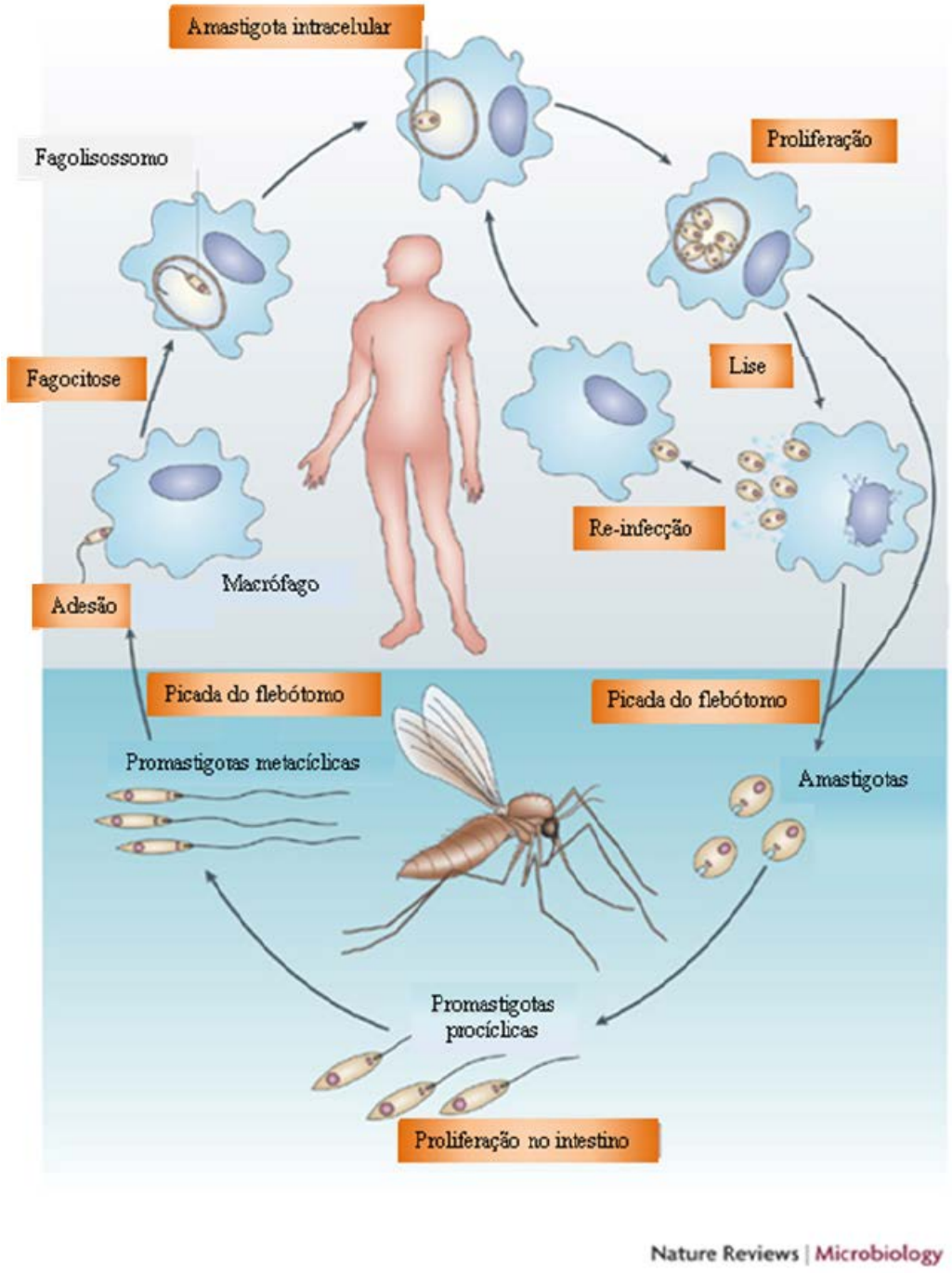

Figura 3. Ciclo de vida da *Leishmania*. (Modificado de Chappuis *et al*; 2007)

1.2 MODELOS DE ESTUDO

1.2.1 Modelo murino

O desenvolvimento de modelos experimentais em camundongos isogênicos para o estudo da leishmaniose tem contribuído para um melhor entendimento desta doença. Os modelos murinos auxiliam no desenvolvimento de novos medicamentos e estratégias terapêuticas uma vez que os camundongos são utilizados nos primeiros ensaios pré-clinicos para avaliação da eficácia terapêutica de compostos frente à infecção por *Leishmania.*. Modelos de resistência e susceptibilidade têm sido estudados utilizando-se diferentes linhagens de camundongos infectados com espécies distintas de *Leishmania* (Bogdan et al., 1996). A resistência ou a susceptibilidade de uma linhagem de camundongo a uma determinada espécie de *Leishmania* depende do tipo de resposta de linfócitos T CD4+, sendo o perfil protetor caracterizado pela produção de interleucina (IL)-12, interferon gama (IFN-γ) e fator de necrose tumoral alfa (TNF-α) nos eventos iniciais da infecção (Scharton-Kersten et al., 1995). A sinergia entre IFN-γ e TNF-α confere proteção à infecção por *Leishmania*, devido à ativação de mecanismos microbicidas, como a liberação de superóxido (O_2^-) por macrófago e neutrófilos, via ativação do complexo NADPH oxidase, e a síntese de óxido nítrico (NO) pela enzima NO sintetase induzível (iNOS) (Titus et al., 1989; Liew et al., 1990; Bogdan e Rollinghoff, 1998).

Por outro lado, ocorre ativação de células T CD4+ e produção de citocinas como a interleucina-4 (IL-4), IL-10 e fator transformador do crescimento beta (TGF-β) em resposta à infecção, modulando a ativação de macrófago e sua capacidade microbicida e, consequentemente, favorecendo a progressão da doença (Liew et al.,

1989; Ding et al., 1990; Bogdan et al., 1991). Essas citocinas atuam modulando a produção de IFN-γ (Tanaka et al., 1993). Foi demonstrado que inibição de IL-10 induz a redução de parasitos nas lesões (Jones et al., 2002; Ji et al., 2003) e aumento da expressão da atividade de iNOS (Jones et al., 2002). Adicionalmente, a inibição de TGF-β reduz o tamanho das lesões e induz o aumento da expressão de mRNA para IFN-γ em camundongos BALB/c infectados com *L. amazonensis* (Barral-Netto et al., 1992).

A resistência ou susceptibilidade de diferentes linhagens de camundongos a espécies distintas de *Leishmania* depende da interação entre *background* genético do parasito, do hospedeiro e o perfil da resposta imunológica induzida pela infecção (Afonso e Scott, 1993; Guevara-Mendoza et al., 1997; Dekrey et al., 1998). Nesse contexto, macrófagos têm um papel central na infecção por *Leishmania*, atuando nas fases indutora e efetora da resposta imune. Além de abrigarem os parasitos, os macrófagos também apresentam antígenos para linfócitos T específicos e liberam citocinas relacionadas a uma resposta protetora como a IL-12 e TNF-α, assim como, citocinas relacionadas com a resposta moduladora como a IL-10 e TGF-β (Barral-Netto et al., 1992; Wang et al., 1994). O curso da infecção depende, principalmente, da capacidade dos macrófagos em eliminar os parasitos intracelulares. Assim, é importante compreender os mecanismos efetores dos macrófagos para que seja possível explorá-los durante o curso do tratamento de pacientes com leishmaniose.

1.3 TRATAMENTO DAS LEISHMANIOSES

A primeira linha de tratamento contra a LV ou LT são antimoniais pentavalentes como o estibogluconato de sódio (Pentostam®) e o antimoniato de meglumina (Glucantime®). Esses medicamentos têm sido usados como primeira linha de tratamento contra a leishmaniose nos últimos 70 anos, sendo administrados diariamente por via parenteral: intravenosa ou intramuscular por cerca de 20 dias por ciclo, o tratamento é longo, desconfortável e bastante tóxico (Singh e Sundar, 2014). Adicionalmente, os antimoniais também podem ser aplicados intralesionalmente em pacientes infectados por *L. braziliensis* (Oliveira-Neto et al., 1997).

O tratamento intralesional com antimoniais pentavalentes levou a taxas de cura, que variam consideravelmente entre pacientes e a depender da espécie de *Leishmania*, com trabalhos reportando taxas, para infecção por *L. mexicana*, de 57% e 100% (Navin et al., 1990; Vargas-Gonzalez et al., 1999) e para infecções causadas por *L. braziliensis* e *L. guyanensis* taxas tão baixas quanto 50,8% e 26,3% (Romero et al., 2001). Além disso, outro dado surpreendente é a baixa correlação entre a taxa de cura observada em pacientes infectados com *L. braziliensis* e o tempo de tratamento com antimoniais pentavalentes (Machado et al., 2002). Pacientes em estágios inicias de LCL (< 20 dias) e submetidos ao tratamento com antimoniais tiveram uma taxa de falha no tratamento de 46% (Machado et al., 2002). É possível que a utilização da menor dose efetiva seja um dos fatores responsáveis pela alta taxa de falha terapêutica, uma vez que existe correlação entre a dose utilizada no tratamento e a resposta do paciente (Llanos-Cuentas et al., 2008). Por

outro lado, o uso de antimoniais em baixas doses, associado à imunoterapia ou ao tratamento tópico com GM-CSF foram altamente eficientes, sendo capazes de curar 100% dos pacientes, inclusive pacientes com LCL refratários ao tratamento convencional (Machado-Pinto et al., 2002; Almeida et al., 2005).

Os principais efeitos colaterais associados ao tratamento com antimoniais são dor muscular, náuseas, vômito, diarreia, dor abdominal, dor de cabeça, anorexia, fadiga, febre e eritema. Sintomas mais graves também são observados como pancreatite e hepatite com o aumento moderado de enzimas pancreáticas e hepáticas. No entanto, a maioria dessas alterações é transitória e retornam para níveis normais ao final do tratamento (Oliveira et al., 2011). Casos de arritmia também foram associados ao tratamento de pacientes com leishmaniose por antimoniais pentavalentes, no entanto, este efeito colateral foi também atribuído ao uso de tiazida, um diurético que leva à redução dos níveis de potássio plasmático, durante o tratamento. Casos de morte associados, principalmente, à insuficiência renal também foram observados em pacientes tratados com antimoniais (Oliveira et al., 2011).

Parasitos resistentes ao tratamento com antimoniais pentavalentes têm surgido com alta frequência principalmente na Índia (Croft, Sundar, et al., 2006). A resistência tem sido relacionada com o fato de o medicamento ser livremente comercializado nesse país. Assim, pacientes fazem uso indiscriminado dos antimoniais, além de comumente iniciarem o tratamento com baixas doses e aumentarem as doses gradualmente com intervalos de até uma semana durante o

tratamento (Croft, Sundar, et al., 2006). Essas práticas são conhecidas por induzirem a seleção de parasitos resistentes.

Anfotericina B, pentamidina, miltefosina e paromomicina são os medicamentos utilizados em uma segunda linha de tratamento. A anfotericina B e sua formulação lipossomal (AmBisome®) são utilizadas como tratamento padrão em algumas partes do mundo onde o tratamento com o antimonial apresenta taxas de cura inferiores a 52% (Alvar, Croft, et al., 2006; Croft, Seifert, et al., 2006; Croft, Sundar, et al., 2006; Croft e Olliaro, 2011). Esse medicamento é um antibiótico administrado via intravenosa e seu mecanismo de ação é dependente de sua associação com os esteróis da membrana plasmática do parasito, abrindo poros na membrana e induzindo a lise celular (Saha et al., 1986).

A formulação lipossomal da anfotericina B, por apresentar menos efeitos colaterais, é empregada, principalmente, contra LMC e LV associadas à coinfecção com HIV. No entanto, o tratamento completo com AmBisome possui altos custos, dificultando o seu uso principalmente em países em desenvolvimento onde a leishmaniose é endêmica (Croft e Olliaro, 2011). O tratamento de pacientes com LC por anfotericina B lipossomal em doses de 3 mg/Kg/dia com 10 a 15 doses apresentou melhores resultados, com uma taxa de cura de 85%, sendo mais eficiente e melhor tolerado do que o tratamento realizado com antimoniais (Solomon et al., 2013). No entanto, em ensaio similar realizado no Brasil evidenciou que anfotericina lipossomal possui uma taxa de cura inferior à do tratamento com antimoniais pentavalentes, mas com efeitos colaterais significantemente mais amenos (Motta e Sampaio, 2012). Em casos de LMC a anfotericina B lipossomal

demonstrou ser mais eficiente do que o deoxicolato de anfotericina mesmo em casos nos quais o tratamento prévio com antimoniais apresentaram falha (Sampaio e Marsden, 1997; Amato et al., 2007).

A pentamidina também é um medicamento de segunda escolha. Apesar de ter tido o seu uso descontinuado na Índia, devido a sua baixa eficácia, esse medicamento é efetivo contra algumas espécies de *Leishmania* causadoras de LT na América do Sul (Croft e Olliaro, 2011). Esse medicamento é recomendado para os casos graves de leishmaniose não responsiva ao tratamento convencional. O tratamento é normalmente realizado com 4 – 10 injeções com doses de 3 – 4 mg/Kg/dia em dias alternados. A pentamidina parece ser bastante eficiente com taxas de cura superiores a 90% em casos de LC causados por *L. guyanensis* (Minodier et al., 2005). No Brasil, resultados similares foram obtidos em ensaios comparando a taxa de cura entre o tratamento com pentamidina e antimoniais. O tratamento com pentamidina durou apenas uma semana, enquanto o com os antimoniais durou 20 dias (De Paula et al., 2003). Por outro lado, ensaios realizados no Peru com pacientes infectados com *L. braziliensis*, a pentamidina demonstrou ser muito menos efetiva com taxas de cura de apenas 35% (Andersen et al., 2005). Em pacientes com LMC e tratados com pentamidina houve uma taxa de cura de 94% com baixo índice de recidiva ao final do tratamento (Amato et al., 1998). Em outro estudo, pacientes com LMC e tratados com pentamidina apresentaram taxas de cura similares às observadas em pacientes tratados com antimoniais, 91% em ambos os casos. No entanto, a taxa de recidiva foi considerada alta, com valores de 25% (Amato et al., 2009). Os principais efeitos colaterais associados com o tratamento por pentamidina são dor muscular, anorexia, dor abdominal, vômito dor

de cabeça dentre outras (Oliveira et al., 2011). Doses mais altas de 7 mg/kg podem levar a rabdomiólise e casos de hipoglicemia severa (Oliveira et al., 2011).

Miltefosina (ImpavidoTM®) é um fosfolipídio alquil, que tem seu efeito leishmanicida ao interferir na síntese de fosfolipídios e esteróis do parasito (Rakotomanga et al., 2007). A miltefosina é o primeiro tratamento oral para LV e mostrou eficiência contra algumas espécies causadoras de LT. O tratamento de pacientes com LCL por miltefosina apresentou resultados variáveis com taxas de cura superiores a 91% para *L. panamensis*. Por outro lado, pacientes provenientes de locais endêmicos para *L. braziliensis* e *L. mexicana* apresentaram taxas de cura de apenas 53% quando tratados por miltefosina (Soto et al., 2004). No Brasil, pacientes infectados com *L. braziliensis* e tratados com miltefosina apresentaram uma taxa de cura de 75%, taxa consideravelmente superior à observada em pacientes tratados com antimoniais. (Machado et al., 2010). O uso de miltefosina para o tratamento de LMC também apresenta resultados variados sendo que a efetividade do tratamento é inversamente proporcional a severidade dos casos de LMC, de forma que pacientes com comprometimento do palato, laringe e faringe apresentaram taxas de cura inferiores às observadas em pacientes que apresentavam apenas lesões nasais (Soto et al., 2001). A miltefosina possui efeitos teratogênicos e não deve ser utilizada por mulheres em idade gestacional (Dorlo et al., 2012). Os principais efeitos colaterais do uso da miltefosina em doses de 50 mg/dia são vômito, náuseas, cinetose, dor de cabeça e diarréia (Soto et al., 2004), além de alterações laboratoriais como aumento de aminotransferase e creatina (Soto et al., 2001).

A paromomicina é um antibiótico promissor da família dos aminoglicosídios no tratamento contra LV quando administrada via intramuscular e contra a LCL quando aplicada de forma tópica. No entanto, a baixa disponibilidade deste fármaco restringe o seu uso em áreas endêmicas, além disso, casos de resistência *in vitro* já foram descritos em diferentes espécies de *Leishmania* (Singh et al., 2012). O tratamento tópico com esse medicamento para casos de LCL causada por *L. mexicana*, *L. panamensis* e *L. braziliensis* apresentou taxas de cura que variaram entre 70 e 90% (Krause e Kroeger, 1994; Arana et al., 2001). O uso tópico desse medicamento associado a antimoniais também apesentou resultados favoráveis com taxa de cura de 90% (Soto et al., 1995). No entanto, o tratamento de pacientes colombianos infectados com *L. panamensis* e *L. braziliensis* com paromomicina não ultrapassou a taxa de cura de 58% (Soto et al., 1998).

O tratamento de pacientes que apresentam LCL com métodos físicos, a exemplo da termoterapia e crioterapia, apresenta resultados interessantes. As taxas de cura desses ensaios clínicos são normalmente iguais ou superiores às observadas nos tratamentos com antimoniais. Estudos comparando a taxa de cura da termoterapia com radiofrequência mostraram-se mais eficientes com uma taxa de cura de 80,7% quando comparada com a alcançada com o uso de antimônio intralesional, cuja taxa é de 55,3% (Sadeghian et al., 2007). Outro estudo realizado em pacientes com LCL causada por *L. major* evidenciou taxas de cura similares quando comparados aos tratamentos por termoterapia e antimoniais intravenosos, porém com efeitos colaterais mais amenos em relação ao tratamento convencional (Aronson et al., 2010). A crioterapia também demonstrou ser eficiente em ensaios limitados com baixos números de pacientes nos quais o tratamento foi capaz de

curar 100% das lesões com poucos efeitos colaterais e em intervalo de tempo inferior ao tratamento convencional com antimoniais pentavalentes (Panagiotopoulos et al., 2005). No entanto, mais estudos randomizados e com controle precisam ser realizados para corroborar os dados observados que utilizam tratamentos físicos contra a LCL.

Ensaios clínicos também são realizados testando a combinação de diferentes tratamentos (Sundar et al., 2011). Tais combinações podem reduzir a seleção de parasitos resistentes, aumentar a eficiência do tratamento mesmo com menores doses e, consequentemente, reduzir os efeitos colaterais que a maioria dessas drogas apresenta. Esses ensaios também utilizam antifúngicos orais como cetoconazol, itraconazol e fluconazol que apresentam efeito leishmanicida (Sundar et al., 2011).

O aparecimento de casos de coinfecção entre leishmaniose e HIV merece destaque e vem ganhando importância nos últimos anos devido ao crescente número de ocorrências. Essa coinfecção tem grande relevância, principalmente, em casos de infecção por LV. Pacientes infectadas por HIV apresentam chances aumentadas de progressão de uma infecção assintomática para casos graves de LV (Alvar et al., 2008). Os testes sorológicos para detecção de LV em pacientes com HIV são menos eficazes (Cota et al., 2012). Adicionalmente, o tratamento convencional com antimoniais tem taxas de cura de 25% (Hurissa et al., 2010) e, mesmo após a cura clínica cerca de 60% dos pacientes apresentam reincidência da doença após um ano (Ter Horst et al., 2008). No nordeste brasileiro, pacientes com LV, infectados simultaneamente por HIV, foram tratados profilaticamente com

anfotericina B, mesmo assim, os casos de recidiva ocorreram em 53% dos pacientes. Um total de 23% dos pacientes coinfectados veio a óbito, enquanto 7% dos pacientes apenas com LV faleceram, evidenciando assim, o risco de morte aumentado em pacientes coinfectados (Nascimento et al., 2011). Ainda não existe um tratamento definitivo para pacientes coinfectados por HIV e LV. Diferentes países usam estratégias distintas, como o uso de anti-retrovirais antes da administração de medicamentos leishmanicidas ou tratamento simultâneo com ambos os medicamentos. No entanto, em ambos os casos os resultados não são satisfatórios e a cura de pacientes com LV coinfectados por HIV continua a representar um grande desafio (Croft e Olliaro, 2011).

Existe urgência na introdução de novos compostos anti-*Leishmania*, assim como o desenvolvimento de esquemas terapêuticos menos tóxicos, mais seguros, quando comparados aos utilizados atualmente. Dentre os alvos moleculares com potencial de serem explorados farmacologicamente, a proteína de choque térmico 90 (HSP90) recebeu destaque. Essa proteína possui alta expressão em parasitos protozoários além de ser responsável diretamente pelo controle de diversas outras proteínas envolvidas em diferentes etapas do ciclo celular do parasito.

1.4 PROTEÍNA DE CHOQUE TÉRMICO 90 (HSP90)

A HSP90 é uma chaperona molecular necessária para o enovelamento e a estabilização de um grande número de proteínas e complexos protéicos (Hartson e Matts, 2012). Essa chaperona liga-se e estabiliza outras proteínas, controlando a ligação e a liberação das mesmas, facilitando, assim, o seu dobramento,

proporcionando que as proteínas sejam endereçadas ao destino correto (Hartl, 1996). As chaperonas não determinam a estrutura terciária das proteínas recém-sintetizadas, mas facilitam seu dobramento e permitem que essas proteínas alcancem sua estrutura final de forma eficiente (Hartl, 1996). Mais de 300 proteínas são conhecidas por necessitarem interagir com a HSP90 para que possam alcançar sua estrutura terciária. Essas proteínas, sobre as quais a HSP90 tem ação de dobramento são coletivamente chamadas de proteínas cliente (lista completa: http://picard.ch/downloads/Hsp90interactors.pdf). A HSP90 participa do dobramento de proteínas cliente das mais diversas famílias estruturais ou funcionais, tais como proteínas ribossomais, telomerases, proteínas de vírus dentre outras, apesar da maioria delas serem cinases, fatores de transcrição ou receptores esteróides.

A HSP90 é altamente conservada e encontrada em organismos de todos os reinos com a exceção de Archea (Gupta, 1995; Johnson, 2012). Existem quatro homólogos de HSP90 distribuídos em diferentes compartimentos celulares: a HSP90 citosólica, proteína 94 regulada pela glicose (do inglês, *glucose-regulated protein 94* – GRP94), proteína 1 associada ao receptor de TNF (do inglês, *TNF receptor-associated protein 1* – TRAP1) e a chaperona molecular HTPG bacteriana (Csermely, 1998). Todos esses homólogos são essenciais para o funcionamento celular com exceção da HPTG que não é essencial em condições fisiológicas, apesar de sua ausência acarretar o retardo do crescimento bacteriano em altas temperaturas (Lund, 2001).

A HSP90 desempenha sua função como um homodímero, no qual cada monômero tem três domínios estruturais distintos: i) domínio N terminal (NTD), que

contém o sítio de ligação ao ATP; ii) domínio intermediário (MD) que associa-se ao NTD através de um conector; iii) domínio C terminal (CTD), que é responsável pela dimerização da proteína (Pearl e Prodromou, 2006). O NTD é altamente conservado entre as diferentes espécies, possui aproximadamente 25 kDa e é o local onde ocorre a ligação entre HSP90 e o ATP ou com as co-chaperonas. As co-chaperonas, por sua vez, são proteínas que ligam-se à HSP90 auxiliando-a a desempenhar seu papel de estabilizador e facilitador do dobramento das proteínas cliente, mas não possuem atividade chaperona (Fig. 4). O MD possui aproximadamente 40 kDa sendo essencial para o reconhecimento e ligação das diferentes proteínas cliente e co-chaperonas. Esse domínio também é importante para a atividade ATPásica do NTD, uma vez que a deleção desse domínio implica em uma redução drástica da atividade ATPásica do NTD (Meyer et al., 2003). CTD possui aproximadamente 12 kDa, é responsável pela homodimerização da HSP90 na sua forma biologicamente ativa e pela interação com co-chaperonas (Nemoto et al., 1995; Pearl e Prodromou, 2006). A HSP90, juntamente com as co-chepronas, formam um heterocomplexo chamado de máquina de dobramento de proteínas.

A HSP90 auxilia a maturação estrutural de proteínas recém sintetizadas em condições de homeostasia celular e estabiliza proteínas quando as células estão em condições de estresse, como as causadas por aumento de temperatura, mudanças de pH e privação nutricional. Nestas condições, HSP90 tem sua expressão aumentada e ao associarem-se às co-chaperonas promovem a estabilização e a renaturação de proteínas desnaturadas durante o período de estresse celular (Schneider et al., 1996; Nathan et al., 1997). O mecanismo pelo qual a HSP90

promove o dobramento e maturação de proteínas cliente não está completamente esclarecido.

O dobramento de proteínas cliente promovido pela HSP90 compreende, primeiramente, a estabilização de proteínas recém sintetizadas pelos ribossomos por um complexo formado pelas co-chaperonas HSP70, HSP40 e ADP (Fig. 4A) (Frydman, 2001). Este complexo associa-se à proteína cliente sendo adicionalmente estabilizado pela *HSP70-interacting protein* (HIP), que facilita a troca de ADP por ATP e, consequentemente, promove liberação da proteína recém sintetizada para a HSP90 (Fig. 4A) (Frydman, 2001; Pearl e Prodromou, 2006). Várias proteínas cliente, quando ligadas ao complexo HSP70/HSP40/HIP podem interagir com a HSP90 através da proteína de organização HSP70/HSP90 (HOP - do inglês *HSP70/HSP90-organizing protein*). Por sua vez, a HOP promove a transferência da proteína cliente para o homodímero da HSP90 e também dissociação do complexo HSP70/HSP40/HIP/HOP (Fig. 4B) (Heinzel et al., 1995). Nesta situação, o homodímero da HSP90 encontra-se aberto, mantendo-se unido pela interação entre os CTDs dos monômeros. Após a entrega da proteína cliente para a HSP90, outras co-chaperonas interagem com a HSP90, formando um complexo heteroprotéico. O ATP se liga ao NTD, levando à mudança conformacional com dimerização da NTD e fechamento do homodímero (Fig. 4D). A proteína p23 é então recrutada para o NTD, estabilizando a sua dimerização e promovendo a hidrólise do ATP. Outra co-chaperona que participa do processo é a ativadora da atividade ATPásica de HSP90 (AHA1 - do inglês *activator of HSP90 ATPase*). A AHA1 estimula a atividade ATPásica da HSP90, promovendo a hidrólise do ATP e induzindo a volta da HSP90 para a sua conformação aberta e finalmente liberando a proteína cliente em seu

estado terciário final (Fig. 4E). A proteína cliente, enquanto associada à HSP90, sofre rearranjos, cujos mecanismos ainda não foram elucidados (Heinzel et al., 1995; Whitesell e Lindquist, 2005). Além disso, é importante salientar que, por mecanismos ainda não descritos, o padrão de interação das co-chaperonas com a proteína cliente e com a HSP90 muda de acordo com a natureza das proteínas cliente em questão. Assim, na montagem de proteínas cinases clientes da HSP90, a co-chaperona proteína controladora da divisão celular 37 kDa (CDC37) substitui a co-chaperona HOP. A CDC37, diferentemente da HOP, inibe a dimerização do NTD e, consequentemente, retarda a hidrólise do ATP. Este atraso da hidrólise do ATP é importante no processo de dobramento apenas das proteínas cinases, mas não para o dobramento de outras proteínas cliente da HSP90 (Caplan et al., 2007). Provavelmente, todas as formas de HSP90 acima descritas têm papéis similares em relação a facilitar a dobradura de proteínas cliente mas, co-chaperonas que regulam a atividade ATPásica de TRAP1, GRP94 ou HTPG ainda não foram identificadas. Adicionalmente, as co-chaperonas não são universais entre os diferentes eucariotos, sendo que genes de diferentes co-chaperonas podem estar ausentes ou presentes entre os diferentes grupos de eucariotos. Desta forma, a função de algumas co-chaperonas pode ser restrita especificamente a conjuntos de proteínas cliente, ser requerida apenas em alguns grupos de organismos eucariotos ou ser redundante com outras co-chaperonas presentes em outros organismos (Zuehlke e Johnson, 2010; Li e Buchner, 2013).

As proteínas cliente da HSP90 não compartilham nenhuma sequência ou estrutura que as identifique como proteínas cliente da HSP90. Além disso, os domínios de interação entre a proteína cliente e a HSP90 ainda não estão definidos,

o que dificulta a identificação das possíveis proteínas cliente. O fato de que os três domínios da HSP90 participam da interação com diferentes proteínas cliente dificulta ainda mais a identificação das proteínas cliente, e é possível que estes domínios de interação mudem à medida que ocorram as mudanças conformacionais da HSP90 durante o dobramento protéico das proteínas cliente (Pratt e Toft, 2003).

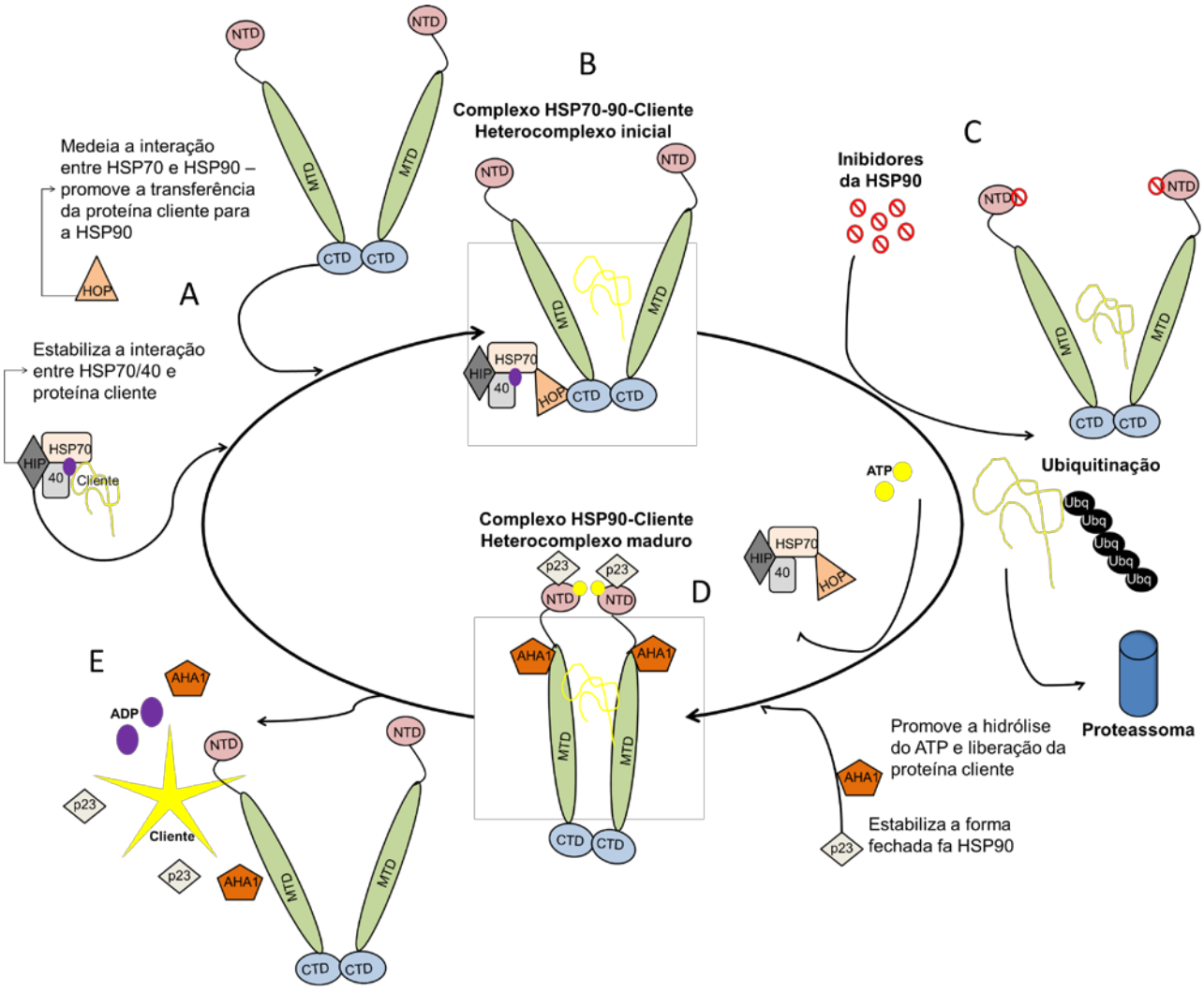

Figura 4. Ciclo da HSP90. O papel da HSP90 é auxiliar a proteína cliente a atingir sua conformação ativa. A proteína cliente inativa é associada a um heterocomplexo inicial, a partir do qual ela pode seguir dois caminhos distintos: i – associar-se ao complexo maduro ou ii – prosseguir pela via da degradação. Quando a proteína cliente associada a HSP90 assume sua conformação ativa ela é liberada da HSP90 e está apta a realizar suas funções biológicas. Inibidores da HSP90 favorecem a degradação das proteínas cliente uma vez que impedem a formação do heterocomplexo maduro.

1.4.1 Inibidores da HSP90

Muitas proteínas cliente da HSP90 estão envolvidas em diferentes vias oncogênicas. Assim, a HSP90 passou a ser reconhecida como potencial alvo molecular contra diferentes tipos de câncer (Jhaveri et al., 2012). A geldanamicina (GA) e o radicicol (Fig. 5) foram os primeiros compostos naturais identificados como

sendo capazes de inibir a atividade da HSP90 (Sharma et al., 1998; Neckers et al., 1999). O GA é um antibiótico natural derivado do fungo *Streptomyces hygroscopicus*, que apresenta atividade antiproliferativa contra células cancerígenas e pertence à família das benzoquinonas ansamicinas. GA interage com sítio de ligação do ATP presente no NTD da HSP90, inibindo sua atividade ATPásica (Fig. 4C) (Grenert et al., 1997; Prodromou, Roe, O'brien, et al., 1997; Prodromou, Roe, Piper, et al., 1997; Roe et al., 1999). A inibição da HSP90 leva a ubiquitinação da proteína cliente com sua subsequente degradação pelo proteassoma (Fig. 4C) (Isaacs et al., 2003). GA tem baixa solubilidade, estabilidade *in vivo* limitada e é hepatotóxico, tornando-o um composto com baixas chances de se tornar comerciável (Schulte e Neckers, 1998).

Compostos semi-sintéticos foram produzidos à partir da GA. O grupamento metoxilo, presente no carbono 17, foi substituído por diferentes aminas . O primeiro inibidor da HSP90 a entrar em ensaios clínicos foi um derivado da geldanamicina, o 17-AAG (17- allylamino -17-demethoxygeldanamycin - tanespimicina) (Fig. 5) que apresenta menor toxicidade, maior afinidade com o sítio de ligação do ATP e, consequentemente, maior poder inibitório sobre a HSP90 (Schulte e Neckers, 1998). O 17-AAG apresentou um maior número de resultados positivos contra câncer de mama e mieloma múltiplo em comparação ao GA (Neckers e Workman, 2012). Problemas de solubilidade, baixas propriedades farmacêuticas e o perfil tóxico relacionado principalmente à porção benzoquinona levou ao desenvolvimento de outro derivado da geldanamicina, o 17-DMAG (17- dimethylaminoethylamino-17-demethoxygeldanamycin - Alvespimicina) (Fig. 5). O 17-DMAG apresenta um grupamento amino ionizável adicionado ao C-17, o que possibilita uma formulação e

farmacocinética melhorados em relação ao 17-AAG, devido à maior solubilidade e bioviabilidade oral (Tian et al., 2004; Hollingshead et al., 2005). No entanto, estudos pré-clínicos indicam que a hepatotoxicidade do 17-DMAG é um fator dose limitante, sendo a máxima dose tolerada do 17-DMAG significantemente menor do que a tolerada pelo 17-AAG (Glaze et al., 2005; Hollingshead et al., 2005). Ainda, outro derivado da geldanamicina, o IPI-504 (Fig. 5) (Retaspimicina) apesar de ser altamente solúvel e teoricamente menos tóxica devido à perda do seu grupamento benzoquinona, possui alta sensibilidade a ambientes oxidativos. Sua rápida interconversão em 17-AAG em células *in vitro* e *in vivo*, sugere que o IPI-504 apresente o mesmo grau de toxicidades que o 17-AAG (Sydor et al., 2006).

Em conjunto, os resultados obtidos com inibidores da HSP90 de primeira geração estimularam a pesquisa e o desenvolvimento de novas moléculas sintéticas capazes de inibir a atividade da HSP90 com maior biodisponibilidade e perfil toxicológico mais ameno. Essas novas moléculas têm a perspectiva de permitir a administração de quantidades pequenas de fármaco, suficientes para que o efeito inibitório seja eficiente e capaz de promover benefícios terapêuticos. Dentre os inibidores de primeira geração, apenas o 17-DMAG e o IPI-504 continuam em ensaios clínicos para tipos limitados de neoplasias (Jhaveri et al., 2014).

Os inibidores da HSP90 de segunda geração que estão em desenvolvimento clínico incorporaram o grupamento resorcinol obtido originalmente na molécula do radicicol ou tem a base de purina obtida a partir do primeiro inibidor sintético da HSP90, o PU3. Outros inibidores da HSP90 possuem uma base distinta e foram descobertos em estudos de larga escala, nos quais bibliotecas de compostos

químicos foram testadas em relação ao seu poder inibitório da atividade da HSP90 (Jhaveri et al., 2014). Todos os inibidores de segunda geração da HSP90 foram desenvolvidos ou descobertos por indústrias farmacêuticas que detêm as patentes desses produtos. Os estudos com esses novos agentes evidenciaram que a toxicidade associada aos inibidores de primeira geração, principalmente a hepatotoxicidade, relaciona-se à presença da porção benzoquinona no antibiótico, e não ao seu efeito inibitório sobre a HSP90. Desta forma, os novos inibidores da HSP90 progridem em ensaios clínicos com resultados animadores, principalmente em neoplasias que foram identificadas por apresentarem um perfil protéico composto por proteínas cliente sensíveis à inibição da HSP90, a exemplo do receptor HER2 expresso em células cancerígenas de neoplasias mamárias (Jhaveri et al., 2014).

A HSP90, além do seu papel central em diversos tipos de neoplasias (Jhaveri et al., 2012; Neckers e Workman, 2012), também mostrou ser um alvo quimioterápico interessante no combate a doenças causadas por microrganismos, devido ao seu papel no desenvolvimento do ciclo celular de parasitos protozoários ou sobre a sua capacidade de sobreviver no interior da célula hospedeira (Shonhai et al., 2011; Roy et al., 2012).

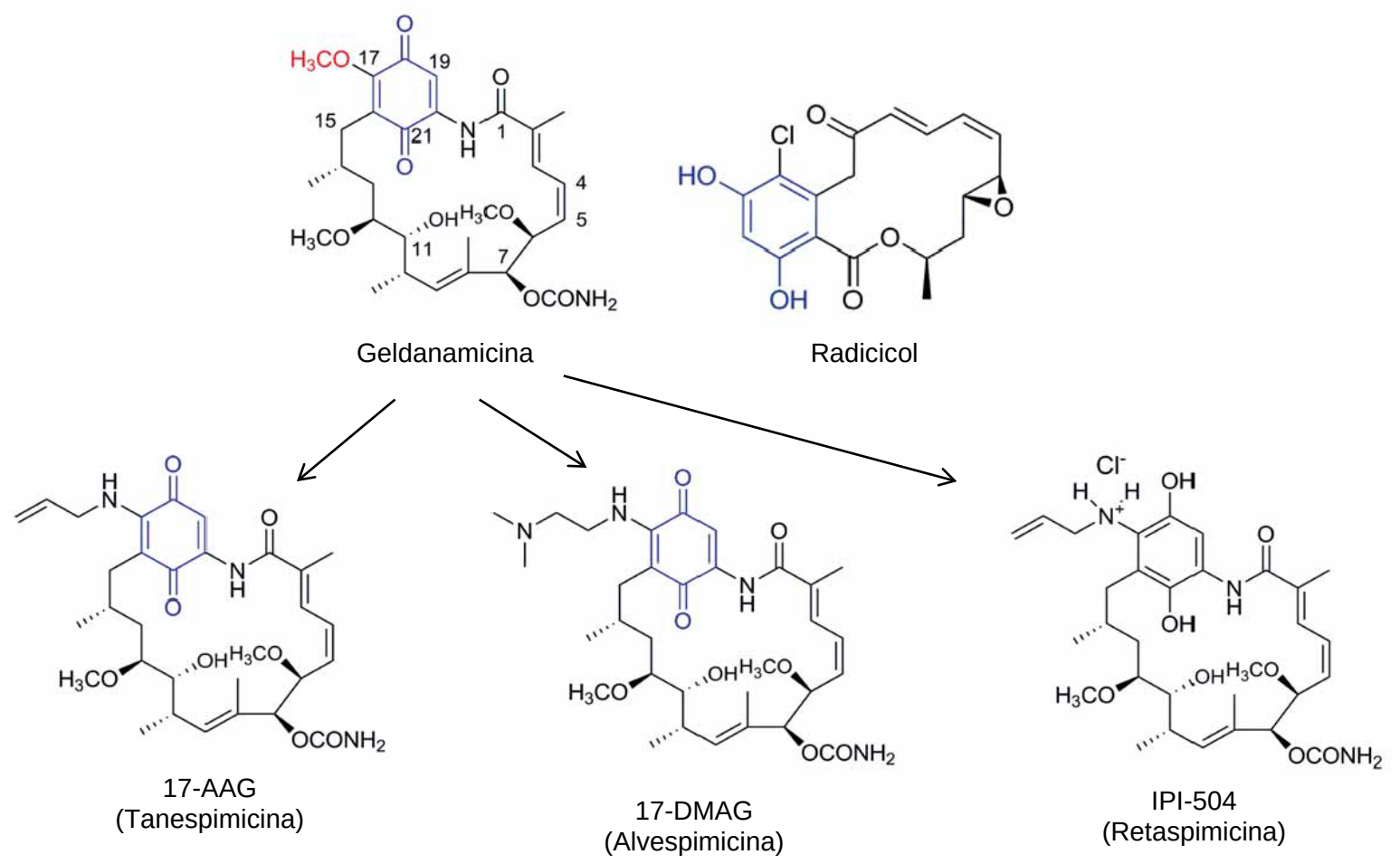

Figura 5. Estrutura química de inibidores da HSP90 de primeira geração. Na geldanamicina e em seus derivados (17-AAG, 17-DMAG) em azul está representado o motivo benzoquinona e em vermelho o grupo metoxi no C17. No Radicicol, em azul está representado o motivo resorcinal.

1.4.2 HSP90 de parasitos protozoários

Em parasitos protozoários, chaperonas moleculares participam de outras funções celulares além da homeostase proteica. A HSP90 está envolvida em processos relacionados ao desenvolvimento de estágios específicos e à patogênese da doença (Shonhai et al., 2011). Nesse contexto, o parasita sofre um estresse devido a mudanças súbitas de temperatura, pH, espécies reativas de oxigênio e o ataque de enzimas hidrolíticas quando infecta células hospedeiras. O aumento da

expressão de chaperonas pelo parasito em resposta ao estresse está relacionado à adaptação às novas condições ambientais, o que ativa o processo de diferenciação celular (Pallavi et al., 2010; Shonhai et al., 2011). Evidencias indicam que as chaperonas influenciam diretamente a virulência do parasito e a sua sobrevivência no interior de macrófagos do hospedeiro vertebrado (Shonhai et al., 2011). Promastigotas de *Leishmania* se tornam mais infectivas em resposta ao choque térmico (Smejkal et al., 1988). Além disso, parasitos do gênero *Leishmania* virulentos respondem com o aumento da expressão de HSPs quando submetidos a condições de estresse em relação a parasitos com virulência atenuada (Salotra et al., 1994), indicando o papel das HSPs na virulência da *Leishmania*.

Em 2003, foi demonstrado que geldanamicina inibe o crescimento das culturas de *Plasmodium falciparum* impedindo a mudança do estado de anel para trofozoíto, evidenciando o potencial terapêutico de inibidors da HSP90 contra malária (Banumathy et al., 2003). Também foi demonstrado que a geldanamicina inibe o crescimento de *P. falciparum in vitro*, apresentando IC_{50} similar a observada pela cloroquina, sendo eficaz contra cepas de parasitos susceptíveis e resistentes à cloroquina (Kumar et al., 2003). Os autores também demonstraram que o tratamento de eritrócitos infectados com doses de até 160 nM de geldanamicina foi capaz de eliminar todas as formas dos parasitos intracelulares (Kumar et al., 2003). A evidência mais forte do efeito microbicida do bloqueio de HSP90 em parasitos protozoários vem de ensaios *in vitro* realizados com HSP90 de *Plasmodium falciparum*. Os autores mostraram que a geldanamicina liga-se com maior afinidade a HSP90 de plasmódio (PfHSP90) do que à HSP90 humana (HuHSP90). Além disso, o mesmo trabalho evidencia que o tratamento com geldanamicina inibe três

vezes mais a atividade ATPásica da PfHPS90 do que a atividade ATPásica da HuHSP90 (Pallavi et al., 2010). Resultados similares foram observados com HSP90 de *Trypanosoma evansi*, reforçando a ideia de que HSP90 de protozoários é mais sensível à inibição do que a HSP90 humana (Pallavi et al., 2010).

Estudos *in vivo* em modelos de malária também forneceram resultados promissores. Nestes estudos, camundongos infectados com *P. berghei* e tratados com 50 mg/kg de 17-AAG por 4 dias apresentaram uma redução de 80% na parasitemia. Além disso, todos os animais controle não tratados morreram após 7 dias de infecção enquanto metade dos animais tratados com 17-AAG sobreviveram ao final do mesmo período (Pallavi et al., 2010). Outro trabalho *in vivo* utilizando o modelo murino de infecção por *P. yoelii* evidenciou que o tratamento com apenas duas doses de 17-AAG ou com o outro derivado da geldanamicina, o 17-PEG-Alkyn-GA, foi capaz de reduzir a parasitemia para níveis abaixo do limite de detecção acompanhada de 80% de sobrevivência dos animais 15 dias após a infecção (Mout et al., 2012). Parasitos da família Apicomplexa aumentam a expressão de HSP90 em bradizoítos de *T. gondii* quando essas células são submetidas ao estresse (Echeverria et al., 2005). Os autores mostraram que a localização intracelular de HSP90 em *T. gondii* difere a depender do estágio do parasito, se taquizoíto ou bradizoíto. Em taquizoítos a HSP90 é encontrada apenas no citosol e em bradizoitos encontra-se no citosol e núcleo (Echeverria et al., 2005). Além disso, a inibição da HSP90 por geldanamicina impede a diferenciação do *Toxoplasma*, sugerindo que a atividade e a localização sub-celular dessa proteína tem um papel central na conversão entre os estágios desse parasito (Echeverria et al., 2005). A

inibição da HSP90 também reduz a invasão e replicação do *Toxoplasma* no interior da célula hospedeira (Ahn et al., 2003).

A HSP90, além de ser importante para diferenciação e viabilidade intracelular de parasitos protozoários, parece exercer um papel importante na resistência ao antimônio. Parasitos de *L. donovani* resistentes ao antimônio apresentam expressão aumentada de HSP90, indicando que esta proteína tem papel na resistência ao tratamento quimioterápico por antimoniais (Matrangolo et al., 2013). Similarmente, parasitos isolados de pacientes e resistentes ao tratamento com antimônio apresentaram aumento da expressão de HSP90 (Vergnes et al., 2007). É possível que maior expressão da HSP90 proteja o parasito dos efeitos tóxicos do antimônio reduzindo a apoptose e aumentando a resistência ao estresse oxidativo (Vergnes et al., 2007).

HSP90 é uma proteína muito abundante em *Leishmania*, representando cerca de 2,8% do total das proteína citoplasmática desse parasito (Brandau et al., 1995). Além disso, o gene que codifica essa proteína está presente em 17 cópias organizadas em cadeia (Hubel et al., 1995). Os primeiros estudos funcionais com HSP90 de *Leishmania* surgiram em 2001 com a utilização dos inibidores geldanamicina e radicicol (Fig. 5). Esses estudos demonstraram que a inibição da HSP90 de promastigotas induz modificações sugestivas do processo de transformação de promastigota em amastigota, como a expressão de proteínas específicas de amastigotas a exemplo da HSP100 e proteínas da família A2, concomitantemente ao arredondamento celular e à diminuição do flagelo. Essas modificações mimetizam o efeito do aumento da temperatura e diminuição do pH,

condições similares às encontradas pelo parasito em compartimentos intracelulares da célula hospedeira. A geldanamicina, também foi capaz de inibir o crescimento do parasito, promovendo parada de crescimeno na fase G2 do ciclo celular (Wiesgigl e Clos, 2001). Os autores sugerem que a disponibilidade de HSP90 presente no parasito estaria relacionada com o desencadeamento do processo de amastigogênese. Em condições de estresse, como no interior do vacúolo parasitóforo, diferentes proteínas do parasito seriam estabilizadas pela HSP90, diminuindo a quantidade de HSP90 livre no interior do parasito. Esta diminuição da quantidade de HSP90 livre no citosol funcionaria como um sensor do ambiente externo possibilitando o parasito reconhecer mudanças ambientais e induzir mudanças de estágios de promastigota para amastigota. Nesse contexto, inibidores da HSP90 agiriam de forma similar, reduzindo a atividade da HSP90 e, consequentemente, levando a transição de promastigota para amastigota (Wiesgigl e Clos, 2001).

O aprofundamento do conhecimento sobre o funcionamento das chaperonas de parasitos protozoários e quais inibidores de HSP90 possuem potencial terapêutico irá acelerar o desenvolvimento e disponibilidade de inibidores mais específicos, capazes de combater com eficiência as diversas doenças causadas por esses parasitos.

2 JUSTIFICATIVA

A leishmaniose é uma doença grave, negligenciada e cerca de 350 milhões de pessoas correm risco de contrair essa doença. Os tratamentos atualmente utilizados contra leishmaniose vêm tendo seu uso limitado, principalmente devido ao aumento dos casos de resistência em regiões endêmicas (Sundar et al., 2000; Sundar, 2001) e aos graves efeitos colaterais observados (Chappuis et al., 2007). Os medicamentos utilizados contra as diferentes formas de leishmaniose têm baixos índices de eficácia, associadas a efeitos colaterais, assim como a longos períodos de tratamento. Esse conjunto de fatores promove desistência e interrupções do tratamento e, em consequência disso, aumento da probabilidade de desenvolvimento de resistência a esses fármacos (Chappuis et al., 2007; Croft e Olliaro, 2011).Esses fatores evidenciam a necessidade do desenvolvimento de novos fármacos ou estratégias terapêuticas para o controle das leishmanioses.

Nos últimos 15 anos, inibidores do HSP90 foram utilizados para compreender a função da HSP90 em parasitos protozoários. Os trabalhos produzidos nesse período relacionam a HSP90 ao controle do ciclo celular, adaptação às condições de estresse e virulência de parasitos. De forma similar ao observado em outros protozoários, existem evidencias que HSP90 de *Leishmania* também desempenha um papel importante no ciclo de vida desse parasito (Shonhai et al., 2011; Hombach e Clos, 2014).

Atualmente os inibidores da HSP90 são considerados como uma promessa no tratamento de diferentes tipos de neoplasias, uma vez que diversas oncoproteínas são proteínas cliente da HSP90. Assim, a estratégia utilizada para

inibir a atividade da HSP90 e, consequentemente, inibir a expressão de várias oncoproteínas com um único fármaco parece promissora. Desta forma, inibidores da HSP90, a exemplo do 17-AAG, foram utilizados em ensaios pré-clinicos e clínicos com resultados promissores contra certos tipos de neoplasias (Jhaveri et al., 2012). Isso significa que, após os ensaios *in vitro* e pré-clínicos, é possível que esses fármacos estejam rapidamente disponíveis para testes clínicos em pacientes com leishmaniose, uma vez que existem informações sobre toxicidade, farmacocinética e esquema terapêutico para doenças neoplásicas (Jhaveri et al., 2012; Jhaveri et al., 2014). Isso poderá facilitar e acelerar o possível uso desses inibidores em pacientes portadores de leishmaniose.

Apesar das informações obtidas sobre a HSP90 de *Leishmania* e da importância desta proteína para a viabilidade deste parasito, estudos ainda não haviam sido conduzidos com o intuito de avaliar a capacidade leishmanicida de inibidores da HSP90. No presente trabalho, avaliamos a atividade leishmanicida do 17-AAG e investigamos o mecanismo de morte induzido por este agente em promastigotas de *Leishmania*.

3 HIPÓTESE

A inibição da HSP90 pelo 17-AAG leva ao controle da infecção por *Leishmania*

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar o potencial leishmanicida do 17-AAG e investigar o mecanismo pelo qual este fármaco induz a morte do parasito.

4.2 OBJETIVOS ESPECÍFICOS

- Determinar o efeito anti-*Leishmania amazonensis* do 17-AAG sobre o crescimento de promastigotas e parasitos em macrófagos murinos infectados *in vitro*
- Avaliar se o tratamento com 17-AAG altera a produção de moléculas leishmanicidas por macrófagos infectados com *Leishmania* spp.
- Determinar o efeito anti-*Leishmania braziliensis* do 17-AAG sobre o crescimento de promastigotas e parasitos em macrófagos murinos infectados *in vitro.*
- Determinar o efeito anti-*Leishmania braziliensis* do 17-AAG em camundongos infectados com *Leishmania in vivo.*
- Identificar alterações morfológicas induzidas pelo tratamento com 17-AAG em parasitos

- Estudar a relação entre alterações na via autofágica e a morte secundária ao tratamento com 17-AAG

5 MANUSCRITOS

5.1 MANUSCRITO I

17-AAG Kills Intracellular *Leishmania amazonensis* while Reducing Inflammatory Responses in Infected Macrophages

17-AAG mata *Leishmania amazonensis* intracellular enquanto reduz a resposta inflamatória de macrófagos infectados

Resumo de resultados: Este trabalho demonstra, pela primeira vez, que um inibidor da HSP90, o 17-AAG, é capaz de matar *Leishmania amazonensis* tanto na forma promastigota quanto na forma amastigota intracelular. Essa morte ocorre por um processo independente da produção de moléculas leishmanicidas, a exemplo do NO e do O_2^- e de citocinas pró-inflamatórias como TNF-α ou MCP-1. Além disso, por microscopia eletrônica de transmissão, lançamos evidencias de que a morte do parasito pode estar relacionada ao processo autofágico.

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17-AAG Kills Intracellular *Leishmania amazonensis* while Reducing Inflammatory Responses in Infected Macrophages

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Abstract

Background: Leishmaniasis is a neglected endemic disease with a broad spectrum of clinical manifestations. Pentavalent antimonials have been the treatment of choice for the past 70 years and, due to the emergence of resistant cases, the efficacy of these drugs has come under scrutiny. Second-line drugs are less efficacious, cause a range of side effects and can be costly. The formulation of new generations of drugs, especially in developing countries, has become mandatory.

Methodology/Principal Findings: We investigated the anti-leishmanial effect of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), an HSP90 inhibitor, *in vitro*. This inhibitor is currently in clinical trials for cancer treatment; however, its effects against intracellular *Leishmania* remain untested. Macrophages infected with *L. amazonensis* were treated with 17-AAG (25–500 nM) and parasite load was quantified using optical microscopy. Parasite load declined in 17-AAG-treated macrophages in a dose- and time-dependent manner. Intracellular parasite death became irreversible after 4 h of treatment with 17-AAG, and occurred independent of nitric oxide (NO) and superoxide (O_2^-) production. Additionally, intracellular parasite viability was severely reduced after 48 h of treatment. Interestingly, treatment with 17-AAG reduced pro-inflammatory mediator production, including TNF-α, IL-6 and MCP-1, yet IL-12 remained unaffected. Electron microscopy revealed morphological alterations, such as double-membrane vacuoles and myelin figures at 24 and 48 h after 17-AAG treatment.

Conclusions/Significance: The HSP90 inhibitor, 17-AAG, possesses high potency under low dosage and reduces both pro-inflammatory and oxidative molecule production. Therefore, further studies are warranted to investigate this inhibitor's potential in the development of new generations of anti-leishmanials.

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Introduction

The leishmaniases are a complex of vector-borne diseases affecting 12 million people worldwide, caused by protozoans of the genus *Leishmania*, which are transmitted by the bite of infected sand flies. This complex of diseases manifests in two main clinical forms: visceral leishmaniasis, which can be fatal if left untreated, and tegumentary leishmaniasis, whose mucocutaneous and cutaneous forms induce lesions that are generally self-healing, but may leave scars or deformities. The worldwide prevalence and severity of leishmaniasis has led the World Health Organization (WHO) to consider it as one of the most serious infectious diseases [1]. Due to the complexity of host-parasite interaction, as well as contradictory results from vaccination models, the control of infection is mainly dependent on chemotherapeutic intervention [2].

A variety of treatment options currently exist. Pentavalent antimonials, such as Pentostam and Glucantime, have been used to treat leishmaniasis for the last 70 years [3,4]. However, decreasing effectiveness due to increased resistance [5] has limited their application. The antimicrobials Pentamidine and Amphotericin B represent the second line of treatment. Pentamidine has demonstrated efficacy against cutaneous leishmaniasis, however, its side effects include cardiotoxicity, renal failure and the development of diabetes at high dosage [6,7]. Liposomal Amphotericin B is the treatment of choice in the USA and Europe, as it has been proven effective against leishmaniasis with minimal side effects. However, its high cost limits the applicability for many patients in developing countries [8]. The antiprotozoal miltefosine has demonstrated efficacy against cutaneous leishmaniasis and visceral leishmaniasis in oral administration; however,

teratogenic effects restrict its widespread usage. In addition, this is a long-term use drug associated with treatment discontinuation and, as a consequence, development of resistance [4,9]. While the antibiotic paromomycin offers the same cure rate as amphotericin B, its poor oral absorption has led to the development of parenteral and topical formulations. However, local pain, ototoxicity and nephrotoxicity are the most frequently reported adverse events associated with this drug [4,9]. Taken together, the inadequacies of these current treatment options implicate the urgent development of new drugs to cure leishmaniasis with greater efficacy, minimal side effects and low cost.

Heat shock protein 90 (HSP90) is highly abundant in mammalian cells and known to be induced during stress responses. This protein is an ATP-dependent chaperone known to be involved in the stabilization, correct folding, and assembly of several client proteins, including kinases, transcription factors and proteins involved in cell-cycle control [10,11,12]. Some of these client proteins are known to be key oncogenic proteins that are upregulated in cancer cells [11,13,14]. Protozoan parasites also express HSP90 [15], which is known to play a crucial role in the stabilization of heat-labile proteins within these cells [16,17]. The modulation of HSP90 by specific inhibitors or by heat shock stress provokes profound modifications in parasite differentiation processes [18]. The use of Geldanamycin (GA), an HSP90-specific inhibitor, to treat erythrocytes infected with *Plasmodium* protozoa arrested intracellular parasite growth, blocked progression of protozoa from the ring stage to the trophozoite stage and resulted in the death of intra-erythrocyte parasites [19,20,21]. A previous study demonstrated HSP-90 inhibition using a GA analog, 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), which reduced *Plasmodium* sp growth in an *in vivo* mouse model of malaria [21]. These authors also demonstrated that 17-AAG inhibited *Trypanosoma evansi* growth *in vitro* and enhanced infected animal survival rates from 0% in the untreated control group to 60% in the 17-AAG-treated group [21]. In addition, the protozoan *Toxoplasma gondii,* when treated with GA, resulted in reduced parasite entry and intracellular growth in host cells [22]. In sum, these data suggest that HSP90 participates in parasite entry and influences survival in host cells [21,22].

In *Leishmania* spp., HSP90 represents 2.8% of the total cell protein content [23]. This protein plays a role in the differentiation process of the parasite from promastigote to amastigote form [18]. Indeed, inhibition of parasite HSP90 by heat shock stress or treatment with specific inhibitors, such as GA or radicicol (RD), induces the arrest of promastigote growth and the transformation of promastigotes into rounded amastigote-like forms, together with size reduction and flagellum loss [18]. Furthermore, the inhibition of HSP90 using GA was able to promote apoptosis-induced parasite death, when the promastigotes were incubated at 37°C pH 5.5, environmental conditions similar to those found in intracellular *Leishmania*-induced parasitophorous vacuoles [24]. Taken together, these data support the notion that HSP90 is a potential target for chemotherapeutic intervention for the control of parasitic diseases [18,25].

The less-toxic GA analog, 17-AAG, is currently in phase II clinical trials for the treatment of several cancers. This analog HSP-90 inhibitor shares many common features with GA [26], however 17-AAG binds to the HSP90 ATP pocket with greater affinity than GA, more efficiently impairing ATP hydrolysis, and, consequently, HSP90 chaperone activity. This effect ultimately promotes the proteasomal cleavage of HSP-90 client proteins more efficiently [27]. This study represents the first attempt to evaluate the effect of 17-AAG on *Leishmania amazonensis*-infected macrophages. The data presented herein demonstrate that when macrophages were treated with nanomolar concentrations of 17-AAG, the clearance of parasite infection *in vitro* was obtained.

Materials and Methods

Ethics Statement

The Animal Care Facility at CPqGM/FIOCRUZ provided male and female CBA and C57BL/6 mice. The animals were housed under specific pathogen-free conditions, fed commercially available rations and given water *ad libitum*. CBA and C57BL/6 mice were euthanized at 6 to 12 weeks of age. The animal husbandry and housing conditions, as well as the experimentation protocols at our facility, comply with the International Guiding Principles for Biomedical Research Involving Animals and have been approved by the CPqGM Institutional Review Board for Animal Experimentation.

Reagents

17-AAG was purchased from InvivoGen (San Diego, California, EUA). Dimethyl sulfoxide (DMSO) (SIGMA, St Louis, MO, USA) was used to prepare a 5 mM stock solution of 17-AAG, stored in aliquots at −20°C, until use. This solution was further diluted in cell culture medium to the desired concentrations at time of use. Other reagents used were: Schneider's insect medium (SIGMA), gentamycin (SIGMA), sodium bicarbonate (SIGMA), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (SIGMA), glutamine (SIGMA), lipopolysaccharide LPS (SIGMA), lucigenin (SIGMA), wortmannin (SIGMA), AlamarBlue® (Invitrogen, Carlsbad, CA, USA), amphotericin B (Fungizone, GIBCO, Carlsbad, CA, USA), fetal calf serum (FCS) (GIBCO), DMEM medium (GIBCO), heparin (ARISTON, Brazil), ciprofloxacin (CLARIS, India) and IFN-γ (R&D systems, Minneapolis, MN, USA).

Parasite Cultures

To avoid a loss of parasite infectivity, the *L. amazonensis* (MHOM/BR/87/BA125), *Leishmania major* (MHOM/RI/−/WR173) and *Leishmania infantum* (MCAN/BR/89/BA262) strains were maintained by serial passages in C57BL/6 mice. Following parasite isolation from the popliteal lymph nodes of infected mice, axenic parasites were maintained by serial passages in Schneider's insect medium supplemented with 10% FCS and 50 μg/mL of gentamycin. Axenic cultures were maintained until a maximum of seven passages.

Inhibiting Effect on Axenic Promastigotes

Axenic promastigotes of *L. amazonensis*, *L. major* or *L. infantum* at a concentration of 5×10^6 parasites/mL, cultivated in Schneider's complete medium, were treated with differing concentrations of the HSP90-inhibitor, 17-AAG (25, 125, 300 or 500 nM). The anti-leishmanial amphotericin B was used as positive control at 270 nM. At 48 h after treatment, the effect of these drugs on parasite growth was evaluated by directly counting live motile parasites using a Neubauer chamber.

Macrophage Cultures, Infection and Treatment

Briefly, thyoglycollate-elicited macrophages were obtained from the peritoneal cavities of CBA mice and cultivated according to modified protocols established by Gomes *et al.* (2003) [28]. All cells were recovered in heparinized saline (20 UI/mL) and centrifuged at 300 × g for 10 min at 4°C. Next, macrophage cultures were maintained at 2×10^5 cells/mL in DMEM complete medium (DMEM medium supplemented with 10% inactivated FCS, 2 g/L of sodium bicarbonate, 25 mM HEPES, 1 mM of glutamine and

0.2% of ciprofloxacin). Cells were cultivated at 37°C in 5% CO_2 for 4–16 h before infection. Next, the antileishmanial activity of 17-AAG against intracellular *L. amazonensis* parasites was assessed in elicited peritoneal macrophages infected with stationary-phase promastigotes (10:1 ratio). After 6 h of incubation with parasites, all macrophage groups were washed and submitted to a variety of treatment protocols: a) macrophages were treated with either 25, 125 or 500 nM of 17-AAG for an additional 6, 24 or 48 h to evaluate drug effects at early time after infection. Positive control cultures were treated with amphotericin B at a variety of concentrations: 250, 500, 1,000, 2,000, and 4,000 nM for 48 h; b) macrophages were incubated for 48 h, then treated with 25, 125 or 500 nM of 17-AAG for an additional 48 h to evaluate drug effects at later stages following infection; c) macrophages were treated with 500 nM of 17-AAG for 2, 4, 8, 12 or 24 h. At the end of each treatment period, all cells were washed and subsequently reincubated in 17-AAG-free medium to obtain a total incubation time of 72 h regardless of treatment period. After 6 h of infection, the control group was incubated in complete DMEM medium containing DMSO at the same concentration used in 17-AAG-treated cultures for 24 h, then washed and incubated for an additional 48 h (total incubation time = 72 h). After 72 h, all macrophage groups were washed and stained with hematoxylin/eosin (H&E) at the end of experimentation procedures. Parasite load was assessed using optical microscopy by quantifying both the percentage of infected cells and the number of parasites per infected cell. For each experimental condition, at least 400 cells were counted per coverslip in triplicate to sextuplicate.

Assessment of Macrophage Viability

Macrophage cultures were maintained at 2×10^5 cells/mL in DMEM complete medium and treated with 125, 500, 1,000, 3,000, 5,000 and 10,000 nM of 17-AAG or its diluent for 48 h. Next, cultures were washed twice and the medium was replaced by DMEM complete medium containing 10% AlamarBlue®. Cells were reincubated for 4 h at 37°C, in 5% CO_2. Next, reagent absorbance was measured at the wavelengths of 570 nm and 600 nm using a spectrophotometer (SPECTRA Max 340 PC). Ethanol-fixed cells were used as positive controls.

Assessment of Parasite Intracellular Viability

First, macrophage cultures were infected for 6 h and either wortmannin for 24 or 48 h, or left untreated. At the end of these incubation times, all cultures were washed and the culture medium was replaced with 1 mL of fresh Schneider's complete medium and the remaining intracellular parasites were incubated at 23°C. At the end of five days, the intracellular amastigotes that had transformed into motile extracellular promastigotes were quantified by determining the number of viable parasites using a Neubauer chamber [29].

Quantification of Superoxide Production by Macrophages

Drug effect on superoxide (O_2^-) production was assessed using: i) a chemiluminescence (CL) assay to monitor O_2^- production during phagocytosis, and ii) a fluorescence assay using an O_2^- specific hydroethidine probe to determine intracellular O_2^- production. Elicited peritoneal macrophages were plated at 10^6 parasites in 2 mL of complete DMEM medium. For both assays, macrophages were stimulated with LPS at 1 μg/mL in the presence or absence of 17-AAG at 500 nM, or with 500 nM of 17-AAG alone, for 20 h. O_2^- production by peritoneal inflammatory macrophages was measured during 20 min after the addition of *L. amazonensis* promastigotes at a 10:1 ratio at 37°C, using a lucigenin (25 μM) chemiluminescence (CL) method in a photon-counting device comprising a gallium arsenide photomultiplier tube (Hamamatsu R943) [30]. Chemiluminescence emissions from sample dishes, incubated at 37°C in a sealed chamber, were reflected and focused onto the photomultiplier tube. The emitted signal was fed directly to a frequency counter unit, and data were collected in units of photon counts per second [30]. For the fluorescent probe assay, hydroethidine at 5 μM was added according to manufacturer protocols (Invitrogen). Cell fluorescence was measured (FACSort; BD Biosciences) and expressed as mean fluorescence intensity (MFI) using a flow cytometer. In both assays, unstimulated macrophages infected with *L. amazonensis* promastigotes were used as negative controls.

Quantification of Cytokine and Nitric Oxide (NO) Production by Macrophages

Elicited peritoneal macrophages were plated at 10^6 cells/mL of complete DMEM medium. Macrophages were stimulated with IFN-γ (100 UI/mL) for 20 h. Next, macrophages were infected with *L. amazonensis* stationary phase promastigotes for 6 h. Then, macrophage cultures were washed to remove all non-internalized parasites, the DMEM cell medium was replaced and IFN-γ stimulation was reapplied together with 500 nM of 17-AAG. These cultures were incubated and cell supernatants were collected at 24 h or 48 h to determine mediator levels or NO production, respectively. Concentration of released mediators was determined using an inflammatory CBAKit (BD Biosciences, San Jose, CA, USA) in accordance with manufacturer protocols. NO production was assessed in cell supernatants by determining nitrite accumulation, using the Griess method [31].

Transmission Electron Microscopy

Macrophages were first infected with *L. amazonensis* for 6 h and treated with 500 nM of 17-AAG for 6, 12, 24 or 48 h. Control macrophages were similarly infected with *L. amazonensis*, but left untreated. Cells were then fixed in a solution containing 2.5% glutaraldehyde grade II, 2% formaldehyde and 2.5 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer adjusted to pH 7.2. Next, cells were post-fixed in the same buffer with 1% osmium tetroxide and 0.8% potassium ferricyanide, then dehydrated in an acetone series and embedded in Polybed resin. Thin sections were taken and stained with uranyl acetate and lead citrate. Observations were performed using a Zeiss 109 or Jeol 1230 transmission electron microscope.

Statistical and Data Analyses

Data are shown as an average of 3–6 independent experiments (mean ± SEM) performed in at least triplicate, or a representative experiment from a series of independent experiments performed in sextuplicate (mean ± SD). The number of experiments performed is indicated in each figure legend. Using GraphPad 5.0 software, normality testing (Kolmogorov-Smirnov) determined the use of a parametric method (Student's *t*-test, one-way ANOVA with Dunnett's Multiple Comparison Test and post-test for linear trend) or non-parametric method (Mann-Whitney test). Results were considered statistically significant if $p<0.05$.

The selective index (SI), based on a nonlinear regression analysis was calculated as follows: $SI = LC_{50}/IC_{50}$, where LC_{50} corresponds to the lethal concentration able to reduce macrophage viability by 50% and the IC_{50} value represents the inhibitory concentration needed to kill 50% of intracellular parasites [32].

Results

17-AAG Reduced Axenic Growth of *Leishmania* and Controlled Parasite Infection

In order to evaluate whether 17-AAG had a direct effect on parasite axenic growth, quantification under microscopy was performed. Amphotericin B was used as positive control against *Leishmania* parasites. The effect of both drugs on parasite growth was quantified at 48 h after treatment and the control exhibited complete inhibition at a concentration of 270 nM. All parasite groups treated with 17-AAG showed significantly reduced axenic growth, and a statistically significant lower number of parasites was measured following 48 h of treatment in comparison to untreated control promastigotes. Treatment with 25 nM of 17-AAG reduced parasite growth by 21.22±4.6% in relation to the control group ($p<0.0001$) (Fig. 1A). Concentrations of 125, 300 and 500 nM reduced parasite growth by 74.74±4.3, 90.57±1.5 and 93.89±1.4%, respectively ($p<0.0001$, Fig. 1A). The IC_{50} value calculated for *L. amazonensis* in axenic culture was 64.56±7 nM. 17-AAG was also able to inhibit the growth of other *Leishmania* species: *L. major* promastigotes at very low concentrations, with an IC_{50} value of 79.6±3.7 nM, and *L. infantum* promastigotes at low concentrations, with an IC_{50} value of 169.1±18.3 nM. These findings indicate that 17-AAG induced a static effect on promastigote axenic growth.

Next, the anti-leishmanial activity of 17-AAG against intracellular *L. amazonensis* parasites was evaluated by determining parasite load at time points representative of early stages of infection: 6, 24 and 48 h. Untreated cells were used as controls, and the percentage of infected cells ranged from 93.2±3.9% to 96.9±2.3% throughout all incubation periods (Fig. 1B). After 6 h of treatment, no statistically significant differences were observed in the percentage of infected cells between the 17-AAG-treated groups and control cells. However, after 24 h of treatment, the percentage of infected cells treated with 125 and 500 nM of 17-AAG fell significantly to 88.4±5.2% ($p<0.05$) and 83.4±3.8%, respectively, in comparison to 96.9±2.3% in the control group ($p<0.0001$, Fig. 1B). The percentage of infected cells decreased further in infected macrophages submitted to a 48-hour treatment incubation time, even at concentrations as low as 25 nM: to 66.1±14.6%, compared to 93.2±3.9% in untreated controls ($p<0.0001$, Fig. 1B). This reduction was much more pronounced in cells treated at concentrations of 125 nM and 500 nM to 13.9±7.3% and 3.0±2.9%, respectively ($p<0.0001$, Fig. 1B).

The number of parasites per untreated cell ranged from 4.4±0.3 to 5.0±0.6 (Fig. 1C). Similarly to what was observed in the percentage of infected cells, no significant differences were observed in the number of parasites when comparing the 17-AAG-treated groups to control cells after 6 h of treatment. However, after 24 h of treatment, a decrease in the number of parasites per macrophage was observed in cells treated with 125 and 500 nM of 17-AAG to 3.4±0.4 and 3.1±0.2, respectively (($p<0.0001$, Fig. 1C) in comparison to control cells: 4.9±1.2. After 48 hours of treatment, a pronounced reduction in the number of parasites per macrophage was observed at concentrations of 25, 125, and 500 nM, to 2.7±0.7, 1.5±0.2, and 1.1±0.1, respectively, in comparison to controls: 4.5±0.3 ($p<0.0001$, Fig. 1C). Amphotericin B was used as a positive control for intracellular parasite death at concentrations of 250, 500, 1,000, 2,000, and 4,000 nM. Even the lowest concentration resulted in complete clearance of intracellular parasites.

The authors also evaluated the toxicity effect of 17-AAG on host macrophages. When treated with 125, 500, 1,000 and 3,000 nM of 17-AAG for 48 h, macrophage viability remained unaltered, as assessed by an AlamarBlue® assay (Fig. 1A). However, treatment with 5,000 nM of 17-AAG reduced host cell viability by 28.9±9.2% ($p<0.05$, Fig. 1A) and treatment with 10,000 nM reduced host cell viability by 45.7±11.7% ($p<0.0001$, Fig. 1A). The resulting LC_{50} value was calculated as 10,830±1,700 nM of 17-AAG, while the IC_{50} value was determined to be 149±7 nM. Hence, the corresponding SI value was calculated as follows: SI = 10,830 nM/149 nM = 72.68.

In order to evaluate the treatment's effect at later stages of infection, 17-AAG was added to axenic cultures after 48 h of infection, the time needed for parasites to transform from promastigote to amastigote form [33]. After 48 h of treatment, all treated groups exhibited a decrease in parasite load. A significant reduction was observed in the percentage of infected cells in a dose-dependent manner: from 85.6±3.4% in controls, to 72.0±6.2% ($p<0.005$) at 25 nM; 42.9±9.4% ($p<0.0001$) at 125 nM; and 1.4±0.8% ($p<0.0001$) at 500 nM (Fig. 1D). A decrease was similarly observed when assessing the number of parasites per macrophage: from 3.0±0.1 in controls, to 2.0±0.1 ($p<0.0001$) at a concentration of 25 nM; to 1.5±0.1 ($p<0.0001$) at 125 nM; and to 1.1±0.2 ($p<0.0001$) at 500 nM (Fig. 1E). In sum, these data show that treatment with 17-AAG effectively reduced the percentage of *L. amazonensis*-infected macrophages, as well as the number of parasites per macrophage, in a time- and dose-dependent manner. Furthermore, this inhibitor was shown to successfully kill parasites that had already differentiated into intracellular amastigotes under infection conditions lasting as long as 96 hours.

The Irreversible Effect of 17-AAG on *Leishmania* Parasites

Next, the reversibility of the inhibitor's effect on parasite load was assessed. Infected macrophages were treated with 500 nM of 17-AAG for 2, 4, 8, 12 or 24 h. At the end of each treatment period, all cells were washed and subsequently reincubated in 17-AAG-free medium to obtain a total incubation time of 72 h, regardless of initial treatment period (Fig. 2). Control groups were incubated in 17-AAG-free medium for 24 h, then washed and incubated for an additional 48 h (total incubation time = 72 h). The cultures treated with 17-AAG for 2 h showed no reduction in either the percentage of infected cells, or in the number of parasites per infected macrophage (Fig. 2A–B). However, after 4 h of treatment an irreversible reduction in the percentage of infected cells was observed: from 87.2±4.2% in the control group to 67.5±6.7 ($p<0.0001$) (Fig. 2A). After 8 h of treatment, this irreversible effect was more pronounced in the percentage of infected cells: to 57.8±8.3 ($p<0.0001$), as well as in the number of parasites per macrophage: from 3.3±0.2 in the control group to 2.0±0.3 ($p<0.05$) (Fig. 3A–B). After 24 h of treatment, the percentage of infected cells fell dramatically to 9.7±3.1%, ($p<0.0001$) (Fig. 2A), as well as the number of parasites per infected cell to 1.1±0.06 ($p<0.0001$) (Fig. 2B). These data indicate that the inhibitory effect of 17-AAG on *Leishmania* parasites *in vitro* is irreversible at treatment times longer than 4 h.

17-AAG Reduced Intracellular Parasite Viability

Next, experiments were performed to evaluate whether 17-AAG was able to significantly reduce intracellular parasite viability. After 24 h of treatment, a significant reduction in the number of viable parasites was observed at concentrations of 25 nM: 2,408±306 parasites/mL, 125 nM: 329±127 parasites/mL, and 500 nM: 0.5±0.2 parasite/mL ($p<0.0001$), in comparison to the untreated control group: 6,535±1,024 parasites/mL ($p<0.0001$) (Fig. 3A). After 48 h, no viable parasites remained in the group treated with 500 nM (Fig. 3B). These findings

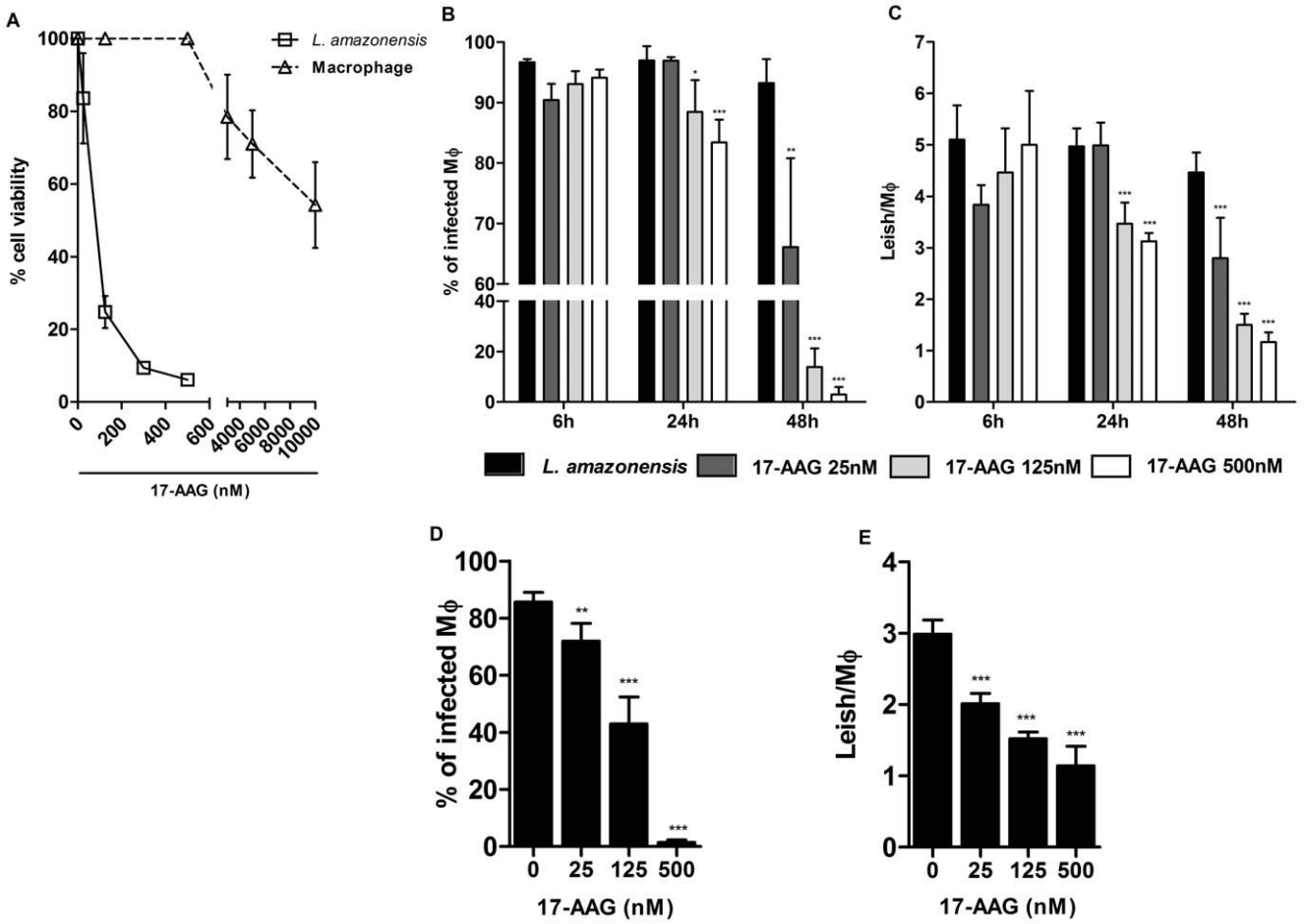

Figure 1. Inhibition of axenic growth of *Leishmania* and reduction in parasite load by 17-AAG. (**A**) Axenic promastigotes were exposed to several concentrations of 17-AAG (25, 125, 500 nM) for 48 h and the number of viable parasites was assessed as described in Materials and Methods. Data are presented as the percentage inhibition of parasite growth related to untreated controls ($4{,}754\times10^7$). Bars represent means ± SD of one representative out of two experiments performed in sextuplicate (one-way ANOVA, Dunnett's Multiple Comparison Test, ***$p<0.0001$, post-test for linear trend, $p<0.0001$). (**B, C**) Drug effects at early times after infection. Following 6 h of incubation with parasites, macrophage cultures were treated for 6, 24 and 48 h with specific concentrations of 17-AAG (25, 125, 500 nM); (**D, E**) Drug effects at later stages after infection. Following 6 h of incubation with parasites, macrophage cultures were reincubated for additional 48 h then submitted to treatment with specific concentrations of 17-AAG (25, 125, 500 nM). Bars represent means ± SD of one representative experiment out of two performed in sextuplicate (one-way ANOVA, Dunnett's Multiple Comparison Test, *$p<0.05$, **$p<0.001$, ***$p<0.0001$, post-test for linear trend, $p<0.0001$).
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demonstrate that the rate of parasite survival was considerably affected by treatment with 17-AAG in a time- and dose-dependent manner.

17-AAG Reduced the Oxidative Response of *Leishmania*-infected Macrophages

In order to investigate the mechanism by which 17-AAG induces intracellular *Leishmania* death, the authors measured the production of leishmanicidal molecules by macrophages, such as superoxide (O_2^-) and nitric oxide (NO). First, O_2^- production was assessed using a CL assay. Figure 4A depicts O_2^- production by stimulated macrophages following an infection time of 20 min. As expected, prior to infection, LPS-stimulated macrophages produced high levels of O_2^-, reaching a maximum value (Rmax) of 424 photons/sec. By contrast, macrophages treated with LPS and 500 nM of 17-AAG produced lower levels of O_2^-, reaching an Rmax of 116 photons/sec. When *L. amazonensis* promastigotes were added to LPS-stimulated cultures, a marked increase in O_2^- production was observed, reaching an Rmax of 1,217 photons/sec (Fig. 4A), 2.6 times higher than the Rmax from LPS-stimulated cell cultures treated with 17-AAG (471 photons/sec, $p=0.02$) (Fig. 4A). Macrophages treated with 17-AAG alone, as well as the untreated control cells, exhibited no alterations in O_2^- production, with an Rmax value of 261 photons/sec in macrophages treated with 17-AAG alone, and 200 photons/sec in untreated macrophages ($p>0.05$, Fig. 4A). In order to confirm that photon release was due to O_2^- production, the rapid decay values of photon emission, in response to the addition of S.O.D. (2.5 UI/mL) was verified at the end of the assay (Fig. 4A).

Intracellular O_2^- production was then determined at later infection times using a fluorescent assay with hydroethidine, an O_2^- specific fluorescent probe. Figure 4B depicts the mean fluorescence intensity (MFI) values emitted by macrophages, which are proportional to O_2^- production by these cells. Unstimulated macrophages treated with 17-AAG (500 nM) for 24 h (MFI = 41.7) produced O_2^- at similar levels to those produced by untreated control macrophages (MFI = 42.8) (Fig. 4B). However, when LPS-stimulated macrophages were

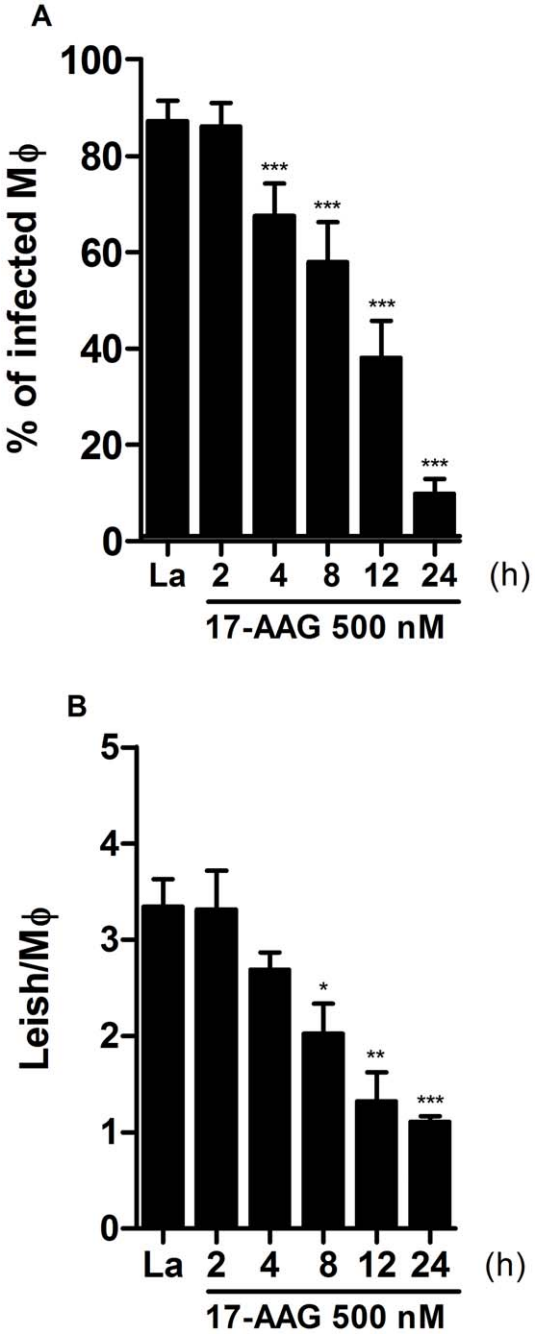

Figure 2. Irreversibility of treatment with 17-AAG on intracellular *Leishmania*. To assessment the reversibility of parasite growth inhibition by treatment with 17-AAG parasite load was determined by quantifying the percentage of infected macrophages (**A**) and the number of parasites per macrophage (**B**) as described in Materials and Methods. Bars represent means ± SD of one representative experiment out of two performed in sextuplicate (one-way ANOVA, ***$p<0.0001$, Dunnett's Multiple Comparison Test, *$p<0.05$, **$p<0.001$, ***$p<0.0001$, post-test for linear trend, $p<0.0001$).
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treated with 17-AAG (500 nM), O_2^- values fell from 106 to 85.3 MFI (Fig. 4B), a reduction that was not statistically significant.

Thereafter, the authors evaluated the effect of 17-AAG (500 nM) on NO production by detecting nitrite levels using the

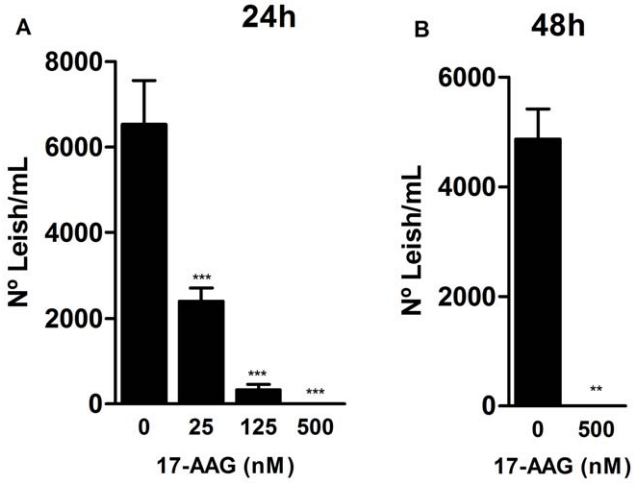

Figure 3. Reduction of parasite intracellular viability by 17-AAG. Treatment's effect on parasite viability was assessed after 24 h (**A**) and 48 h (**B**) of infection, as described in Materials and Methods. Bars represent means ± SD of one representative experiment out of two performed in sextuplicate (one-way ANOVA, ***$p<0.0001$, Dunnett's Multiple Comparison Test, *$p<0.05$, **$p<0.001$, ***$p<0.0001$, post-test for linear trend, $p<0.0001$; Mann Whitney test, **$p<0.001$).
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Griess reaction. Untreated control cells, both uninfected and infected with *L. amazonensis* promastigotes, produced NO at levels under the detection limit of the curve. Uninfected macrophages previously stimulated with IFN-γ released 34.7±3.8 μM of NO in culture medium. The addition of 17-AAG to these cell cultures reduced NO production to 17.7±2.2 μM ($p<0.0001$, Fig. 4C). Similarly, the addition of 17-AAG to infected cells previously stimulated with IFN-γ reduced NO production to levels significantly lower than those produced by infected macrophages previously stimulated with IFN-γ: from 47.3±1.8 μM to 19.7±1.6 μM ($p<0.0001$, Fig. 4C).

In sum, these findings suggest that O_2^- and NO play no role in the induction of intracellular *Leishmania* death triggered by 17-AAG.

17-AAG Modulates Cytokine Production by Infected Macrophages

Next, the authors evaluated the effects of 17-AAG (500 nM) on the production of pro-inflammatory cytokines by infected macrophages. Negative control macrophages, both non-activated and uninfected, i.e. not previously stimulated with IFN-γ, secreted the following cytokines in culture supernatants: 8.3±0.5 pg/mL of IL-6; 39.1±2.0 pg/mL of IL-10; 15.5±2.4 pg/mL of IL-12; 15.7±4.4 pg/mL of TNF-α, as well as the chemokine MCP-1:5.4±6.0 ng/mL (Fig 5A–E). Non-activated *L. amazonensis*-infected macrophages secreted mediators at levels similar to those produced by control cells: 49.1±3.7 pg/mL of IL-10; 17.7±1.7 pg/mL of IL-12; 17.9±2.2 pg/mL of TNF-α and 6.2±0.8 ng/mL of MCP-1, except for IL-6 which increased production to 16.7±3.0 pg/mL (Fig 5A–E). By contrast, non-activated infected macrophage cultures treated with 17-AAG exhibited a statistically significant reduction in the secretion of all produced cytokines: 8.3±0.3 pg/mL of IL-6; 40.8±2.0 pg/mL of IL-10; 11.4±0.7 pg/mL of TNF-α, as well as MCP-1:0.6±0.3 ng/mL (**$p<0.001$; ***$p<0.0001$), with the exception of IL-12, which was released at 15.5±2.4 pg/ml, values similar to those produced by untreated and infected macrophages ($p = 0.1$) (Fig. 5A–E).

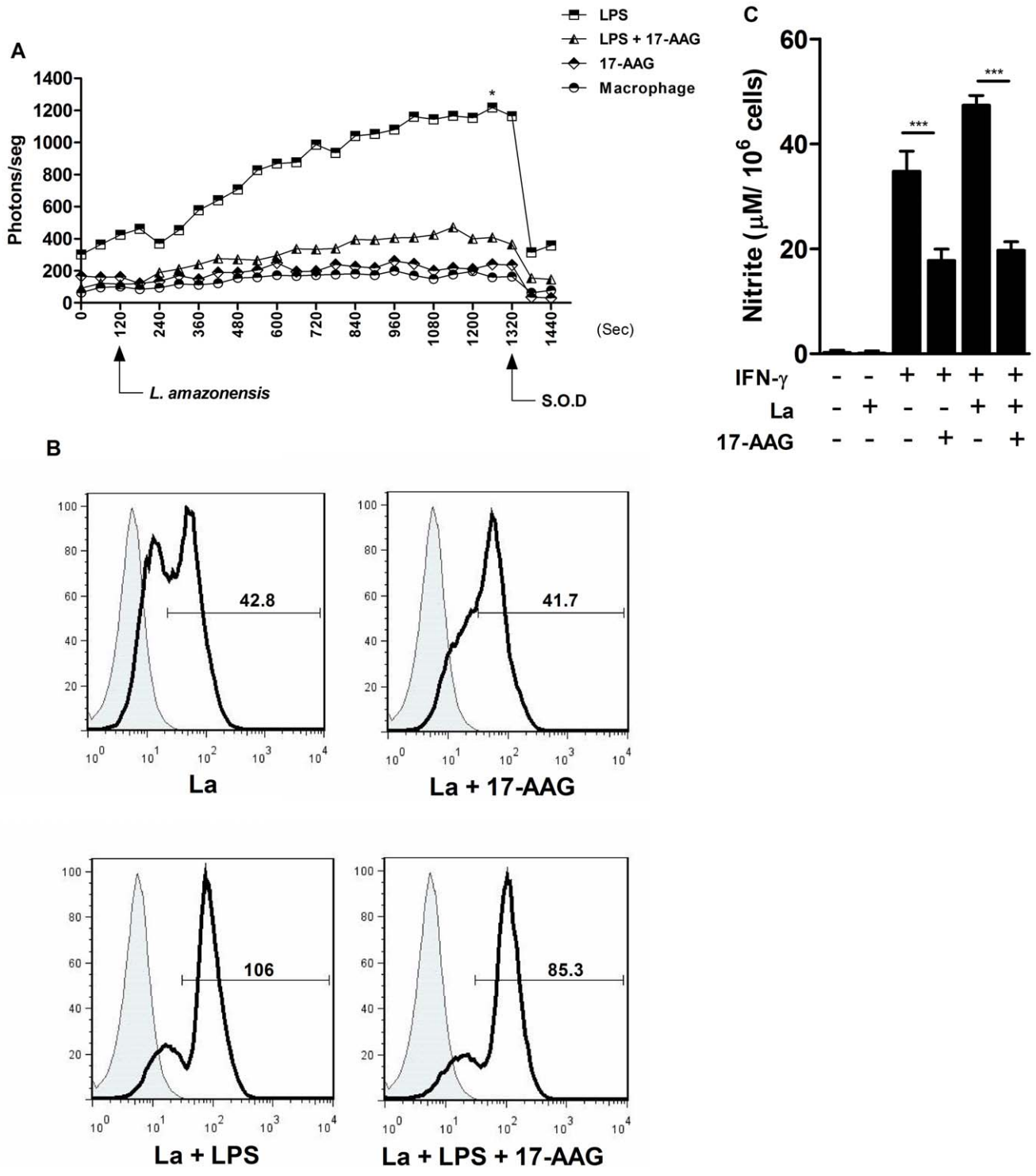

Figure 4. Reduced O_2^- and NO production in macrophage cultures treated with 17-AAG. (**A**) O_2^- production was measured at early stages of infection using a lucigenin-derived CL method. Points on the graph represent photon emissions per second by macrophage cultures 2 min prior to the addition of *L. amazonensis* promastigotes, as well as throughout the incubation period of 20 min. Data are derived from one representative experiment out of four performed in uniplicate (Mann Whitney test, p = 0.028); (**B**) Intracellular O_2^- production was assessed by determining cell fluorescence in the presence of hydroethidine (5 μM) and expressed as MFI using a flow cytometer. The histogram overlay depicts the MFI of hydroethidine-labeled cells (solid lines) in comparison to unlabeled control cells (shaded areas). Data are derived from one representative experiment out of three performed in uniplicate (Mann Whitney test, $p = 1$). (**C**) NO production was measured by detecting nitrite in the supernatants of 17-AAG-treated cells, as described in Materials and Methods. Bars represent NO production measurement expressed as means ± SD of one representative experiment out of four performed in triplicate or more (Student's *t* test, ***$p < 0.0001$).
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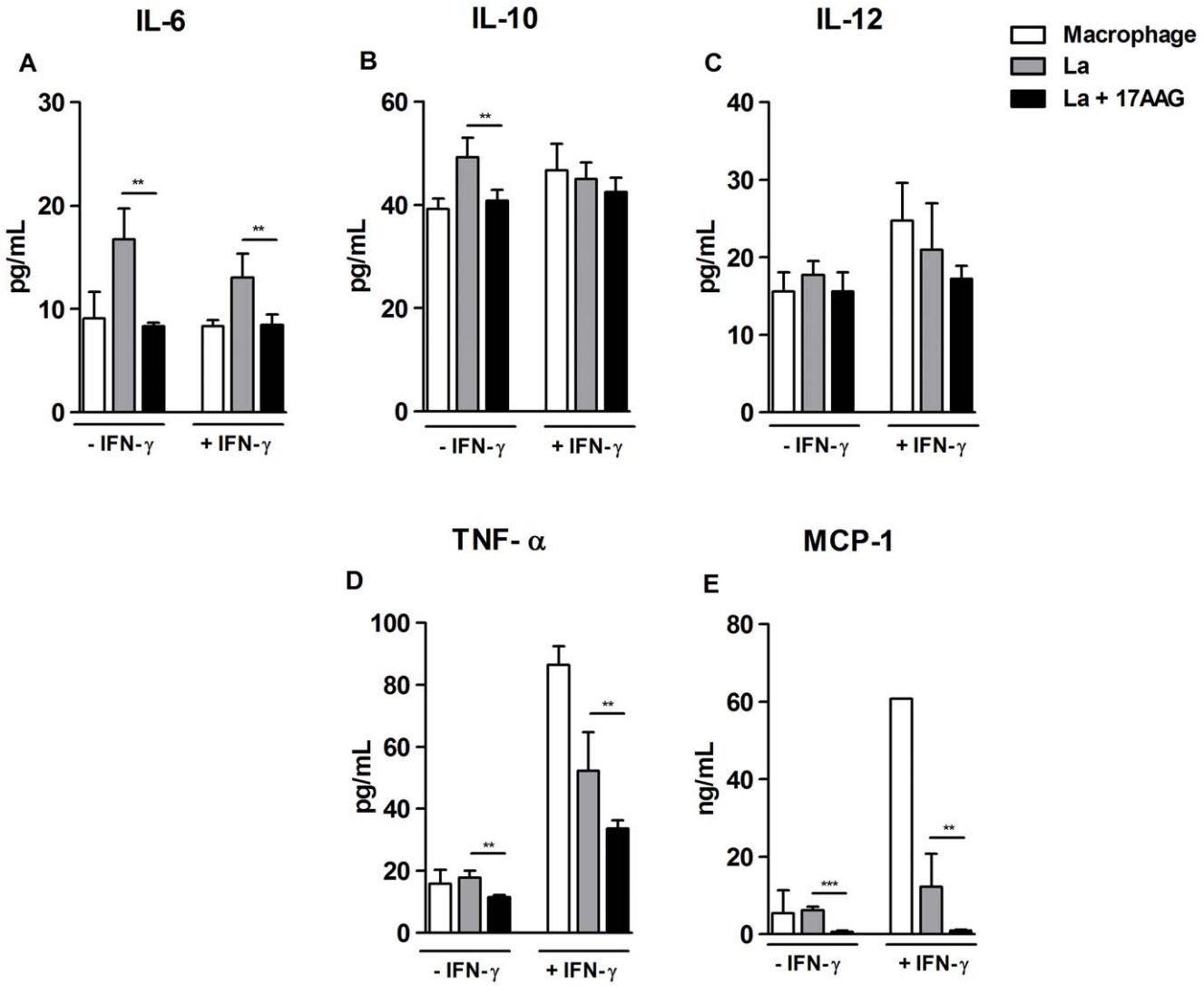

Figure 5. Modulation of mediator production by treatment with 17-AAG. Mediators released by 17-AAG-treated cells were measured in cell supernatants using an inflammatory CBAKit, as described in Materials and Methods: (**A**) IL-6; (**B**) IL-10; (**C**) IL-12; (**D**) TNF-α; (**E**) MCP-1. Bars represent means ± SD of a single experiment performed in sextuplicate (Student's *t*-test and Mann-Whitney, $^{**}p<0.001$, $^{***}p<0.0001$).
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Uninfected macrophages that were activated, i.e., stimulated with IFN-γ, secreted elevated levels of mediators: 24.7±4.8 pg/mL of IL-12; 86.4±5.9 pg/mL of TNF-α, and 60.7 ng/mL of MCP-1, yet IL-6 and IL-10 production remained unaltered. When 17-AAG was added to both activated and infected macrophages, the following statistically significant reductions in secretion levels were observed in comparison to untreated cells: IL-6, from 13.0±2.3 ($p=0.0012$) to 8.4±1; TNF-α, from 52.1±2.5 pg/mL ($p=0.0055$) to 33.6±2.7 pg/mL; and MCP-1, from 12.2±8.4 ng/mL ($p=0.0084$) to 0.9±0.2 ng/mL (Fig 5A,D–E). In sum, 17-AAG did not enhance macrophage production of either inflammatory cytokines or the MCP-1 chemokine, indicating that the mechanism involved in 17-AAG-induced parasite killing is unrelated to macrophage activation.

Treatment with 17-AAG Induced Ultrastructural Alterations in Intracellular Parasites

To further examine the mechanism involved in parasite killing, the authors used electron microscopy to investigate the presence of ultrastructural morphological alterations in intracellular parasites resulting from treatment with 17-AAG (500 nM). After 6 and 12 h of treatment, most intracellular parasites showed features similar to those found in control untreated macrophages (Fig. 6A). However, after 12 h of treatment, some intracellular parasites presented morphological ultrastructural alterations (Fig. 6). First, several small vesicles were observed in the cytoplasm of parasites, some even containing cytoplasmic material inside (Fig. 6B, arrows). It appeared as though the vacuoles had grown in size and that intravacuolar materials had been degraded (Fig. 6C–D). At 24 h after treatment, the intracellular parasites presented a large number of vesicles occupying most of the cytoplasm, even though preserved subpellicular microtubules, as well as intact nuclei and well-preserved mitochondria were observed (Fig. 6E–F). At 48 h, membrane-bounded structures were found inside parasitophorous vacuoles, probably corresponding to the remains of dead parasites (Fig 6G). By contrast, most of the untreated cells contained well-preserved round amastigotes within parasitophorous vacuoles (data not shown). At 12 and 24 h after treatment, several

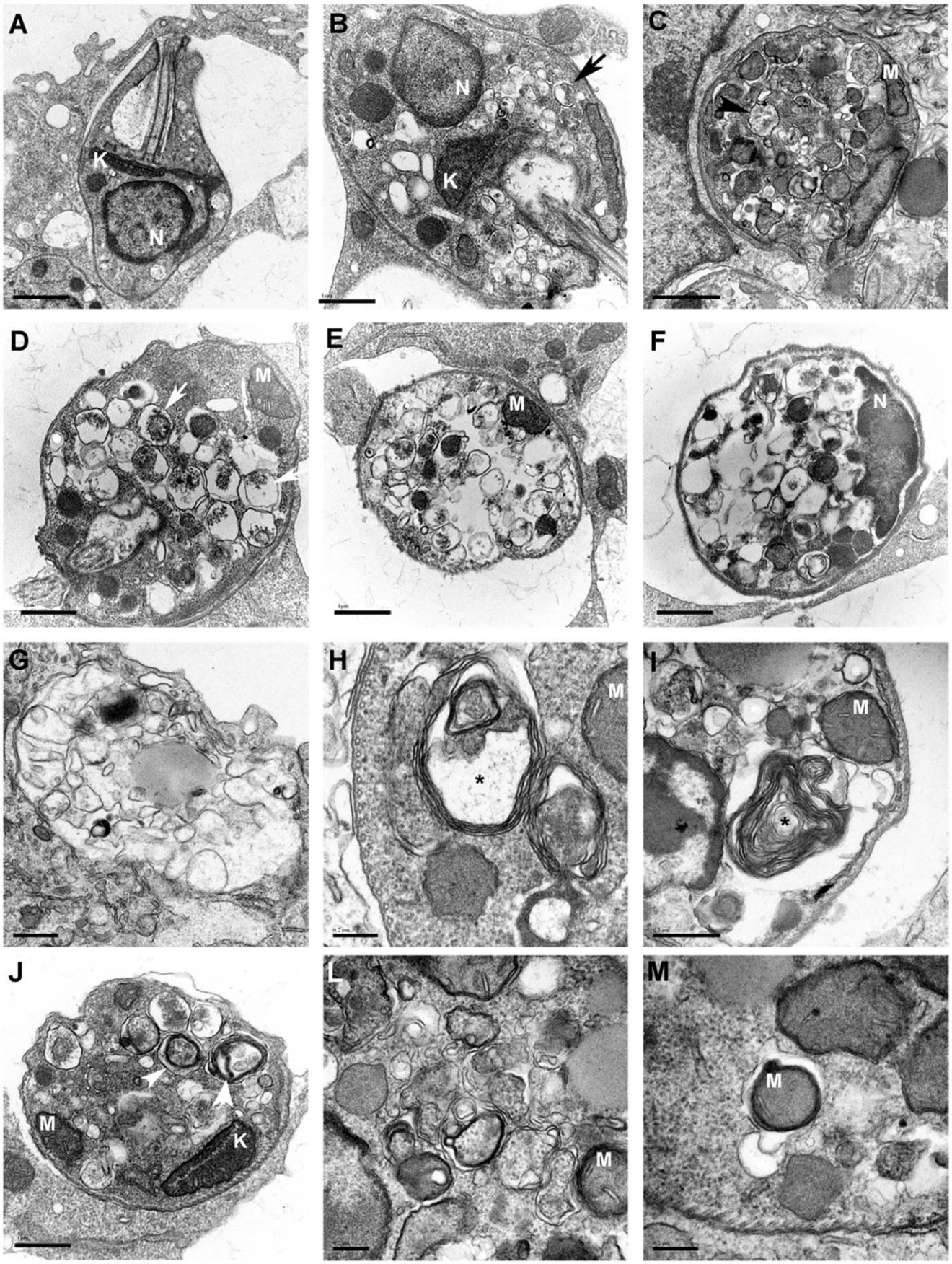
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Figure 6. Alterations suggestive of autophagy in intracellular parasites treated with 17-AAG. Transmission electron microscopy was used to investigate ultrastructural morphological alterations in intracellular parasites inside 17-AAG-treated macrophages. (**A**) Control infected macrophages. After 12 h of treatment, several morphological alterations were seen in intracellular parasites, including: (**B**) numerous small vesicles some containing cytoplasmic material inside **(black arrow)**, (**C**) vacuoles larger in size **(black arrow-head)**, (**D**) with intravacuolar materials degraded **(white arrow)**. After 24 h of treatment, the intracellular parasites presented a large number of vesicles occupying most of the cytoplasm containing well-preserved nuclei, mitochondria, and subpellicular microtubules (**E–F**). After 48 h of treatment, no preserved parasites inside cells were observed, yet empty vesicles, and membrane-bounded structures with an electron-density similar to parasite cytosol in parasitophorous vacuoles were seen (**G**). At 12 and 24 h after treatment, several alterations were also visible in parasite cytoplasm, including myelin figures **(*)** (**H–I**), vesicles with double-layered membranes **(white arrow-head)** (**J–M**), and portions of mitochondria inside membrane-bounded structures (**M**). The nuclei (**N**) and mitochondria (**M**) and kinetoplast (**K**) remained intact in all groups.
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alterations suggestive of autophagy were also visible in the cytoplasm of parasites, including myelin figures (Fig. 6H–I), vesicles with double-layered membranes (Fig. 6J–L) and portions of mitochondria inside membrane-bounded structures (Fig. 6M).

In order to assess the possibility of autophagy being involved in parasite death, infected macrophages were simultaneously treated with 17-AAG in addition to wortmannin (300 nM), which inhibits autophagy by a mechanism dependent on phosphatidylinositol 3-kinase (PI3K). After 48 h of infection, the number of viable parasites was assessed by replacing culture medium with Schneider's complete medium at the end of incubation period. In cells treated with 17-AAG (500 nM) and wortmannin (300 nM), the number of viable parasites (706.7±126.9 parasites/mL) was two times higher than in macrophages treated with 17-AAG alone (345±154.4 parasites/mL). Nonetheless, no statistically significant difference was observed between these groups ($p = 0.08$). We observed the same result when *Leishmania* were pre-treated with wortmannin and when different concentrations of 17-AAG were used (25 and 125 nM), suggesting that 17-AAG induced parasite death by way of a mechanism independent of PI3K.

Discussion

Protozoan parasites undergo drastic environmental changes throughout their development process, and evidence indicates that HSP90 is a fundamental molecular chaperone throughout the life cycle of a variety of protozoa, including *Trypanosoma cruzi*, *Toxoplasma gondii* and *Plasmodium falciparum* [34]. It follows, then, that HSP90 may be a candidate target for the chemotherapeutic treatment of parasitic diseases [11,18,21,25,34]. The present study represents the first attempt to investigate the effects of an HSP90 inhibitor, namely 17-AAG, on axenic cultures of *L. amazonensis* promastigotes, as well as on *L. amazonensis* parasites within infected macrophages.

We demonstrated that the number of axenic *L. amazonensis* promastigotes remained stable in a time- and dose-dependent manner after cultures were treated with 17-AAG (Fig. 1A). Additionally, the promastigotes treated with this inhibitor became rounded in shape and lost flagellum (data not shown). A previous study demonstrated that the growth of *L. donovani* was inhibited when axenic cultures were treated with GA, a 17-AAG analog, due to parasite growth arrest in the G2 cell cycle phase [18]. In this previous study, treatment with GA further induced the transformation of *L. donovani* promastigotes into rounded amastigote-like forms, alterations similar to those observed in promastigotes submitted to conditions mimicking the parasitophorous vacuole microenvironment, where these parasites transform and multiply in their amastigote forms. These findings, taken together with those of the present study, clearly demonstrate that HSP90 inhibitors directly induce a cytostatic effect on axenic cultures of *Leishmania* spp. promastigotes, as well as promote the transformation of promastigote forms into amastigote-like forms [18].

In the present study, treatment with 17-AAG drastically reduced the intracellular viability of *L. amazonensis* (Fig. 3). Similarly, previous studies demonstrated that a GA-induced inhibition of HSP90 blocked the growth of other protozoa, such as *P. falciparum*, within erythrocytes both *in vitro* [19,20,35] and in an *in vivo* experimental malaria model [21]. These findings reinforce the notion that parasite HSP90 is essential to intracellular parasite growth and complete differentiation process [36]. The present study detected an inhibitory effect from 17-AAG even when administered at low concentrations of 125 to 500 nM, under brief exposure periods and in late stages of infection. This inhibitory action, associated to a low SI value, suggest that 17-AAG may be appropriate for use in *in vivo* studies, which could prove advantageous.

Furthermore, there is evidence that 17-AAG is liposoluble and that this inhibitor accumulates intracellularly in cancer cells [37]. Although the present study did not seek to address the intracellular distribution of 17-AAG in infected cells, this inhibitor may have an enhanced intracellular effect, however this remains unverified. It is possible that 17-AAG accumulates in *Leishmania*-induced parasitophorous vacuoles, which are membrane-bound compartments with lysosomal characteristics, an acidic pH and abundant hydrolytic enzymes [38,39]. This environment is ideal for the destruction of parasites subjected to the action of both microbicidal molecules and antileishmanial drugs.

Alternatively, it is also possible that 17-AAG acts on host HSP90 instead of directly affecting parasite HSP90, thereby promoting the production of molecules known for microbicidal action, including O_2^- and NO [40], as well as pro-inflammatory mediators [40,41]. However, the present study demonstrated that infected macrophages treated with 17-AAG produced reduced levels of O_2^- and NO (Fig. 4), as well as pro-inflammatory mediators, including TNF-α, IL-6 and MCP-1 (Fig. 5). Additionally, previous studies demonstrated that HSP90 inhibitors similarly induced anti-oxidative [42,43,44,45] and anti-inflammatory responses in *in vivo* and *in vitro* infection models not involving *Leishmania* [46,47,48]. Taken together, these findings directly contradict the literature, which clearly evidences the production of proinflammatory mediators TNF-α, IL-1β, IL-6, IL-12, IFN-γ and MCP-1 being associated with the control of *Leishmania* spp infection [40,49,50,51]. To the best of our knowledge, this is the first report of treatment with an HSP-90 inhibitor being associated with low levels of pro-inflammatory molecule production by *Leishmania*-infected macrophages in association with the clearance of intracellular parasites. Therefore, the findings presented herein indicate that 17-AAG-induced *L. amazonensis* killing in the absence of host pro-inflammatory molecule production, thereby contradicting the known role these molecules play in control of parasite infection. This reinforces the notion that 17-AAG directly affects parasite survival while overriding the drug's potential toxicity with respect to host cells.

To investigate the mechanism of parasite killing at the ultrastructural level, the authors employed transmission electron

microscopy. Images revealed alterations in intracellular parasites suggestive of autophagy (Fig. 6). Autophagy is a naturally occurring process in *Leishmania* infection, which plays an important role in the differentiation process from promastigote to amastigote [52,53]. The present study showed that infected macrophages treated with wortmannin, a PI3K inhibitor known to disrupt the autophagic process [52], were unable to reverse induced parasite death following treatment with 17-AAG. The inability of wortmannin to revert the autophagic process may be related to the fact that 17-AAG irreversibly affected parasite viability at early stages of treatment. Furthermore, the lack of reversibility in the autophagic process may be related to the possibility that wortmannin is unable to access the PI3K target molecule present in parasites within parasitophorous vacuoles. Since the autophagic process leads to the release of energy and is triggered in cells subjected to stress conditions [54], it is possible that the parasite death observed herein did not result from the onset of autophagy, but rather that the autophagic-like process was related to *Leishmania*'s attempt to evade the action of 17-AAG. The absence of nuclear or mitochondrial changes in the parasites themselves leads us to suggest that apoptosis played no role in this particular mechanism of parasite death. Nonetheless, the underlying mechanism involved in 17-AAG-induced parasite death remains to be clarified.

In sum, the authors propose that the treatment of cutaneous leishmaniasis with 17-AAG may represent a promising therapeutic strategy for the elimination of intracellular *Leishmania*, as well as offering the added benefits of reduced levels of O_2^- and NO, in addition to lower proinflammatory mediator production. This treatment method would be especially advantageous in lesions typical of cutaneous and muco-cutaneous leishmaniasis, which are characterized by an intense inflammatory response, low parasite counts, elevated expressions of IFN-γ and TNF-α, as well as pronounced tissue damage as a result of intensified oxidative molecule production [55,56,57]. Although 17-AAG is currently in clinical trials for the treatment of neoplasia [58,59,60], this medication has yet to receive approval for human treatment despite many advances made in recent years with respect to its formulation in an effort to reduce toxicity and increase the threshold dosage [58]. It is our hope that these trials, in conjunction with the findings presented herein, will provide a basis for this inhibitor's eventual application in the treatment of patients suffering from leishmaniasis.

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Author Contributions

Conceived and designed the experiments: ALdOAP CESG PSTV. Performed the experiments: ALdOAP CESG CLV JGBL. Analyzed the data: ALdOAP VMB LARdF PSTV. Contributed reagents/materials/analysis tools: VMB PSTV. Wrote the paper: ALdOAP PSTV.

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5.2 MANUSCRITO II

Chemotherapeutic Potential of 17-AAG against cutaneous leishmaniasis caused by *Leishmania Viannia braziliensis*

Potencial quimioterapeutico do 17-AAG contra leishmaniose cutânea causada pela *Leishmania Viannia braziliensis*

Resumo de resultados: Nesse trabalho, foi avaliado o efeito anti-*Leishmania* do 17-AAG, investigando a capacidade desse inibidor de HSP90 em reduzir a infecção *in vitro* e *in vivo* de *Leishmania braziliensis*. Observamos que 17-AAG em concentrações nanomolar é capaz de eliminar completamente os parasitos intracelulares, confirmando que o 17-AAG é um fármaco com potencial quimioterápico contra diferentes espécies de *Leishmania*. Esse trabalho descreve o primeiro ensaio pré-clinico da literatura utilizando um inibidor da HSP90 contra leishmaniose. O tratamento com 17-AAG de camundongos infectados resultou em uma redução do tamanho da lesão cutânea e da carga parasitária no local da infecção.

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Chemotherapeutic Potential of 17-AAG against Cutaneous Leishmaniasis Caused by *Leishmania (Viannia) braziliensis*

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Abstract

Background: Leishmaniasis remains a worldwide public health problem. The limited therapeutic options, drug toxicity and reports of resistance, reinforce the need for the development of new treatment options. Previously, we showed that 17-(allylamino)-17-demethoxygeldanamycin (17-AAG), a Heat Shock Protein 90 (HSP90)-specific inhibitor, reduces *L. (L.) amazonensis* infection *in vitro*. Herein, we expand the current knowledge on the leishmanicidal activity of 17-AAG against cutaneous leishmaniasis, employing an experimental model of infection with *L. (V.) braziliensis*.

Methodology/Principal findings: Exposure of axenic *L. (V.) braziliensis* promastigotes to 17-AAG resulted in direct dose-dependent parasite killing. These results were extended to *L. (V.) braziliensis*-infected macrophages, an effect that was dissociated from the production of nitric oxide (NO), superoxide (O^{-2}) or inflammatory mediators such as TNF-α, IL-6 and MCP-1. The leishmanicidal effect was then demonstrated *in vivo*, employing BALB/c mice infected with *L. braziliensis*. In this model, 17-AAG treatment resulted in smaller skin lesions and parasite counts were also significantly reduced. Lastly, 17-AAG showed a similar effect to amphotericin B regarding the ability to reduce parasite viability.

Conclusion/Significance: 17-AAG effectively inhibited the growth of *L. braziliensis*, both *in vitro* and *in vivo*. Given the chronicity of *L. (V.) braziliensis* infection and its association with mucocutaneous leishmaniasis, 17-AAG can be envisaged as a new chemotherapeutic alternative for cutaneous Leishmaniasis.

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Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All data are included within the manuscript.

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Introduction

Leishmaniasis is a widespread group of parasitic diseases caused by protozoa of the genus *Leishmania*, that is transmitted by the bite of female sand flies. Currently, about 12 million people are at risk of leishmaniasis and there are an estimated 1.5–2 million new cases each year [1]. There are two main clinical manifestations: visceral leishmaniasis, affecting mainly the spleen and liver and cutaneous leishmaniasis, affecting the skin. CL caused by *Leishmania (V.) braziliensis* is particularly distinguished from other leishmaniasis by its chronicity, latency and tendency to metastasize in the human host [2]. In 1–5% of patients, mucocutaneous leishmaniasis may develop due to the ability of *L. (V.) braziliensis* to persist within lesion scars after spontaneous or chemotherapy-mediated healing and to its ability to metastasize to the nasal mucosal [3,4]. In this case, extensive tissue destruction is observed, resulting from the potent cell-mediated immune response triggered by parasite replication [5]. More rarely, parasite invasion of the bloodstream results in disseminated skin lesions [6]. Brazil along with nine other countries account for 70–75% of the global estimated CL incidence [7].

The drugs of first choice for leishmaniasis chemotherapy are Pentavalent Antimonials (Sb^{+5}) [8], which interfere with the oxidative metabolism of intracellular *Leishmania* [5,9,10]. These compounds are significantly toxic and have been associated with drug resistance [11,12]. Amphotericin B and Paramomycin, two other drugs available [13–15], also display limitations with regards to toxicity, cost and/or duration of treatment [16]. In the current scenario, the identification of new chemotherapeutic compounds is urgently needed, especially since vaccines against leishmaniasis are not yet available.

Heat Shock Proteins (HSPs) form complexes that act as chaperones, binding other proteins, denominated client proteins. These multimolecular complexes are involved in regulating

Author Summary

Antimony-containing compounds are the main drugs used to treat leishmaniasis but the severe associated side effects pose the need for alternative chemotherapeutic options. Herein, we evaluated the ability of 17-AAG (a Heat Shock Protein 90 inhibitor) to kill *Leishmania (Viannia) braziliensis* parasites, a species that causes both cutaneous and mucocutaneous Leishmaniasis in Brazil. Heat Shock Protein 90 (HSP90) is associated with important biological processes; inhibition of this molecule interferes with parasite survival and, hence, it can be exploited as a chemotherapeutic target. We show that exposure to 17-AAG induced killing of *L. brazilensis* parasites in both its extracellular and intracellular forms. This effect was not dependent on the activation of the host cell. More importantly, treatment of mice infected with *L. (V.) braziliensis* also modulated lesion development and decreased parasite growth at the infection site. Collectively, our results show that targeting HSP90 is a promising alternative for development of novel chemotherapeutic options for leishmaniasis.

protein folding, intracellular protein transport and repair or degradation of proteins partially denatured due to stress, for example [17,18]. Among the HSPs, HSP90 is one of the most abundant cellular chaperones and many of its client proteins are involved in cell signaling, proliferation and survival [19]. It is essential for oncogenic transformation and exploited by malignant cells to support cancer-associated kinases and transcription factors [20]. HSP90 also plays an important role in protozoans such as *Leishmania* and *Trypanosoma*, which critically rely on HSP90 for survival in alternating environments associated with their complex life cycles [21]. Therefore, HSP90-inhibitors become interesting candidates for leishmaniasis chemotherapy.

Treatment of *L. donovani* parasites with geldanamycin (GA), a HSP90-specific inhibitor, arrested promastigote growth and differentiation into amastigotes [22]. It also reduced gluthathione levels, increasing the production of reactive oxygen species (ROS) and promoting apoptosis [23]. Recently, we reported on the effects of 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) on *L. (L.) amazonensis* [24]. 17-AAG is a HSP90-specific inhibitor analogous to geldanamycin (GA) [25]. Macrophages infected with *L. (L.) amazonensis* and treated with a low dose of 17-AAG displayed significantly smaller parasite loads, an effect that was not mediated by activation of the macrophage inflammatory response [24].

In the present work, we expanded our previous observations to the effects of 17-AAG on *L. (V.) braziliensis*, the etiological agent of both cutaneous and mucocutaneous leishmaniasis in Brazil. Experiments were performed *in vitro* and *in vivo*, employing an experimental model [26]. 17-AAG was efficient at reducing *L. (V.) braziliensis* promastigote growth and macrophage infection. More importantly, 17-AAG was equally efficient *in vivo*, highlighting its potential as a novel chemotherapy agent against CL caused by *L. (V.) braziliensis*.

Methods

Ethics statement

Female BALB/c mice, 6–8 weeks of age, were obtained from CPqGM/FIOCRUZ animal facility where they were maintained under pathogen-free conditions. All animal work was conducted according to the Guidelines for Animal Experimentation of the Colégio Brasileiro de Experimentação Animal and of the Conselho Nacional de Controle de Experimentação Animal. The local Ethics Committee on Animal Care and Utilization (CEUA) approved all procedures involving animals (CEUA-L001/12-CPqGM/FIOCRUZ).

17-AAG and amphotericin B

17-AAG (17-(allylamino)-17-demethoxygeldanamycin) (Invivogen) was dissolved in Dimethyl sulfoxide (DMSO) (SIGMA) to a 5 mM stock solution, stored at -20°C in aliquots. For in vitro use, the stock solution was diluted in cell culture medium to the desired concentration at the time of use. For *in vivo* treatments, a stock solution was prepared at 100 mg/ml and diluted to 20 mg/kg at the time of use. Amphotericin B (Fungizone, Life Technologies) was dissolved in DMEM medium to a 250 ug/ml stock solution. The stock solution was diluted in cell culture medium to the desired concentration at the time of use.

Parasite culture

L. (V.) braziliensis (MHOM/BR/01/BA788) [26] was cultured at 26°C in Schneider's insect medium (Invitrogen) supplemented with 10% inactive Fetal Bovine Serum (FBS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin (all from Invitrogen).

L. (V.) braziliensis promastigotes viability assay

Axenic *L. (V.) braziliensis* promastigotes (1×10^6 parasites/ml), cultivated in supplemented Schneider medium, were treated with increasing concentrations of 17-AAG (25, 75, 125, 250, 500 or 625 nM). After 48 h, parasite viability was evaluated by direct counting of live motile parasites using a Neubauer chamber. In some experiments, promastigotes were treated with the half maximal inhibitory concentration (IC_{50}) (65 nM). After 48 h, promastigotes were washed three times with PBS and were further cultured for 24 and 48 h in supplemented Schneider medium, devoid of 17-AAG. The number of viable promastigotes was determined by direct counting.

Macrophage infection with *L. (V.) braziliensis* and treatment with 17-AAG

BALB/c mice were injected i.p. with 3% thioglycolate. Five days after injection, peritoneal lavage was performed using 8 ml DMEM medium supplemented with 10% Fetal Calf Serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (all from Invitrogen). To obtain monolayers, cells (6×10^5 cells/ml) were place into glass coverslips within the wells of a 24-well plate and were left to adhere for 2 h, at 37°C and 5% CO_2. Non-adherent cells were removed by gentle and extensive washing with PBS; purity was routinely above 99%. Remaining cells (3×10^5 cells/ml) received 3×10^6 cells/ml of stationary-phase *L. (V.) braziliensis* promastigotes and were incubated at 37°C in supplemented DMEM medium. After 24 h of infection, glass coverslips containing infected macrophages were washed to remove non-internalized parasites and cells were treated with different concentrations of 17-AAG (25, 100, 250 and 500 nM) for 12–72 h. Control groups were incubated in supplemented DMEM medium containing DMSO only. Glass coverslips were washed and stained with H&E and the intracellular amastigotes were counted by light microscopy. The results are shown as the percentage of infected cells and the number of intracellular amastigotes was counted in 400 macrophages. Cultures were performed in quintuplicate. Alternatively, infected macrophages were washed extensively and the medium was replaced with

0.5 ml of supplemented Schneider medium, devoid of 17-AAG. Cells were cultured at 26°C for an additional 5 days and the number of viable parasites was determined by direct counting. In some experiments, infected macrophages were treated with the half maximal inhibitory concentration (IC_{50}) (220 nM) of 17-AAG or with amphotericin B (0.25 μg/ml; 0.27 μM) for 24 h. Parasite viability was determined by direct counting.

Viability of 17-AAG-treated murine macrophages

Macrophages (2×10^5 cells/ml), obtained as above, were treated increasing concentrations of 17-AAG (39–20,000 nM) or with DMSO for 72 h. Next, cultures were washed twice cells were incubated with supplemented DMEM containing 10% Alamar-Blue (Invitrogen). Cells were incubated for another 4 h and reagent absorbance was measured at the wavelengths of 570 nm and 600 nm using a spectrophotometer (SPECTRA Max 340 PC). Ethanol-fixed cells were used as positive controls.

Detection of NO, reactive oxygen species, cytokines and chemokines

Macrophages (3×10^6 cells/ml), obtained as above, were stimulated with IFN-γ (100 UI/ml) (Sigma) and were infected with *L. (V.) braziliensis* (3×10^7 cells/ml) for 24 h. Macrophage cultures were then washed to remove non-internalized parasites and fresh culture medium containing IFN-γ and 220 nM of 17-AAG was added. Cultures supernatants were collected 48 h later. Griess reaction was used to measure nitric oxide (NO) production by determining concentration of its stable reaction product nitrite (NO_2^-) [27]. Superoxide (SO) production was determined by adding hydroxylamine (Sigma) (0.5 mM) [28,29] to infected macrophages. Hydroxylamine converts superoxide into nitrite, which is then be quantitated by the Griess reaction, as described above. Background levels of nitrite generated by the release of NO were determined in parallel, without the addition of hydroxylamine. Production of TNF-α, IL-6, IL-10 and CCL2/MCP-1 was evaluated using an inflammatory Cytometric Bead Array (BD Biosciences) following the manufacturer's instructions. Data were acquired and analyzed using a FACSort flow cytometer and FCAP Array (V.3.0) (BD Biosciences).

Intradermal infection with *L. (V.) braziliensis and in vivo* treatment with 17-AAG

BALB/c mice were inoculated with stationary-phase *L. (V.) braziliensis* promastigotes (10^5 parasites in 10 μl of saline) in the left ear dermis using a 27.5-gauge needle. Four weeks post-infection, mice (n = 10) were treated 3 times/wk for 3 weeks with 17-AAG (20 mg/kg of 17-AAG diluted in DMSO i.p.). The control group (n = 10) received i.p. injections of DMSO in the same concentrations used in 17-AAG treated animals (n = 10). Lesion size was monitored weekly for 10 weeks using a digital caliper (Thomas Scientific). Parasite load was determined using a quantitative limiting-dilution assay as described elsewhere [26]. Briefly, infected ears and lymph nodes draining the infection site were aseptically excised six weeks post-infection and homogenized in Schneider medium. Homogenates were serially diluted in supplemented Schneider complete and seeded into 96-well plates. The number of viable parasites was determined from the highest dilution at which the promastigotes could be grown after up to 2 weeks of incubation at 26°C.

Statistical analysis

The half maximal inhibitory concentration (IC_{50}) of 17-AAG on *L. braziliensis* promastigotes and on intracellular *L. braziliensis* amastigotes were determined from sigmoidal regression of the concentration-responses curves, respectively, using Prism (GraphPad Prism V. 6.0). The selectivity index of 17-AAG was calculated as the ratio between the CC_{50} for murine macrophages and the IC_{50} for intracellular *L. braziliensis* amastigotes.

Data are presented as the mean ± standard error of the mean. Kolmogorov-Smirnov was used for normality analysis. Parametric (One-way ANOVA followed by Dunnett's Multiple Comparison Test or post-test for linear trend or by Bonferroni) or non-parametric analysis (Mann-Whitney) tests were also performed using Prism (GraphPad software, V. 6.0) To evaluate disease burden in mice, ear thickness of mice treated with 17-AAG or DMSO was recorded weekly for each individual mouse. The course of disease for 17-AAG-treated and control mice was plotted individually, and the area under each resulting curve was calculated using a non-parametric test (Mann-Whitney). p-values ≤ 0.05 were considered significant.

Results

Exposure to 17-AAG reduces the viability of *L. (V.) braziliensis* promastigotes

Initially, we investigated the effects of 17-AAG on axenic *L. (V.) braziliensis* promastigotes. Parasites were incubated with increasing concentrations of 17-AAG for 48 h and viability was quantified by direct counting. All 17-AAG-treated cultures showed a significantly lower number of parasites in comparison to the control, treated with vehicle (DMSO) alone (Figure 1A) (One-way ANOVA, $p<0.001$). After 48 h of treatment, 17-AAG (25 nM) reduced parasite viability by 13% (Figure 1A) (compared to DMSO-treated cultures) and increasing concentrations of 17-AAG (75–625 nM), maximized killing effects. At the highest concentration tested (625 nM) parasite viability was reduced by 98% when compared to DMSO-treated cultures (Figure 1A). These results also indicate that parasite viability was reduced in a dose-dependent effect (One-way ANOVA, $p<0.001$ followed by test for linear trend $p<0.001$). The DMSO concentration used was not toxic as parasite viability was similar in cultures left untreated (Lb) or treated with DMSO only (Figure 1A). Based on these results, IC_{50}, after 48 h of 17-AAG treatment, was established at 65 nM (Figure S1). To evaluate whether the effect of 17-AAG on *L. (V.) braziliensis* promastigotes was reversible, parasites were treated with 17-AAG (65 nM) for 48 h, washed and subsequently re-incubated in 17-AAG-free medium, for an additional 24 and 48 h. Parasite numbers were significantly reduced ($p<0.01$) in cultures kept for both 24 h (Figure 1B) and 48 h (Figure 1C). These results show that the effect of 17-AAG on *L. (V.) braziliensis* promastigotes is irreversible.

17-AAG reduces parasite load in *L. (V.) braziliensis*-infected macrophages

Next, we investigated the effect of 17-AAG on intracellular *L. (V.) braziliensis* amastigotes. BALB/c macrophages were infected with *L. (V.) braziliensis* and cells were treated with a range of 17-AAG concentrations (25–500 nM) for 12–72 h. At each time point, cells were fixed and the parasite load was assessed by light microscopy. At the initial time points (12 and 24 h), we did not detect significant alterations in treated cultures versus control cultures (DMSO-treated) (Figure 2A and B). After 48 h, 17-AAG (25 nM) reduced the infection rate to 85% (Figure 2A) and intracellular amastigotes were reduced to 82% (compared to the percentages obtained in DMSO-treated cultures) (Figure 2B). With increasing concentrations of 17-AAG (100–500 nM), these effects became more pronounced and, at 500 nM, the percentage of infected cells was reduced to 63% (Figure 2A) and of

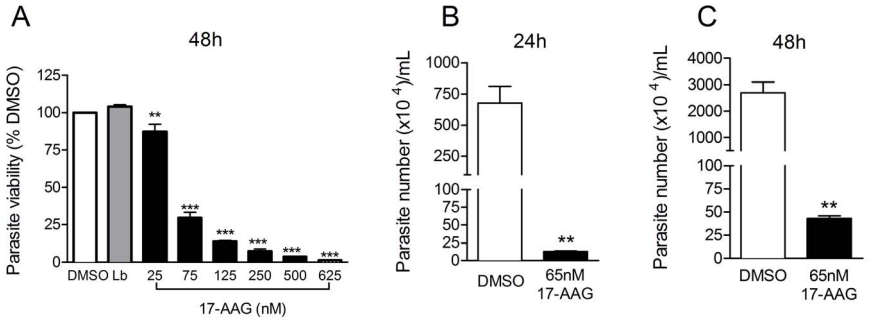

Figure 1. 17-AAG induces killing of *Leishmania (V.) braziliensis* promastigotes in a dose-dependent and irreversible manner. *L. (V.) braziliensis* promastigotes were exposed to increasing concentrations of 17-AAG, to vehicle alone (DMSO) or were left unexposed (Lb) for 48 h. (A) The number of viable parasites was evaluated by direct counting. *L.(V) braziliensis* promastigotes were treated with 65 nM (IC_{50}) of 17-AAG for 24 h (B) and (C) 48 h. After washing, promastigotes were cultured for additional 48 h and the number of viable parasites was evaluated. Data, shown as mean ±SEM, are from one of two independent repeats (**$p<0.01$ and ***$p<0.001$).
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intracellular amastigotes to 43% (Figure 2B) (One-way ANOVA, $p<0.001$) (again compared to the percentages obtained in DMSO-treated cultures). After 72 h of treatment, these effects were maximal: 500 nM of 17-AAG decreased the infection rate to 20% (Figure 2A) whereas intracellular amastigotes were reduced to 11% (Figure 2B). The absolute percentages of infection, following treatment with different concentrations of17-AAG and the absolute numbers of amastigotes/100 cells over time are shown in Figures S2A and S2B, respectively. As with promastigotes (Figure 1A), effects observed with 17-AAG on intracellular macrophages were dose-dependent (One-way ANOVA, $p<0.001$ followed by test for linear trend $p<0.001$). IC_{50}, after 72 h of 17-AAG treatment, was determined as 220 nM (Figure S3); additionally, 17-AAG employed at different concentrations (125, 220 and 500 nM) did not compromise macrophage viability as assayed by MTT (Figure S4). Cytotoxicity against murine macrophages was determined upon treatment of non-infected macrophage cultures with 17-AAG with a calculated CC_{50} of 3.6 nM. The selectivity index of 17-AAG was established at 16.6.

17-AAG reduces the viability of intracellular *L. (V.) braziliensis* amastigotes

Although 17-AAG treatment of *L. (V.) braziliensis*-infected macrophages for 24 h did not significantly modify the infection rate (Figure 2), we asked whether it would alter amastigote viability. Cells were infected and treated with a range of 17-AAG concentrations (25–500 nM). Intracellular parasite survival was determined following the replacement of DMEM for Schneider culture medium and direct counting of surviving *L. (V.) braziliensis*. Five days after medium replacement, treatment with increasing concentrations of 17-AAG for 24 h significantly reduced the number of viable *L. (V.) braziliensis* parasites (Figure 3A) (One-way ANOVA, $p<0.001$), indicating once more a dose-dependent effect (test for linear trend $p<0.001$). This effect was also time-dependent as exposure to 220 nM (IC_{50}) of 17-AAG for longer periods (48 and 72 h) also significantly decreased the number of *L. (V.) braziliensis* promastigotes (Figure 3B). Therefore, exposure of infected macrophages to 17-AAG negatively impacted on the survival of *L. (V.) braziliensis*.

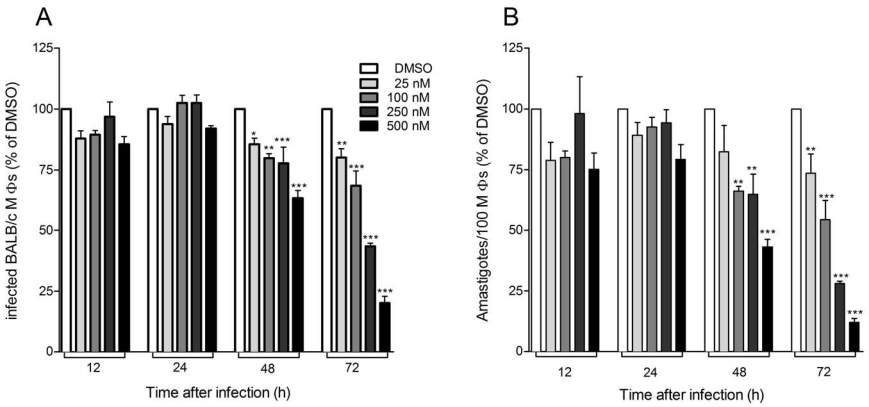

Figure 2. Treatment with 17-AAG controls *L. (V.) braziliensis* replication inside macrophages. *L. (V.) braziliensis*- infected macrophages were treated with increasing concentrations of 17-AAG or with vehicle alone (DMSO). After 12–72 h, glass coverslips were stained with H&E and assessed for the percentage of infected macrophages (A) and the number of amastigotes per 100 macrophages (B) by light microscopy. Data, shown as mean ±SEM, are shown as the percentage of DMSO -treated cultures, from one of three independent repeats (*$p<0.05$; **$p<0.01$ and ***$p<0.001$).
doi:10.1371/journal.pntd.0003275.g002

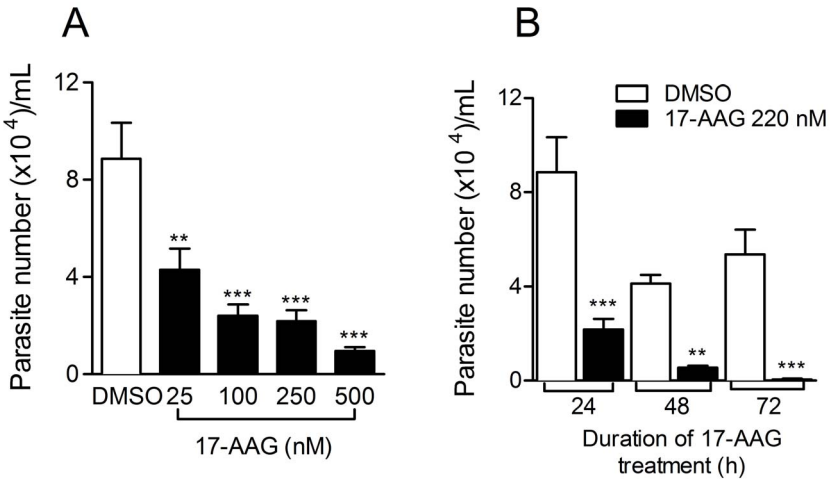

Figure 3. Treatment with 17-AAG reduces intracellular *L. (V.) braziliensis* viability. *L. (V.) braziliensis*- infected macrophages were treated for 24 h with increasing concentrations of 17-AAG or with vehicle alone (DMSO). The number of viable parasites was evaluated by further culture (5 days) in Schneider medium, free of 17-AAG (A). Infected macrophages were treated with 220 nM (IC_{50}) of 17-AAG for 24–72 h. The number of viable parasites was evaluated by further culture for five days in Schneider medium, free of 17-AAG (B). Data, shown as mean ±SEM, are from one of two independent repeats (**$p<0.01$ and *** $p<0.001$).
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17-AAG down-modulates the production of inflammatory mediators by *L. (V.) braziliensis* -infected macrophages

Macrophage activation and production of nitric oxide and superoxide are key steps towards elimination of intracellular *Leishmania* [30]. In macrophages infected with *L. (V.) braziliensis* and treated with 220 nM 17-AAG (IC_{50}), production of nitric oxide (Figure 4A) and superoxide (Figure 4B) were lower compared to cells exposed to DMSO. 17-AAG-treatment also down-modulated the production of TNF-α (Figure 4C), IL-6 (Figure 4D) and CCL2/MCP-1 (Figure 4E) by *L. (V.) braziliensis*-infected macrophages.

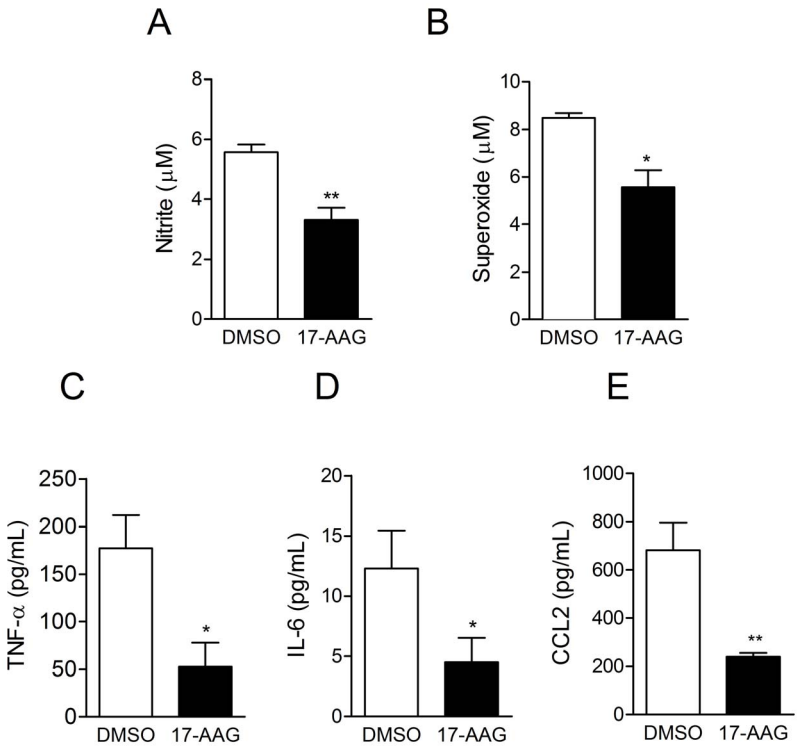

Figure 4. Treatment with 17-AAG down regulates ROS and cytokine production in *L. (V.) braziliensis-infected* cells. *L. (V.) braziliensis*-infected macrophages were treated with 17-AAG (220nM) + IFN-ɣ. After 48h, supernatants were assayed for nitrite production (A) and for presence of (B) superoxide, following addition of hydroxylamine. The presence of secreted (C) TNF-α, (D) IL-6 and (E) CCL2 was determined in culture supernatants by Cytometric Bead Array, after 24 h of treatment. Data, shown as mean ± SEM, are from one of two independent repeats (**$p<0.01$; *$p<0.05$).
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Therefore, the leishmanicidal effect of 17-AAG is uncoupled from the production of microbicidal molecules and from the production of pro-inflammatory cytokines.

In vivo control of CL caused by L. (V.) braziliensis

Next, we tested the *in vivo* effect of 17-AAG against CL caused by *(V.) L. braziliensis*. These experiments were performed in an mouse model that reproduces aspects of the natural infection such as the presence of an ulcerated lesion, parasite dissemination to lymphoid areas and the development of a Th-1 type immune response [26]. BALB/c mice were inoculated with *L. (V.) braziliensis* in the ear dermis and lesion development was monitored weekly. Four weeks after infection, mice were treated with 17-AAG or with vehicle (DMSO) alone, three times a week, for three weeks. The ear thickness of mice treated with 17-AAG was significantly smaller compared to controls (Figure 5A). Disease burden, calculated by the area under the curve (AUC) obtained for the two experimental groups, was also significantly ($p<0.05$) different (Figure 5B), demonstrating the ability of 17-AAG to control lesion development, *in vivo*. 17-AAG treatment significantly ($p<0.05$) reduced the parasite load at the infection site, six weeks post inoculation (Figure 5C). However, treatment with 17-AAG was not able to reduce the parasite load within draining lymph nodes (dLN) (Figure 5D).

Comparison of the *in vitro* killing effects of 17-AAG and amphotericin B

In order to further characterize 17-AAG as an anti-leishmanial, its effect on infected macrophages was compared to that exerted by amphotericin B. Macrophages were infected with *L. (V.) braziliensis* and treated with 220 nM 17-AAG (IC_{50}) or with amphotericin B (AMB) (0.27 μM). Forty-eight hours after medium replacement (Figure 6), cultures treated with either 17-AAG or amphotericin B displayed significantly lower parasite numbers ($p<0.01$) in comparison with controls, treated with vehicle (DMSO) alone.

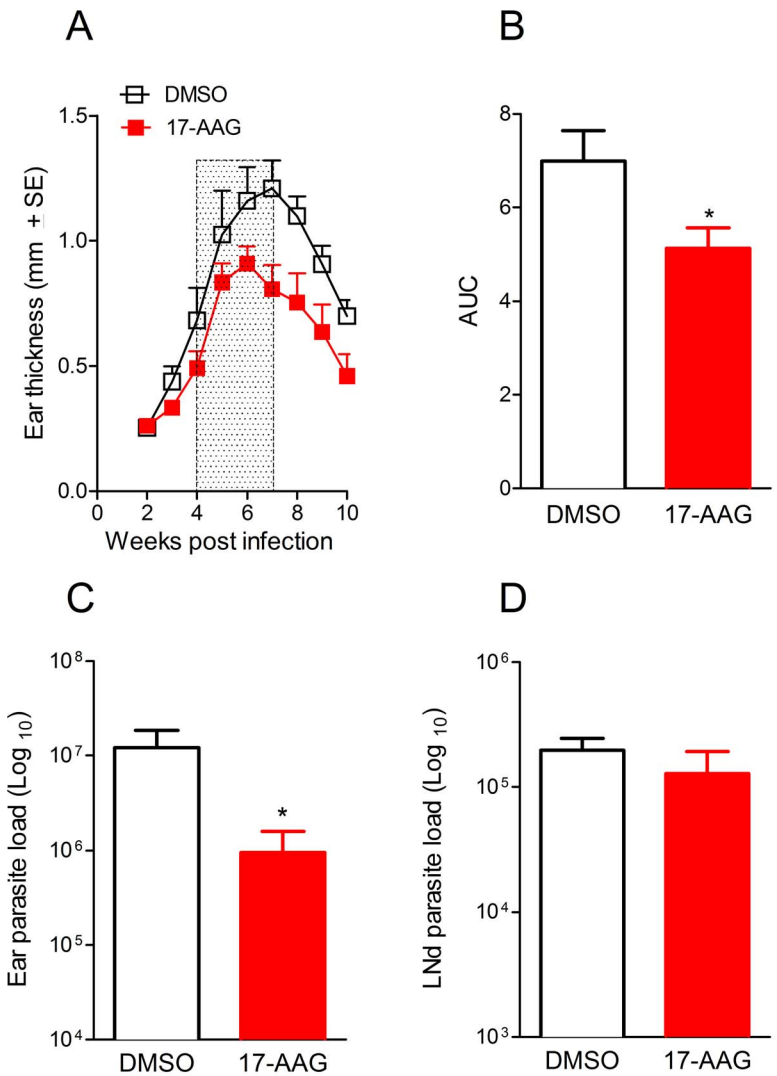

Figure 5. *In vivo* treatment with 17-AAG decreases *L. (V.) braziliensis* infection. Mice were infected with *L. (V.) braziliensis* and four weeks later, mice were treated with 17-AAG, 3x a week for 3 weeks (boxed area) or with vehicle (DMSO) alone. (A) The course of lesion development was monitored weekly. (B) Disease burden [shown as Area Under the Curves (AUC) depicted in (A)] in mice treated with 17-AAG or injected with DMSO. Parasite load was determined at the infection site (C) and at the dLN (D), 6 weeks later, by limiting dilution analysis. Data, shown as mean ±SEM, are from one of two independent repeats, each performed with 10 mice in each group (**$p<0.01$; *$p<0.05$).
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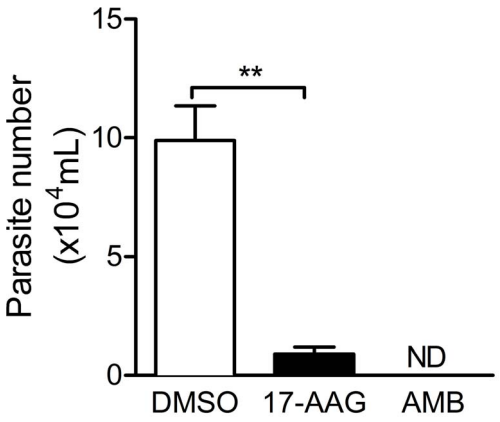

Figure 6. Comparison of the effects of 17-AAG and amphotericin B on the growth of intracellular *L. (V.) braziliensis*. *L. (V.) braziliensis*-infected macrophages were treated with 17-AAG or with amphotericin B (AMB) for 48 h. The number of viable parasites was evaluated by further culture in Schneider medium, free of 17-AAG. Data, shown as mean ±SEM, are from one of two independent repeats (***$p<0.001$). (ND, not detected).
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Discussion

HSP90 is a molecular chaperone fundamental for the life cycle of a variety or protozoa [31] and, as such, inhibitors of HSP90 have been suggested as novel chemotherapeutic agents against malaria [32], filariasis [33,34] and schistosomiasis [34]. Recently, we showed that 17-AAG, a HSP90 inhibitor, reduced *L. (L.) amazonensis* infection *in vitro* [24]. Herein, we investigated the potential of 17-AAG as a chemotherapeutic agent against *L. (V.) braziliensis*, the main etiological agent of CL and MCL in Brazil [35]. We confirmed the effects of 17-AAG against this *L. (V.) braziliensis* promastigotes and we extended these findings to a preclinical model of CL.

Initially, we investigated the *in vitro* effects of 17-AAG, against both axenic promastigotes and intracellular amastigotes. Treatment of *L. braziliensis* promastigotes with the lower dose of 17-AAG (25 nM) already decreased promastigote viability. Herein, the IC_{50} determined for *L. (V). braziliensis* was comparable to that described for *L. (L). amazonensis* (65 nM) whereas in experiments performed with *L. (L). major,* the IC_{50} was established at 80 nM [24]. 17-AAG was equally effective at reducing intracellular amastigote numbers and the viability of surviving *L. (V). braziliensis* promastigotes. These effects were not associated with an increase in the microbicidal functions of macrophages as levels of NO, superoxide and TNF-α were diminished in the presence of 17-AAG. These results are in accordance with our previous report [24]. Additionally, the lack of amastigote replication in control macrophages could be attributed to innate microbocidal properties of macrophages that allow *L. (V). braziliensis killing*, as observed with *L. (V). guyanensis* and *L. (L.) major* [36–38]. In CL patients, an exacerbated inflammatory immune response is associated with the development of mucocutaneous leishmaniasis (rev. in [39]) whereas subclinical patients, who do not develop the disease, have a more controlled immune response [40]. Therefore, the possibility of selectively inducing parasite killing without contributing to overt inflammation is an important advantage for the treatment of CL using 17-AAG. Of note, macrophages treated with 17-DMAG alone displayed reduced production of IL-6, TNF-a and NO [41] whereas 17-AAG prevented iNOS expression upon stimulation with LPS or IFN-g [42].

Geldanamycin (GA), a HSP90 inhibitor analogous to 17-AAG, induces an anti-oxidative and attenuated inflammatory response in sepsis [43], autoimmune encephalitis [44], experimental atherosclerosis [45] and endotoxin-induced uveitis [46]. The proposed mechanism for these effects is the reduced nuclear translocation of NF-κB, reflecting in decreased production of IL-6, TNF-α and NO [41]. Although we cannot extrapolate the complexities of *in vivo* situations cited above, *L. (L.) amazonensis*-infected macrophages treated with 17-AAG displayed parasite killing, in spite of a diminished production of inflammatory mediators [24]. It has been shown that in BALB/c mice infected with *L. (V). braziliensis*, the density of INOS+ cells was higher when compared to *L. (L). amazonensis*-infected mice [47]. So, different responses to NO between *L. (V.) braziliensis* and *L. (L.) amazonensis* could also impact on the killing effect exerted by 17-AAG. In our previous work, [24], we showed that *L. (L) amazonensis* amastigotes displayed structural alterations following exposure of infected macrophages to 17-AAG. Visible alterations in the cytoplasm of parasites such as the presence of myelin figures, vesicles with double-layered membranes and mitochondrial segments inside membrane-bounded structures were the suggestive indications of autophagy, a process that naturally occurs in *Leishmania* and which plays an important role in the transition from promastigote to amastigote [48]. It is possible that inhibition of HSP90 activity interferes with cell cycle progression, blocking differentiation or expression of stage specific protein and, consequently, affecting survival in the intracellular environment.

17-AAG was also effective *in vivo*: mice infected with *L. (V.) braziliensis* and treated with 17-AAG showed a significantly smaller disease burden in parallel to a smaller parasite load at the infection site. However, 17-AAG was not able to alter parasite load at the draining lymph nodes (dLN), a site where *L. (V.) braziliensis* parasites persist following lesion healing [26]. In this experimental model, parasite persistence is associated with the presence of regulatory T cells (Tregs) that accumulate within dLNs of *L. (V.) braziliensis*-infected mice [49] and these Tregs control Th1 responses by IL-10-dependent mechanisms [50]. Although 17-AAG treatment controlled parasite replication at the infection site and promoted lesion healing, parasite persistence within distal sites such as the dLNs may have important effects with regards to maintenance of immunity to *Leishmania* [51] and/or development of mucotutaneous leishmaniasis, deserving further investigation.

Currently, the drugs available for the treatment of CL are limited and among them, pentavalent antimonials have been the choice for over 60 years. However, treatment is long (20-30 days), patients develop several side effects and, in the recent years, the number of cases refractory to treatment has increased [52,53]. In the case of therapeutic failure, second-line drugs such as amphotericin B can be employed as well as combination of two available drugs [54]. Advantages of a combination treatment include increased efficacy, less drug resistance, lower drug dosage and a general decrease in side effects [55]. Herein, 17-AAG was as effective as amphotericin B at decreasing the parasite load within infected macrophages. Experimentally, the treatment of *L. infantum* and *L. panamensis* promastigotes with 17-AAG plus edelfosine improved the anti-leishmanicidal activity of the latter [56]. *In vitro* synergism was also observed for the combinations of paramomycin and amphotericin B against *L. (V.) braziliensis* [57]. *In vivo*, association of tamoxifen with amphotericin B yielded an additive effect in mice infected with *L. (L.) amazonensis* [58]. The combination of GA with fluconazole showed synergistic activity against *Candida albicans* isolates resistant to fluconazole alone [59]. Thus, we propose that combinations of 17-AAG and

amphotericin B may be further investigated for the treatment of CL caused by *L. (V.) braziliensis*.

Herein, we reported that 17-AAG reduces *L. (V.) braziliensis* infection *in vitro* and *in vivo*. 17-AAG shows excellent bioavailability when given to mice by the i.p. route [60]. At 60 mg/kg, 17-AAG caused no changes in appearance, appetite, waste elimination, or survival of treated animals as compared to vehicle-treated controls. We employed with 20 mg/kg, a dose well below that reported as having any harmful effects, as those decribed by Solit et al. (equal or above 75 mg/kg) [61]. Given that HSP90 inhibitors, analogous to 17-AAG, have entered clinical trials with cancer patients [62], we propose that 17-AAG could be further investigated as a novel target for chemotherapy against cutaneous leishmaniasis.

Supporting Information

Figure S1 Determination of IC_{50} values for 17-AAG in *L. braziliensis* promastigotes. Cells were treated in sextuplicate with 17-AAG for 48 hours with varying concentrations of 17-AAG. Following treatment, parasite viability was evaluated by direct counting. IC_{50} values (nM) were determined using GraphPad Prism.
(TIF)

Figure S2 Treatment with 17-AAG controls *L. braziliensis* replication inside macrophages. *L. braziliensis*- infected macrophages were treated with increasing concentrations of 17-AAG or with vehicle alone (DMSO). After 12–72 h, glass coverslips were stained with H&E and assessed for the percentage of infected macrophages (A) and the number of amastigotes per 100 macrophages (B) by light microscopy. Data, shown as mean ±SEM, are from one of three independent repeats (*$p<0.05$; **$p<0.01$ and ***$p<0.001$).
(TIF)

Figure S3 Determination of IC_{50} values for 17-AAG in macrophages infected with *L. braziliensis* promastigotes. Infected macrophages were treated in sextuplicate with 17-AAG for 72 hours with varying concentrations of 17-AAG. Following treatment, glass coverslips were stained with H&E and assessed for the presence of amastigotes by light microscopy. IC_{50} values (nM) were determined using GraphPad Prism.
(TIF)

Figure S4 Cell viability following macrophage exposure to 17-AAG. Thioglycolate-elicited macrophages were exposed to with different concentrations of 17-AAG or to DMSO (vehicle) alone for 24 h. Cell viability was evaluated by MTT assay.
(TIF)

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Author Contributions

Conceived and designed the experiments: DMS ALOAP FSC VMB PSTV CIdO. Performed the experiments: DMS ALOAP FSC. Analyzed the data: DMS ALOAP VMB PSTV FSC CIdO. Contributed reagents/materials/analysis tools: VMB PSTV CIdO. Wrote the paper: DMS ALOAP VMB PSTV CIdO.

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5.3 MANUSCRITO III

17-AAG reduces autophagosome fusion and induces autophagy-dependent cell death of *Leishmania* parasites

17-AAG reduz a fusão de autofagossomos e induz morte cellular dependente de autofagia em parasitos de *Leishmania*

Resumo de resultados: Nesse trabalho foi investigada a capacidade do 17-AAG em induzir a formação de vacúolos autofágicos em parasitos do gênero *Leishmania* e se a formação dos autofagossomos está relacionada com o processo de morte induzido por este fármaco. Nossos resultados indicam que 17-AAG é capaz de induzir especificamente a formação de autofagossomos nos parasitos após 48 h de tratamento. Observamos também que parasitos transgênicos incapazes de formar autofagossomos são mais resistentes à morte celular induzida pelo 17-AAG, evidenciando o papel da via autofágica na morte celular da *Leishmania*. Além disso, nossos resultados indicam que parasitos incapazes de formar autofagossomos acumulam mais proteína ubiquitinada após o tratamento com 17-AAG ou MG132, indicando uma relação entre a degradação de proteínas ubiquitinadas e a ativação da via autofágica.

TITLE

17-AAG reduces autophagosome fusion and induces autophagy-dependent cell death of *Leishmania* parasites

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ABSTRACT

17-AAG is a semisynthetic Heat Shock Protein 90 (HSP90) inhibitor derivate from geldanamycin. It is the first geldanamycin derivate to enter clinical trials against several types of cancer with encouraging results. HSP90 is thought to be an excellent drug target against parasitic diseases once it is described as a protein responsible for cell cycle control, differentiation, virulence and drug resistance in different species of protozoan parasites. We have previously demonstrated the leishmanicidal effect of 17-AAG using *in vitro* and *in vivo* model of cutaneous leishmaniasis. In this paper we aimed to investigate the

mechanism of cell death induced by 17-AAG treatment. Using transgenic parasites we were able to identify that 17-AAG treatment induces autophagosome formation that does not fuse with its known cargo, the glycosomes, or with lysosomes, tough it does not mature into autolysosome. Moreover, ATG5 knock-out (ΔATG5) parasites, that do not form autophagosomes, are more resistant to 17-AAG induced cell-death with an IC50 90% higher than WT *Leishmania*. As expected, treatment with 17-AAG and MG132, a proteasomal inhibitor, induced an accumulation of ubquitinated protein in parasite cytoplasm, however, we detected an overall accumulation of ubiquitinated proteins in ΔATG5 in comparison with WT parasites, suggesting that autophagy plays a role in degradation of ubiquitinated proteins. Our study suggests that 17-AAG induces an autophagy-dependent cell death of *Leishmania*, once parasites still die even when autophagy is blocked in ΔATG5 parasites, moreover HSP90 controls the expression of hundreds of client proteins causing a single mechanism of death unlikely. Here we show results that corroborates with previeous data of 17-AAG as molecule with leishmanicidal effect and we also show evidences of the mechanism of death induced by this compound in *Leishmania*.

Introduction

Leishmania spp. are protozoan parasites that infect a variety of vertebrate hosts, including humans and dogs (Banuls et al 2007, Herwaldt 1999). The disease caused by these protozoa is generally called leishmaniasis, which can present as different types of clinical manifestations, including localized skin lesions that heal spontaneously, disseminated skin

lesions that spread, a mucocutaneous form of the disease and visceral leishmaniasis, which may lead to death if left untreated (WHO 2010).

The WHO classifies leishmaniasis as one of the neglected tropical diseases which affect millions of people worldwide. Financial investment in new therapeutic strategies has been scarce (WHO 2010), resulting in pentavalent antimonials being the drug of choice for 70 years in Brazil. However, antimonial therapy requires parenteral administration at high dosages and involves a lengthy course of treatment that may result in a number of serious side effects (Oliveira et al 2011). In recent years, increases in resistant cases have been reported, effectively restricting the use of pentavalent antimonials as antileishmanials (Croft et al 2006a, Croft et al 2006b). Thus, it is important to highlight the need to develop and test new drugs capable of replacing or complementing existing strategies for leishmaniasis treatment.

Heat shock protein 90 (HSP90) has been considered as a potential molecular target for the treatment of parasitic diseases (Angel et al 2013, Roy et al 2012, Shonhai et al 2011). HSP90 inhibitors, such as geldanamycin or 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), have demonstrated inhibitory effects on the differentiation process of *Leishmania donovani in vitro*. Moreover, these inhibitors have also been shown to have anti-parasitic activity *in vitro*, as well as in pre-clinical testing against a variety of infectious diseases (Ahn et al 2003, Meyer & Shapiro 2013, Pallavi et al 2010, Petersen et al 2012, Santos et al 2014). Geldanamycin and 17-AAG are members of a family of benzoquinone ansamycin antibiotics, which bind selectively to the HSP90 ATP pocket, preventing ATP hydrolysis and, consequently,

conformational changes in this molecular chaperone. In addition, it is known that more than 200 client proteins require the participation of this heat shock protein for folding and to achieve their tertiary structure. The inhibition of HSP90 by 17-AAG or geldanamycin has been ultimately proven to induce the accumulation of misfolded proteins in cancer cells, which are then ubiquitinated and degraded within proteasomes (Isaacs et al 2003). In mammalian cells, the accumulation of ubiquitinated proteins can cause proteasome overload, which leads to the formation of protein aggregates and, subsequently, activation of the autophagic pathway. This mechanism protects mammalian cells against the harmful effects of protein aggregate accumulation in the cytoplasm (Driscoll & Chowdhury 2012, Tyedmers et al 2010, Wolff et al 2014).

In previous studies, we have demonstrated the antileishmanial effect of 17-AAG *in vitro* (Petersen et al 2012) and *in vivo* (Santos et al 2014). We found that dosages of 17-AAG ranging from 125-500 nM were capable of clearing over 90% of intracellular parasites at treatment times lasting no more than 48 hours (Petersen et al 2012). The effects of this HSP90 inhibitor were observed to be dose- and time-dependent, with a selective index of 70. Moreover, 17-AAG is able to eliminate both promastigotes, which colonize the insect vector, as well as amastigotes, which are found within vertebrate host cells. Nonetheless, the mechanism by which HSP90 inhibition causes parasite cell death remains unclear.

Electron microscopy revealed ultrastructural changes in intracellular parasites treated with 17-AAG *in vitro*. The following observed alterations provide evidence for the activation of the autophagic pathway in parasites:

progressive cytoplasmic vacuolization, the formation of double-membrane vacuoles, the presence of myelin figures and vacuoles containing cytoplasmic material—all in the absence of significant alterations in cellular nuclei, mitochondria or plasma membranes (Petersen et al 2012).

Autophagy is a conserved process among all eukaryotic cells that is triggered by nutritional deprivation (Mizushima & Komatsu 2011) or pharmacological stress (Cheng et al 2013). This regulated intracellular catabolic process is responsible for the turnover of most long-lived proteins and organelles by the aggregation of cytosolic organelles and membrane material into double membrane compartments called autophagosomes. Three main types of autophagy have been described: chaperone-mediated autophagy (Dice 2007), microautophagy (Mijaljica et al 2011), and macroautophagy, which is often simply referred to as autophagy, as will be the case in this text. Autophagy plays an important role in cellular homeostasis and remodeling throughout cell development and differentiation, as well as in cell survival in response to different types of stress (Klionsky & Emr 2000, Mizushima & Klionsky 2007, Mizushima & Komatsu 2011, Yorimitsu & Klionsky 2007). In parasites of the genus *Leishmania*, autophagy has been identified as essential to the differentiation of promastigotes into amastigotes (Besteiro et al 2006).

The formation of autophagosomes takes place in successive steps by the recruitment and activation of proteins from the ATG (AuTophaGy-related genes) family (Duszenko et al 2011, Meijer et al 2007). Among the different ATGs recruited to the autophagosomal membrane, Atg8 is remarkable due to its presence throughout the process of autophagic vacuole formation (Noda et

al 2000, Obara et al 2008a, Obara et al 2008b) and, as such, it is considered a marker of these organelles (Klionsky et al 2012). In *Leishmania* parasites, evidence has been presented that, in order for ATG8 to participate in autophagosome formation, ATG12 firstly conjugates with ATG5, and the assembly of this complex is essential to the closure of autophagic vesicles (Besteiro et al 2007, Besteiro et al 2006, Williams et al 2012, Williams et al 2009). The fact that the ATG12-ATG5 conjugation system colocalizes with ATG8-labeled vesicles and that ATG8 is a structural component whose conjugation and deconjugation is strictly controlled by the cell indicates that autophagosome formation is dependent on ATG8 (Nair et al 2012, Williams et al 2012, Williams et al 2009). Similarly to mammals, *Leishmania* autophagosomes may acquire cargo and fuse with lysosomes, thereby forming autolysosomes (Besteiro et al 2007, Besteiro et al 2006), in which intravacuolar components are degraded, resulting in the generation of small molecules (Levine & Klionsky 2004, Meijer & Codogno 2004)

We hypothesized that parasite death occurs in *Leishmania* spp. following treatment with 17-AAG due to activation of the autophagic pathway. *Leishmania* parasites expressing the autophagosome marker GFP-ATG8 were treated with 17-AAG to assess the presence of puncta structures characteristic of autophagosomes. Furthermore, treated double mutant parasites expressing GFP-ATG8 and glycosomal or lysosomal markers were used to evaluate whether autophagosomes successfully complete the maturation process. Additionally, parasites knockout for ATG5 (ΔATG5) were used to evaluate whether the puncta structures observed following treatment

were in fact autophagosomes, and whether parasite death induced by 17-AAG is related to autophagy. Finally, the possible effects of 17-AAG on proteasome overload were evaluated by determining the amount of ubiquitinated proteins in treated parasites.

Material and Methods

Leishmania culturing

Leishmania major (MHOM/JL/80/Friedlin) were cultivated in modified HOMEM medium (Gibco, Carlsbad,CA, USA) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Gibco,,) and 1% (v/v) penicillin streptomycin solution (Sigma, St. Louis, MO, USA) at 25°C until the mid-log phase was achieved, corresponding to 5 x 10^6 parasites/mL.

Generation of parasite mutants expressing fluorescent markers

The generation of all parasite mutants was performed as previously described using the following plasmid constructions: i) green fluorescent protein-ATG8 (GFP-ATG8) plasmid in accordance with (Besteiro et al 2006), ii) the glycosome containing SQL motif labelled with RFP plasmid as described by (Cull et al 2014) and iii) the proCPB lysosomal-marker labeled with RFP plasmid designed by (Huete-Perez et al 1999). Plasmids were transfected into *L. major* parasites using a nucleofector transfection system according to manufacturer instructions (Lonza, Basel, Switzerland) to obtain the following parasite lines: i) GFP-ATG8, ii) double-mutant parasites expressing both GFP-ATG8 and the glycosome containing SQL motif labeled with RFP, and iii) double-mutant parasites expressing both GFP-ATG8 and the proCPB lysosomal-marker labeled with RFP. Null mutant ATG5 parasites expressing GFP-ATG8 (ΔATG5[GFP-ATG8]) and ΔATG5::ATG5 parasites reexpressing ATG5 in the ΔATG5 null mutant were generated as previously described by (Williams et al 2012) and used as controls. To select parasites that successfully expressed the desired proteins, an appropriate antibiotic was used to treat each transfected parasite line: G418 (Neomycin) at 50 μg/mL; Hygromycin at 50 μg/mL; Blasticidin S at 10 μg/mL; Phleomycin at 10 μg/mL (all from InvivoGen, San Diego, CA, USA**).**

Parasite treatment with 17-AAG or Pentamidine

In accordance with each experimental treatment protocol, parasites were submitted to treatment procedures using the antileishmanial 17-AAG (300 or 500 nM) or pentamidine (10, 20 and 30 μM) for up to 72h. At the end

of each treatment period, parasites were pelleted by centrifugation for 3 min at 1,000 x g and then washed thrice in PBS for medium removal. Cells were then resuspended in PBS and a 10 µL suspension was spread thinly over a slide covered with a 22 x 40 mm coverslip, then sealed with nail varnish to perform fluorescence microscopy.

Western blot for ubiquitinated protein assessment

The accumulation of ubiquitinated proteins in parasites treated with 17-AAG (300 or 500 nM) for 24, 48 or 72 h was evaluated by Western blot analysis. As a positive control, parasites were treated with the proteasome inhibitor MG132 (1 or 3 µM) for 24, 48 or 72 h. Following treatment, parasites were pelleted by centrifugation for 3 min at 1,000 x g, and then washed thrice in PBS for medium removal. Protein extraction was performed for Western blot analysis as described below.

Parasites were lysed with laemmli buffer (2-Mercaptoethanol 0.1%, Bromophenol blue 0.0005%, Glycerol 10%, SDS 2%, Tris-HCl 63 mM (pH 6.8)) at a proportion of 10 µL of buffer for 10^6 parasites, then boiled for 5 min and cell extracts were stored at -20°C. Proteins were transferred from a 12% polyacrylamide gel, following electrophoresis, to a Hybond-C nitrocellulose membrane (Amersham, GE Healthcare, Little Chalfont, UK). Transfer was carried out by semi-dry blotting using a BioRad Trans-Blot SD Semi-Dry Transfer Cell at 30 volts for 45 min, with membranes and filter papers soaked in transfer buffer (20 mM Tris-HCl, 15 mM glycine, 20 % (v/v) methanol, in distilled water). Membranes were subsequently incubated in a blocking solution of 5% (w/v) milk powder in TBST buffer (25 mM Tris-HClpH 8, 125

mM NaCl, and 0.1 % Tween) for 1 hour at room temperature or overnight at 4°C under agitation. After blocking, each membrane was incubated with 1:1000 FK2 anti-ubiquitin antibody (LifeSensors, Malvern, PA, USA) diluted in fresh TBST buffer with 3% milk for 1 hour at room temperature. Secondary anti-mouse antibody conjugated with horseradish peroxidase (HRP) (Promega, Madison, WI, USA) was diluted at 1:10000 in fresh TBST buffer. After washing three times in TBST, each membrane was first incubated with an ECL (Enhanced Chemiluminescence) solution (SuperSignal West Pico Chemoluminescent Substrate Kit, Pierce, Rockford, IL, USA) in accordance with manufacturer instructions, and then exposed on Kodak photographic film. An antibody against elongation factor 1α (EF1α) (Millipore, Germany) was used for loading control.

Assessment of Autophagosome Formation by Fluorescence Microscopy

Fluorescence microscopy was used to assess the presence of GFP-ATG8-labelled vesicles, characteristic of autophagosomes, which appear as puncta structures (Cull et al 2014, Williams et al 2012). Immediately after mounting for up to 1h to ensure cell viability, parasites smears were observed under a DeltaVision Core deconvolution microscope (Applied Precision Inc.,Issaquah (WA), USA) using GFP or mCherry filters. Imaging was performed using a Photometrics CoolSNAP HQ2 camera at 100X magnification. For cell visualization, DIC images were obtained under polarized light. For florescence imaging, a 3-µm Z-stack was used with a 0.2 µm interval between each image in the stack. Deconvolution was performed

using a conservative ratio method with 10 iterations. All images were processed and analyzed using SoftWoRx image analysis software (Applied Precision Inc).

The percentage of parasites containing GFP-ATG8-labelled vesicles, as well as the number of GFP-ATG8-labelled vesicles within these parasites, were recorded. At least 300 parasites were counted for each combination of treatment and time point, with no less than three independent experiments considered.

Confocal Microscopy

To evaluate the effect of 17-AAG treatment on the autophagosome maturation process, parasites were treated with 17-AAG (500 nM) for 24 or 48h and autophagosome colocalization with glycosomal cargo or with lysosomes was evaluated. Double mutant *L. major* promastigotes expressing both GFP-ATG8 and the glycosome-containing SQL motif labeled with RFP, or promastigotes expressing GFP-ATG8 in addition to the proCPB lysosomal-marker labeled with RFP were treated with 17-AAG as described above. Parasites were then imaged using a DeltaVision Core deconvolution microscope as described above. Images were submitted to colocalization analysis. The SoftWoRx image analysis (Applied Precision, Inc) colocalization finder tool was used to analyze individually captured images. This tool uses Pearson's correlation coefficient (*r*) to calculate the correlation between two fluorophores within the selected area, with values close to zero indicating no colocalization and values close to one indicating complete colocalization (Bolte & Cordelieres 2006). When colocaliation of a GFP-labeled

autophagosome with an RFP-lysosome or RFP-glycosome was suspected, the region of the image containing the suspected area was selected, and colocalization analysis was performed on this region. The fluorophores of a region with an $r > 0.5$ were considered as colocalized.

Parasite Viability

Axenic parasites in mid-log phase were treated with serial dilutions of 17-AAG for 48 h at concentrations ranging from 10 to 15625 nM, or with DMSO (control). Next, AlamarBlue (Invitrogen, Carlsbad, California, USA) was added to obtain a final concentration of 10% and the solutions were then reincubated for another 24h at 24°C. Finally, reagent absorbance was measured at the wavelengths of 570 and 600 nm using a spectrophotometer (SPECTRA Max 340 PC).

Parasite Growth Curve

Leishmania cell lines (WT, ΔATG5 and ΔATG5::ATG5) at a concentration of 10^5 cells/mL were incubated in 10 mL of HOMEM supplemented with 10% (v/v) heat-inactivated fetal calf serum and 1% (v/v) penicillin streptomycin solution. Cells were treated or not with 100 nM of 17-AAG and counts were performed daily for at least 13 days. Parasite numbers were recorded and plotted.

Statistical analysis

The half maximal inhibitory concentration (IC_{50}) of 17-AAG on *L. major* WT, ΔATG5 and ΔATG5::ATG5 promastigotes was determined by performing sigmoidal regression on each respective concentration-response curve. Data

are presented as the mean ± standard deviation of the mean under parametric (One-way ANOVA followed by Dunnett's Multiple Comparison) or non-parametric analysis (Mann-Whitney), in which *p*-values ≤ 0.05 were considered significant. All data were analyzed using the Prism software program (GraphPad software, V. 6.0 La Jolla, California).

Results

17-AAG induces autophagosome formation in promastigote forms of *Leishmania*

A previous study observed morphological alterations in *Leishmania* parasites suggestive of activation of the autophagic pathway in infected cells treated with 17-AAG, resulting in parasite death by an unknown mechanism. To confirm 17-AAG induces activation of autophagic pathway in parasites, fluorescence microscopy was used to assess the presence of GFP-ATG8-labelled vesicles characteristic of autophagosomes. Treatment with 17-AAG at concentrations of either 300 or 500 nM indeed resulted in an increased number of parasites containing vesicles marked as green ATG8-labelled puncta structures at later time points (Fig. 1A). After 48 h of treatment, 32% and 36.6% of the counted parasites exhibited puncta structures at concentrations of 300 or 500 nM, respectively, while these alterations were seen in only 19.1% of control parasites treated with DMSO (Fig. 1B; $p < 0.05$). This percentage increased after 72 h of treatment, with 37.5% of the parasites treated with 300 nM and 49.6% treated with 500 nM showing these structures, yet similar alterations were seen in only 20% of control parasites (Fig. 1B; $p < 0.05$). Similar results were observed when counting the number of GFP-

ATG8- labelled vesicles per parasite. After 48 h, parasites treated with 500 nM of 17-AAG had a mean of 1.7 puncta structures per parasite, while a mean of 1.3 was observed in controls (Fig. 1C; $p < 0.05$).

Null mutant ATG5 parasites expressing GFP-ATG8 (ΔATG5[GFP-ATG8]) treated with 17-AAG did not show any fluorescently labeled puncta structures, confirming that puncta structures observed in GFP-ATG8 parasites treated with this antileishmanial were indeed autophagosomes (Fig. 1E).

Although GFP-ATG8 parasites treated with 500 nM of 17-AAG induced the appearance of puncta structures in 35.5% of the parasites after 48 h of treatment (Fig. 1D; $p < 0.05$), the formation of autophagosomes was not observed following treatment with pentamidine at the three time points evaluated. The induction of parasite death was confirmed at all pentamidine concentrations tested (10, 20 or 30 μM), indicating that autophagosome formation in parasites is not directly related to the induction of parasite death.

17-AAG inhibits the autophagosome maturation process

Since the inhibition of endocitic vesicles and autophagosome fusion with lysosomes was previously shown to be involved in parasite death (Amaravadi & Thompson 2007, Cervantes et al 2014, Hoppe et al 2004), we evaluated whether treatment with 17-AAG was able to inhibit the maturation process in autophagosomes.

In double mutant parasites expressing GFP-ATG8 and the glycosome containing SQL motif labelled with RFP, the percentage of autophagosomes that colocalized with glycosomes was similar under treatment with 17-AAG

(500 nM for 24 h) 30.4% compared to controls, 22.6% (Fig. 2A & B). By contrast, after 48 h of treatment, this percentage was 20.5%, significantly lower than that observed in control parasites, 41.6% (Fig. 2B; $p = 0.028$).

Treatment with 17-AAG was observed to also influence autophagosome fusion with lysosomes. Double mutant parasites expressing GFP-ATG8 in addition to the proCPB-RFP lysosomal-marker treated with 17-AAG (500 nM) for 24 and 48 h exhibited a remarkably lower rate of autophagosome-lysosome colocalization after 24h, 8.2%, (Fig. 2C & D) and 48h, 12%, (Fig. 2D; $p = 0.028$) in comparison to untreated controls, 35.5% and 38.5%, respectively.

Taken together, these findings clearly demonstrate that parasite autophagosomes did not undergo the maturation process from autophagosomes to autolysosomes when treated with 17-AAG due to the inhibition of autophagosomes to acquire glycosome cargo and subsequently fuse with lysosomes.

Null ATG5 mutant parasites resist 17-AAG-induced cell death

In order to evaluate whether autophagy plays a role in 17-AAG parasite death, IC50 values were determined for ΔATG5 parasites treated with 17-AAG for 48h, which were then compared to WT and ΔATG5::ATG5, a ΔATG5 parasite line that re-expresses *ATG5.* The IC50 for ΔATG5 parasites treated with 17-AAG (174.3 ± 15.1 nM) was 90.6% higher than that determined for WT (95 ± 23 nM) (Fig. 3A, $p < 0.05$), and 80% higher than what was seen in ΔATG5::ATG5 (104.3 ± 16.4 nM) parasites (Fig. 3A, $p > 0.05$) (Table 1). Accordingly, when cultivated in medium containing 100 nM of 17-AAG for

seven days, ΔATG5 parasites grew faster than either WT or ΔATG5::ATG5 (Fig. 3B). This marked growth was especially noticeable during the log phase (between days 3 – 5) of parasite growth, when the number of ΔATG5 parasites was 500% higher than that of WT or ΔATG5::ATG5 (Fig. 3B). In contrast, no differences in parasite growth rates were observed among the three parasite lines when cultivated in 17-AAG-free medium (Fig. 3B).

These findings provide convincing evidence that ΔATG5 parasites, which are unable to form autophagosomes, demonstrate considerable resistance to 17-AAG induced cell death.

Increased amounts of ubiquitinated proteins in ΔATG5 parasites

Since proteasome overload subsequent to an increase in misfolded ubiquitinated proteins may contribute to parasite death, the amount of ubiquitinated proteins was determined in WT, as well as ΔATG5 and ΔATG5::ATG5 parasite mutants, upon treatment with 17-AAG. Low basal levels of ubiquitinated proteins were seen in all WT, ΔATG5 and ΔATG5::ATG5 untreated parasites (Fig 4). Treatment with 17-AAG (500nM) for 48h caused an overall increase in the amounts of ubiquitin-labeled proteins in all three parasite cell lines (Fig. 4). As expected, when MG132 (3 μM, 48h) was used as a positive control to inhibit the proteasomal pathway, an enhancement in the amount of ubiquitinated proteins in WT, ΔATG5 and ΔATG5::ATG5 parasites was observed. Notably, among these three lines evaluated, the null mutant ΔATG5 demonstrated higher amounts of ubiquitinated proteins after treatment with either 17-AAG or MG132 in comparison to WT and ΔATG5::ATG5 (Fig. 4).

With respect to autophagosome formation in GFP-ATG8 parasites, 21.3% or 22.4% were found to bear puncta structures when treated for 24h with 1 or 3 μM of MG132, respectively (Fig. 5 A and B), in comparison to 12.7% in the untreated control group. At 48h, 21% or 26% of these parasites, respectively, showed puncta structures, while just 7% of untreated parasites had these structures (Fig. 4B, $p < 0.05$).

In order to confirm whether the observed puncta structures were in fact autophagosomes and not another type of vesicle, (ΔATG5[GFP-ATG8]) parasites were treated with 1 or 3 μM of MG132 for 24, 48 and 72h. No puncta structures were observed, even after 72h of treatment (data not shown). Taken together, these findings indicate that the accumulation of ubiquitinated proteins seen following treatment with 17-AAG or MG132 results in the activation of the autophagic pathway.

Discussion

Eukaryotic cells with the ability to multiply continuously, such as cancer cells, as well as unicellular organisms, e.g. protozoan parasites, synthesize greater amounts of several molecular chaperones during the multiplication or differentiation process, including HSPs. HSP90 plays an important role in regulating the life cycles of a variety of unicellular and multicellular organisms (Angel et al 2014, Devaney et al 2005, Shonhai 2010, Wenkert et al 2010) by controlling the expression of hundreds of proteins involved in a diversity of functions in eukaryotic cells (Hartl 1996, Hartl et al 2011) (for a full list of HSP90 client proteins http://picard.ch/downloads/Hsp90interactors.pdf). In addition, these housekeeping proteins are known to assist general protein

folding and prevent nonfunctional side reactions, such as the nonspecific aggregation of misfolded or unfolded proteins (Neckers 2002, Theodoraki & Caplan 2012). Because it assists in the maintenance of homeostasis in many cell types, HSP90 has become a molecular target for therapeutic intervention, most notably in cancer cells, in addition to a variety of parasite species (Angel et al 2014, Angel et al 2013, Pesce et al 2010, Roy et al 2012, Shonhai 2010, Shonhai et al 2011).

The suppression of HSP90 activity in cancer cells by 17-AAG or geldanamycin causes the inhibition of a range of proteins, ultimately resulting in cell death. Similarly to what has been described in cancer cells, the inhibition of HSP90 in parasites may also interfere in the correct folding of many proteins, thereby producing serious alterations in cellular homeostasis and the induction of autophagy. Several previous studies have shown that HSP90 inhibitors, such as geldanamycin, 17-AAG and radicicol, demonstrate microbicidal efficacy in the treatment of protozoan parasitic diseases capable of affecting humans (Angel et al 2013, Bayih & Pillai 2014, Petersen et al 2012, Roy et al 2012, Santos et al 2014, Shonhai et al 2011). To investigate whether *Leishmania* cell death following treatment with HSP90 inhibitors occurs as a result of activation of the autophagic pathway, we further investigated the mechanisms underlying cell death.

In a previous study, electron microscopic observations revealed that 17-AAG induced morphological alterations in parasites characteristic of autophagy. The present report firstly sought to confirm that autophagy was induced when parasites were treated with 17-AAG by verifying the presence

of autophagosomes in treated parasites (Fig. 1). We found that GFP-ATG8-labelled parasites, when treated with 17-AAG, exhibit an increase in the overall number of parasites bearing autophagosomes, as well as in the number of autophagosomes per parasite (Fig. 1). We also found that ΔATG5[GFP-ATG8] parasites, which are unable to form autophagosomes but carry the GFP-ATG8 plasmid, did not form any detectable puncta structures when treated with 17-AAG, suggesting that the structures observed in GFP-ATG8 treated parasites were indeed autophagosomes.

Next, we investigated whether the autophagosomes induced by treatment with 17-AAG were actually undergoing the maturation process that has been previously described following activation of the autophagic pathway in *Leishmania*. A recent study described the critical role played by autophagy in *Leishmania* with respect to glycosome turnover under normal and nutrient-deprived growth conditions (Cull et al 2014). These authors observed the formation of double-layered membrane structures in both double mutant parasites expressing GFP-ATG8 and the glycosome containing SQL motif labeled with RFP, as well as in parasites expressing GFP-ATG8 and the proCPB lysosomal-marker labeled with RFP. They also found puncta structures colocalizing with either the RFP-SQL motif or RFP-proCPB lysosomal-marker in each of these double mutant parasite lines, which strongly suggests that autophagosomes acquire glycosomes that are subsequently delivered to lysosomes (Cull et al 2014). The present study found that treatment with 17-AAG induced an overall reduction in the degree of colocalization between autophagosomes and glycosomes, as well as between autophagosomes and lysosomes, when compared to controls (Fig.

2). HSP90 is known to control the expression of hundreds of proteins involved in the most diverse cell functions (Hartl 1996, Hartl et al 2011). A previous report described that HSP90 is involved in controlling vesicle trafficking and fusion by folding proteins, such as GDI, which are responsible for recycling RAB proteins from vesicle membranes back into the cytoplasm for use in new fusion cycles (Chen & Balch 2006). In addition, the VAP-33 proteins, which are known to form a complex with HSP90 and also play a role in the transport of glycoproteins from the Golgi complex to other cellular compartments, are similarly regulated by HSP90 (Lotz et al 2008). Accordingly, it is possible that HSP90 inhibition causes the unfolding or incorrect folding of parasite proteins involved in vesicle trafficking and fusion. Since autophagy is a degradative pathway critical to the depletion of senescent organelles and degraded proteins, it is feasible to suggest that inhibiting the fusion of newly-formed autophagosomes and lysosomes could result in the trapping of non-degraded proteins and organelles within autophagosomes, thereby depriving cells of critical small molecules (Amaravadi & Thompson 2007). This deprivation, in itself, would be a major factor leading to parasite death.

Since we observed that ΔATG5 parasites, which are unable to form autophagosomes, demonstrated 90.6% greater rates of survival compared to WT parasites under treatment with 17-AAG (Fig. 3A), it is safe to assume that the activation of the autophagic pathway contributed to *Leishmania* cell death. Although this process has been well documented in the literature as a successful adaptive strategy that is a protective mechanism activated under physiological stress stimuli, such as starvation, stress oxidative response and accumulation of misfolded, undegraded proteins (Choi et al 2013, Degenhardt

et al 2006, Lum et al 2005), the induction of uncontrolled autophagy can be harmful to eukaryotic cells (Mizushima et al 2008), including mammals and unicellular parasites.

In order to investigate the actual participation of autophagy in eukaryotic cell death, properly controlling the genetic or chemical inhibition of autophagy is critical (Klionsky et al 2012, Levine & Yuan 2005). In fact, several studies have described autophagic vesicles as early morphological alterations that appear immediately before parasite death, thusly associating these with autophagy, i.e. the mechanism responsible for parasite death (Bera et al 2003, de Almeida Nogueira et al 2013, Delgado et al 2009, Tiuman et al 2014). For instance, a study showed that treatment with 3-methyladenine, a known inhibitor of autophagy, caused *Leishmania* death, indicating the protective effect conferred by autophagy in parasites treated with an antiparasitic drug (Sengupta et al 2011). By contrast, other authors have demonstrated that the inhibition of autophagy by 3-methyladenine actually reverted the *T. cruzi* autophagic phenotype, in addition to inhibiting parasite death caused by naphthoimidazoles (Menna-Barreto et al 2009). In sum, these findings indicate that the true role played by autophagy with respect to the mechanism responsible for causing protozoan parasite death in response to antiparasitic drugs remains to be elucidated (Sengupta et al 2011).

Since ΔATG5 parasites do eventually die after treatment with 17-AAG, it is appropriate to consider that other mechanisms besides autophagy may be contributing to *Leishmania* death. Since the inhibition of HSP90 in cancer cells has been proven to enhance the accumulation of ubiquitinated proteins

in the cytosol (Isaacs et al 2003) and, subsequently, proteasome overload, which results in the accumulation of unfolded and misfolded proteins (Theodoraki & Caplan 2012, Tyedmers et al 2010), it is possible that a similar event takes place in 17-AAG-treated parasites. It has been proven that, in cancer cells, as well as in other cell types, the accumulation of unfolded and misfolded proteins leads to protein aggregate formation, thereby inducing autophagy (Janen et al 2010). The present report showed that treating *Leishmania* parasites with 17-AAG led to an accumulation of ubiquitinated proteins at levels similar to what were observed in parasites treated with MG132, a proteasome inhibitor (Fig. 4). In addition, we also showed a considerable accumulation of ubiquitinated proteins in ΔATG5 null mutant parasites treated with either 17-AAG or MG132, compared to WT or ΔATG5::ATG5. It is possible that, similarly to what has been reported in cancer cell models, the overall increase in the amount of ubiquitinated proteins by inhibition of parasite HSP90 by 17-AAG is causing proteasome overload . Subsequently, the autophagic pathway becomes activated, functioning as a protective mechanism to clear the accumulation of misfolded proteins. In cancer cells, the degradation of misfolded proteins and aggregates by way of the activation of the autophagic pathway takes place because the adaptor proteins, p62 and NRP1, link ubiquitin from the ubiquitinated protein with ATG8 present in the internal surface of the nascent autophagosome membrane (Duszenko et al 2011, Kraft et al 2010, Pankiv et al 2007). In yeast, although these adaptor proteins have not yet been identified, similar motifs that interact with ATG8 have been identified in ATG19, a protein that participates in the yeast autophagic pathway (Hutchins

& Klionsky 2001, Scott et al 2001, Yuga et al 2011). Our data support the idea that a similar mechanism is taking place in *Leishmania* parasites where unfolded and protein aggregates are being delivered to autophagosomes. This represents an initial attempt to demonstrate that the accumulation of ubiquitinated proteins and the induction of autophagy are related in *Leishmania* genus parasites.

Together, these findings strongly suggest that 17-AAG induces *Leishmania* cell death through activation of autophagic pathway that forms autophagosomes unable to complete autophagolysosomal maturation where probably degradation of material to obtain essential elements doesn't take place. Moreover, ubiquitinated proteins accumulated in treated parasites induce possibly overload of proteasome degradative pathway that can, additionally, activate uncontrolled autophagic pathway contributing to parasite death.

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Table 1. The IC_{50} concentration (nanomolar) determined for 17-AAG against WT, ΔATG5 and ΔATG5::ATG5 *Leishmania*

Figure Legends

Figure 1. Evaluation of autophagosomes in promastigotes of *Leishmanaia* after treatment with 17-AAG. (A) Axenic promastigotes of *Leishmania* expressing ATG8-GFP were treated or not with 17-AAG for 24 or 48h and imaged for fluorescence microscopy. Arrows point to autophagosomes. The percentage (B) of cells bearing autophagosomes and the number of autophagosomes per cell (C) were calculated at 24, 48 and 72h after treatment with 17-AAG. (D) Comparison of the percentage of cells bearing autophagosomes after treatment with pentamidin or 17-AAG for 24 and 48h. (E) GFP-ATG8-ΔATG5 parasites were treated with 17-AAG and imaged for fluorescence microscopy. Bars represent mean ± SD of three independent experiments (Kruskal-Wallis test, Dunn's Multiple Comparison Test, * $p < 0.05$)

Figure 2. Analysis of fusion between autophagosomes and glycosomes or lysosomes. (A) Axenic promastigotes of *Leishmania* expressing ATG8-GFP and RFP-SQL were treated or not with 17-AAG for 24 or 48h and imaged for fluorescence microscopy. Arrows point to colocalization between these two fluorophores. (B) Quantification of autophagosome-glycosome colocalization after treatment of *Leishmania* with 17-AAG, data is presented as the percentage of cells containing at least one colocalization data of GFP-ATG8 and glycosome. (C) Axenic promastigotes of *Leishmania* expressing ATG8-GFP and proCPB-RFP were treated or not with 17-AAG for 24 or 48h

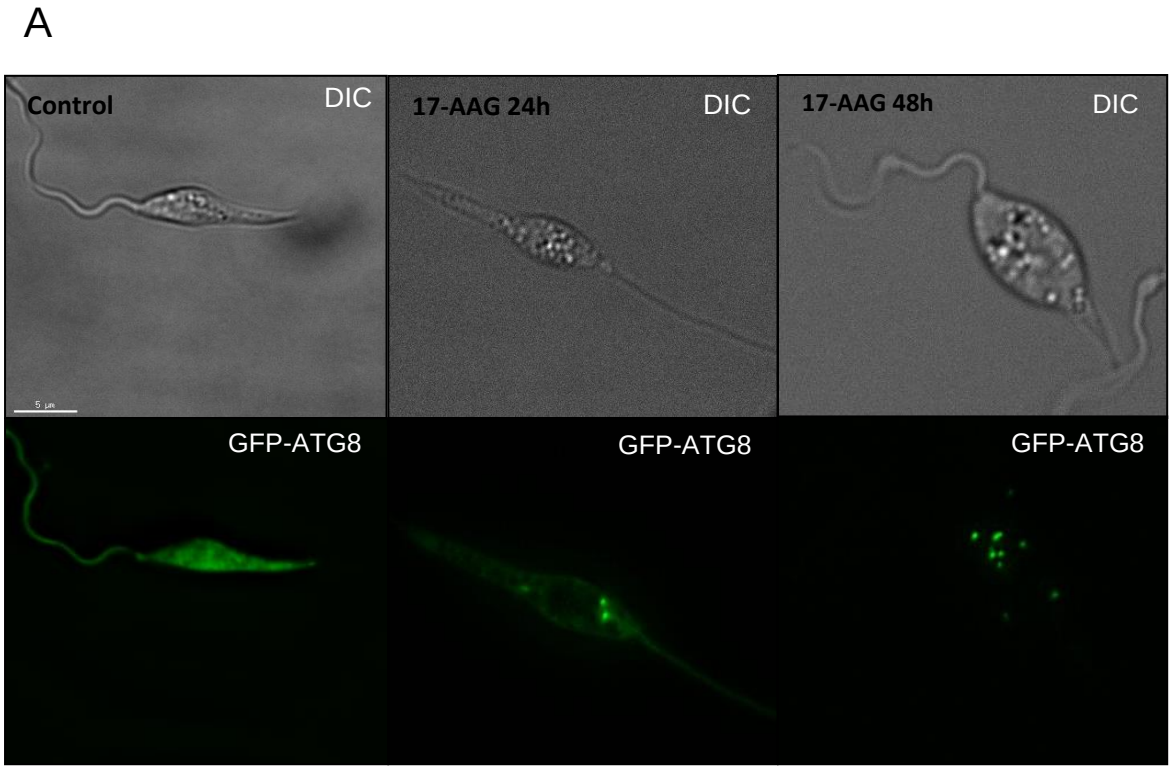
and imaged for fluorescence microscopy. Arrows point to colocalization between these two fluorophores. (D) Quantification of autophagosome-lysosome colocalization after treatment of *Leishmania* with 17-AAG, data is presented as the percentage of cells containing at least one colocalization data of GFP-ATG8 and lysosome. Bars represent mean ± SD of three independent experiments (Mann Whitney test, *p = 0.0286)

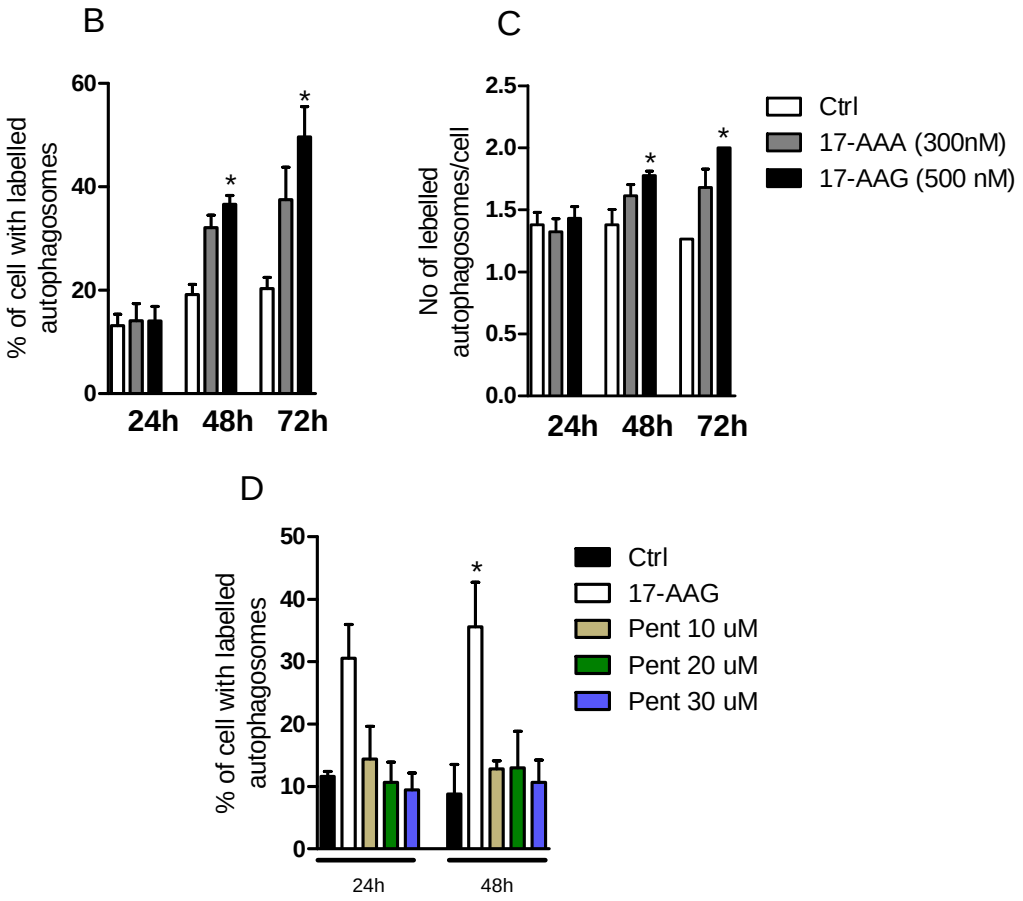
FIGURE 3. Effect of 17-AAG on the survival and replication of WT, ΔATG5 and ΔATG5::ATG5 *Leishmania*. (A) Calculated IC50 of 17-AAG in percentage of WT parasites. Promastigotes of *Leishmania* were treated with different concentrations of 17-AAG and IC_{50} was calculates as described in the material and methods section. Calculated values were converted to percentage of WT cells. Bars represent mean ± SD of four independent experiments (Kruskal-Wallis test, Dunn's Multiple Comparison Test, *p = 0.0244) (B) Growth curve of WT, ΔATG5 and ΔATG5::ATG5 treated or not with 17-AAG. Parasites were treated or not with 17-AAG and counted every day for at least 13 days. Dots represent mean ± SD of three independent experiments.

Figure 4. WT, ΔATG5 and ΔATG5::ATG5 ubiquitinated proteins profile after 17-AAG or MG132 treatment. Protein extracts from WT, ΔATG5 and ΔATG5::ATG5 parasites treated with 17-AAG or MG132 for 48h were blotted against ubiquitinated proteins. Lane A - WT; lane B - ΔATG5; lane C - ΔATG5::ATG5. EF1α was used as loading control.

Figure 5. Evaluation of autophagosomes in promastigotes of *Leishmanaia* after treatment with MG132. (A) Axenic promastigotes of *Leishmania* expressing ATG8-GFP were treated or not with MG132 for 24 or 48h and imaged for fluorescence microscopy. Arrows point to autophagosomes. The percentage (B) of cells bearing autophagosomes was calculated at 24 and 48h after treatment with MG132. Bars represent mean ± SD of three independent experiments (Kruskal-Wallis test, Dunn's Multiple Comparison Test, * $p < 0.05$).

Figure 1.


A
Control
DIC
17-AAG 24h
DIC
17-AAG 48h
DIC
GFP-ATG8
GFP-ATG8
GFP-ATG8


B
% of cell with labelled autophagosomes
60
40
20
0
24h
48h
72h
*
*
C
No of lebelled autophagosomes/cell
2.5
2.0
1.5
1.0
0.5
0.0
24h
48h
72h
*
*
Ctrl
17-AAA (300nM)
17-AAG (500 nM)
D
% of cell with labelled autophagosomes
50
40
30
20
10
0
24h
48h
*
Ctrl
17-AAG
Pent 10 uM
Pent 20 uM
Pent 30 uM

E

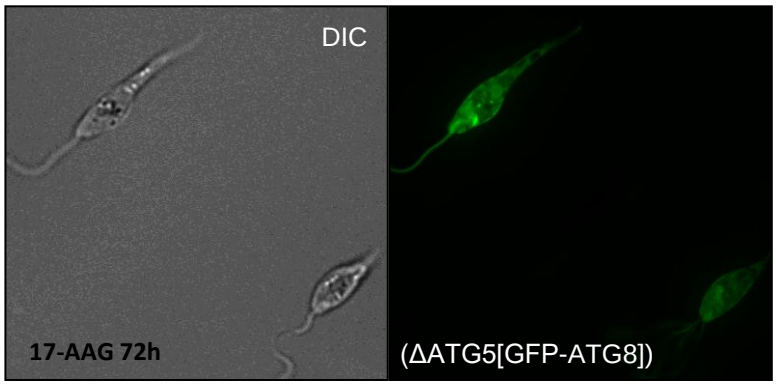
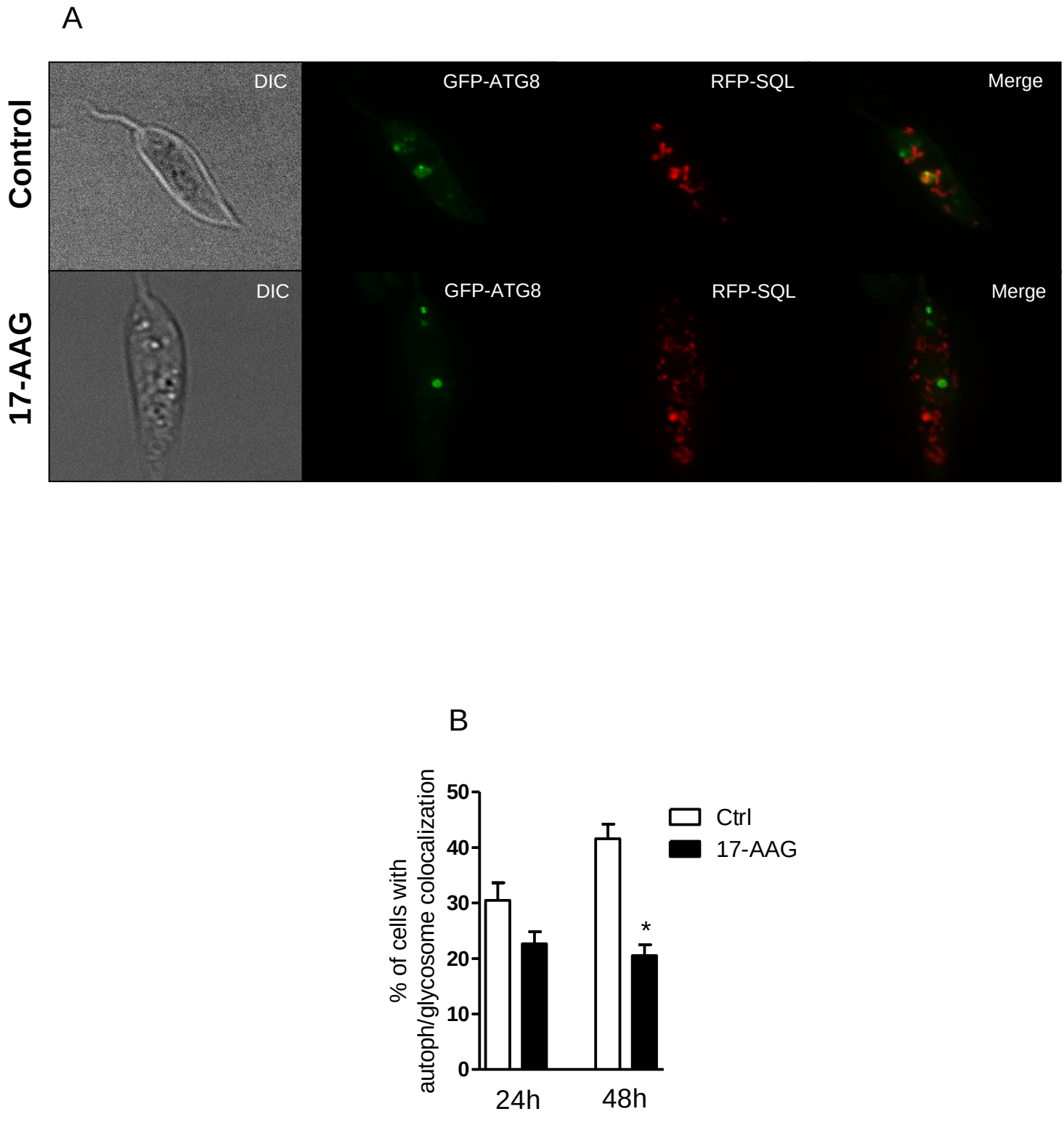
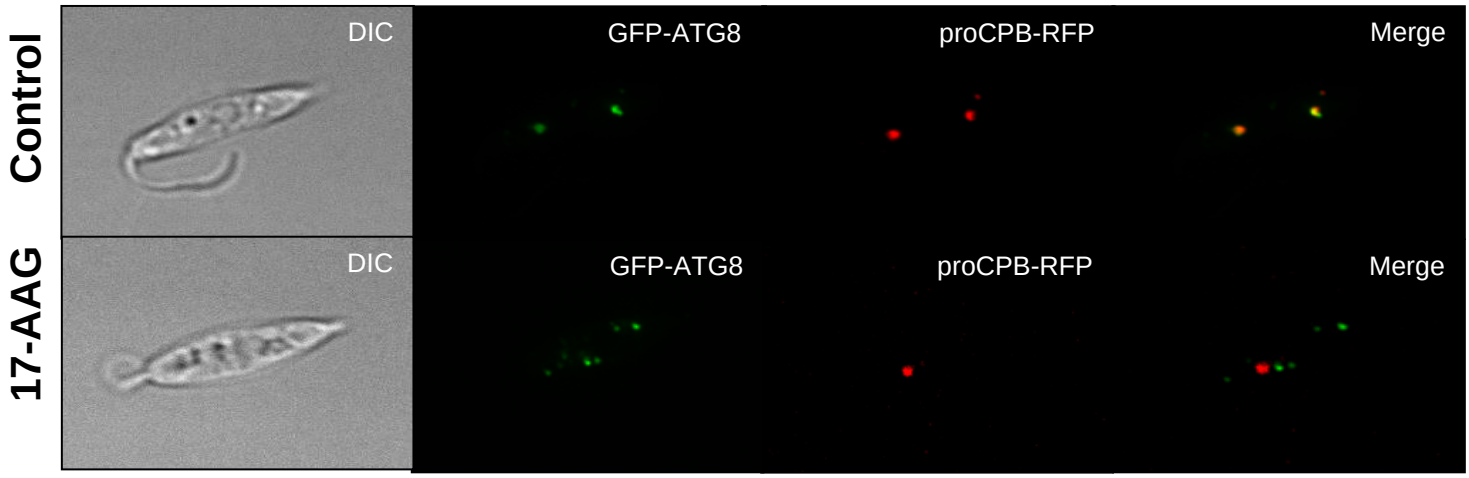


Figure 2.


A
Control
17-AAG
DIC
GFP-ATG8
RFP-SQL
Merge
DIC
GFP-ATG8
RFP-SQL
Merge
B
% of cells with autoph/glycosome colocalization
50
40
30
20
10
0
24h
48h
*
Ctrl
17-AAG

C


Control
17-AAG
DIC
GFP-ATG8
proCPB-RFP
Merge
DIC
GFP-ATG8
proCPB-RFP
Merge

D

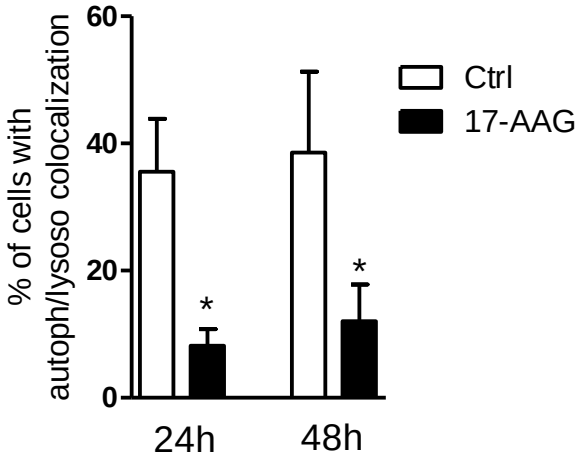

% of cells with autoph/lysoso colocalization
60
40
20
0
*
*
24h
48h
Ctrl
17-AAG

Table 1.

Leishmania	IC_{50} 17-AAG (nM)
WT	95 ± 23
ΔATG5	174.3 ± 15.1
ΔATG5::ATG5	104.3 ± 16.4

Figure 3.

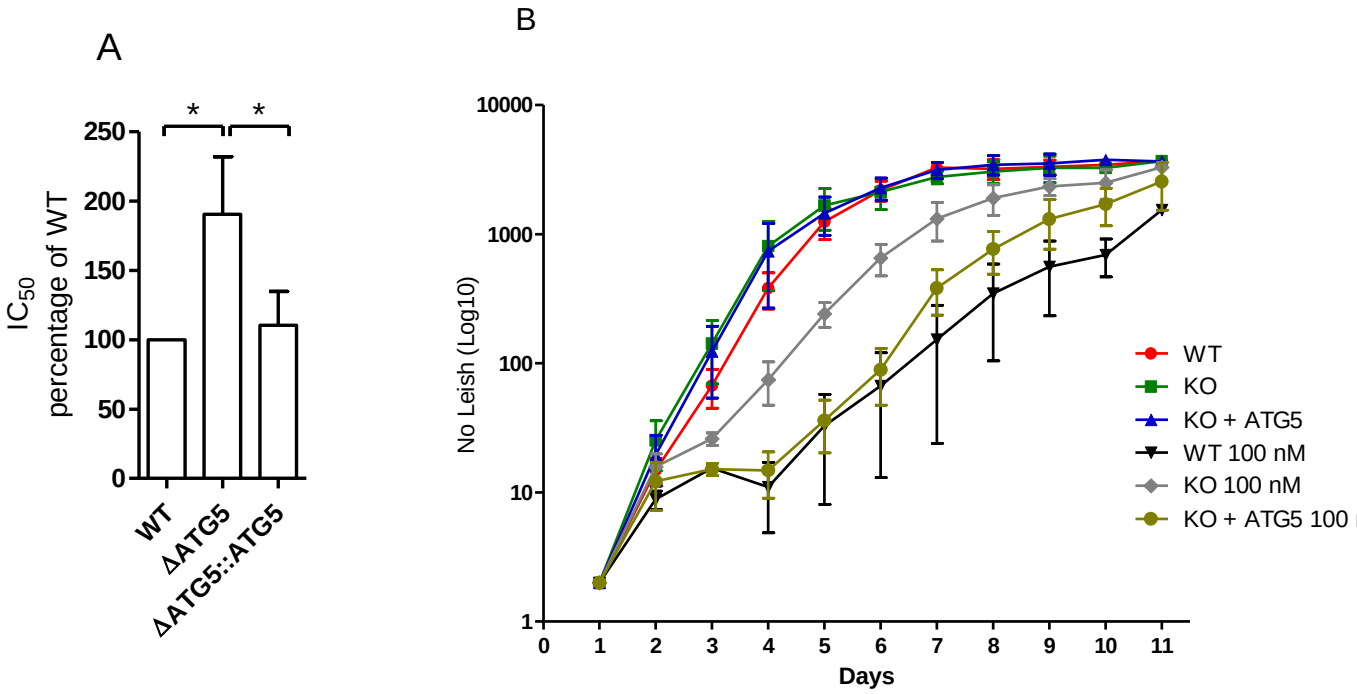


Figure 4.

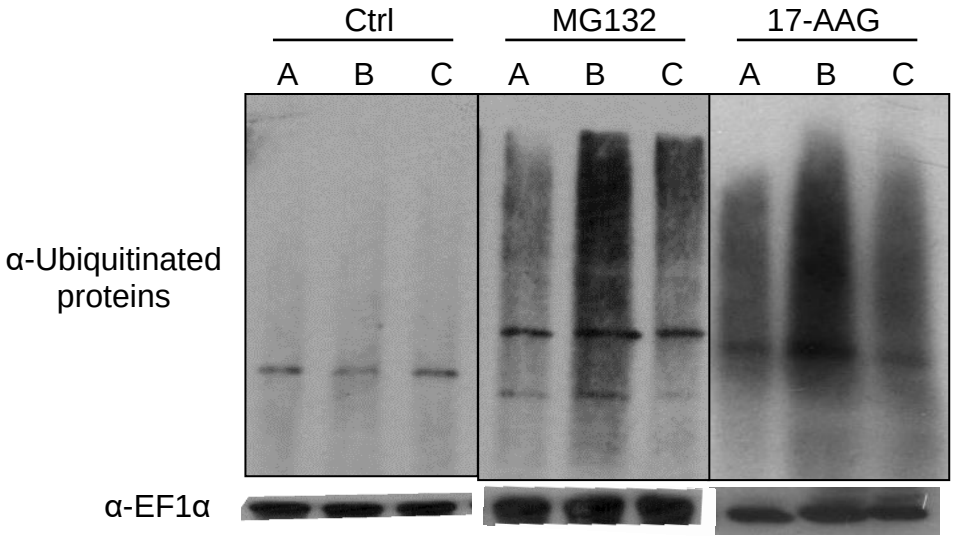
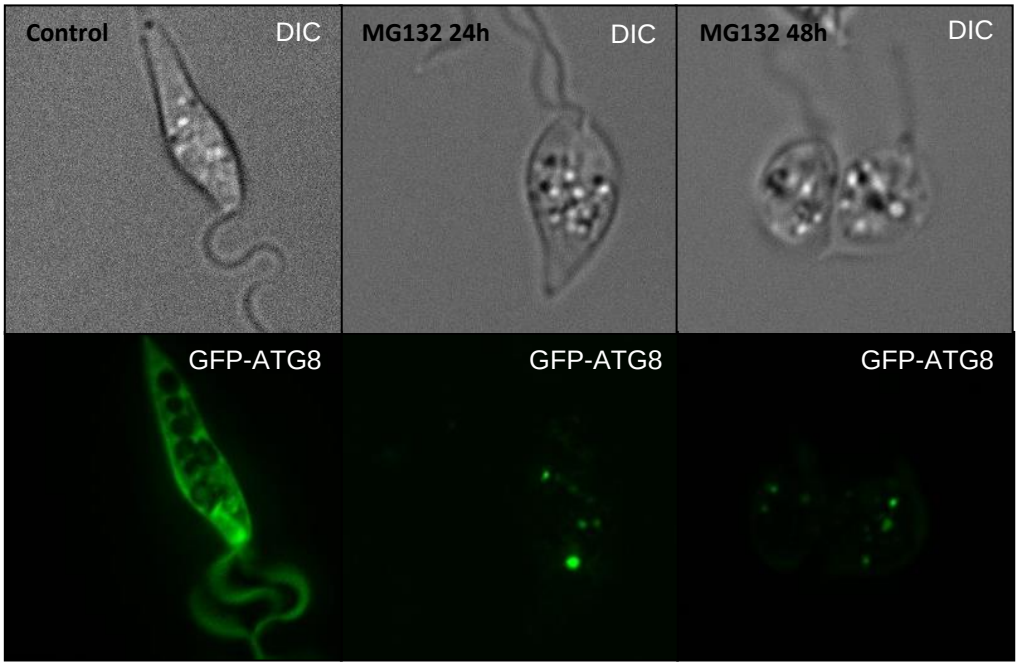
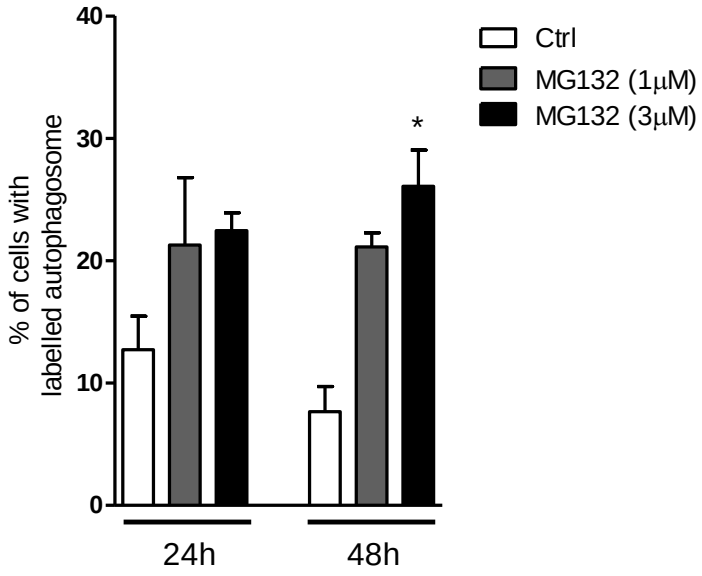

Ctrl
MG132
17-AAG
A
B
C
A
B
C
A
B
C
α-Ubiquitinated proteins
α-EF1α

Figure 5.

A


Control
DIC
MG132 24h
DIC
MG132 48h
DIC
GFP-ATG8
GFP-ATG8
GFP-ATG8

B


40
30
20
10
0
% of cells with labelled autophagosome
Ctrl
MG132 (1μM)
MG132 (3μM)
*
24h
48h

6 DISCUSSÃO GERAL

Apesar dos mais de 70 anos de investigação e investimentos para o entendimento e controle da leishmaniose, poucos avanços foram traduzidos de forma prática que facilitasse ou minimizasse as dificuldades dos pacientes acometidos por essa doença. As diferentes formas da doença continuam sendo endêmicas em países subdesenvolvidos ou em desenvolvimento e continuam acometendo, principalmente, a parcela mais pobre e sujeita aos quadros mais graves da leishmaniose (Alvar, Yactayo, et al., 2006).

O tratamento convencional da leishmaniose está associado a efeitos colaterais graves, altos custos para os sistemas de saúde e à falha terapêutica. A leishmaniose, em particular a LT, é provavelmente passível de ser controlada por um programa eficiente de vacinação, uma vez que a cura de uma infecção primária leva à resistência do hospedeiro a infecções subsequentes (revisado por (Kedzierski et al., 2006). Estudos com modelos animais reforçam a ideia de que é possível atingir proteção através da vacinação, no entanto, tais vacinas não se mostraram eficientes quando testadas em campo (Kedzierski et al., 2006). A complexidade da interação parasito-hospedeiro em humanos e resultados controversos em relação às vacinas indicam que o tratamento contra a leishmaniose ainda reside nos quimioterápicos.

Nessa tese avaliamos a atividade leishmanicida do 17-AAG, inibidor da HSP90, e observamos que: i – 17-AAG apresenta atividade anti-*Leishmania* contra as formas promastigotas e intracelulares de *L. amazonensis* e *L. braziliensis*; ii – 17-AAG apresenta efeito leishmanicida em modelo murino de LT; iii – a morte da

Leishmania, induzida pelo tratamento com 17-AAG é parcialmente dependente do processo autofágico. Apesar de inibidores da HSP90 ainda não terem sido aprovados para uso em humanos pela "*Food And Drug Administration*" norte americana ou outros órgãos controladores de qualquer país, vários desses inibidores foram e estão sendo testados em ensaios clínicos de diferentes estágios quanto ao seu potencial quimioterápico contra diferentes tipos de neoplasias (Jhaveri et al., 2012; Jhaveri et al., 2014; Sidera e Patsavoudi, 2014).

Inibidores da HSP90, a exemplo da geldanamicina, radicicol e 17-AAG, foram testados em relação à sua atividade microbicida anteriormente com resultados positivos (Shonhai et al., 2011; Roy et al., 2012; Angel et al., 2013; Bayih e Pillai, 2014). A maior e mais direta evidência de que inibidores da HSP90 possuem efeito microbicida devido a sua capacidade de ligar-se à HSP90 de parasitos veio do estudo de Pallavi e colaboradores de 2010. Os autores evidenciaram que geldanamicina se liga com grande afinidade a PfHSP90, inibindo a atividade ATPásica dessa chaperona com concentração três vezes menor do que a concentração necessária para inibir a atividade ATPásica da HuHSP90, indicando que a HSP90 do *Plasmodium* é mais sensível à inibição. Os autores também evidenciaram que a inibição da HSP90 por 17-AAG foi capaz de inibir o crescimento do parasito *in vitro* e *in vivo* (Pallavi et al., 2010). Em outro trabalho, o tratamento de *T. gondii* com inibidor da HSP90 bloqueou sua capacidade de invadir células hospedeiras e de transição entre as fases taquizoíto para bradizoíto e desta para taquizoíto (Ahn et al., 2003; Echeverria et al., 2005). Mesmo em fungos existem evidências de que a HSP90 pode ser utilizada como potencial alvo terapêutico. Em casos de pacientes com candidíase invasiva, pacientes tratados com anfotericina B

e anticorpos contra HSP90 apresentaram uma melhora clínica mais rápida quando comparado a pacientes que receberam apenas o tratamento com anfotericina B (Pachl et al., 2006). Estudos utilizando inibidores da HSP90 contra *Leishmania* foram realizados no início dos anos 2000; no entanto, esses estudos buscavam caracterizar e avaliar o papel da HSP90 no controle do ciclo celular do parasito (Wiesgigl e Clos, 2001). Esses estudos evidenciaram que a HSP90 é, pelo menos parcialmente, responsável pelo controle da mudança de estágio do ciclo celular de promastigota para amastigota, e concentrações maiores de geldanamicina inibem o crescimento do parasito na fase G2 do ciclo celular (Wiesgigl e Clos, 2001). Em conjunto, esses dados fornecem suporte à ideia de que a HSP90 é um alvo molecular contra doenças parasitárias. Nesse contexto, no entanto, o uso de inibidores da HSP90 como potenciais quimioterápicos para o tratamento das leishmanioses nunca havia sido investigado, até o presente estudo. Observamos um efeito leishmanicida do 17-AAG contra a forma promastigota e intracelular do parasito, mesmo após 96 h de infecção quando o parasito intracelular se diferenciou completamente em amastigotas. Esse efeito leishmanicida necessita de inibidores na concentração nanomolar, como observado para outros parasitos (Pallavi et al., 2010). Além disso, notamos que macrófagos possuem um IC_{50} superior ao observado no tratamento de promastigotas isoladas, indicando que *Leishmania* é mais sensível ao tratamento com inibidores da HSP90 do que células de mamífero. Ensaios realizados utilizando geldanamicina ou ainda radicicol, conhecido inibidor da HSP90 pertencente a outra classe química, foram capazes de eliminar *Leishmania* intracelular (dado não mostrado).

A principal vantagem do uso da HSP90 como alvo molecular contra doenças parasitáriasbaseia-se na estratégia de que uma única droga pode inibir a expressão de diversas proteínas como cinases, fatores de transcrição e proteínas controladoras do ciclo celular do parasito (Taipale et al., 2010). Os parasitos protozoários, a exemplo da *Leishmania*, têm uma grande expressão e dependência funcional da HSP90, assim, a inibição da HSP90 afetaria o metabolismo e funcionamento dos parasitos em uma extensão maior do que as células do hospedeiro. Além disso, a adaptação do parasito a um ambiente intracelular, ácido, com baixa disponibilidade de oxigênio e nutrientes leva o parasito a uma situação de estresse, reforçando a ideia de que, nessa situação, o parasito depende do bom funcionamento de chaperonas como a HSP90.

Nosso grupo de pesquisa trabalha com o modelo de susceptibilidade, no qual, macrófagos de camundongos infectados por *L. amazonensis* apresentam altas taxas de infecção em tempos tardios como 48 e 72h. A sobrevivência intracelular da *L. amazonensis* está associada a um estado de inativação da célula hospedeira (Gomes et al., 2003). As células infectadas por *L. amazonensis* produzem baixos níveis de H_2O_2 e a *Leishmania* é capaz de sobreviver, mesmo após a ativação dos macrófagos com IFN-γ e TNF-α (Gomes et al., 2003). Assim, foi demonstrado que *L. amazonensis* inativa e resiste aos mecanismos microbicidas, sugerindo que o contato inicial do parasito com o macrófago leva à produção de fatores que podem determinar o curso da infecção (Gomes et al., 2003). Utilizando esse modelo de infecção fomos capazes de evidenciar o potencial leishmanicida do 17-AAG (Petersen et al., 2012). O uso do 17-AAG eliminou tanto as formas promastigotas extracelulares quanto a forma amastigota intracelular associado a uma redução de

moléculas inflamatórias a exemplo do TNF-α, IL-6 e MCP-1 (Petersen et al., 2012). A inibição da HSP90 está associada a uma diminuição geral da resposta inflamatória em diferentes modelos de estudo como na sepsis, encefalite autoimune, aterosclerose e uveíte (Dello Russo et al., 2006; Chatterjee et al., 2007; Poulaki et al., 2007; Madrigal-Matute et al., 2010). O provável mecanismo que explique esse efeito anti-inflamatório observado após o tratamento com 17-AAG é a redução da translocação nuclear do NF-κB (Shimp et al., 2012). Foi descrito que a proteína IκB cinase (IKK) que é responsável por fosforilar o IκB, direcionando essa proteína para degradação via proteassoma e, consequentemente, liberando o NF-κB para translocação nuclear, é uma proteína cliente da HSP90 (Broemer et al., 2004; Hinz et al., 2007). Assim, o tratamento com inibidores da HSP90 leva a redução da expressão de IKK e, consequentemente, à redução da translocação do NF-κB para o núcleo que o que pode justificar a redução da produção de IL-6, TNF-α e NO, como observado em nosso trabalho.

Ao mesmo tempo, a redução da produção de NO pode ser explicada pelo fato de que iNOS é uma proteína cliente da HSP90 e foi demonstrado que a inibição da HSP90 reduz a produção de NO devido a diminuição da expressão de iNOS (Kone et al., 2003; Xu et al., 2007; Ding et al., 2013). A HSP90 também participa da ativação da NADPH oxidase e da produção de superóxido. Assim, foi demonstrado que HSP90 estabiliza e transloca a proteína Rac1 do citoplasma para a membrana de células epiteliais promovendo a ativação da NADPH oxidase (Cha et al., 2010). Outros trabalhos também evidenciam a importância da HSP90 na estabilidade da NADPH oxidase e produção de superóxido em diferentes tipos celulares (Chen et al., 2011; Chen et al., 2012). Esse grupo de pesquisa evidenciou que a inibição

genética ou farmacológica da HSP90 reduz a produção de superóxido derivado da Nox5. Os autores também demonstraram que Nox5 interage com a HSP90 e que o tratamento com inibidores da HSP90 reduzem a expressão da Nox5, sugerindo que essa molécula é uma proteína cliente da HSP90. Adicionalmente, foi demonstrado que inibição farmacológica da HSP90 em macrófagos e neutrófilos causa a redução da expressão das proteínas NOX1 e 2 com subsequente redução da produção de superóxido. Esses dados corroboram com nossos estudos que claramente mostraram uma redução da produção de superóxido ou NO, mesmo em macrófagos estimulados com IFN-γ (Manuscritos I e II) (Chen et al., 2011; Chen et al., 2012).

O efeito leishmanicida do 17-AAG, além de ter sido avaliado em modelo de infecção por *L. amazonensis* (Manuscrito I), também foi avaliado em modelo de infecção por *L. braziliensis* (Manuscrito II). A primeira espécie de *Leishmania* em geral causa LCL, enquanto a segunda é conhecida por causar infecções crônicas e latentes com cerca de 5% dos pacientes evoluindo para o quadro mais grave da doença, a LMC (Schubach et al., 1998; Mendonca et al., 2004). Existem evidencias que diferentes espécies de protozoários respondem de forma distinta à inibição da HSP90, desta forma, apesar de *L. amazonensis* e *L. braziliensis* pertencerem ao mesmo gênero, seria plausível sugerir que as respostas desses parasitos frente ao tratamento com 17-AAG fossem distintas (Giannini e Battistuzzi, 2015). Por outro lado, o tripanosomatídeo *T. cruzi*, independente da forma de epimastigota ou tripomastigota, quando tratado com geldanamicina tem seu ciclo celular bloqueado na fase G1. Além disso, ao contrário de *Leishmania*, o bloqueio da HSP90 de *T. cruzi* não induz diferenciação ou mudança do estágio do parasito (Graefe et al., 2002). Assim, esses dados em conjunto sugerem fortemente que as HSP90 são

conservadas em parasitos de mesmo gênero, apesar de apresentarem diferenças funcionais em parasitos de gênero diferentes (Giannini e Battistuzzi, 2015).

Os resultados obtidos com o modelo de *L. braziliensis* corroboram a ideia de o bloqueio da HSP90 é eficiente para o controle da infecção por *Leishmania* em modelos experimentais *in vitro* (Santos et al., 2014). Nossos dados mostraram que IC_{50} de 17-AAG de promastigotas tanto de *L. amazonensis* quanto de *L. braziliensis* é 65 nM (Manuscritos I e II). Os IC_{50} calculados para *Leishmania* intracelular também tiveram valores similares e foram calculados como sendo 149 nM para *L. amazonensis* (Manuscrito I) e 220 nM para *L. braziliensis* (Manuscrito II). Os valores obtidos por Kumar e colaboradores em 2003 (Kumar et al., 2003) mostraram um IC_{50} de apenas 20 nM em culturas eritrocitárias de *P. falciparum* quando tratados com geldanamicina, o que corrobora a alta afinidade desse inibidor pela HSP90 de *P. falciparum* (Pallavi et al., 2010). Nesse contexto, os nossos dados reforçam os observados na literatura de que inibidores da HSP90, mesmo em baixas concentrações, são capazes de inibir o crescimento e promover a morte de protozoários parasitos. Essa constatação é de extrema importância, uma vez que a toxicidade de inibidores da HSP90 é fator limitante em ensaios clínicos em pacientes com diferentes tipos de neoplasias. Nesses casos, as concentrações necessárias para se atingir o resultado esperado, como por exemplo, estabilização ou redução do tamanho de um tumor, são altas e não são bem toleradas pelos pacientes (Jhaveri et al., 2012; Jhaveri et al., 2014). Alternativamente, é possível que as doses necessárias para o tratamento de pacientes com doenças parasitárias sejam baixas, como indicado pelos achados dos estudos (Manuscrito I e Manuscrito II) aqui apresentados.

As evidências mostradas nos dois manuscritos indicam que o efeito leishmanicida do 17-AAG ocorre diretamente sobre o parasito intracelular: i) macrófagos murinos quando tratados com 17-AAG não ativam a capacidade leishmanicida; ii) reduzem a produção de citocinas pró-inflamatórias; iii) reduzem a produção de moléculas microbicidas como NO e superóxido; iv) o efeito leishmanicida do 17-AAG ocorre diretamente sobre promastigotas de cultura axênica. As imagens analisadas por microscopia eletrônica de transmissão (Manuscrito I) revelaram alterações ultraestruturais nos parasitos sugestivas de autofagia, o que nos levou investigar se o mecanismo de morte induzido pelo 17-AAG estava relacionado com a ativação da via autofágica (Manuscrito III).

Os parasitos transgênicos tratados com 17-AAG formaram vacúolos autofágicos que não amadurecem na via endocítica. Comparativamente em células eucarióticas mais complexas o tratamento com geldanamicina ou 17-DMAG inibiu o tráfego de vesículas entre o reticulo endoplasmático e o complexo de Golgi (Chen e Balch, 2006). Esses autores observaram que o Inibidor de Dissociação do Nucleotídeo Guanina (GDI) é estabilizado pela HSP90. Esse regulador negativo de pequenas proteínas G impede a liberação das pequenas proteínas G da membrana, mantendo a pequena proteína G inativa. Assim, a inibição farmacológica da HSP90 impede a reciclagem para o citoplasma de proteínas Rab ligadas à membrana de vesículas, dificultando novos ciclos de transporte (Chen e Balch, 2006). Outros autores também observaram que a HSP90 forma um complexo com a proteína de membrana VAP-33, que é uma proteína que, através de vesículas, exerce um papel importante no transporte de glicoproteínas a partir do complexo de Golgi. Esses dados mostram que a HSP90 influencia na regulação do transporte intracelular de

vesículas (Lotz et al., 2008). É possível que uma inibição similar esteja acontecendo nos parasitos tratados com 17-AAG, dificultando a coordenação do tráfego de vesículas no citoplasma do parasito.

Parasitos *knockout* para o gene ATG5 (ΔATG5), são cerca de 90% mais resistentes à morte induzida pelo tratamento com 17-AAG, indicando que a formação de vacúolos autofágicos é um importante fator para o mecanismo de morte induzido por esse fármaco. Esse tipo de morte que ocorre independente da ativação de caspases, sem características de necrose e com presença persistente do processo autofágico tem sido atribuído a uma redução da disponibilidade de organelas e proteínas essenciais para a manutenção da célula (Yu et al., 2004; Amaravadi e Thompson, 2007). A presença de características autofágicas em parasitos submetidos a condições de estresse é comum e foi observada por diferentes autores (Bera et al., 2003; Delgado et al., 2009; Sengupta et al., 2011; De Almeida Nogueira et al., 2013; Tiuman et al., 2014). Existe, no entanto, uma grande dificuldade em relacionar a indução da autofagia e a morte celular (Levine e Yuan, 2005; Klionsky et al., 2012), principalmente devido ao processo autofágico estar normalmente relacionado à sobrevivência e homeostase celular (Lum et al., 2005; Degenhardt et al., 2006).

Sengupta e colaboradores (Sengupta et al., 2011) demonstraram que a morte de parasitos de *L. donovani,* após o tratamento com criptolepina, uma indoloquinolina conhecida por sua atividade anti-malárica, apresentou alterações morfológicas como formação de autofagossomo e que o tratamento com 3-methiladenina, um conhecido inibidor de autofagia, aumenta a morte celular do

parasito induzida pela criptolepina. Esses resultados sugerem um papel protetor da autofagia uma vez que a inibição da mesma aumenta a morte celular (Sengupta et al., 2011). Nossos achados com *Leishmania* tratadas com 17-AAG são similares aos observados em *Trypanosoma cruzi* quando tratados com naftoimidazóis (Menna-Barreto, Correa, et al., 2009). A morte desses parasitos após o tratamento com naftoimidazóis foi associada a diferentes alterações estruturais, incluindo mitocondriais, no Complexo de Golgi e formação de autofagossomas (Menna-Barreto et al., 2005; Menna-Barreto, Correa, et al., 2009; Menna-Barreto, Salomao, et al., 2009). O tratamento desses parasitos com zVAD, conhecido inibidor de apoptose, não impediu a morte dos parasitos após o tratamento com naftoimidazóis (Menna-Barreto, Correa, et al., 2009), no entanto, o tratamento com o inibidor de autofagia 3-methiladenina bloqueou completamente a morte do parasito, sugerindo a autofagia como provável causa de morte celular (Menna-Barreto, Correa, et al., 2009).

O mecanismo pelo qual inibidores de HSP90 induzem a formação de vacúolos autofágicos ainda não foi investigado em protozoários, mas sim em modelos de células neoplásicas. Foi descrito que a geldanamicina é capaz de induzir autofagia por um mecanismo dependente da inibição da via da mTOR. Os autores mostraram que, nesse modelo, a autofagia age como um mecanismo de proteção contra a morte celular induzida pela geldanamicina, protegendo as células cancerígenas contra apoptose, uma vez que o tratamento com 3-methiladenina promoveu a morte celular por apoptose (Mori et al., 2015). Como os tripanossomatídeos possuem todas as proteínas necessárias para a ativação do processo autofágico, inclusive proteínas homólogas a mTOR (Barquilla e Navarro,

2009), é possível que de forma similar à observada em células de mamífero a inibição farmacológica da HSP90 também leve a indução de autofagia de forma dependente da inibição de mTOR de *Leishmania*. Em mamíferos, também existem evidências que a degradação específica da IkB cinase ocorre pela via autofágica após inibição da HSP90 (Qing et al., 2006). Diferentemente da maioria das proteínas cliente da HSP90 que são degradadas via proteassoma (Isaacs et al., 2003), IkB cinase é degradada via autofagia por um mecanismo dependente de ATG5 (Qing et al., 2006). É possível que algumas das proteínas cliente da HSP90 de protozoários sejam degradadas por um mecanismo similar ao observado com a IkB cinase em células de mamíferos com formação de vacúolos autofágicos, explicando a formação de autofagossomos após o tratamento de *Leishmania* com 17-AAG. Mas a falta de conhecimento sobre quais são as proteínas cliente da HSP90 em protozoários e os seus mecanismos de controle ainda limitam o nosso conhecimento de como essas proteínas são degradadas. Os nossos achados indicam que a morte celular induzida pelo 17-AAG é dependente de autofagia. Esse tipo de morte descrita por Klionsky e colaboradores em 2012 (Klionsky et al., 2012) aplica-se a nossas observações, uma vez que a inibição genética da autofagia retardou, mas não preveniu completamente a morte do parasito. Apesar de nós não termos excluído a necrose ou apoptose como possíveis mecanismos da morte celular em parasitos de *Leishmania* tratados com 17-AAG, nossos dados suportam a de que a formação de vacúolos autofágicos favorece a ação leishmanicida do 17-AAG. No entanto, o mecanismo pelo qual ocorre a morte do parasito ainda precisa ser esclarecido. Uma vez que a HSP90 atua controlando a expressão de diversas proteínas relacionadas com diferentes vias celulares (Taipale et al., 2010), além de

agir estabilizando proteínas lábeis em situações de estresse celular (Theodoraki e Caplan, 2012) é pouco provável que haja uma única via ou um único mecanismo responsável pela morte celular da *Leishmania* após o tratamento com 17-AAG. É possível que os parasitos ΔATG5, por não formarem autofagossomos, sejam mais resistentes aos efeitos induzidos pelo tratamento com 17-AAG em comparação com os parasitos WT. No entanto, os mesmos parasitos ΔATG5 morrem ao serem tratados com concentrações maiores de 17-AAG, indicando que um segundo mecanismo, além da ativação desregulada da via autofágica, contribua com a morte celular induzida pelo 17-AAG.

A observação de que o tratamento de parasitos com MG132 induz a formação de autofagossomas, associado ao fato de que parasitos ΔATG5 não são capazes de degradar proteínas ubiquitinadas com a mesma eficiência que parasitos selvagens é provavelmente o achado mais importante do terceiro manuscrito. Nossos dados mostram que o tratamento de *Leishmania* com 17-AAG induziu um acúmulo de proteínas ubuquitinadas (Manuscrito III). Foi demonstrado em células de mamífero que o acúmulo de proteínas ubiquitinadas é capaz de induzir a ativação da via autofágica como um mecanismo de proteção celular contra danos causados pela exposição de porções hidrofóbicas de agregados proteicos (Tyedmers et al., 2010; Theodoraki e Caplan, 2012). Esse mecanismo de homeostasia da célula funciona junto com a ação do proteassoma no controle de qualidade proteica (Cuervo et al., 2010; Tyedmers et al., 2010; Theodoraki e Caplan, 2012). Em levedura, proteínas ubiquitinadas podem associar-se à ATG8 da face interna de vacúolos autofágicos em formação através da proteína adaptadora ATG19 que medeia a entrega de alvos selecionados para o vacúolo em um processo

constitutivo chamado de *cytoplasm-to-vacuole targeting* (Cvt) (Hutchins e Klionsky, 2001; Scott et al., 2001; Yuga et al., 2011). Adicionalmente, foi demonstrado que esse processo é mecanicamente equivalente à autofagia seletiva observada em células de mamíferos (Ichimura et al., 2008; Noda et al., 2008; Nakatogawa et al., 2009), em que ocorre a seleção do componente celular que será capturado pelo vacúolo autofágico. É possível que um mecanismo similar esteja acontecendo em *Leishmania* de forma que as proteínas ubiquitinadas no seu citoplasma após tratamento com 17-AAG ou MG132 estejam sendo direcionadas para os vacúolos autofágicos.

Outra observação importante foi a identificação do efeito leishmanicida do 17-AAG em ensaios *in vivo* no modelo de LCL. Observamos que camundongos tratados com 17-AAG apresentaram um tempo de cura da lesão reduzido em relação aos animais tratados apenas com DMSO, além da redução da carga parasitária na lesão (Manuscrito II). Outros trabalhos também têm reportado resultados positivos e interessantes em ensaios pré-clinicos. Camundongos infectados com *P. berghei* e tratados com 17-AAG tiveram a parasitemia reduzida para valores inferiores a 10% e uma taxa de sobrevivência de 50% enquanto 100% dos animais controle, não tratados, vieram a óbito (Pallavi et al., 2010). Neste mesmo estudo, utilizando o modelo de *P. yoelii*, o tratamento com 17-AAG zerou a parasitemia com uma taxa de sobrevivência de 60%, enquanto animais controle não tratados tiveram uma taxa de 100% de óbitos (Pallavi et al., 2010). Outro trabalho, utilizando o modelo de *P. yoelii* também demonstrou uma redução completa da parasitemia, associada a uma sobrevivência de 75% dos animais, em comparação com 100% dos animais controle que vieram a óbito (Mout et al., 2012). Os animais

curados apresentaram um alto grau de resistência contra novos desafios com uma proteção elevada de anticorpos contra diferentes proteínas do parasito (Mout et al., 2012). Em outro trabalho recente, utilizando camundongos infectados com *T. brucei*, agente etiológico da doença do sono, os autores evidenciaram que o tratamento desses animais com 17-DMAG pelas vias intraperitoneal ou oral por gavagem reduzem a parasitemia para limites abaixo do detectável e aumentam a sobrevida dos animais para 100 e 75% respectivamente, enquanto 100% dos animais controle não tratados vieram a óbito após apenas quatro dias de infecção (Meyer e Shapiro, 2013). Os resultados observados em nossos ensaios não foram tão robustos quanto os descritos para outros parasitos protozoários, uma vez que observamos a redução da carga parasitária na derme de 10^7 nos animais controle para 10^6 nos animais tratados e nenhuma alteração da carga parasitária do linfonodo drenante. É possível que a persistência dos parasitos de *L. braziliense*s no local inicial da infecção dificulte a ação do 17-AAG, uma vez que, em estudos de distribuição tecidual, camundongos tratados via intraperitoneal com 40 mg/kg de 17-AAG apresentaram um pico de 24 µg/g em musculo esquelético e 6 h após o tratamento os níveis de 17-AAG foram zero (Egorin et al., 2001). Assim, este fármaco teria dificuldade de atingir os tecidos periféricos, a exemplo da derme e epiderme (Egorin et al., 2001). É possível que o uso de curativos dérmicos ou pomadas contendo 17-AAG sejam mais eficientes para o tratamento de LCL, uma vez que o fármaco poderia atingir concentrações maiores no local da infecção.

A identificação de que a HSP90 é um alvo molecular para o tratamento de doenças causadas por parasitos protozoários causou uma explosão na busca de novos inibidores e formas mais eficientes de identificá-los (Kumar et al., 2007; Wider

et al., 2009; Angel et al., 2013; Meyer e Shapiro, 2013; Pizarro et al., 2013; Shahinas et al., 2013; Rochani et al., 2014; Giannini e Battistuzzi, 2015). Utilizando a abordagem de quimioinformática foi possível projetar análogos da geldanamicina com alta afinidade para HSP90 de protozoários e afinidade reduzida para HSP90 humana, sugerindo que esses novos análogos podem ser candidatos a drogas (Singh e Atri, 2013). Esses análogos, no entanto, ainda precisam ser sintetizados e passar por ensaios pré-clinicos para avaliação da sua atividade microbicida. Em outro estudo recente, os autores testaram 15 inibidores da HSP90 derivados de três diferentes estruturas moleculares em *T. brucei, T. cruzi, P. falciparum* e *Giardia lamblia*. O IC_{50} foi calculado para todos os inibidores e as drogas com melhor desempenho foram selecionadas para ensaios *in vivo* com resultados promissores (Giannini e Battistuzzi, 2015). Trabalhos ainda mais complexos e em larga escala foram realizados na tentativa de identificar compostos específicos, capazes de inibir com maior afinidade a HSP90 de *Plasmodium* do que a HSP90 humana. Nesse estudo, os autores utilizaram uma avaliação robótica, em larga escala, utilizando cerca de 4.000 compostos pré-aprovados pela *Food and Drug Administration* – FDA americana contra a HSP90 de *P. falciparum*. Dos 4.000 compostos avaliados três foram selecionados e prosseguiram para ensaios *in vitro* utilizando eritrócitos parasitados e todos os compostos testados apresentaram IC_{50} com concentrações na faixa de nanomolar inclusive contra *Plasmodium* resistente à cloroquina (Shahinas et al., 2010). Trabalhos em larga escala também têm sido conduzidos na tentativa de identificar inibidores mais específicos da HSP90 de *T. brucei*. Os autores expressaram HSP90 recombinante e através de um ensaio de varredura fluorimétrica diferencial identificaram diferentes compostos capazes de inibir a

HSP90 de *T. brucei*, com pouco efeito inibitório sobre a HSP90 humana. Adicionalmente, nesses estudos foram realizados ensaios de interação estrutural que indicam ser possível o desenvolvimento de novas drogas com maior especificidade e poder de interação com HSP90 de parasitos em relação à HSP90 humana (Pizarro et al., 2013).

Em conjunto, os dados da literatura associados com os resultados obtidos por nossa equipe reforçam a idéia de que a HSP90 é um excelente alvo molecular que deve ser extensivamente explorado no combate a doenças parasitárias. O grande número de compostos presentes no mercado e o fato de uma grande parcela desses compostos terem sido explorados previamente em relação a doenças neoplásicas irá facilitar e acelerar o desenvolvimento dos estudos em relação às doenças parasitárias. Nosso estudo vem a somar com os dados da literatura, reforçando a ideia de que inibidores da HSP90, além de sua utilização no combate a cânceres, podem também ser utilizados contra infecções causadas por *Leishmania*.

7 CONCLUSÕES

- 17-AAG mostrou ser um agente com efeito anti-*Leishmania*
- 17-AAG causa morte de *Leishmania* em macrófagos tratados por um mecanismo independente da produção de moléculas ativadoras de macrófagos.
- A morte do parasito induzida por 17-AAG depende parcialmente da ativação da via autofágica
- A via autofágica de *Leishmania* tem um papel importante na degradação de proteínas ubiquitinadas

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