INGRID RABITE GARCIA

AVALIAÇÃO *IN SILICO* DA INTERAÇÃO DO DERIVADO DE HEPARINA NÃO ANTICOAGULANTE DOCIPARSTAT SODIUM (DSTAT) COM LPG3 E DE SEU EFEITO *IN VITRO* NA INFECÇÃO DE MACRÓFAGOS POR *Leishmania chagasi*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Biologia Celular e Estrutural, para obtenção do título de *Doctor Scientiae.*

Orientador: Eduardo de Almeida M. da Silva

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Eduardo de Almeida Marques da Silva
Orientador

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RESUMO

GARCIA, Ingrid Rabite, D.Sc., Universidade Federal de Viçosa, junho de 2023. **Avaliação *in silico* da interação do derivado de heparina não anticoagulante Dociparstat Sodium (DSTAT) com LPG3 e de seu efeito *in vitro* na infecção de macrófagos por *Leishmania chagasi***. Orientador: Eduardo de Almeida Marques da Silva.

Contextualização: Há uma necessidade urgente de novos tratamentos para combater a Leishmaniose Visceral (LV). A proteína LPG3 de *Leishmania chagasi* foi considerada por nosso grupo de pesquisa como potencial alvo para novos fármacos contra LV.O bloqueio dessa proteína pela heparina sódica reduziu a internalização de formas promastigotas do parasita em macrófagos. No entanto, o uso da heparina sódica na LV é limitado devido a sua potente atividade anticoagulante. **Objetivo**: Investigar *in silico* e *in vitro* a hipótese de que o bloqueio da proteína LPG3 pela heparina não-anticoagulante (DSTAT) interfere no processo de infecção parasitária. **Métodos**: Primeiramente, previmos *in silico* a interação de DSTAT com a proteína LPG3, bem como sua afinidade de ligação, utilizando *docking* molecular do tipo receptor-ligante. Em seguida, DSTAT foi utilizada em ensaios *in vitro* para testar a toxicidade em macrófagos RAW 264.7, sua atividade leishmanicida contra formas promastigotas de *L. chagasi* e sua atividade antileishmania em processos de adesão e internalização de formas promastigotas de *L. chagasi* em macrófagos. **Resultados:** O *docking* molecular revelou a ligação de DSTAT no sítio previsto para heparina na proteína LPG3 com uma afinidade de ligação ligeiramente maior que a heparina sódica. Nos ensaios *in vitro*, as concentrações testadas de DSTAT não foram tóxicas para os macrófagos e a maior concentração selecionada (300 µg/mL de DSTAT) não apresentou efeito leishmanicida contra formas promastigotas de *L. chagasi*. Por outro lado, DSTAT (100 µg/mL) levou à redução na internalização de *L. chagasi* em macrófagos, sem interferir na adesão parasitária. **Conclusão:** Tratamento com heparina não-anticoagulante DSTAT interfere na infecção de macrófagos por formas promastigotas de *L. chagasi*, provavelmente pelo bloqueio da proteína LPG3 do parasito, sem causar toxicidade para ambas células.

Palavras-chave: LPG3. Leishmaniose Visceral. *Leishmania chagasi*. Glicosaminoglicanos. Heparina.

ABSTRACT

GARCIA, Ingrid Rabite, D.Sc., Universidade Federal de Viçosa, June, 2023. ***In silico* evaluation of the interaction of the non-anticoagulant heparin derivative Dociparstat Sodium (DSTAT) with LPG3 and its *in vitro* effect on macrophage infection by *Leishmania chagasi***. Adviser: Eduardo de Almeida Marques da Silva.

Background: There is an urgent need for new treatments to combat Visceral Leishmaniasis (VL). The LPG3 protein from *Leishmania chagasi* was considered by our research group as a potential target for new drugs against VL. The blockade of this protein by sodium heparin reduced the internalization of promastigotes forms of the parasite in macrophages. However, the use of heparin sodium in VL is limited due to its potent anticoagulant activity. **Objective:** We to investigate *in silico* and *in vitro* the hypothesis that the blockade of the LPG3 protein by non-anticoagulant heparin (DSTAT) interferes in the process of parasitic infection. **Methods**: First, we predicted *in silico* the interaction of DSTAT with the LPG3 protein, as well as their binding affinity, through receptor-ligand molecular docking. Then, DSTAT was used in *in vitro* assays to test its toxicity on RAW 264.7 macrophages, its leishmanicidal activity against promastigotes of *L. chagasi* and its antileishmanial activity in processes of adhesion and internalization of *L. chagasi* promastigotes in macrophages. **Results:** Molecular docking revealed DSTAT binding to the heparin predicted site on the LPG3 protein with a slightly higher binding affinity than heparin sodium. In the *in vitro* assays, the DSTAT concentrations tested were not toxic to macrophages and the highest selected concentration (300 µg/mL of DSTAT) did not present leishmanicidal effect against *L. chagasi* promastigotes forms. On the other hand, DSTAT (100 µg/mL) led to a reduction in *L. chagasi* internalization in macrophages, without interfering with parasite adhesion. **Conclusions:** Treatment with non-anticoagulant heparin DSTAT interferes with the infection of macrophages by promastigotes of *L. chagasi*, probably by blocking the parasite's LPG3 protein, without causing toxicity to both cells.

Keywords: LPG3. Visceral leishmaniasis. *Leishmania chagasi*. Glycosaminoglycans. Heparin.

LISTA DE ABREVIATURAS

LV – Leishmaniose Visceral

LPG3 – Lipophosphoglycan 3 protein

DSTAT – Dociparstat sodium

TP – Tempo de Protrombina

TTPa – Tempo de Tromboplastina Parcial ativado

GAGs – Glicosaminoglicanos

PLH – Proteína Ligante de Heparina

PLH*Lc* – Proteína Ligante de Heparina de *Leishmania chagasi*

LPG3 – Lipophosphoglycan 3 gene

HNF – Heparina não fracionada

HBPM – Heparina de baixo peso molecular

HUBPM – Heparina de ultrabaixo peso molecular

HBP – Heparin Binding Protein

HS – Heparan Sulfate

Anti-rLPG3 – Antibody anti- recombinant Lipophosphoglycan 3 protein

rLPG3 – Recombinant Lipophosphoglycan 3 protein

MTT – 3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

IdoA – Iduronic Acid

APRESENTAÇÃO

A presente tese foi elaborada de acordo com as normas estabelecidas pela Pró Reitoria de Pesquisa e Pós-Graduação da Universidade Federal de Viçosa – UFV. O corpo do trabalho compreende em uma introdução geral e um artigo. O artigo intitulado "*In silico* evaluation of the interaction of the non-anticoagulant heparin derivative Dociparstat Sodium (DSTAT) with LPG3 and its *in vitro* effect on macrophage infection by *Leishmania chagasi*." será submetido à revista *International Journal for Parasitology.*

SUMÁRIO

INTRODUÇÃO GERAL

A Leishmaniose é uma doença tropical negligenciada causada por diferentes espécies de parasitas protozoários do gênero *Leishmania* (1). No hospedeiro mamífero, formas promastigotas metacíclicas (formas infectantes flageladas) do parasito são introduzidas na pele durante o repasto sanguíneo de flebotomíneos fêmeas infectadas. Após serem fagocitadas por macrófagos e outros tipos de células fagocitárias, as formas promastigotas metacíclicas se transformam em formas amastigotas (formas intracelulares sem flagelos externalizados) que.se multiplicam por divisão simples e continuam infectando outras células fagocíticas mononucleares (2-4). Dependendo da espécie infectante de *Leishmania* e da resposta imune do hospedeiro (5), as manifestações clínicas da leishmaniose podem variar de infecções assintomáticas até lesões em sítios cutâneos (leishmaniose cutânea – LC), mucosos (leishmaniose mucocutânea – LMC) ou em órgãos internos (leishmaniose visceral – LV) (6).

A forma clínica mais grave da doença em humanos é a LV, também conhecida como calazar (7). Estima-se que, anualmente, ocorram entre 50.000 a 90.000 novos casos da doença em todo mundo, a maioria deles no Brasil, África Oriental e Índia (8). A doença é causada pelas espécies *Leishmania donovani* e *Leishmania infantum* (sinônimo de *Leishmania chagasi* na América do Sul) (9, 10). O sistema retículo-endotelial do baço, medula óssea, fígado e nódulos linfáticos são os principais alvos do parasito e consequentemente, as manifestações clássicas de LV incluem hepatoesplenomegalia e pancitopenia, além de febre crônica e perda de peso (11). Embora a anemia seja a manifestação hematológica mais comum, outras alterações hematológicas estão associadas à LV como, por exemplo, alterações das funções plaquetárias e anormalidades de coagulação (TP e TTPA prolongados) (12).

Se não tratada, a LV é fatal em mais de 95% dos casos (13). Por décadas, os derivados antimoniais têm sido os medicamentos utilizados no tratamento da LV. Porém, a toxicidade e o aumento da resistência parasitária às drogas disponíveis contribuíram para o uso de drogas alternativas como miltefosina, paromomicina ou anfotericina B que, no entanto, também estão sendo associadas à toxicidade, a custos elevados, a regimes de longo prazo e ao surgimento de resistência (14). Portanto, há uma necessidade urgente de novos tratamentos para combater a LV. No Brasil, o

Ministério da Saúde recomenda três opções terapêuticas para o tratamento de LV: antimoniato de N-metilglucamina (primeira escolha), desoxicolato de anfotericina B (segunda escolha) e anfotericina B lipossomal. Os medicamentos de primeira e segunda escolha são altamente tóxicos e, portanto, são necessários testes específicos durante o tratamento para monitorar as funções do fígado, rim, pâncreas e coração (15). Embora as formulações lipossomais de anfotericina B sejam menos tóxicas, são mais caras e, por isso, no Brasil, são disponíveis mediante solicitação ao Ministério da Saúde somente para pacientes com algumas comorbidades, crianças menores de 1 ano e pacientes com mais de 50 anos (16).

Para o desenvolvimento de novos fármacos, é necessário uma melhor compreensão da interação *Leishmania* spp.-hospedeiro. Este conhecimento permite a descoberta de alvos cuja modulação ou bloqueio podem interferir no curso da infecção/doença (17). Sulfatos de heparana são glicosaminoglicanos que exibidos na forma de proteoglicanos, na matriz celular ou ao redor da célula hospedeira, interagem com proteínas da superfície do parasita *Leishmania* e podem contribuir para invasão da célula hospedeira, como relatado para alguns vírus, bactérias e outros parasitas (18). Os receptores de *Leishmania* envolvidos no reconhecimento dos glicosaminoglicanos (GAGs – preferencialmente moléculas como a heparina e o sulfato de heparana) são do tipo proteínas ligantes de heparina (PLH) e foram registrados pela primeira vez como fatores de virulência de *L. donovani*, mediando infecções em macrófagos pelo parasito (19).

Nosso grupo de pesquisa purificou, localizou e caracterizou uma PLH de formas promastigotas de *L. chagasi* (PLH*Lc*). Por imunomarcação, foi possível visualizar sua ampla distribuição na superfície do parasito e, internamente, próxima ao cinetoplasto. Além disso, prévia incubação de formas promastigotas de *L. chagasi* com heparina promoveu diminuição na frequência de internalização do parasito em macrófagos RAW "*in vitro*" (20), o que sugere a participação da PLH*Lc* no processo de infecção parasitária. Após sequenciamento proteico, a PLH*Lc* foi identificada como produto do gene lipofosfoglicano 3 (*LPG3*). Posteriormente, foi demonstrada sua capacidade de se ligar à heparina com afinidade micromolar e seu sítio envolvido nessa ligação foi previsto por intermédio de análises *in silico* (21). Diante desses resultados, a proteína LPG3, tanto na forma nativa quanto na recombinante, foi

considerada por nosso grupo de pesquisa como potencial alvo de teste como antígeno vacinal e para formulação de novos fármacos contra LV.

A heparina, um polissacarídeo altamente sulfatado da família dos glicosaminoglicanos, está envolvida em várias atividades biológicas importantes devido à sua capacidade de interagir com várias proteínas, sendo a inibição da cascata de coagulação sua principal atividade (22). De acordo com a sua massa molecular, a heparina pode ser classificada em três tipos: heparina não fracionada (HNF, massa molecular média ~15.000 Da), heparina de baixa massa molecular (HBPM, massa molecular média ~ 6.000 Da) e heparina de ultrabaixa massa molecular (HUBPM, massa molecular média < 2.000 Da). Embora a forma não fracionada seja a mais utilizada como anticoagulante, ela apresenta várias limitações, incluindo efeitos colaterais como: trombocitopenia induzida por heparina (TIH) e sangramento (23). A atividade anticoagulante dessas heparinas se deve a uma sequência pentassacarídica específica que se liga e ativa a antitrombina (AT), um inibidor de fatores da coagulação. Nas preparações de heparina, apenas uma fração das cadeias polissacarídicas contém a estrutura pentassacarídica específica e, portanto, se ligam a AT com alta afinidade e produzem atividade anticoagulante significativa (24). Nos últimos anos, houve um grande interesse no uso não anticoagulante das heparinas para tratar uma variedade de doenças que não fazem parte dos distúrbios trombolíticos (25), uma vez que a heparina demonstra ter propriedades anti-inflamatórias e antimetastáticas mas, no entanto, complicações associadas à sua potente atividade anticoagulante limitam seu uso nessas aplicações (26).

Vários estudos estão voltados para o desenvolvimento de terapias medicamentosas que removem a atividade anticoagulante da heparina para obter heparina não-anticoagulante (27). O processo de obtenção das heparinas não-anticoagulantes, a partir da heparina não fracionada, consiste na remoção das cadeias contendo a sequência de ligação à antitrombina ou na inativação dessa sequência por modificações em seus grupos ou resíduos. Entre as heparinas não-anticoagulantes, as mais amplamente estudadas são as heparinas seletivamente dessulfatadas e as heparinas “glycol-split” (28).

Considerado por alguns autores como uma heparina não-anticoagulante/derivado não-anticoagulante da heparina (29-31), Dociparstat sodium

(DSTAT; 2-O, 3-O heparina dessulfatada) é um derivado da heparina que apresenta propriedades anti-inflamatórias robustas e está atualmente, em estágio avançado de desenvolvimento para o tratamento de leucemia mieloíde aguda (32). Esse glicosaminoglicano é derivado quimicamente da heparina não-fracionada pelo processo de dessulfatação seletiva nas posições 2-oxigênio (2-O) do ácido α-L-idourônico e 3-oxigênio (3-O) da D-glucosamina (33). Como consequência dessas modificações, há uma perda na capacidade de interagir com a antitrombina e o fator Xa e, portanto, ocorre uma redução significativa da atividade anticoagulante. No entanto, ainda é conservada a capacidade de interagir com muitas proteínas carregadas positivamente (34)**.**

A caracterização da proteína LPG3 de *L. chagasi* como uma PLH e a sua participação no processo de infecção parasitária despertam o interesse em desenvolver estratégias contra a LV que envolvam o bloqueio da LPG3 pela heparina. No entanto, a heparina tem seu uso limitado na leishmaniose visceral devido à sua ação anticoagulante que, somada às alterações hematológicas da doença, pode resultar em episódios hemorrágicos nos pacientes. Uma alternativa seria o uso de uma heparina sem ou com baixa atividade anticoagulante que compartilha com a HNF a capacidade de interagir com outras proteínas.

Assim, no presente estudo, testamos *in silico* e *in vitro* a hipótese de que DSTAT pode se ligar no sítio de ligação da heparina em LPG3 e que o bloqueio da proteína LPG3 por DSTAT pode prejudicar o processo de infecção de macrófagos por formas promastigotas de *L. chagasi*, na busca de uma nova possibilidade terapêutica contra LV.

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ARTIGO

IN SILICO EVALUATION OF THE INTERACTION OF THE NON-ANTICOAGULANT HEPARIN DERIVATIVE DOCIPARSTAT SODIUM (DSTAT) WITH LPG3 AND ITS *IN VITRO* EFFECT ON MACROPHAGE INFECTION BY *Leishmania chagasi*

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Abstract

LPG3 is a protein widely distributed on the outer surface of promastigote forms of *Leishmania chagasi* that acts as a heparin-binding protein (HBP). The blockade of this protein with sodium heparin generates a partial reduction in the internalization of the parasite in macrophages, suggesting the participation of the LPG3 protein in the infection process. In the present study, we tested *in silico* and *in vitro* the hypothesis that blockade of LPG3 protein by heparin with low anticoagulant activity (DSTAT) interferes in the process of parasitic infection. Molecular docking revealed DSTAT binding at the predicted heparin site on the LPG3 protein with slightly higher binding affinity than sodium heparin. *In vitro* assay demonstrated that the DSTAT concentrations equal or below 300 µg x mL^{-1} were not toxic to macrophages and this highest selected concentration of DSTAT did not present a leishmanicidal effect against promastigote forms of *L. chagasi.* On the other hand, DSTAT (100 µg x mL^{-1}) lead to a reduction in the internalization of *L. chagasi* promastigote forms in macrophages, without interfering with parasite adhesion. Together, these results suggest that LPG3 protein blockade by DSTAT prevents macrophage infection by *L. chagasi* promastigotes, without toxicity to both cells. This potential antileishmanial activity of DSTAT supports further investigations for its use as an adjunctive treatment against visceral leishmaniasis caused by *L. chagasi* parasite.

Keywords: LPG3, Visceral Leishmaniasis, *Leishmania chagasi*, Glycosaminoglycans, Heparin,

1. Introduction

Visceral leishmaniasis (VL) is the most severe form of leishmaniasis, with potential lethality, especially when untreated (1, 2). *Leishmania infantum* (syn. *Leishmania chagasi*) (3) is the protozoan parasite responsible for cases of VL in the Mediterranean Basin, Middle East, Central Asia, South and Central America (4). The parasite, in the metacyclic promastigote form, is transmitted during the blood meal performed by female sandflies infected. After be phagocyted by macrophages and other host phagocytes, it transforms into an amastigote, a replicative stage, which spreads the infection to the spleen, bone marrow, liver, and lymph nodes (5-7). Thus, the symptoms observed in patients who progress to clinical disease are enlargement of the spleen and liver, fever, weight loss, and hematological disorders, usually anemia, neutropenia and thrombocytopenia that can result in hemorrhage (8).

Since the 1940s, pentavalent antimonials have been used in the treatment of VL. However, the effectiveness of treatment with antimonials has been compromised by drug toxicity, long-term regimen and increased parasite resistance. Widely used in many parts of the world against VL, amphotericin B is an antifungal agent that is effective against the *Leishmania* parasite but also toxic to the patient. Although its toxic effects are reduced by liposomal formulations, the high cost of this drug makes it difficult to access treatment (9-11). Other treatment options for VL include the miltefosine, paromomycin, and pentamidine, which have side effects, toxicity, and variable efficacy (12). Given this limited arsenal, there is a need for new antileishmanial treatments that needs to be both effective against parasite infection and safe for the patient.

Knowledge about parasite-host interactions allows the discovery of targets whose modulation or blockade may interfere with the course of infection/disease (13). Heparin-binding proteins (HBPs) are receptors of *Leishmania* parasites that recognize and bind to glycosaminoglycans (GAGs), especially highly sulfated ones like heparin and heparan sulfate (HS) (14). Our research group characterized the LPG3 protein from *L. chagasi*, widely distributed on the outer surface of promastigote forms, as a HBP. The blockade of this protein with heparin generated a partial reduction in the internalization of the parasite in macrophages, suggesting the participation of the LPG3 protein in the process of parasitic infection (15, 16).

Heparin, known for its anticoagulant activity, also can inhibit the infection of host cells by pathogens that bind to the HS present on cell surface (17-19). However, the use of heparin in the treatment of VL is limited due to the risk of bleeding associated with its strong anticoagulant activity (20-22). An alternative is the removal or reduction of the anticoagulant activity of heparin through the selective desulfation process (23). DSTAT is a 2-O, 3-O desulfated heparin, derived from unfractionated heparin, with significantly reduced anticoagulant activity, but which still shares many other properties with heparin, including the ability to interact with many positively charged proteins (24, 25). Therefore, in the present study, we tested *in silico* and *in vitro* the hypothesis that the DSTAT can bind to LPG3 and that the blockade of the LPG3 protein by the DSTAT can interferes in the process of parasitic infection. *In silico*, DSTAT interacted with the LPG3 protein at the heparin binding predicted site with slightly higher binding affinity than heparin sodium. *In vitro*, DSTAT prevented the infection of macrophages by *L. chagasi*, without toxicity for both cells. Together, the results suggest that the antileishmanial activity of DSTAT demonstrated here is due to the blockade of the LPG3 protein by DSTAT binding.

2. Material and methods

2.1. Drugs

DSTAT solution was supplied in sterile 10 mL vials at a concentration of 50 mg x mL^{-1} (CHIMERIX, INC., Durham, NC, USA). Heparin sodium 5000 IU/mL (Cristália, São Paulo, SP, Brazil) and Amphotericin B solution 250 µg/mL (SIGMA-ALDRICH, St. Louis, MO, USA) were used as reference drugs. All drugs were directly diluted in the culture medium for the use in *in vitro* experiments.

2.2. Leishmania *cultivation*

L. chagasi promastigote forms (strain MHOM/BR/75/M2682) were cultivated in Dulbecco's Modified Eagle medium (DMEM; SIGMA-ALDRICH, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Vitrocell Embriolife, Campinas, SP, Brazil) inactivated at 56ºC/30 min, 10% human urine, 0.01 mM adenine (SIGMA-

ALDRICH), 100 U x mL^{-1} penicillin G (SIGMA-ALDRICH), 0.25% hemin solution (SIGMA-ALDRICH), 2 mM L-glutamine (Synth, São Paulo, SP, Brazil), and 0.8% hepes (Ludwig Biotecnologia, Alvorada, RS, Brazil), pH 6.8.The cultures were kept at 25°C and passages were performed weekly (Supplementary Fig.S1). To perform the experiments of infection, parasites from the beginning of the stationary phase of growing were used, obtained according to the supplementary figure S1. The culture was centrifuged at 1540 g/4°C/10 minutes, the parasites were washed with phosphate buffered saline (PBS) and resuspended in fresh *Leishmania* culture medium.

2.3. RAW 264.7 macrophages culture

RAW 264.7 macrophages were cultured in DMEM medium (SIGMA-ALDRICH) supplemented with 10% FBS (Vitrocell Embriolife) inactivated at 56ºC/30 min, 100 U x mL^{-1} penicillin G (SIGMA-ALDRICH), and 0.8% hepes (Ludwig Biotechnology), pH 7.2, at 37°C and 5% CO_2. The cells were maintained in the culture medium until they reach confluent monolayers before being collected for further experiments.

2.4. Molecular docking

Receptor-ligand molecular docking was performed using the 3D model of the LPG3 protein predicted and refined by Martins *et al*. (2018) (16). The 2D chemical structures of the ligands heparin sodium (PubChem CID: 772) and DSTAT were retrieved from database PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/92044406) and (https://pubchem.ncbi.nlm.nih.gov/substance/363714334), respectively.

After preparation and 3D conversion of the binders using the Marvin Sketch program version 20.18.0, ChemAxon (https://www.chemaxon.com), the dockings between the LPG3 protein and the ligands were performed using the AutoDockVina included in the PyRx 0.8 software (26). A grid box of dimensions x = 25.1674640819, y=17.4073350682,z=27.862251566 and center x=28.9073528342, y=28.2036361177, z = 7.05853734212 was constructed around the amino acid residues of the heparin-binding site (SER-457, ASN-458, ARG-460, GLN-536, THR-538, ASP-539 and ASP-659) predicted in the previous study (16). Heparin sodium was used as positive control.

The number of conformations obtained for each ligand was nine. The conformation chosen was the one with the highest binding affinity within the heparin-binding site. The PyMOL 1.2r3pre (27) and the BIOVIA Discovery Studio 2016 (28) were used to visualize the anchored structures and to create the 2D diagram of ligand interactions, respectively.

2.5. Production and purification of anti-rLPG3 polyclonal antibodies

A quantity of 345 µg of the purified recombinant protein rLPG3 in PBS, pH 7.2 (volume of 500 µL), was emulsified in 500 µL of incomplete Freund's adjuvant (SIGMA-ALDRICH). Then, a single young rabbit, acquired from the rabbit hutch at the Federal University of Viçosa (UFV), was subcutaneously immunized with 200 µg of the purified protein (580 µL of the emulsion). Before this first dose, a blood sample from the animal was collected through a peripheral cut in the ear (pre-immune serum). Fifteen and thirty days after the first inoculation, the animal received boosters consisting of a half of the first dose used. Two weeks after the last booster, a new blood sample was collected from the animal by peripheral cut in the ear to obtain the hyperimmune serum. Total IgG was purified by the caprylic acid method (29).The pre-immune serum and hyperimmune sera were evaluated by Western blot analysis, which revealed the recognition of the recombinant protein rLPG3 only by the hyperimmune serum (Supplementary Fig. S2).

2.6. Analysis of cytotoxicity in macrophages RAW 264.7

The cell viability of RAW 264.7 macrophages was evaluated by the MTT method (30) 48 hours after the treatment with DSTAT. Initially, macrophages were plated in 96-well plates at a concentration of 5 x 10^4 cells/well and incubated for 4 hours at 37°C and 5% CO_2 to allow their adhesion to the surface of the plate. The culture was washed twice with PBS, pH 7.2 to remove non-adherent cells. Adherent cells were treated with different concentrations of DSTAT (ranging from 1.17 to 300 µg x mL^{-1}) in quadruplicates and incubated for a further 48 hours at 37°C and 5% CO_2. Wells containing untreated macrophages or macrophage culture medium alone were used

as positive cell viability control and blank, respectively. At the end of the 48 hours of incubation, 50 µL of 0.5 mg x mL^{-1} sterile 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; SIGMA-ALDRICH) were added to each well and the plates were incubated for 2 hours at 37°C and 5% CO_2. The reaction was stopped by the addition of 100 µL of 10% SDS in 0.01 M HCl to each well and the absorbance were read under incidence of light at 570 nm. Results were expressed as the percentage of viable cells calculated in relation to the positive control (untreated macrophages) considered to have 100% cell viability.

2.7. Leishmanicidal activity of DSTAT against promastigote forms of L. chagasi

To investigate the leishmanicidal effects of DSTAT, promastigote forms of *L. chagasi* in logarithmic phase of growth (see supplementary figure 1) were plated in 96-well plates (4 x 10^6 cells/well) in the presence or absence of DSTAT (300 µg x mL^{-1}/well), in quadruplicates. Wells containing untreated promastigote forms of *L. chagasi* or only *Leishmania* culture medium were used as positive cell viability control and blank, respectively. Amphotericin B (3.08 µg x mL^{-1}/well) was used as the reference leishmanicidal drug. After 48 hours of incubation at 25°C, the cell viability of the parasite was evaluated by the MTT method, as described in the previous experiment. The absorbances were read under incidence of light at 570 nm and the percentage of viable cells for each group was calculated in relation to the positive control (untreated promastigotes) considered to have 100% cell viability.

2.8. Adhesion and internalization assays of L. chagasi *in macrophages*

For the *L. chagasi* adhesion and internalization assays on RAW 264.7 macrophages, 5 x10^5 macrophages/well were plated in 24-well plates coated with circular glass coverslips. Cells were kept at 37°C and 5% CO_2 overnight to adhere to the coverslips. Then, wells were washed twice with PBS, pH 7.2 to remove the non-adherent macrophages and the macrophages were infected with 2.5 x 10^6 promastigote forms of *L. chagasi*/well. The plates were incubated at 37°C and 5% CO_2 for 30 minutes or 3 hours for the adhesion or internalization tests, respectively. The parasites were pre-treated with DSTAT at different concentrations (300 µg x mL^{-1} or

100 µg x mL^{-1}), heparin sodium (2 IU x mL^{-1}) or polyclonal rabbit anti-rLPG3 antibodies (50 µg/mL x mL^{-1}) for 30 minutes at 25°C. Heparin sodium was used as a blocking agent for LPG3 protein from *L. chagasi* and untreated parasites were used as a control group. After the last incubation period, the coverslips were washed with PBS, pH 7.2, removed and stained using the panoptic kit (RENYLAB, Barbacena, MG, Brazil), following the manufacturer's protocol. The percentage of macrophages with attached or internalized parasites and the number of attached or internalized parasites in 100 macrophages were determined by counting 200 cells, in duplicate, using an optical light microscope (Nikon eclipse E200).

2.9. Statistical analyzes

Data were analyzed using the unpaired Student's t test in GraphPad Prism 7.00 software (GraphPad Software, Inc., San Diego, CA, USA). Differences with *p*-values < 0.05 were considered statistically significant.

3. Results

3.1. Molecular docking of DSTAT with LPG3

Molecular docking using the 3D model of the LPG3 protein revealed the best coupling position for DSTAT and heparin at the predicted heparin-binding site at the intermolecular interface of contact between two LPG3 protein monomers (Figure 1). The values of binding affinity and interactions of heparin and DSTAT with amino acid residues of the LPG3 protein are summarized in the Table 1.

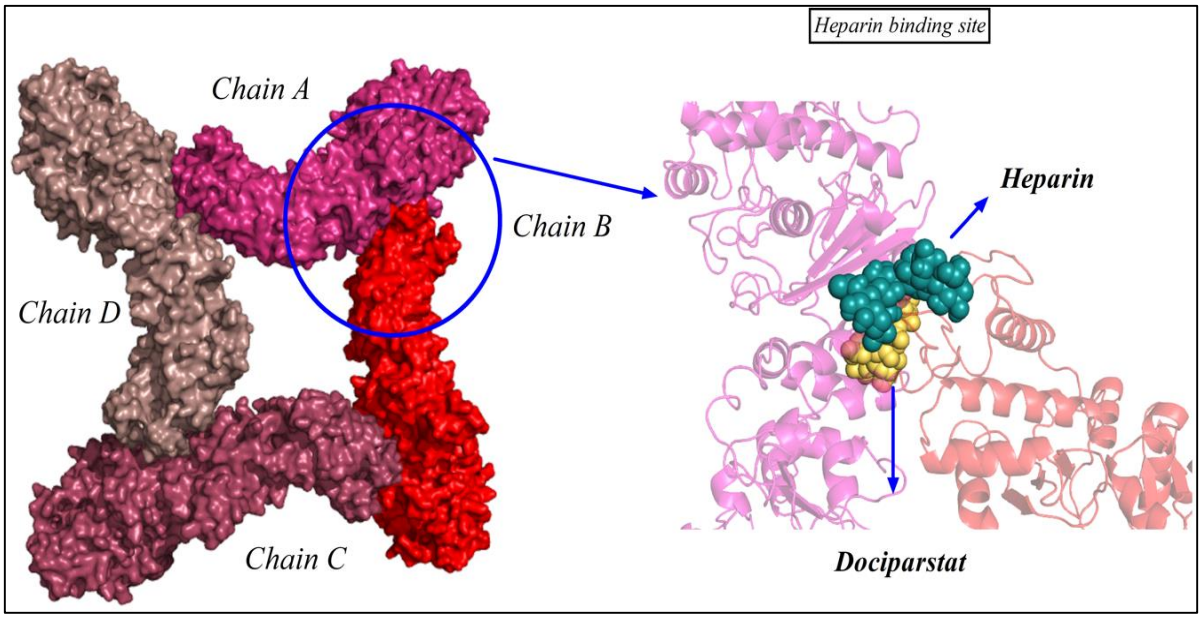

Figure 1. The best coupling position for DSTAT at the predicted binding site for heparin at the intermolecular interface of contact between two LPG3 protein monomers. The figure shows the LPG3 protein organized as a tetramer and its heparin-binding site where Dociparstat was also able to bind. Heparin is shown in blue and Dociparstat in yellow.

Table 1. Binding affinity and interactions of heparin and DSTAT with LPG3.

Ligand	***Interacting Residues***	***Binding affinity (Kcal/mol)***
Heparin	SER-457, ARG- 460, GLN-536, THR-538, ASP-539, ASP-659 e LEU-455.	-6.0
DSTAT	SER-457, ARG- 460 GLN-536, THR-538, GLN-729, GLU-726, LEU-455.	-6.6

Both ligands heparin and DSTAT interacted with the same number of residues and shared hydrogen bond interactions with five amino acid residues (SER-457, ARG-460, GLN-536,THR-538 and LEU-455), suggesting the colocalization of the ligands at this site (Figure 2). However, DSTAT showed a greater number of hydrogen bonding interactions with the amino acid residues at the site. Furthermore, when we compared heparin and DSTAT bindings in the protein target, we observed that the predicted binding affinity values for heparin (-6.0 Kcal/mol) and for DSTAT (-6.6 Kcal/mol) are close, but DSTAT has a little better energy affinity than heparin (Table 1).

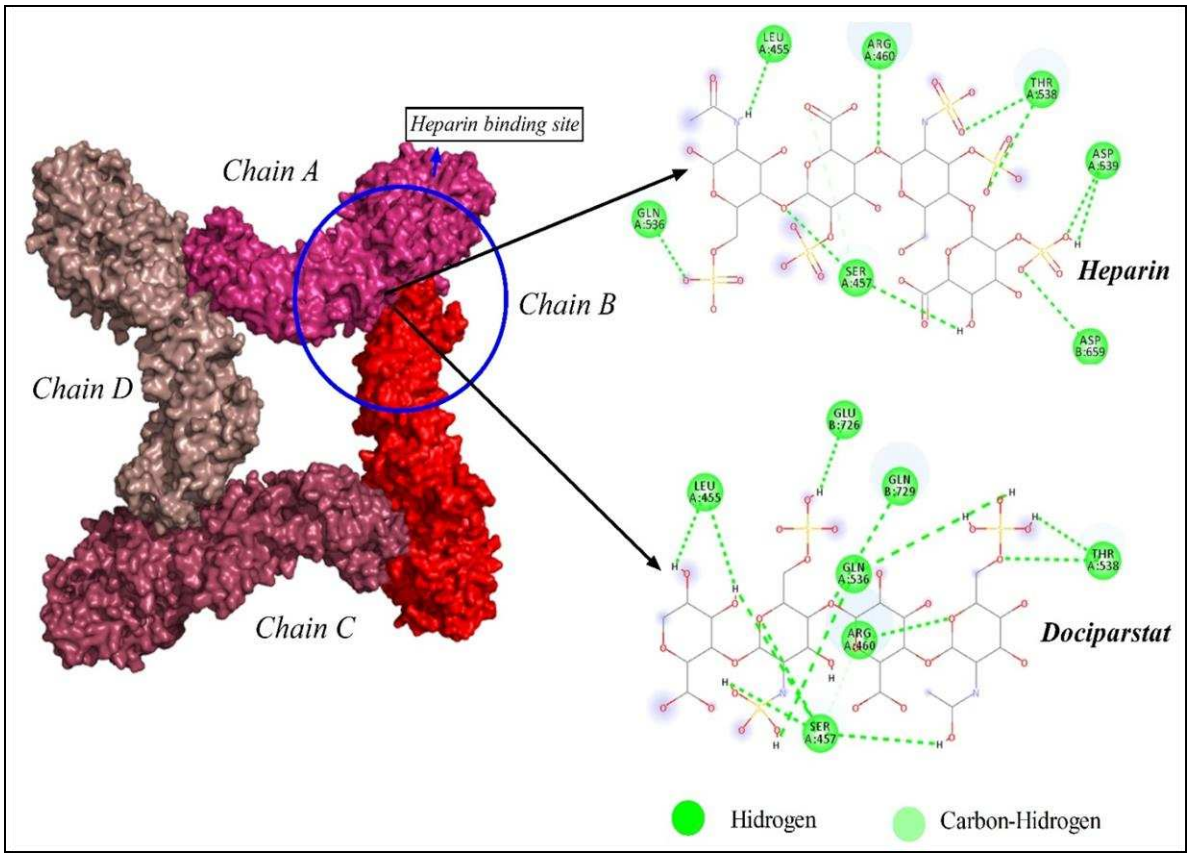

Figure 2. Representation of the LPG3 protein amino acid residues that interact with the ligands heparin and DSTAT in the heparin-binding site. 2D diagram of the interactions between the amino acid residues of the LPG3 protein (green circles with amino acid numbering) and the ligands heparin and Dociparstat (central carbon structures).

3.2. DSTAT is not toxic to RAW 264.7 macrophages

The toxicity of DSTAT to RAW 264.7 macrophages was evaluated for different drug concentrations (1.17 to 300 µg x mL^{-1}) by the MTT method. This assay was made to select the highest concentration of DSTAT that do not kill more than 10% of the

macrophages in relation to the control group (macrophages without treatment). The cells were treated with different concentrations of DSTAT by 48 hours, and it was observed that, for all concentrations tested, the cell viability was greater than 90% in relation to the control group (Figure 3). Therefore, the highest concentration selected for the investigation of the leishmanicidal activity of DSTAT against promastigote forms was 300 µg x mL^{-1}.

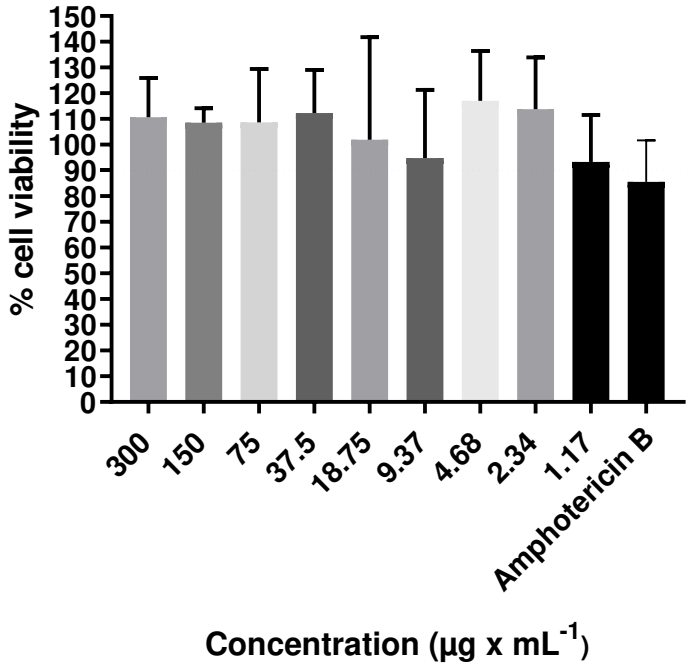

Figure 3. Cell viability of RAW 264.7 macrophages after treatment by 48 hours with various concentrations of DSTAT. The cell viability of macrophages was evaluated by the MTT method. The percentage of viable cells in each group was calculated in relation to the control group (macrophages without treatment) considered to have 100% cell viability. Amphotericin B (3.08 µg x mL^{-1}) was used as the reference leishmanicidal drug. Each bar represents the mean ± standard deviation of three independent experiments in quadruplicates. The horizontal line delimits a range of 90% cell viability in relation to the control group (macrophages without treatment).

3.3. DSTAT did not show in vitro *leishmanicidal activity against promastigotes of* L. chagasi

The cell viability assay by the MTT method using promastigote forms of *L. chagasi* revealed that DSTAT at a concentration of 300 µg x mL^{-1} was not able to reduce the cell viability of the parasite in relation to the untreated group (100% cell viability). On the other hand, promastigote forms of *L. chagasi* treated with the

reference leishmanicidal drug, Amphotericin B (3.08 µg x mL^{-1}), showed significantly lower cell viability than the group treated with 300 µg x mL^{-1} of DSTAT (Figure 4).

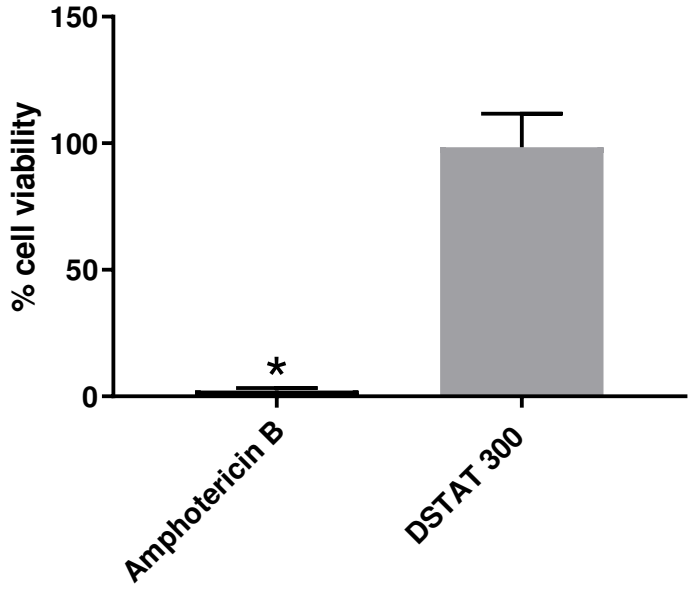

Figure 4. **Evaluation of the *in vitro* leishmanicidal effect of DSTAT against promastigote forms of *L. chagasi*.** Promastigote forms of *L. chagasi* were treated with 300 µg x mL^{-1} of DSTAT for 48 hours and cell viability was evaluated by the MTT method. The percentage of viable cells was calculated in relation to the control group (untreated promastigotes) considered to have 100% cell viability. Amphotericin B (3.08 µg x mL^{-1}) was used as the reference leishmanicidal drug. Each bar represents the mean ± standard deviation of three independent experiments in quadruplicates. The asterisk indicates statistically significant difference ($p < 0.05$) between groups (unpaired Student's *t*-test).

3.4. Effects of DSTAT treatment in adhesion and internalization of promastigotes forms of L. chagasi *in RAW 264.7 macrophages*

Adhesion and internalization assays were performed to investigate the influence of the DSTAT in the infection of the macrophages, considering that the blocking of the LPG3 by DSTAT can interfere in the process of the interaction host cell/parasite. Promastigote forms of *L. chagasi* were treated with DSTAT in different concentrations (300 or 100 µg x mL^{-1}), anti-rLPG3 antibody (50 µg x mL^{-1}) and heparin sodium (2 IU x mL^{-1}), prior to the infection in RAW 264.7 macrophages. Anti-rLPG3 antibody (50 µg

x mL^{-1}) and heparin sodium (2 IU x mL^{-1}) were used as controls according to the previous study by Martins *et al*. (2015). Assays for adhesion or internalization of the parasites in the macrophages were made as described in the methodology. We observed that, in the adhesion assay, there was no significant reduction in the percentage of macrophages with attached parasites or in the number of parasites attached in 100 macrophages in the pre-treated groups in relation to the untreated group (Figures 5 and 6). On the contrary, groups pre-treated with polyclonal anti-rLPG3 rabbit antibodies (50 µg x mL^{-1}) exhibited a significant increase in the percentage of macrophages with attached parasites. The same profile was observed considering the number of parasites attached in 100 macrophages, when the parasites were co-treated with DSTAT 300 µg x mL^{-1} and anti-rLPG3 antibody.

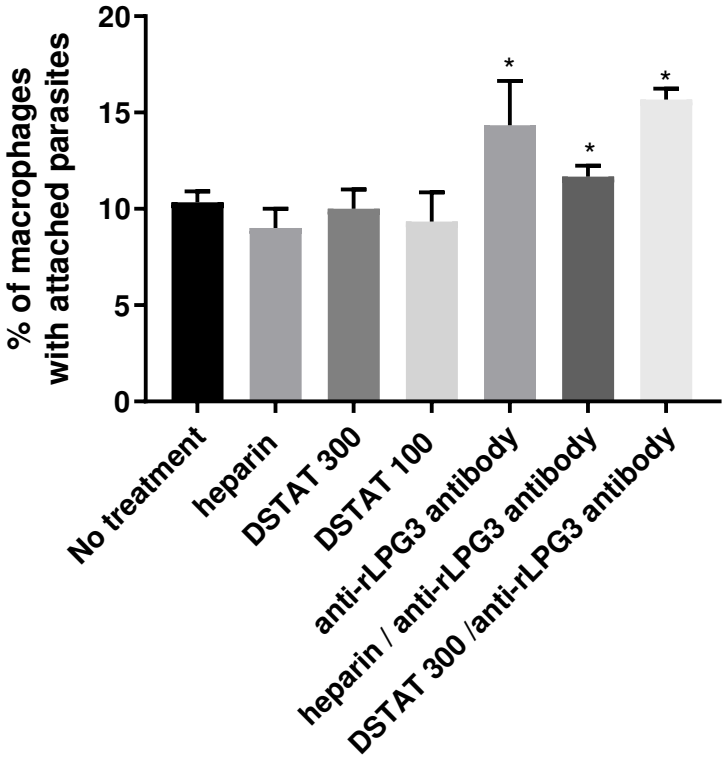

Figure 5. Adhesion assay of *L. chagasi* pre-treated with DSTAT in RAW macrophages. Promastigote forms of *L. chagasi* were treated with DSTAT (300 or 100 µg x mL^{-1}), heparin sodium 2 IU x mL^{-1} or anti-rLPG3 antibody 50 µg x mL^{-1} for 30 minutes, before macrophage infection. The percentage of macrophages with attached parasites was determined by the counting of 200 cells. The untreated group was used as a control. Each bar represents the mean ± standard deviation of three independent experiments in duplicates. The asterisk indicates that there are statistically significant differences ($p < 0.05$) in relation to the untreated group (unpaired Student's *t*-test).

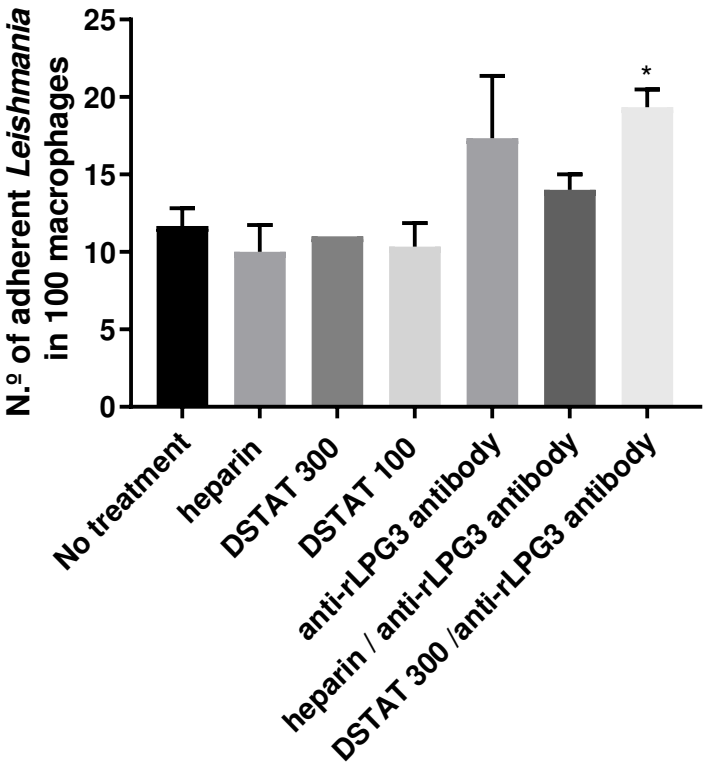

Figure 6. Adhesion assay of *L. chagasi* pre-treated with DSTAT in RAW macrophages. Promastigote forms of *L. chagasi* were treated with DSTAT (300 or 100 µg x mL^{-1}), heparin sodium 2 IU x mL^{-1} or anti-rLPG3 antibody 50 µg x mL^{-1} for 30 minutes, before macrophage infection. The number of parasites adhered in 100 macrophages was determined by the counting of 200 cells. The untreated group was used as a control. Each bar represents the mean ± standard deviation of three independent experiments in duplicates. The asterisk indicates that there are statistically significant differences ($p < 0.05$) in relation to the untreated group (unpaired Student's *t*-test).

When pre-treated promastigotes were submitted to the internalization assays, there was a significant reduction in the percentage of macrophages with internalized parasites in the groups pre-treated with 100 µg x mL^{-1} of DSTAT and with 2 IU x mL^{-1} of heparin sodium in relation to the untreated group (Figure 7). On the contrary, in the groups of parasites pre-treated with rabbit anti-rLPG3 polyclonal antibodies (50 µg x mL^{-1}), there was a significant increase in infection, even in those previously co-treated with 2 IU x mL^{-1} of heparin sodium or 300 µg x mL^{-1} of DSTAT.

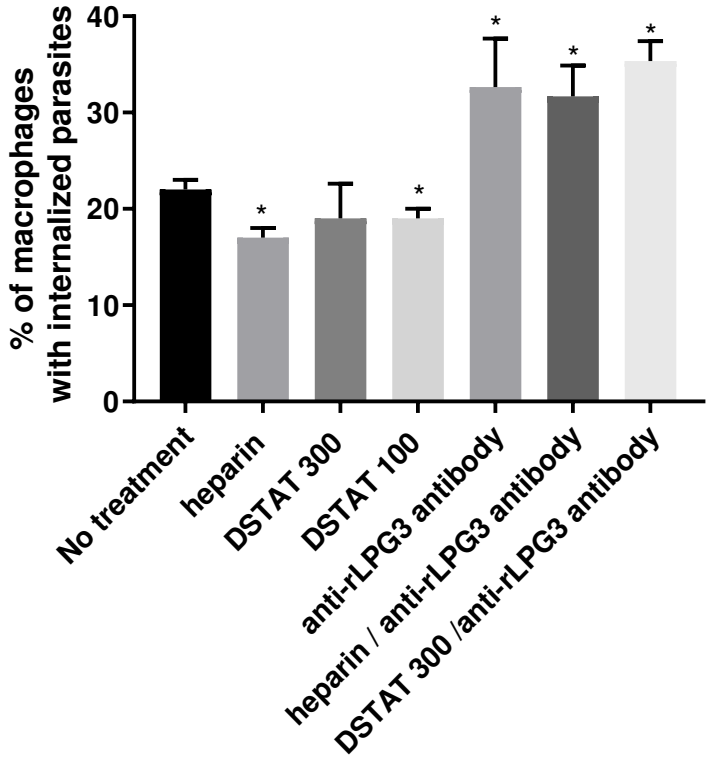

Figure 7. Internalization assay of *L. chagasi* pre-treated with DSTAT in RAW macrophages. Promastigote forms of *L. chagasi* were treated with DSTAT (300 or 100 µg x mL^{-1}), heparin sodium 2 IU x mL^{-1} or anti-rLPG3 antibody 50 µg x mL^{-1} for 30 minutes, before macrophage infection. The percentage of macrophages with internalized parasites was determined by the counting of 200 cells. The untreated group was used as a control. Each bar represents the mean ± standard deviation of three independent experiments in duplicates. The asterisk indicates that there are statistically significant differences ($p < 0.05$) in relation to the untreated group (unpaired Student's *t*-test).

The parasite load represented by the number of parasites internalized in 100 macrophages was significantly lower only in the group pre-treated with 2 IU x mL^{-1} of heparin sodium in relation to the untreated group. In all groups with parasites pre-treated with antibodies there was an increase in the parasite load in relation to the untreated group (Figure 8).

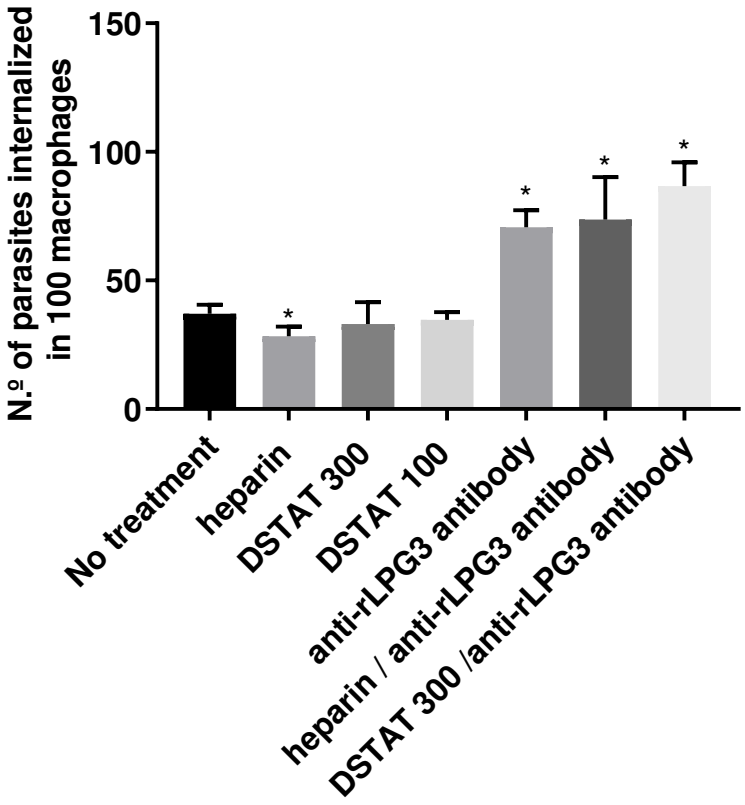

Figure 8. Internalization assay of *L. chagasi* pre-treated with DSTAT in RAW macrophages. Promastigote forms of *L. chagasi* were treated with DSTAT (300 or 100 µg x mL^{-1}), heparin sodium 2 IU x mL^{-1} or anti-rLPG3 antibody 50 µg x mL^{-1} for 30 minutes, before macrophage infection. The number of parasites internalized in 100 macrophages was determined by the counting of 200 cells. The untreated group was used as a control. Each bar represents the mean ± standard deviation of three independent experiments in duplicates. The asterisk indicates that there are statistically significant differences ($p < 0.05$) in relation to the untreated group (unpaired Student's *t*-test).

4. Discussion

LV is one of the main parasitic diseases with outbreak potential and mortality (31) and therefore, it must be treated. Considering the therapeutic options available, the safety of anti-*Leishmania* drugs is a major concern (32). Although other treatments against LV have emerged, most of them still have serious side effects, such as nephrotoxic, hepatotoxic, and teratogenic effects (33, 34). Thus, patient safety remains a major challenge in the treatment of LV. In this work, the toxicity of glycosaminoglycan DSTAT was evaluated against macrophages, the main host cells to the *Leishmania* parasite (35). None of the tested DSTAT concentrations ranging from 1.17 µg x mL^{-1} to 300 µg x mL^{-1} showed toxicity to RAW 264.7 macrophages. Thus, the concentration of 300 µg x mL^{-1} of DSTAT was selected for investigation of the leishmanicide activity against

promastigotes of *L. chagasi.* Although, similarly, DSTAT showed no leishmanicidal effect against these evolutive forms of the parasite. On the other hand, the ability of glycosaminoglycans to inhibit HBP- mediated infections *in vitro* is known (15, 36, 37) and therefore, heparin-like molecules can be used as HBP blocking agents, decreasing infection in macrophages by *leishmania.*(14).

Heparin is a highly sulfated glycosaminoglycan with a negative charge (38). The known anticoagulant effect of heparin is mainly due to the binding of its specific pentasaccharide sequence to the antithrombin protein, an inhibitor of coagulation factors. (21,39). However, many other proteins can interact with heparin producing various pharmacological effects, such as anti-inflammatory, antimestatatic, anti-angiogenic, antitumoral and antiviral effects (40). Despite this, the use of this drug in these applications may not be recommended due to its potent anticoagulant activity, which may cause bleeding (41, 42).Non-anticoagulant derivatives of heparin, obtained through elimination or at least reduction of anticoagulant activity, are being studied for these therapeutic applications (23,42,43). DSTAT is a heparin with low anticoagulant activity chemically derived from non-fractional heparin (UFH) by selective desulfation in the 2-oxigens positions (2-O) of α-L-idoronic acid and 3-O from D-glucosamine-NN sulfate. The basic conditions used for 2-O-desulfation lead to the loss of the 3-O-sulfate group in the pentasaccharide sequence of UFH, which in turn, markedly reduces anticoagulant activity. Nevertheless, selective desulfation in these locations does not significantly alter other UFH shared physical properties, such as cargo dependent interactions with multiple positively charged proteins (24, 25, 44, 45). LPG3, in turn, is a HBP that participates in the macrophage infection by *L. chagasi* promastigotes. Its heparin-binding domain contains several basic amino acid residues that give it a positive charge (15, 16). In this context, we investigate *in silico* the interaction of 2-O, 3-O desulfated heparin (DSTAT) with LPG3 protein. Molecular docking revealed DSTAT coupling to the heparin-binding site on the LPG3 protein. Although predominantly electrostatic interactions were expected between the basic amino acid residues of the site and glycosaminoglycan sulfate/carboxylate groups (46), there was a predominance of hydrogen bonds type interactions in the DSTAT-LPG3 complex, which are also important to stabilize the interaction of heparin molecule, a well-known LPG3 blocker, with the protein (16, 38). Both LPG3 ligands also shared interactions with amino acid residues from the heparin site. However, a greater number of hydrogen

bonding interactions and a slightly higher binding affinity were predicted for the DSTAT-LPG3 complex. Therefore, the results of the *in silico* analysis suggest that DSTAT can bind or block the *L. chagasi* LPG3 protein that participates in the process of parasitic infection, just as heparin does.

This hypothesis was tested in *in vitro* assays of adherence and internalization of the parasite in macrophages. These experiments are justified by the evidences that HBPs from *Leishmania* spp*.* are involved in the processes of adhesion and internalization of the parasite into host cells (14, 37).Heparin sodium was used as a blocker of the LPG3 protein. Pre-treatment with heparin sodium caused a partial reduction in the internalization of the parasite in macrophages, as described in the study by Martins *et al*. (2015). In our study, we also observed a significant reduction in the parasitic internalization, without interfering in the adhesion of the parasites to macrophages. In malaria disease, heparin sodium is characterized as a promising antimalarial drug because it also binding to a surface protein of the protozoan *Plasmodium falciparum*, inhibiting parasite entry into red blood cells. Despite being a potential antimalarial agent, its use in the treatment of malaria has been discontinued due to side effects such as intracranial bleeding. An alternative was to test modifications in the heparin molecule to achieve the balance between low anticoagulant activity and significant antimalarial activity (21, 47-49). Therefore, in the search for the balance between significant anti-*Leishmania* activity and low anticoagulant activity, 2-O, 3-O desulfated heparin with reduced anticoagulant activity (DSTAT) was used in the pretreatment of promastigotes of *L. chagasi* in our experiments. The concentration of 100 µg x mL^{-1} of DSTAT significantly decreased the percentage of macrophages with internalized parasites compared to the control group (untreated parasites). Probably, the infection was prevented by blocking LPG3 protein by DSTAT, as suggested in the *in silico* analysis and reported for heparin sodium. In addition, corroborating our finding, was demonstrated that promastigotes of *Leishmania infantum* in stationary phase are able to binding with heparin even after 2-O desulfation in IdoA (50).

Multiple receptor-ligand interactions are involved in adhesion and internalization of *Leishmania* promastigotes by macrophages (51). Fcγ receptors, one of the best characterized host cell receptors, are responsible for antibody-coated pathogen recognition (52, 53). These receptors bind to the Fc portion of antibodies and then

induce opsonized target phagocytosis (54). In agreement with the presupposition and, similarly to the study of Martins *et al*. (2015), the pretreatment of *L. chagasi* promastigotes with anti-rLPG3 polyclonal antibodies favored adherence and the internalization of parasites in macrophages. Not even the co-treatment of the parasites with heparin sodium was able to prevent the increase in the infection in macrophages. The characteristic of polyclonal antibodies to recognize several binding sites (epitopes) on the target protein (55) is, probably, the reason the pretreatment with heparin sodium did not prevented the binding of the anti-rLPG3 polyclonal antibody to the LPG3 protein.

In the present study, the experiments were directed to the promastigote forms of *L. chagasi*, instead of the amastigotes, which are responsible for the spread of infection in the mammalian host. Although potential HBPs candidates in amastigotes have not yet been reported, has been shown that amastigotes forms of *Leishmania amazonensis* and *Leishmania major* have a higher affinity for heparin than the promastigotes forms (37, 56). In this sense, it is interesting to plan *in vitro* studies involving the treatment of amastigotes of *L. chagasi* with DSTAT, as well as *in vivo* studies for evaluation of DSTAT as adjunctive treatment in VL.

5. Conclusion

The development of parasite resistance, the toxicity and side effects of drugs used to treat leishmaniasis stimulate the search for alternative treatments. DSTAT is a heparin with low anticoagulant activity that, in our study, prevented infection of macrophages by promastigote forms of *L. chagasi*, without causing toxicity to both cells. The antileishmanial activity demonstrated here is, probably, due to the blockade of the parasitic LPG3 protein by DSTAT binding, resulting in the reduction of internalization of the parasite by macrophages. Based on this premise, further *In vitro* and *in vivo* studies considering amastigote forms of the parasite and randomized clinical trials using the DSTAT in combination with lower doses of leishmanicidal drugs may provide a new strategy for the control of VL.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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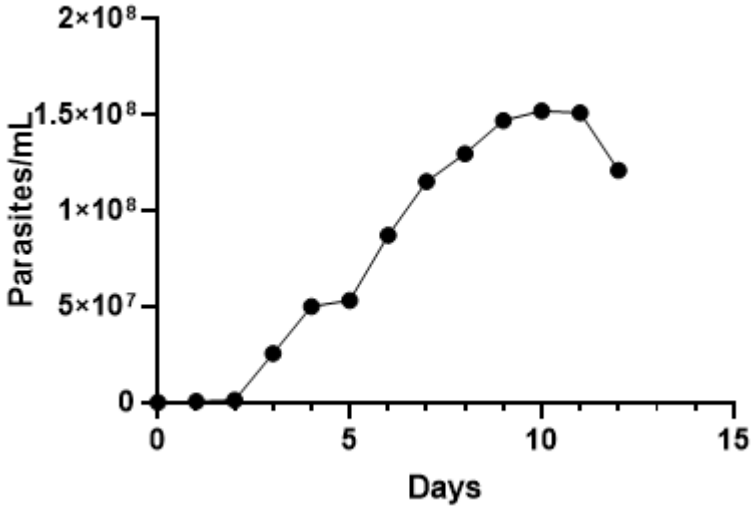
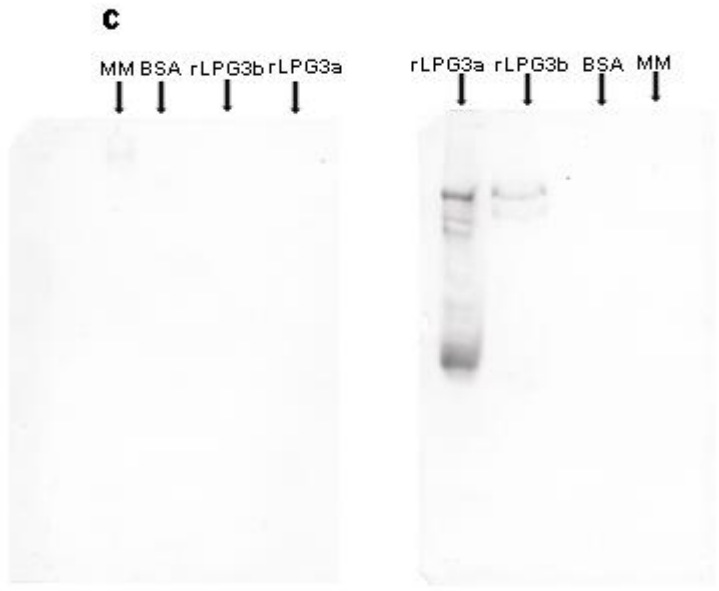
Supplementary data

The following are the Supplementary data to this article:



Supplementary figure 1. *In vitro* growth curve of *L. chagasi* strain MHOM/BR/75/M2682 in liquid medium. The parasites are in the final of logarithmic phase of growth until the ninth day of culture, then passing to the stationary phase. Promastigotes forms from the beginning of the stationary phase (tenth day of culture) were used for macrophage infection.



Supplementary figure 2. ***Western blotting of L. chagasi LPG3 recombinant protein samples*****.** Recombinant LPG3 protein purified in sample **a** (rLPG3a), Recombinant LPG3 protein purified in sample **b** (rLPG3b), bovine serum albumin (BSA) and Molecular Mass standard (MM) stained with anti-rLPG3 total IgG antibody. Control (C): rLPG3a, rLPG3b, MM and BSA stained with pre-immune serum total IgG.

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